Assessment of antimicrobial compounds by microscopy techniques

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The increasing rate of resistance to conventional antibiotics by bacteria and other human pathogens have raised the interest of health departments and pharmaceutical industries to develop new lead compounds to fight against persistent infections. Many bacteria resistance strategies rely on the guard keeping of membrane and cell wall integrity.

Antimicrobial proteins and peptides (AMPPs) are among the selected lead compounds to fight against microbial resistances. Although antimicrobial peptides are nowadays far from reaching the antimicrobial potency of current drugs, they have advantages that make them particularly interesting: (a) the ability to avoid the development of bacteria resistance mechanism thanks to an unspecific mechanism of action (b) a broad range of activity and (c) a low toxicity to the host. In addition to AMPPs, other drugs have been developed in the past years. Compounds like peptoids, cyclic peptides and lipoglycopeptides are also able to kill bacteria at a nanomolar range.

Microscopy imaging includes a group of powerful techniques that can be used to analyze the antimicrobial action of these compounds. Transmission (TEM) and scanning electron microscopy (SEM) conventional procedures and cryo-methods allows visualizing morphological changes at the membrane and cell wall ultrastructure at native conditions. By combining microscopy with labelling protocols it is possible to track and quantify the protein and peptide distribution throughout the cell. Electron microscopy and atomic force microscopy (AFM) can give us a perspective at a molecular level to understand each antimicrobial compound mechanism of action. Moreover, other complementary microscopy techniques can be used to analyze the characteristic properties of each drug. Of great interest is confocal microscopy, which provides, at a first glance, an overall picture of the entire cell condition. By using live–dead staining protocols, the cell integrity and the overall morphology of the cell population can be simultaneously followed. Thus it is possible to assess the kinetics of the antimicrobials effect on bacteria cultures and, at the same time, monitor cell agglutination and other population behaviours. Despite the noticeable interest to describe AMPPs mechanism at ultrastructural level, the subcellular information is still scarce.

Therefore, it can be concluded that microscopy techniques are very powerful to assist the mechanism of action of antimicrobials and can provide us with valuable information on both the cell and culture morphology.

Keywords: antibiotics; antimicrobials; scanning electron microscopy; transmission electron microscopy: confocal microscopy; bacteria cell wall; atomic force microscopy.

1. Introduction

The alarming growing rate of bacteria resistance to classical antibiotics has urged the scientific community to develop new drugs in order to efficiently control resistant microorganisms [1, 2]. The main goal is to achieve antimicrobial compounds with broad and potent antimicrobial activity but without undesirable side effects. In addition, these new compounds should be inexpensive and easy to use, chemically stable and must minimize the risk of microbial resistance [3]. Among these new compounds, antimicrobial peptides and derivatives have emerged as an important field of research in the fight of microbial resistance [4-6].

Antimicrobial proteins and peptides (AMPPs) generally display a high content of cationic and hydrophobic amino acids [7]. It is widely believed that they act through nonspecific binding to biological membranes even though the exact nature of these interactions is still unclear [8]. Many AMPPs bind in a membrane -parallel orientation, interacting only with one face of the bilayer, thus perturbing the membrane integrity. Some peptides and phospholipids may translocate or form multimeric transmembrane channels promoting the membrane depolarization [9, 10]. In addition to direct killing of bacteria, AMPPs display some immunomodulatory functions that may be related to infection clearance, including the ability to act as chemokines and/or induce chemokine production, inhibiting lipopolysaccharide induced pro -inflammatory cytokine production and modulating the responses of dendritic cells and cells of the adaptive immune system [11, 12].

Although AMPPs differ widely in sequence and source, several themes in their three - dimensional topology are predominant, and peptides have been categorized accordingly. They are folded to have both a hydrophobic face, comprising non -polar amino acid side chains and a hydrophilic face of polar and positively charged residues [13]. Regardless of this amphipathic nature, polypeptides may differ widely in length and amino acid composition and they can be fitted into four major classes: β -sheet structures, stabilized by two or more disulfide bonds, α-helices, extended helices (polyproline helices), with a predominance of one or more amino acids, and loop structures. Additionally, some modified structures can also be found, i.e. cyclic peptides as θ -defensins or lipopeptides as polymixin [14].
The precise mechanism by which AMPPs kill bacteria is still not clearly understood. However, antimicrobial molecules, as components of the innate immune defense system, require a selective toxicity that discriminates between the host and microbial cells. Therefore, they must preferably target common structures among pathogens, and act mainly at the cell surface [15].

The selective toxicity of most AMPPs is based on the differences between prokaryotic and eukaryotic cell membranes. The polypeptide–membrane interaction is determined by the physicochemical properties of both elements. Bacterial membranes display a negatively charged exposed leaflet, while in eukaryotic cells the outer leaflet is neutral and most negative lipids are displayed in the inner leaflet [15].

Most cationic AMPPs need electrostatic interactions to bind selectively with anionic membranes. They tend to form amphipathic secondary structures, which can be accommodated into membranes and permeabilize the lipid bilayer. This process is mainly dependent on the lipidic membrane composition. The first selective binding step would depend on the electrostatic interactions. Then, hydrophobic interactions between non-polar amino acids and the membrane core would be the main driving force for the membrane destabilization process. A variety of processes at the membrane level can lead to the bacteria killing, through membrane depolarization, pore formation or membrane lysis.

Amphipathic lytic polypeptides can act by three main different mechanisms, classified as “barrel-stave”, “carpet-like” and “toroidal pore” models [7]. In the “barrel stave” model, the helices insert into the hydrophobic core of the membrane and form transmembrane pores. Therefore, AMPPs can bind to both zwitterionic and charged phospholipid membranes. The mechanism involves the insertion by hydrophobic interactions in the lipid bilayer core, oligomerization and transmembrane pore formation. The pore formation can occur at a low peptide concentration, but also shows usually a sigmoidal dependence, above a certain threshold concentration [16].

In the “carpet-like” model, the peptide–membrane initial interaction is electrostatically driven between the AMPPs, positively charged and the cytoplasmic membrane, negatively charged. In this model, the polypeptides would interact with the lipid head groups and accumulate in the membrane surface. Finally, they would permeate the membrane when a local threshold concentration has been reached [8]. A high local AMPP concentration is usually needed on the membrane surface. In this model, the membrane leakage is due to the alteration of the bilayer curvature, triggered by a partial peptide insertion, in order to reorientate its hydrophobic residues towards the membrane hydrophobic core [17]. In some AMPPs there are two domains linked by a flexible hinge. This hinge, usually favoured by the presence of one or more proline residues, can help to induce a membrane curvature. In many cases, the “carpet like” mechanism ends up with membrane micellization. Some AMPPs are also able to induce membrane fusion. Most of these are rather hydrophobic and prone to self-association and they tend to form oligomeric structures in the membrane-bound state [18].

In the “toroidal pore” mechanism, a first step of membrane binding is needed, driven by electrostastical forces, and placing AMPPs in a random fashion towards the water–lipid interface. In a second stage, AMPPs interact to form clusters in the membrane surface. This step may happen prior to or concomitant with membrane binding. After having clustered, some AMPPs begin to embed deeper in the membrane, causing large deviations from membrane planarity. Finally, when one of the most deeply embedded AMPP connects with the other interface. A water pore is formed and one or more AMPPs, together with some lipid molecules move across the membrane, causing a pore relaxation into a toroidal shape [19].

Cytoplasmic–membrane permeabilization by AMPPs seems to be a widespread ability but, in many cases, did not appear to be the unique killing mechanism [20]. In this line, AMPPs can bind to the negatively charged bacteria cell wall, suggesting that this step may be a key event in the bactericidal mechanism. In addition, recognition of microbial motives is crucial in the host defense immune response. It has been described that the innate immune defense system can react against pathogen associated molecular patterns (PAMPs), which are absent in the host [21]. These microbial motifs are mainly lipopolysaccharides (LPS), teichoic acids (TA) and peptidoglycans (PGN). For example, AMPPs are able to displace divalent cations, promoting a local disturbance and altering the physicochemical properties of the outer membrane. In this way, the transient “cracks” could increase the outer membrane permeability and allow the peptide uptake itself [22, 23].

To study the mechanism of action of AMPPs high–resolution techniques are needed to observe particular characteristics at a molecular level. Thus, microscopy techniques have proved to be suitable to study the behaviour of these peptides in intact cells but also in model systems, such as lipid vesicles or planar bilayers. Combination of fluorescence and labelling techniques with electronic microscopy can give a very detailed picture of the mechanism of action of these peptides. Moreover, recent improvements in these techniques, as single particle detection and time resolution, can give a new wide range of opportunities to study new AMPPs and derived peptide–like antimicrobial drugs.

Here, we review some microscopic methodologies used to characterize the properties of AMPPs and summarize most of the current information on conventional and high-resolution imaging and analysis. Moreover, we also report information on the suitability of available imaging techniques for fluorescence and electronic microscopy including novel techniques to expand our knowledge about AMPPs structure and function in microbial cells.
2. Antimicrobial peptide’s effect on model systems

As described before, the main target for AMPs usually concerns the lipid bilayer, where peptides can promote the cell depolarization and/or internalize in the cytosol in order to interact with inner targets [24, 25]. Many biophysical techniques can be conducted using model membranes in order to understand the peptide–membrane interaction mechanism. Moreover, the ability of AMPPs to block LPS toxicity has raised the interest on the molecular aspects related to the interaction of AMPPs and LPS [11]. In this context, microscopy techniques can provide us with detailed information about peptide interaction with both phospholipid and LPS bilayers.

2.1 Lipid bilayers

2.1.1 Atomic force microscopy (AFM).

Nowadays, AFM has emerged as a powerful technique able to characterize lipid bilayers and other biological soft materials. The high resolution provided by AFM is not accessible by other techniques as conventional transmission electron microscopy (TEM), scanning electron microscopy (SEM) and fluorescence microscopy. In particular, AFM can provide information about lipid domain dynamics and mechanical properties of phospholipid bilayers [26]. Fluorescent and electronic microscopies work with samples prepared in solution. In contrast, AFM needs the sample to be attached to a regular surface as mica or glass.

Basically, two methods can be used to construct supported lipid bilayers (SLB): the Langmuir–Blodgett and the fusion of lipid vesicles methodology. Briefly, in the Langmuir method the lipid vesicles are compressed in an air–water interface, the solvent is evaporated and the monolayer transferred onto a solid surface. Next, a second lipid layer is transferred to create a supported bilayer. In the fusion of lipid vesicles technique, a population of small unilamellar vesicles (SUWs) is deposited in a freshly cleaved mica surface and leaved for 45-60 minutes at a temperature between 40 and 60°C. During this time, vesicles fuse over the mica surface and create a SLB (See references [27, 28] for a full description of these techniques).

The mechanical properties of SLBs are strongly modified by ionic strength and temperature [29]. It has been described that the presence of divalent, but also monovalent cations can enhance the deposition of lipid bilayers on hydrophilic surfaces. In this way, a high ionic strength can induce a fast and improved deposition of the bilayer on mica substrates with optimal mechanical properties [30]. Temperature is also a critical parameter that affects the bilayer structure. Natural membranes usually contain a high amount of insaturated phospholipids that induce a global fluidic state, which is strongly dependent on temperature. For example, DMPC 1 bilayers are in a gel (ordered) phase at low temperatures but undergo a gel to liquid (disordered) phase transition as temperature increases, modifying extensively the mechanical properties of the bilayer [29, 31].

Thus, the physicochemical parameters of the sample must be accurately taken into account to obtain reproducible and traceable results and to correctly understand and interpret the effects of AMPPs on lipid bilayers, as its mechanism of action is highly dependent on membrane characteristics.

In general, AMPPs induce a line tension reduction at the lipid domain boundaries, promoting the coalescence of the liquid–ordered domains into a larger and less round domains [32]. AMPPs usually display a non–membranolytic activity at low concentration but induces a slight reorganization of the bilayer. As can be seen in Figure 1A, the antimicrobial peptide melittin only induces low topographical changes in the phase–separated DSPC:DOPC 1:1 bilayers 1. Similar effects can be observed by a temperature increase or addition of amphiphilic molecules in the media [33, 34]. However, at high peptide concentrations, membrane remodelling seems to be concentration dependent and a drastic disruption of bilayers can be observed in many cases. Some peptides, like melittin, can disrupt the lipid bilayer without showing any domain specificity (Figure 1A) [32].

2.1.2 Fluorescence microscopy

In order to obtain additional details on the AMPPs–membrane interaction, polarized total internal reflection fluorescence microscopy (pTIRFM) can be used. This technique is able to map the order of a collection of fluorescent probes located in a supported bilayer interface and can quantify the molecule distribution by analyzing the orientational order parameter \( \langle P^2 \rangle \). Moreover, co–localization of fluorescence imaging and topography can be used to determine time–dependent structural and orientational changes in the bilayer environment [35, 36].

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1 DMPC: D–myristoyl–phosphatidylcholine.
3 \( \langle P^2 \rangle \) can be calculated pixel by pixel using the formula \( \langle P^2 \rangle = \frac{I_x - R^{xy} I_y + I_z}{I_x - R^{xy} I_y - 2 I_z} \), where \( I_x, I_y \), and \( I_z \) are the squares of the x-, y- and z–direction evanescent electric field vector amplitudes, respectively, calculated using the Fresnel equations using the angle of incidence (\( \alpha \)) and the relative refraction index \( n=n_1/n_2 \) between the mica substrate \( n_1 \) and the medium \( n_2 \). RFD is the fluorescence detected dichroic ratio, the ratio of...
SLBs for TIRFM imaging are formed using the vesicle fusion method on a glass–mica substrate. In order to detect SLBs by TIRF microscopy, extrinsic cell membrane labelling protocols can be employed using fluorescent dyes as Dil and BODIPY-PC 4.

The pTIRFM order parameter evaluates the localization of peptides within the supported bilayers and analyzes preferred orientation with respect to the membrane normal providing information about peptide orientation that will be greater in the case of pore or aggregate structures and lower if the peptide acts via a “carpet–like” model [35]. As it can be seen in Figure 1B, DOPE:DOPG bilayers appear brighter in the p–polarized rather than the s–polarized image and present a \( \langle P_2 \rangle \) value similar to lipid ordered phase membranes. After addition of 5 µg/ml of indolicidin, a decrease in the \( \langle P_2 \rangle \) parameter is observed, suggesting a more disordered arrangement of the fluorescent dipoles. AFM images taken in the same region of interest confirm the results observed by pTIRFM [35].

2.2 Lipid vesicles

Liposome interactions with AMPPs may be studied by means of negative staining in order to visualize changes in the form, aggregation or size of the lipid vesicles. However, the artefacts produced by the sample treatment limit the information that can be obtained with this technique [37]. Cryo-methods may be applied to attain more accurate information and a low altered visualization of lipid vesicles. Briefly, cryo–TEM and freeze fracture for TEM are based on the sample ultrafast freezing in a cryogen such as liquid ethane. Routine procedures of sample preparation consist in the deposition of a liposome small volume between two copper platelets, rapidly freeze by cryogen immersion, fracture at low temperature (-150°C) and high vacuum (10^{-9} mbar) and shadow both with a 2 nm layer of a heavy metal (Pt, Ta) and 20 nm layer of carbon. Then, samples are taken to room temperature and organic material is eliminated for morphological studies [38, 39]. Cryo–TEM and freeze fracture techniques can provide information at high resolution (until 3-4 nm). Moreover, replicas may also be used for immunolabelling techniques in order to localize antigenic molecules in the inner and outer surface layers.

2.3 Lipopolysaccharide (LPS) bilayers

Bilayers composed of lipopolysaccharides (LPS) can also be studied using AFM. To construct such bilayers, mica supports are coated with polyethilenimine (PEI) to minimize electrostatic repulsions between LPS and mica. The bilayers can then be prepared by using the vesicle fusion method [27, 28]. Samples can be observed in solution or dried, after removing the buffer with deionized water. These structures have similar thickness as detected by X–ray scattering (until 3-I4 nm). Moreover, replicas may also be used for immunolabelling techniques in order to localize antigenic molecules in the inner and outer surface layers.

Although studies on the interaction of AMPPs with lipid bilayer are extensively conducted, much less is known about LPS bilayers. LPS bilayers present a thickness about 7-I9 nm whereas lipid membranes have a 4-I6 nm average thickness. The difference is due to the presence of the polysaccharide core in LPS [41]. As can be seen in Figure 1C, fully hydrated and partially hydrated films appear different by AFM. In both cases the flat area remains but more defects are observed in the dried sample and a less coverage is detected (from ~ 85% in fully hydrated samples to ~ 35% in dried samples) [41].

Roes et al. [42] have assayed the interaction of the lipopeptide polymyxin B on LPS monolayers and observed the peptide intercalation followed by drastic changes in the lipid domain organization, in good agreement with the results obtained with lipid bilayers and monolayers. In addition, it should be interesting to conduct experiments in order to reveal the interaction mechanism of AMPPs with Gram -negative cell wall structures. As LPS is a potent endotoxin, more details in the binding mode of drugs to LPS could provide valuable information to develop new strategies to prevent sepsis. However, no fluorescently labelled LPS supported bilayer has been yet developed in order to properly analyze its characteristics under conventional and pTIRF microscopy.

3. Antimicrobial peptide’s effect on bacteria cells

Microscopy methodologies are particularly useful to complement biophysical studies on the mechanism of action of AMPPs at the bacteria membrane and cell wall and provide a physiological approach of live bacteria. Microscopy imaging includes a group of powerful techniques that can analyze in vivo the antimicrobial action of these compounds. By AFM, conventional SEM and TEM procedures and cryo –methods we are able to visualize morphological changes at the membrane and cell wall ultrastructure at native conditions. Using labelling protocols we can track and quantify the AMPPs distribution throughout the cell, whereas specific techniques allow visualizing the fluorescence intensity values (I) in a region of interest when the substrate is oriented perpendicular (p) and parallel (s) to the excitation light, \( I^{p} = \frac{F_p}{F_s} \).

BODIPY-PC: 4,4–difluoro –3a,4’–diaze –s –indacene –phosphatidylcholine; Dil: 1,1’–dilinoleyl-3,3,3’,3’–tetramethylindocarbocyanine perchlorate.

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location of cell heterosaccharides, proteins or lipidic structures. Thus electron microscopy can give us a very detailed perspective on the AMPPs mechanism of action. Moreover, other complementary microscopy techniques, as confocal microscopy, can provide a direct scenario of the cell life status.

3.1 Confocal microscopy to assess bacteria cell viability

Although characterization of samples at a nanometer scale can provide very detailed data, it is also necessary to monitor the overall integrity and viability of the bacteria cells after exposure to antimicrobial compounds. The assessment of bacteria cell viability is a good indicator to characterize the action of AMPPs. Using confocal microscopy, the kinetics and morphological evolution of bacteria cell population can be monitored at real time and compared with bacteria viability. Confocal microscopy can be performed directly in solution, thus the sample is not subject to artefacts due to sample fixation or drying, like electronic microscopy or AFM.

The mostly used fluorescent stains to assess cell viability are SYTO9 and propidium iodide (PI) [43]. Briefly, SYTO9 stains the DNA of all bacteria, either with intact or damaged membranes, whereas PI only stains damaged bacteria, displacing the SYTO9 when both dyes are present. Using an appropriate amount of both stains, bacteria with intact membranes will appear green, whereas bacteria with damaged membranes will appear red. Statistical analysis on the sample can be performed in order to derive quantitative measurements about viability (fluorescence signal integration of SYTO9 or PI signal) or aggregation (number of cells present in an isolated aggregate or number of aggregates in a region of interest). Moreover, this assay can be also complemented with fluorescence spectroscopy [44] and cytometer analysis.

![Figure 1](image-url).

**Figure 1.** Summary of microscopy techniques used to study the interaction between AMPPs and lipid bilayers. A) AFM imaging of the melittin peptide effect on DSPC:DOPC (1:1) bilayers (left panel) and SM:Ch:DOPC \(^5\) (3.5:3:3.5) bilayers (right panel) at different peptide concentrations: 0.0, 0.5, 2.5, and 7.5 µM. The scale bar spans 2 µm. B) pTIRFM–AFM combined image that shows the indolicidin effect on DOPE:DOPG \(^6\) (3:1) supported lipid bilayers labelled with BODIPY-PC. The top panel (a) shows the lipid bilayer prior to peptide treatment and the bottom panel (b) after the addition of 5 µg/ml of indolicidin. In both panels, top figures represent the p – and s –polarized images and the order parameter combined image. Bottom images represent different magnifications of the section using AFM. pTIRFM figures are complemented with a section line profile of the order parameter and

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\(^5\) SM: sphingomyelin; Ch: Cholesterol; DOPC: Di-oleoyl-phosphatidylcholine

\(^6\) DOPE: Di-oleoyl-phosphatidylethanolamine

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AFM images with a height profile for the same section line. C) AFM image of LPS supported bilayers scanned in water (left panel) and air (right panel). Each image is complemented with a depth section analysis along the horizontal line as indicated in the image. Figures were adapted with permission (A) from [45], (B) from[35], and (C) from [41].

3.2 High resolution techniques to analyze AMPPs action on bacteria

3.2.1 Atomic force microscopy (AFM) analysis of the bacteria envelope.

Atomic force microscopy (AFM) can provide high –resolution images of bacteria cells treated with AMPPs, avoiding any fixation with aggressive chemical compounds that may destabilize its morphology and metabolic condition.

In AFM bacteria are generally deposited on freshly cleaved mica, although other substrates can be used as glass slides or gold coupons [45-47]. Positively charged surfaces are preferred in order to minimize charge repulsion between the support surface and the negatively charged bacteria cell wall.

Samples can be dried or scanned in solution. Although samples in solution are closer to physiological conditions, dried samples present a higher resolution to distinguish structural details. Moreover, it has to be taken into account that some crystals may appear in the sample if dried. The best option to elude this problem is to rinse the samples with ultrapure water before depositing the sample in the glass slide. However, the incubation time with ultrapure water must be minimized because osmotic effects can promote bacteria lysis. If incubation with ultrapure water is not possible, a low saline buffer must be employed and negative controls should be carefully prepared in order to avoid artifacts. Even in dried samples it has to be considered that the thin water layer that remains between the sample surface and the AFM tip could induce a meniscus effect that can lower the image resolution because of transient adhesions between the tip and the sample [48].

Some important steps in the AMPP’s antimicrobial mechanism of action can be observed with AFM. In fact, it is worthwhile to perform different experiments at different protein concentration, below and above the minimal inhibitory concentration at 50% (MIC50) [45]. When the cells are incubated below the MIC50 value, some changes in the bacteria morphology can be observed. An important detail is the bacterial surface corrugation or bacteria roughness. This parameter measures the integrity of the cell wall, especially in Gram –negative bacteria. Many peptides promote an increase of the surface corrugation that may be caused by peptide incorporation in the bacteria cell wall and causes a wrinkle effect due to an increase in the surface area. This increase in the surface corrugation can oscillate from 140%, in the PGLa case, and up to >300% in the melittin case. Generally, surface corrugation is increased with the peptide concentration even when greater effects on cell morphology are observed [45, 49].

Changes in the bacteria cell wall structure can also be observed by AFM. In general, lesions and blebbings can be observed, located first at the bacteria apical end. This can be explained by the different membrane lipid composition throughout the cell surface. As the cardiolipin domains are concentrated in the poles and septal regions of bacteria, peptides could present a greater affinity for these regions [45, 50].

The release of vesicles with peptide aggregates can also be detected in some cases. These observations are linked to the cell wall damage and are usually related to a “carpet –like” mechanism in which micellization of the lipid bilayers can occur after peptide aggregation on the membrane [45].

Indentations in the bacteria surface can also be observed, which illustrate again the damage at the cell wall level. This indicates a disruption of the outer membrane of bacteria and probably causes an exposition of the peptidoglycan layer in the Gram –negative case that is usually linked to a micellization process. Disruption of the outer membrane causes, in general, a release of bacteria debris outside the cell, which is thought to belong to bacteria periplasm. In this case, peptides could cause the cell outer membrane destabilization and release of the periplasm content to the exterior of the cell [45, 48]. If pore –like structures are formed at this level, they can be shown at a higher magnification using SLBs, as in the case of melittin, where pores of approximately 65 nm can be observed [51].

At higher protein concentrations (above the MIC50) a greater leakage and a large amount of debris and partially disintegrated cells, indicate disruption of the inner membrane and an evident damage at the membrane level. In this case the bacteria is severely damaged and the membrane fully collapsed [45, 48].

In order to gain insight in stiffness and deformability of bacteria, nanoindentation experiments can be carried out to measure force –distance curves at different peptide concentrations and obtain information about the membrane mechanical properties [46, 52]. As we are working with soft and deformable samples, lower slopes in the force curves

\[ R_a = \frac{1}{n} \sum_{i=1}^{n} Z_i \]

where \( n \) is the number of measurements (numbered from \( i=1 \) to \( i=n \)) and \( Z \) is the height measurement.

Cardiolipin is a negatively charged phospholipid that can be found in bacteria and also in mitochondria.
are observed if compared with rigid surfaces. Considering the interaction as two springs connected in series, the equivalent spring constant can be computed and the results obtained can be related to the whole bacteria integrity.

It is worthwhile to comment that AFM analysis in bacteria are especially interesting in Gram–negative bacteria as the technique can provide us with a great amount of data concerning the characteristic cell wall structure [52]. Less is known on Gram–positive bacteria. E.g. in S. aureus, fewer details can be observed concerning the overall structure of peptidoglycan matrix, surely due to its rigidity. In this line, only the presence of cell debris and marked morphological changes can be observed [53].

Recently, Fantner et al. [54] have developed an increased time resolution AFM methodology that is able to characterize the initial steps of antimicrobial peptides on individual E. coli cells. Using this technique, the authors concluded that the peptide CM15 acts in a two–stage process, an incubation phase (that takes from seconds to minutes) and an execution phase that promotes the 50% damage in less than one minute, thus being the incubation phase the limiting stage in the antimicrobial action of CM15.

The high time resolution achieved (13 s per image) together with the high image resolution of AFM (on the nanometer scale) can provide an exciting new field of research on the AMPPs mechanism at the molecular level.

3.2.2 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to study cell envelope

Conventional TEM and SEM microscopy are frequently selected to visualize the ultrastructural damage on both cell wall and cytoplasmatic membrane of entire microbes when fixed material can be used [55-57].

SEM provides high resolution images to visualize protuberances or blebs related to a local destabilization of the bacterial cell envelope by AMPPs. In contrast, global damage of the cell wall can be detected as a generalized disturbance on its morphology [44, 55, 56]. Also, fibrous material, probably arising due to the leakage of cell content and cell debris scattered around the bacteria can also be observed in bacteria treated with AMPPs [58].

At ultrastructural level, a simple negative staining for TEM of bacterial cells can report evidences on the mechanism of membrane disruption by AMPPs [55, 56]. Ultrathin sections obtained by conventional procedures, namely fixation with aldehydes, post-fixation with osmium tetroxide, dehydration and embedding in Epoxy resin, allow the observation of membrane and cytoplasmatic alterations. Treatment with AMPPs can induce several external and internal changes such as membrane bleb, ruffling or detachment, the presence of electrodense dots or fibers, hypodense cytoplasmic release and cell vacuolization [55, 57]. The outer membrane detachment observed is generally related to the extremely high affinity of AMPPs to LPS, the main component of the gram-negative bacteria cell wall [57, 59].

3.2.3 Labelling methods for subcellular studies

SEM and TEM analysis can be complemented with immunolabelling techniques to quantify and localize compounds at the subcellular level. Generally speaking, immunolabelling methods may be divided on pre – and post –embedding labelling. Considering that pre –embedding methods often results in a poor ultrastructural preservation, post – embedding labelling are usually used for EM [60, 61].

The most common method consists on the localization of antigenic molecules by means of a secondary antibody bound to colloidal gold. Different methods of sample preparation often include several steps using chemical compounds or physical treatments in order to maintain antigenicity of samples. Two of the most common electrodense tags are metallotioneins and quantum dots [59, 62].

3.2.4 TEM tomography and cryo–electron tomography (CET): 3D visualization of entire cells

Tomography techniques by TEM have been greatly developed and allow a 3D reconstruction of, for example, molecules and organelles with conventional techniques and cryomethods [63]. Although there is a lack of information on this methodology applied to the study of AMPPs, these techniques may be suitable for reconstruction and could complement the information obtained with other topographic studies [38].

CET is a high-resolution method with unique potential to visualize cell components at a nanoscale level that include cryoultramicrotomy, a technique that requires complicate sample preparation that may introduce artifacts on the sample. An alternative method that avoids mechanical deformation is the use of focused ion beam (FIB) instrumentation for frozen-hydrated specimens or for samples prepared following conventional methods [63]. FIB milling allows the direct accessibility to selected targets in frozen–hydrated samples at nearly native condition [63]. In this way, CET tomography is the most promising technique for imaging the three-dimensional architecture of hydrated samples at nanoscale level, with a resolution better than 10 nm [64].

A major challenge in electron tomography is to visualise macromolecular structures at a resolution that allows the unambiguous identification within the cellular context. A prerequisite for reaching this goal is a minimum standard in

\[^9\] The equivalent spring constant can be computed using the equation \( k_{eq} = \frac{k_s \Delta d}{1 - s} \), where \( k_s \) is the cantilever spring constant, \( s = \frac{\Delta d}{\Delta z} \), \( d \) is the cantilever deflection and \( z \) is the scanner displacement.
sample preparation, which entails non-destructive modifications to sample geometry while maintaining the vitreous cell state. The use of focused ion beam (FIB) technology promises a significant improvement over conventional methods [65] specially due to the absence of mechanical deformation.

3.2.5 Correlative light and electron microscopy (CLEM)

Correlative microscopy is namely the methodology used to visualize the same sample at light microscopy and EM. For this, fluorescence microscopy and electrodense nanoparticles that present poor interaction with biological compounds have been used for single-particle or single-molecule studies-level (for example: quantum dots, nanofluorogold and metallotioneins). Although information concerning these techniques for the study of AMPPs is scarce, Lepthin et al. [59] reported the action of an antimicrobial peptide labelled with a quantum dot. These authors provide the first study on the combination of high-resolution imaging (single particle observation) and biochemical and biological functional assays.

Correlative cryo-fluorescence microscopy is used to navigate on large cellular volumes and to localize specific cellular targets [63]. Nowadays, it is not an extensively used methodology as it often implies the use of non-easily available technology [63] limiting its regular application.

These methods may constitute a source of information for specific questions on the study of AMPPs in biological systems as they become more available to the scientific community.

4. Particular cases

4.1 Sushi peptides S1 and S3.

The heavy chain of C. Rotundicauda Factor C protein (CrFC) contains some repeated domains of approximately 60 amino acids that contain several disulfide bonds conferring to the domain a characteristic folding. It has been demonstrated that the N-terminall fragment of CrFC presents a high affinity for LPS and that Sushi 1 and 3 (S1 and S3) are the main contributors to this binding [66, 67]. It has also been described that a predominance of lysines and arginines, together with hydrophobic residues are needed to construct these binding regions and a consensus motif BHB(P)HB (B = basic ; H = hydrophobic ; P = polar) has been proposed. Moreover, synthetic peptides containing this motif display also LPS binding activity similar to the Sushi peptides [68].

It has been thoroughly described that the adoption of a defined secondary structure is important for antimicrobial peptides in general and also for S1 and S3 peptides in particular. In this case, S1 acts as a monomer and presents a random structure in aqueous solution, but adopts an alpha helical conformation in presence of anionic phospholipids, as described for many antimicrobial peptides [48]. In contrast, S3 is active as a dimer and its secondary structure remains unchanged in a physiological buffer, but the content of α-helix increases slightly in the presence of anionic phospholipids, as described for many antimicrobial peptides [48]. The reason for this difference lies in the intrinsic secondary structures of S1 and S3 peptides, where an intermolecular disulfide bond probably stabilizes S3.

The action of peptide S3 on Gram-negative bacteria has been carefully described in the literature and it has been characterized by microscopy techniques [50, 59]. The analysis by AFM shows that, the peptide S3, at low concentrations, is able to produce indentations on the cell surface and induce micellization, thus revealing the disruption of the bacteria outer membrane. The cell debris are present mainly around the apical zone of the cell, as mentioned above and thus it is possible that the bacteria leakage could initiate from the apical ends (Figure 2A).

At higher concentrations, a huge leakage effect can be observed in the samples, probably originated from the cytoplasm and thus showing that bacteria are severely damaged and the cell morphology is fully collapsed (Figure 2A). With all this information, the authors conclude that peptide S3 could act via a “carpet –like” mechanism. The results were reproduced in both, E. coli and P. aeruginosa, thus pointing out that the peptide mechanism is not strain dependent but acts as a general disassembler of bacteria membrane and cell wall structures.

Sushi peptide S1 has also been characterized by colloidal gold nanoparticles labelling and analyzed by TEM microscopy [59] (Figure 2B). In this case, the nanoparticles were found to be located mainly in the LPS cell wall, although some particles could also be found in the phospholipid bilayer and the cytoplasm. To effectively calculate the location of gold –labelled peptides, a statistical analysis of the data has been conducted. In the case of S1 peptide, approximately the 75 % of the labelled peptide was located in the outer leaflet of the LPS cell wall and the remainder were nearly equally distributed among cytosol, periplasmic space and the inner leaflet of the lipid bilayer [59]. These results show that peptide S1 has an important affinity for the LPS bilayer, as described in several other studies in the literature, but also has the ability to penetrate the cell, destabilize the lipid bilayer and reach the cytosol where it could undergo several effects on the cell machinery.
4.2 Eosinophil cationic protein (ECP)

Eosinophil Cationic Protein (ECP) is a human host defense ribonuclease involved in inflammatory processes mediated by eosinophils [70]. ECP possesses bactericidal, antiviral and anti-parasitic activities and inhibits mammalian cell growth [71, 72]. Its ribonucleolytic activity does not appear to be necessary for the antibacterial capacity [73].

ECP presents a high isoelectric point (pI), due to the high number of arginines (19 out of 133 residues in the mature protein), thus displaying a high positively charged surface that may be the responsible for the low catalytic activity of ECP [71].

ECP is also cytotoxic for tracheal epithelium and ECP deposits, related to tissue damage, are observed after eosinophil degranulation in inflammatory disorders [71]. It has been reported recently that ECP can bind and aggregate on the eukaryotic cell surface, altering the membrane permeability and modifying the cell ionic equilibrium without internalization. By this way, ECP can promote chromatin condensation, reversion of membrane asymmetry, induce the production of reactive oxygen species and trigger the activation of caspase-like activity [72].

ECP has the ability to agglutinate bacteria, an interesting property that entails the bacteria aggregation. Moreover, ECP is able to agglutinate *E. coli* prior to cell leakage, as observed by confocal microscopy (Figure 3A) using the SYTO9/PI staining protocol [44]. The treated bacteria have been also observed by TEM and SEM microscopy [56]. TEM microscopy shows that ECP can promote outer cell membrane detachment and spilling off the cytoplasmic content (Figure 3B). Moreover, the cell wall appears to be severely damaged and shows ondulations indicating a loss of integrity. SEM microscopy shows also the cell wall damage, and reveals a characteristic aggregational behaviour also observed by confocal microscopy (Figure 3C). The cell wall appears severely damaged, in agreement with the TEM micrographs and shows aggregated material on the cell surface.

All the characteristics observed by microscopic techniques are in good agreement with biophysical studies in lipid vesicles, where membranes are first aggregated and then leaked by ECP [37, 38]. Also freeze fracture replicas and TEM microscopy showed that ECP is able to aggregate lipid vesicles and promote micellization [38]. Moreover, it has been shown that the ECP N-terminus is able to reproduce the antimicrobial properties of the entire protein [57], as observed in several antimicrobial proteins.

![Figure 2](image.png)

**Figure 2.** High-resolution structure analysis of the action of Sushi peptides S1 and S3 on *E. coli* cells. (A) AFM topographical images of untreated *E. coli* cells (a), treated with a low peptide concentration (b), medium peptide concentration (c) and high peptide concentration (d). The right part of the panel depicts schematically the process that takes place in the cell at each peptide concentration. The right panel (B) shows the localization of S1 peptide in a transmission electron micrograph of *E. coli* section. (a) Control cell, treated with gold nanoparticles alone, (b) *E. coli* cells treated with peptide S1 labelled with gold nanoparticles and (c) amplification of the regions of interest depicted in (a) and (b). Figures were adapted with permission (A) from [48] and (B) from [59].
5. Conclusions

On the search of optimized strategies for antimicrobial protein and peptide studies, new and updated microscopy techniques open up new possibilities for visualizing molecular structure and cellular functioning. Electron and light microscopy methods applied to biological samples have been improved, offering an interesting battery of quantitative and qualitative tools for structure, ultrastructure, cytochemistry, localization and tomography for researchers in the AMPPs studies. Currently available data on the antimicrobial damage on cells, liposomes and lipid layers is mainly based on electron microscopy conventional procedures.

However, imaging at ultrastructural levels combined with cryo–methods allows improving the knowledge of AMPPs structure and their effects in cells at native conditions. High–resolution techniques reaching nanoscale level to obtain high quality imaging of structure, 3D reconstruction, and localization of molecules, cell compartments and entire cells will become an essential tool for future research both on their own and as a complement of data obtained with spectroscopy, biochemistry, or crystallography techniques.

Figure 3. Characterization of the antimicrobial mechanism of action of ECP by microscopy techniques. (A) Assessment of the E. coli cells viability by confocal microscopy after addition of 5 mM of ECP. The left images represent the PI channel, the central image corresponds to the SYTO9 channel and the right image is an overlapped image of both dyes. The cell viability is followed at different times (a) 0h, (b) 10 min, (c) 30 min and (d) 2 h. (B) TEM image of E. coli cells treated with ECP at a 4 µM concentration for 3h. The bottom image corresponds to a magnification of the upper image (C) SEM image of E. coli cells treated with ECP at a 4 µM concentration for 45 min. The top images correspond to different magnification of control E. coli cells whereas bottom images represent treated cells. Images were adapted with permission (A) from [44], (B) and (C) from [56]

References


