Shifting gear in antimicrobial and anticancer peptides biophysical studies: From vesicles to cells

Journal:	*Journal of Peptide Science*
Manuscript ID:	PSC-14-0228.R1
Wiley - Manuscript type:	Review
Date Submitted by the Author:	n/a
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Keywords:	Cell, Vesicle, Peptide, Membrane, Biophysics, Spectroscopy, Antimicrobial, Anticancer

Shifting gear in antimicrobial and anticancer peptides biophysical studies: From vesicles to cells**

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Running title: Shifting gear from vesicles to cells

**) Invited Article for the Anniversary Issue 2015 of Journal of Peptide Science.

The review is based on a part of the Zervas Award Lecture delivered by Prof. Miguel Castanho at the 33rd European Peptide Synmposium in Sofia, 31 August – 5 September, Bulgaria 2014.

ABSTRACT

Despite the intensive study on the mechanism of action of membrane-active molecules such as antimicrobial and anticancer peptides, most of the biophysical work has been performed using artificial model systems, mainly lipid vesicles. The use of these systems allows full control of the experimental parameters and to obtain molecular-level detail on the action of peptides but the correlation with biological action is intangible. Recently several biophysical methodologies have been translated to studies using bacterial and cancer cells. Here we review biophysical studies on the mechanism of action of antimicrobial and anticancer peptides performed directly on cells. The data in these studies allow to correlate vesicle- and cell-based studies and fill the vesicle-cell interdisciplinary gap.

Keywords: Cell, Vesicle, Peptide, Membrane, Biophysics, Spectroscopy, Bacteria, Antimicrobial, Anticancer, Lipid

Abbreviations: SLM, supported lipid bilayers; BLM, black lipid membranes; AMPs, antimicrobial peptides; LPS, lipopolysaccharide; AFM, atomic force microscopy; CD, Circular Dichroism; LTA, lipotheicoic acids; NMR, nuclear magnetic resonance; MIC, minimum inhibitory concentration; FACS, fluorescence activated cell sorting; ACPs, anticancer peptides; CPPs, cell-penetrating peptides.

INTRODUCTION

Over the years biophysicists have studied intensively the mode of action of membrane-active molecules. The need for quantitative data has driven these researchers to use artificial systems, mainly vesicles, so that full control of the experimental parameters is achieved. Vesicles are made of artificial mixtures of lipids, which means that the researcher controls the fluidity, the thickness, the concentration, the charge, and the chemical nature of the lipids, as well as the presence, or absence, of sterols. Depending on the mode of preparation of vesicles, unilamellar systems of controlled size or multilamellar systems are obtained. Moreover, there are methods that enable loading vesicles with chemical species such ions and fluorophores.

One of the very few limitations in work with vesicles is the serious difficulty of preparing vesicles of asymmetrical lipid bilayers. In cases in which asymmetrical bilayers

are needed, biophysicists resort to planar lipid bilayers totally or partially supported such as the SLM (Supported Lipid Bilayers) [1] or BLM (Black Lipid Membranes) [2], respectively. Vesicles prepared directly from biological membranes may lose their organization and were not generally adopted.

Vesicles and other artificial lipid bilayer systems enabled collecting massive amounts of molecular-level detail data on the action of antimicrobial peptides (AMPs). A reasonably well structured understanding of the molecular events implied in membrane perturbation and disruption by AMPs has emerged. Most AMPs have high affinity for anionic lipid membranes, change conformation upon contact with the lipids, and undergo critical events at specific local peptide-to-lipid ratios. These critical events are typically a change in orientation and/or oligomerization in the membrane [3], so they occur at high membrane coverage by the peptides.

The details of AMP-induced membrane disruption in bacteria are rarely known and the validity of extrapolating knowledge on AMP action in vesicles to bacteria is largely elusive. Bridging the gap between microbiology and biophysics remains a challenge yet to be met. Being up to the challenge is a matter of both biophysicists and microbiologists leaving their reductionist disciplinary approach engage interdisciplinary work, not an intrinsic limitation of the techniques and methodologies available to tackle the gap.

It is our purpose with this discussion paper to demonstrate that quantitative biophysical studies of AMPs in bacteria are possible and important recent advances have been achieved to elucidate the molecular events that take place at bacterial membranes, and their (dis)similarities with their counterparts in vesicles.

LIPID BILAYERS: SIMPLE BUT UBIQUITOUS

Over millions of years of molecular, cellular, and species evolution, it is amazing that cell membranes have converged, without exception, to lipid bilayer-based structures. The chemical nature of the lipids varies, the presence of non-lipid molecules varies, charge and fluidity vary, but lipid bilayers are the essence of any biological membrane. This makes lipid bilayers the gold reference standard model for biological membranes. Biophysicists praise their simplicity, microbiologists undervalue them but their importance is not ignored.

If AMPs accumulate in the bacterial membranes as in vesicles or not, if they change conformation and supra-molecular organization as in vesicles or not, if they disrupt membrane integrity as in vesicles or not, if they collapse bacteria as they collapse the

morphology of vesicles or not, *etc.* are matters of vivid debate. Discussions are fueled more by intuition rather than objective evidence-based opinion. However, important yet dispersed, data in the literature is frequently overlooked.

BACTERIAL MEMBRANES: MORE THAN BILAYERS

Bacterial membranes are complex (Figure 1) when compared to human cells for instance. In Gram-negative bacteria the presence of a highly organized lipopolysaccharide (LPS) layer constitutes a barrier difficult to mimic in artificial systems. In Gram-positive bacteria is not possible to isolate the intact peptidoglycan wall and use it as single component of an artificial system. Therefore, it is not possible to work with perfect mimics of LPS membranes and peptidoglycan shells. This prevents researchers from working with isolated lipid bilayers, isolated layers of LPS and isolated peptidoglycan meshes forming a closed shell. It is thus impossible for biophysicists to extrapolate the biological action of AMPs from the data obtained with realistic models of each membrane component. The only way for biophysicists to unravel the biological action of AMPs is to apply their techniques directly on bacteria, which demands adaptations and methodological refinement. Although not trivial, successful adaptations have been reported for Atomic Force Microscopy (AFM), Fluorescence Spectroscopy, Circular Dichroism (CD), Infrared Spectroscopy, among others [4-7]. Some of these studies will be visited in the following sections.

Although there are no artificial systems that match the supramolecular organization of LPS and peptidoglycan in membranes, it is possible to work with extracts of biological components of bacterial membranes. Extracts are not realistic from the structural point of view but serve as simple models to study the interaction of peptides with these kinds of molecules. Given the architecture of bacterial membranes, a pertinent question to be asked is: can LPS (Gram-negative bacteria) and peptidoglycan (Gram-positive bacteria) serve as electrostatic barriers that capture AMPs and prevent their interaction with the inner lipid bilayer? Or, on the contrary, these layers saturate and cannot retain excess AMPs, which remain available to permeabilize the inner bilayers? In the end, answering these questions is a matter of concentrations (of AMPs and membrane components) and affinities of AMPs towards the different membrane components.

Omiganan, an AMP of clinical relevance [8, 9], was studied for its interaction with peptidoglycan extracts quantitatively. It was observed that there is extensive interaction between the peptide and peptidoglycan. While the results could, at first glance, leave the

impression that Gram-positive bacteria cell walls trap AMPs and prevent them from interacting with lipid membranes, in typical efficacy assays the concentration of peptidoglycan is very small and the quantity of peptide need to saturate the bacterial wall is negligible. Both aqueous phase and membrane-bound peptide concentrations are, in practice, the same, in the presence or absence of the wall, defeating its role as peptide trap. Henriques *et al*. [10] used Limulus amebocyte lysate to evaluate the binding of the AMP Sub3 to LPS and lipotheicoic acids (LTA). The data was used to propose a model of action of Sub3 in which it targets the outer membrane of bacteria through electrostatic attractions, causing permeation. The interaction with the inner membrane is not lytic: Sub3 translocates the inner membrane to bind to intracellular targets. The 21 kDa peptide based on the N-terminal region of the neutrophil bactericidal/permeability-increasing protein ($rBPI_{21}$) binds LPS aggregates [11, 12], which was hypothesized to facilitate LPS clearance by macrophage phagocytosis and/or blocking of LPS specific receptor recognition [12]. Later, the same authors confirmed by AFM Force spectroscopy that soluble LPS decrease the interaction of $rBPI_{21}$ with bacteria, especially *S. aureus* [13].

BACTERIA IN BIOPHYSICAL STUDIES

Several groups translated biophysical methodologies usually applied in artificial model systems to bacteria to study the molecular events that take place when AMPs interact with bacterial membranes (Table 1). This trend has been growing and important contributions have been published recently. In fact, this trend encompasses the fast development of cell biophysics in general. Whole-cell Nuclear Magnetic Resonance (NMR) [14], AFM [15], single particle tracking microscopies [16], isothermal titration calorimetry [17], zeta-potential light scattering [18] and fluorescence spectroscopy [19] are some examples of biophysical techniques recently applied to study molecular events directly on cells.

The specific case of AMPs action in bacteria has been addressed by Alves *et al.* [4] who studied *E. coli* morphologic collapse induced by two AMPs by AFM in standard conditions of bacterial growth and density. The collapse of the structure of the cell occurs at an AMP concentration that coincides with the minimum inhibitory concentration, MIC. Moreover, this is concomitant with electroneutralization of bacterial surfaces, as measured by zeta-potential light scattering. This parallels the action of AMP in vesicles, in which membrane coverage and electroneutralization has been quantitatively determined [20]. The

similarities observed between vesicles-based and bacteria-based work, even when precise quantitative reasoning is applied, has led to the proposal that biological action parameters, such as the MIC, can be estimated within reasonable limits from data on lipid affinity obtained in vesicles, namely:

$$MIC = \frac{\sigma}{K_p \gamma_L}$$

γ_L is the volumic mass of the lipids, σ is the peptide:lipid ratio on the saturated membrane itself, and K_p is the peptide lipid-aqueous environment partition constant. This thesis is detailed in references [3, 18, 21].

The need for peptides to saturate bacterial membranes and to cause permeabilization and inactivation explains why it is so rare to find AMP with MIC below 1 μm, an intriguing question that upsets peptide drug medicinal chemists. It is hypothesized that AMP with MIC below 1 μM have targets other than the membranes. Some may translocate membranes [22] and reach intracellular targets.

Independent studies by other groups corroborated the finding that AMPs act on bacteria under the same general principles as in vesicles. Stella's group has recently demonstrated [7] that the AMP PMAP 23 is active only when bound peptides completely saturate bacterial membranes, which occurs at micromolar total peptide concentration under the experimental conditions used to measure bactericidal activity. Stella *et al.* used steady-state and time-resolved fluorescence spectroscopy. Using a different technique, CD, Romanelli [5], with a completely independent study from the others previously described, also found important similarities between AMPs action in vesicles and bacteria: AMPs change conformation upon binding to membranes. LPS in bacteria are able to induce the conformational changes also observed in lipid bilayer vesicles.

The question remains on if it is possible for peptides to reach micromolar concentrations *in vivo* in a biological or pharmacological context. However, it is known that human neutrophil peptide 1, HNP-1, for instance, may reach concentrations between 30 μM and nearly 50 μM during infection [23]. For topical administration, reaching concentrations above μM is not a problem but for systemic administration this may be a challenge. Whilst the toxicology of peptides is usually not very stringent, the fast clearance from plasma does not favor high circulating concentration [24]. Chemical modification of peptides to improve pharmacokinetics is possible but it is still a challenge [24, 25].

The process of membrane permeabilization by AMPs was studied from the kinetic point of view by Freire *et al.* [26]. The action of an AMP on *E. coli* was assumed to be described by two consecutive steps: cooperative binding to the membrane and permeabilization. An innovative variant of Fluorescence Activated Cell Sorting (FACS), time-resolved FACS, was used in association to the Syto-9/Propidium Iodide "live-dead" assay [27] to follow the kinetic course of membrane permeabilization and allow retrieving the kinetic constant, associated to membrane association and subsequent membrane permeabilization as well as a cooperativity factor. Although the results are in general agreement with AMPs mechanism of action previously proposed based on vesicle work, the results varied among strains meaning that the details of the AMP action, mainly cooperativity, depends on the specific membrane composition. It is worth highlighting that a mutant strain with LPS having very short saccharide moieties are resistant to the AMP. This probably results from low electrostatic attraction of the AMP at the bacterial outer surface and/or abrogation of the conformational changes that trigger membrane permeabilization [5], which prevents the AMP from reaching and perturbing the inner lipid bilayer. This is in agreement with proposals that AMPs may fuse or at least perturb outer and inner membranes of bacteria [12]. Gee *et al*. [6] compared the action of a fluorescently labeled AMP in vesicles and bacteria using time-lapse fluorescence lifetime imaging and assigned the differences to this dynamic interplay between the effects of AMP in inner and outer membranes. A study of Dathe's group [28] with a cyclic peptide had shown that the LPS in *E. coli* are important for the peptide binding and partition. Absence of bacterial coverage by the AMP abrogates membrane permeabilization. The interaction with the outer membrane modulates the guidance of the AMP to the inner membrane, which is determinant for antimicrobial activity.

FROM BACTERIA TO TUMOURS

Following the discovery that the surface of cancer cells is rich in anionic lipids, a great deal of effort is being concentrated in developing AMPs as anticancer peptides, ACPs [29]. Very few studies are available on the action of ACPs on tumor cell membranes, some of which are summarized in Table 2. Only a subset of AMPs displays anticancer activity and there is strong controversy on whether AMPs and ACPs act the same way [30]. The outer surfaces of cancer cell membranes are not as anionic as in bacteria. Given the role of electrostatics in the mode of action of AMPs it is legitimate to question if ACPs follow the

mechanism of AMPs to permeabilize membranes. In particular, there were concerns that ACPs could accumulate on the surface of cancer cells to the extent observed in bacteria. Gaspar *et al.* [31] used zeta-potential light scattering spectroscopy with lung carcinoma A549 cells to demonstrate that ACPs SVS-1 is active at stages preceding membrane neutralization, in contrast with AMPs that target membranes. HNP-1 has a plethora of effects on cellular morphology and stiffness, membrane ultrastructure and charge on solid and hematological tumor cells [32]. For HNP-1, AFM and zeta-potential measurements show a preferential binding to solid tumor cells from human prostate adenocarcinoma when compared to human leukemia cells. AFM in particular revealed induction of apoptosis concomitant with cellular membrane defects at very low peptide concentrations.

In bacteria, saturation of outer membranes leads to permeabilization and AMP interaction with the inner membrane (Figure 2). ACPs appear to diverge from the mode of action of most AMP, as they translocate membranes prior to saturation of membranes and reach intracellular targets (Figure 2). In these cases, killing may occur by interaction with intracellular targets. It is worth reminding that AMPs are potential Cell-Penetrating Peptides, CPPs [22]. ACPs seem to result from the combination of antimicrobial and cell-penetrating properties in the same peptide.

CONCLUSION

Despite being only a small number of studies when compared to vesicle-based studies, cell-based biophysical studies on the action of AMPs and ACPs constitute important seminal work with a clear growing trend. Recent data sheds light on the molecular-level detail of the mechanism of action of AMPs and ACPs in cells. The vesicle-cell interdisciplinary gap is finally being bridged. The similarities and dissimilarities between the action of AMPs and ACPs in vesicles and cells are finally being unraveled (Figure 2). This demands power to adapt techniques and methodologies, as well as commitment of biophysicists to accept the challenge of performing solid quantitative work in cells.

ACKNOWLEDGMENTS

M. Castanho thanks EPS for the Zervas Award 2014. Fruitful discussion with Professors Lorenzo Stella and Alessandra Romaneli during the 33rd European Peptide Symposium were inspiring for this paper. Projects Ciência Sem Fronteiras PVE 171/2012 (CAPES, Brazil)

and MSCA-RISE-2014-Nr644167 are acknowledged. D. Gaspar and J. Freire are recipients of fellowships SFRH/BPD/73500/2010 and SFRH/BD/70423/2010 from FCT, Portugal, respectively. A.S. Veiga acknowledges FCT, Portugal, for funding within the FCT Investigator Programme (IF/00803/2012).

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Table 1 – Selected recent illustrative papers on the application of biophysical techniques directly in bacteria to study the action of AMP

Technique	AMP	Bacterium	Short description	Ref.
AFM and Zeta-potential Light Scattering	pepR and BP100	*E. coli*	Zeta-potential measurements of live bacterial cells in the presence of AMP. At MIC, the surface of the cells becomes neutral and the membranes collapse. Quantitative surface roughness analyses were performed.	[4]
FLIM	Fluorescently-labelled melittin derivative K-14 AlexaFluor-430	*E. coli*	Direct imaging of the action of the lytic action of AMP in living bacterial cells	[6]
AFM	R-BP100 and RW-BP100	*E. coli* and *S. aureus*	Antibacterial efficiency follows the affinity for bacterial membrane and is mainly driven by electrostatic interactions.	[33]
AFM, Zeta-potential Light Scattering, flow cytometry, fluorescence spectroscopy, and confocal imaging	Sub3	*E. coli* and *S. aureus*	Sub3 targets the anionic outer membrane of Gram-negative bacteria by electrostatic attraction, permeates the outer membrane, and translocates the inner membrane to reach intracellular targets. A similar mechanism is proposed for Gram-positive, with lipotheicoic acids replacing the liposaccharides as electrostatic attractors of AMP	[10]
Steady-sate and time-resolved fluorescence spectroscopy	PMAP-23	*E. coli*	The number of AMP required to kill a bacterium was estimated.	[7]
CD	Magainin 2 and cecropin A	*E. coli*	Study of AMP conformational changes upon bacterial binding.	[5]
Flow cytometry/ Membrane potential-sensitive dyes; AFM (imaging and force spectroscopy), and Zeta-potential light scattering	$rBPI_{21}$	*E. coli* and *S. aureus*	Surface perturbation on cells is followed by lysis. $rBPI_{21}$ has a binding pocket that may participate on the binding to Gram-negative bacteria.	[13]
Time-resolved FACS	pepR	*E. coli*	Describes and analizes the kinetics of bacterial AMP-induced permeabilization. Quantitative kinetic parameters on AMP binding to bacterial membrane, cooperativity and permeabilization are retrieved.	[26]

Table 2 - Selected recent illustrative papers on the application of biophysical techniques directly in tumor cells to study the action of ACP

Technique	ACP	Cell	Short description	Ref.
FACS and Fluorescence Microscopy	NK-2	Human neuroblastoma (NB LA-N-1 and SH-SY5Y); Human colorectal adenocarcinoma (SW480); Human lymphoma (U-937) and Human leukemia (K-562, Jurkat and MOLT-4)	The cytotoxic activity of the peptide was monitored by a Propidium Iodide (PI)-uptake assay.	[34]
FACS, Confocal microscopy and TEM	Lactoferricin B	Human neuroblastoma (Kelly cells, SK-N-DZ and IMR-32)	FACS and microscopy techniques allowed the observation of mitochondrial morphology alterations and mitochondrial membrane potential.	[35]
SEM, and Fluorescence Microscopy	Epinecidin-1	Human fibrosarcoma (HT1080)	Cell death occurred by induced lysis of cell membrane mediated after 48h of treatment by necrosis inhibitory activity.	[36]
FACS, SEM and Confocal fluorescence microscopy	Polybia-MPI	Human leukemia (K562, HL-60, L1210)	The killing mechanism of polybia-MPI AMP involves plasma membrane perturbation. Human cancer cells died after acute injury and bursting in alignment with a necrotic mode of action.	[37]
SEM, TEM And Zeta-potential Light Scattering	SVS-1	Human lung carcinoma (A549), Human epidermal carcinoma (KB), Human breast carcinoma (MCF-7 and MDA-MB-436)	Disruption of cancer cell membrane occurs via pore formation and was monitored with microscopy techniques. Cell membrane surface charge was measured with Zeta-potential and showed that cell death precedes full cell membrane neutralization.	[31, 38]
Confocal microscopy	β-hairpin AMPs (gomesin, protegrin, tachyplesin and polyphemusin II) and their linear analogues	Human erythroleukemia K562 cell	AMP-induced cell death over time was monitored through Ca^{2+} cell levels.	[39]
AFM	CB1a	Human lung carcinoma (NCI-H460 and NCI-H520)	Cancer cell death results mainly from cell surface damage. Cellular biomechanical properties are also affected by the peptide and measured using AFM.	[40]
FACS	Corn peptides	Human hepatocellular	CPs induced S cell-cycle arrest.	[41]

	(CPs)	carcinoma (HepG2)		
FACS, Fluorescence Microscopy	BEPTII and BEPTII-1	Human prostate adenocarcinoma (PC-3)	The inhibition of PC-3 cellular proliferation and apoptotic death was followed with flow cytometry and fluorescence microscopy analysi.s	[42]
SEM, Fluorescence Microscopy	A_9K	Human cervical cancer (HeLa) and Human leukemia (HL-60)	Microscopy techniques were used for evaluating peptide's selectivity, entrance and effect on cancer cell membranes.	[43]
FACS, AFM and Zeta-potential Light Scattering	HNP-1	Human prostate adenocarcinoma (PC-3) and Human leukemia (MOLT-4)	Cellular death, morphology, stiffness, and membrane ultrastructure and charge was followed using FACS, AFM and zeta-potential measurements.	[32]
FACS, Fluorescence Microscopy and TEM	Mastoparan	Murine melanoma (B16F10), Human melanoma (A2058), Human cervical carcinoma (SiHa), Human leukemia (Jurkat), Human breast carcinoma (MCF-7, MDA-MB-231, SK-BR-3), Human glioblastoma (U87)	Cell death was evaluated by the loss of mitochondrial membrane potential, chromatin condensation and morphological changes associated with apoptosis.	[44]

FIGURE CAPTIONS

FIGURE 1 - Schematic view of bacterial membranes and their models. Large unilamellar vesicles of lipid bilayers mimic the inner membrane of bacterias both in structure and composition. LPS and peptidoglycan aggregates mimic composition of outer bacterial membranes but not structure, which limits their application to AMP binding studies

FIGURE 2 – Permeabilization of vesicles and bacteria usually demands saturation of membranes. In standard antibacterial activity assays this occurs in or above micromolar concentration ranges. In bacteria, saturation of outer membranes leads to permeabilization and AMP interaction with the inner membrane. Some AMPs are able to translocate membranes at lower concentrations and reach the interior of vesicles or bacteria. In these cases, killing may occur by interaction with intracellular targets. This mechanism is more often associated to ACP.

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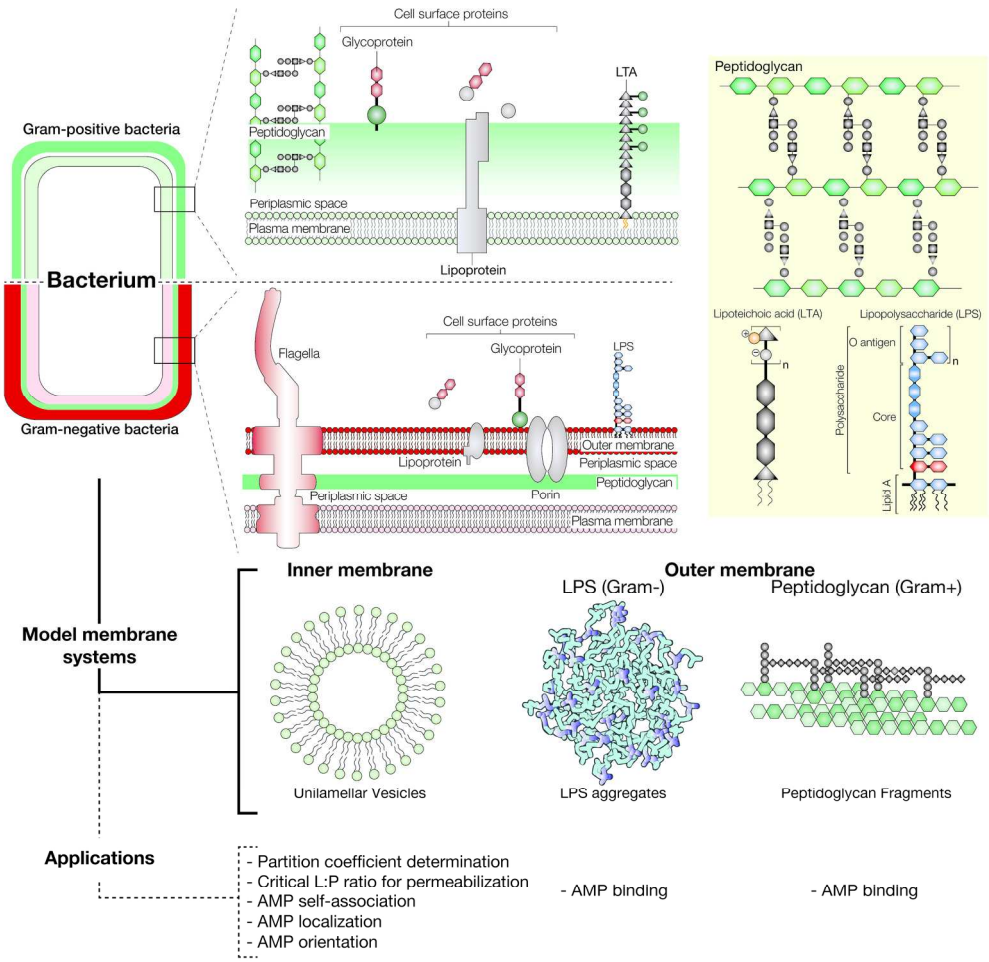

Schematic view of bacterial membranes and their models. Large unilamellar vesicles of lipid bilayers mimic the inner membrane of bacterias both in structure and composition. LPS and peptidoglycan aggregates mimic composition of outer bacterial membranes but not structure, which limits their application to AMP binding studies.
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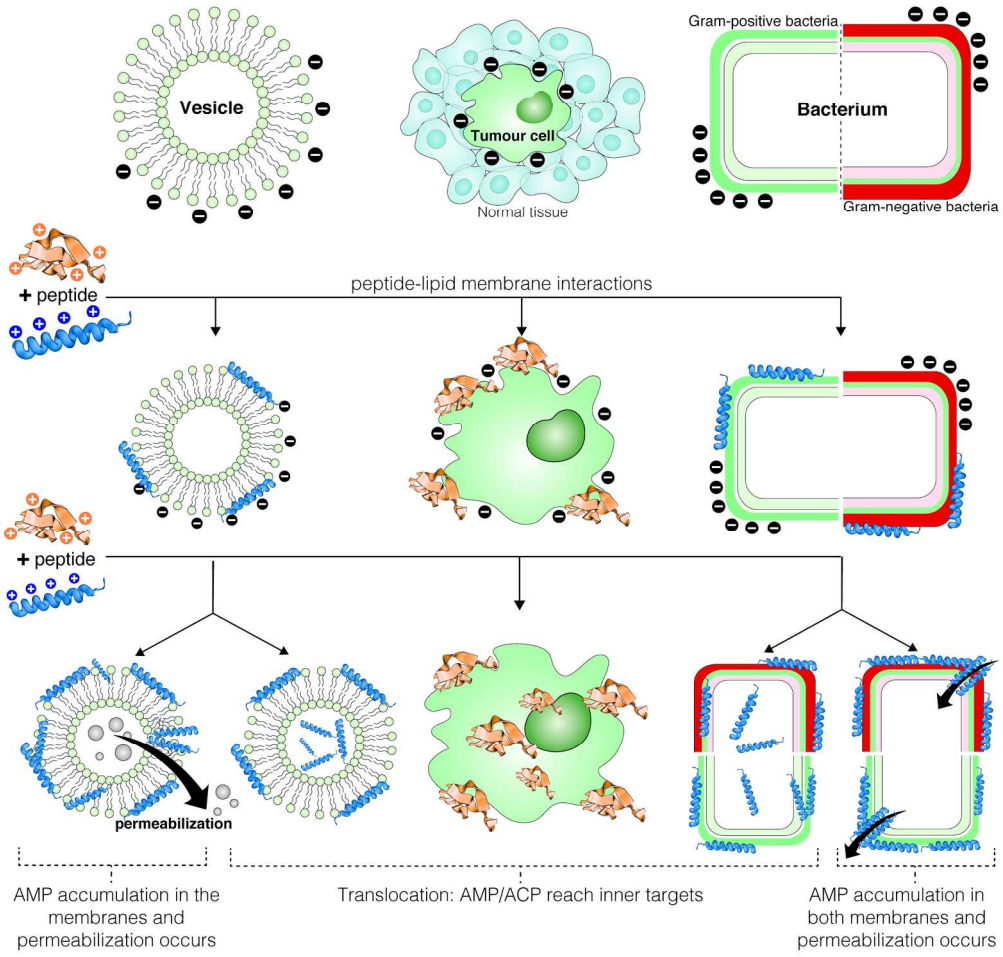

Permeabilization of vesicles and bacteria usually demands saturation of membranes. In standard antibacterial activity assays this occurs in or above micromolar concentration ranges. In bacteria, saturation of outer membranes leads to permeabilization and AMP interaction with the inner membrane. Some AMPs are able to translocate membranes at lower concentrations and reach the interior of vesicles or bacteria. In these cases, killing may occur by interaction with intracellular targets. This mechanism is more often associated to ACP.
182x174mm (300 x 300 DPI)

João Miguel Freire was born in Constância, Portugal in 1987 and obtained his degree in Biochemistry (University of Lisbon, Portugal) in 2008. After finishing his Master thesis in Biophysics (University of Lisbon, 2010) he is currently pursuing his Ph.D. degree in Biomedicine/Biophysics at Instituto de Medicina Molecular (School of Medicine, University of Lisbon, Portugal). His work is majorly centered on structural viral proteins and membrane-active peptides derived from them. He has been enrolled in understanding Dengue virus infection mechanism; and while studying such process to develop viral protein-based innovative drug candidates. Viruses are very simple biological entities, though very elegant and complex in their nature of living. In addition he has special interest in working on new biophysical methodologies to study and access membrane-active peptides mode-of-action at a molecular level.

Diana Gaspar was born in Porto, Portugal, in 1982, graduated in Pharmaceutical Sciences (University of Porto, Portugal) in 2006 and in 2010 obtained her Ph.D. degree in Pharmaceutical and Medicinal Chemistry (University of Porto, Portugal). Since 2011 she is a postdoctoral researcher at the Institute of Molecular Medicine (IMM, Lisbon, Portugal) where she currently focuses on the study and development of biologically active peptides as new anticancer agents. Additionally, she is interested in the characterization of cell-cell communication in metastatic cancer. Her research combines a wide variety of experimental methodologies from biophysical to imaging techniques.

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Miguel Castanho was born in Santarém, Portugal, in 1967. He graduated in Biochemistry (University of Lisbon, Portugal, 1990), has a Ph.D degree in Molecular Biophysics (Technical University of Lisbon, Portugal, 1993) and habilitation in Physical Biochemistry (University of Lisbon, Portugal, 1999). He became a group leader in the Faculty of Sciences at the University of Lisbon, Portugal, where he started working on the mechanism of action of membrane active peptides at the molecular level. M Castanho is now in the Instituto de Medicina Molecular, in the School of Medicne; University of Lisbon. His work includes the development of methodologies aiming at specific functional and structural information, related to cell-penetrating, antimicrobial, anticancer, and viral fusion inhibitor peptides, and neuropeptide drugs. In 2014 Miguel was one of the recipients of the Zervas award of the European Peptide Society

João Freire
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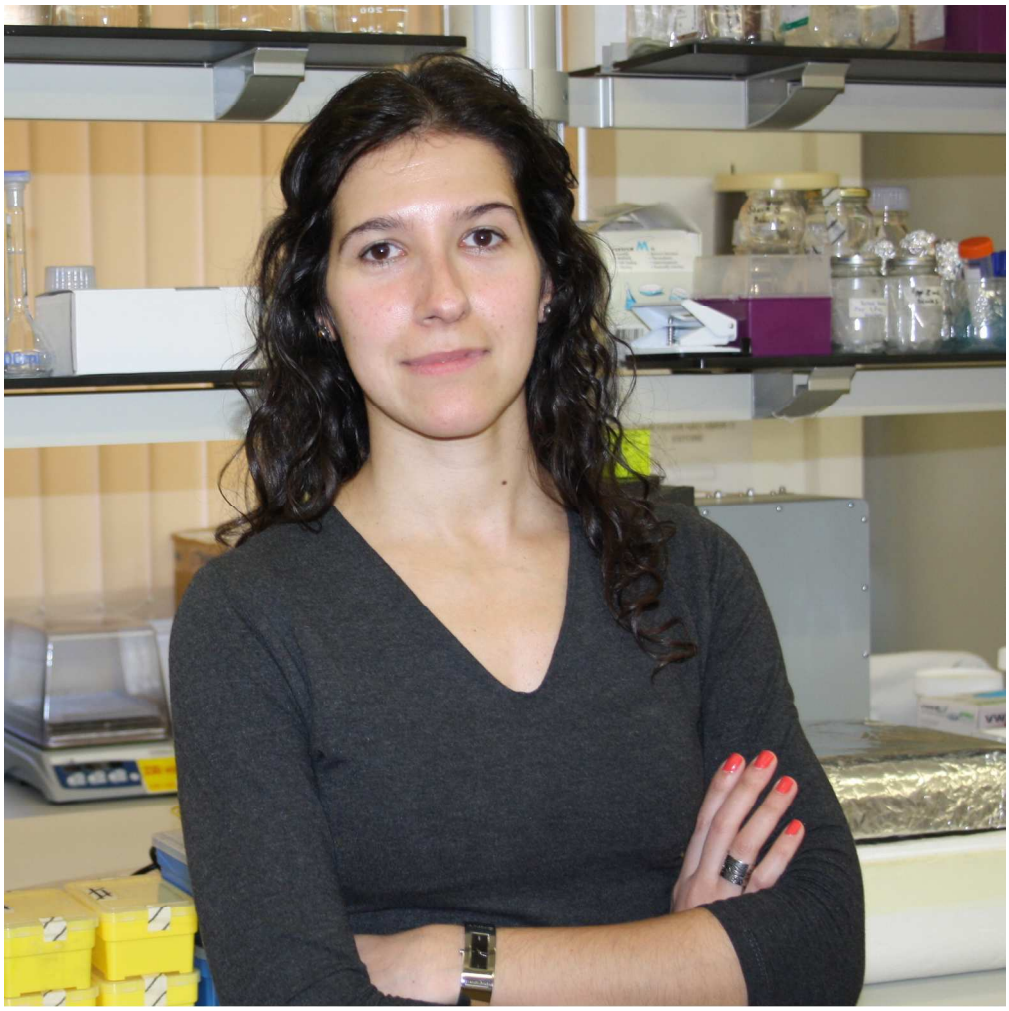

Diana Gaspar
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Ana Salomé Veiga
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Miguel Castanho
114x138mm (300 x 300 DPI)