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Zeta potential beyond materials science: Applications to bacterial systems and to the development of novel antimicrobials

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ABSTRACT

This review summarizes the theory of zeta potential (ZP) and the most relevant data about how it has been used for studying bacteria. We have especially focused on the discovery and characterization of novel antimicrobial compounds. The ZP technique may be considered an indirect tool to estimate the surface potential of bacteria, a physical characteristic that is key to maintaining optimal cell function. For this reason, targeting the bacterial surface is of paramount interest in the development of new antimicrobials. Surface-acting agents have been found to display a remarkable bactericidal effect and have simultaneously revealed a low tendency to trigger resistance. Changes in the bacterial surface as a result of various processes can also be followed by ZP measurements. However, due to the complexity of the bacterial surface, some considerations regarding the assessment of ZP must first be taken into account. Evidence on the application of ZP measurements to the characterization of bacteria and biofilm formation is presented next. We finally discuss the feasibility of using the ZP technique to assess antimicrobial-induced changes in the bacterial surface. Among these changes are those related to the interaction of the agent with different components of the cell envelope, membrane permeabilization, and loss of viability.

1. Introduction

Antibiotic resistance has become a major threat to public health and a serious challenge in clinical practice. Although effective treatments for most infections are still available, the overuse of antimicrobials over decades has accelerated resistance to commonly prescribed therapeutic agents. The gradually reduced effectiveness of antibiotics due to the emergence of multidrug-resistant pathogens, as well as the lack of alternative compounds, emphasizes the need to develop new classes of drugs and dosage forms [1–3].

In this context, antimicrobial peptides (AMPs), metallic nanoparticles, and vegetable extracts (including essential oils) appear as novel tools to fight bacterial infections [4]. However, in order to translate these compounds or engineered derivatives into clinical practice, we must first adequately understand the precise mechanism by which their interaction with bacteria results in cell damage. With this purpose, biophysical techniques have been applied to model membranes, thus providing very detailed information about how these

Abbreviations: AFM, atomic force microscopy; AgNPs, silver nanoparticles; AMPs, antimicrobial peptides; CPNP, charged chitosan-propolis nanoparticles; CTAB, cetyltrimethylammonium bromide; DLVO, Derjaguin–Landau–Verwey–Overbeek; EDL, electrical double layer; ExDLVO, extended Derjaguin–Landau–Verwey–overbeek; HO, Hückel–Onsager; HS, Helmholtz–Smoluchowski; L-Ara4N, 4-amino-4-deoxy-L-arabinose; LPS, lipopolysaccharide; LVO, lavender essential oil; MIC, minimal inhibitory concentration; PEtN, phosphoethanolamine; ZP, zeta potential.

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Table 1
Applications of zeta potential measurements in bacterial systems.

Application	Bacteria studied	References
Detection of colistin-resistant isolates	*Escherichia coli*	[14–16]
	Acinetobacter baumanii	[17]
Differentiation between virulent and nonvirulent strains	*Mycobacterium tuberculosis*	[18]
Biofilm formation	*Staphylococcus aureus*	[19]
	S. aureus and *Pseudomonas aeruginosa*	[20]
	Enterobacter faecalis	[21]
	Pseudomonas putida	[22]
Bacterial flocculation	*Rhodopseudomonas acidophila*	[23]
Membrane damage induced by essential oils	*Klebsiella pneumoniae*	[24]
Cell surface permeability and loss of viability induced by nanoparticles	*Staphylococcus epidermidis*	[25]
Membrane permeability and viability	*E. coli* and *S. aureus*	[26]
Effects of heat treatment	*P. aeruginosa* and *S. aureus*	[27]
Effects of ozone sterilization	*Vibrio parahaemolyticus*	[28]
Effects of UV irradiation	*P. aeruginosa*, *E. coli*, *S. aureus*	[29]
Interaction with positive nanoparticles	*Bacillus subtilis* and *E. coli*	[30]
	B. subtilis, *Staphylococcus carnosus*, *Neisseria subflava*, *Stenotrophomonas maltophilia*	[31]
Interaction with negative nanoparticles	*E. coli* and *S. aureus*	[10]
Interaction with cationic antimicrobial peptides	*E. coli*	[32]
	E. coli and *S. aureus*	[33] [11,34,35]
Interaction with essential oils	*E. coli* and *S. aureus*	[36]
	S. aureus	[37]
Effects of ethanol and freeze-drying	*Lactibacillus plantarum*	[38]
	Oenococcus oeni	[39]

systems interact with antimicrobial compounds. In fact, we have gained from such studies most of our knowledge of the mechanism of action of these new compounds at the molecular level [1].

Nevertheless, studying antimicrobial compound interactions with model membranes in a controlled environment may be an over-simplification [5]. In this regard, it is still unclear whether any of the mechanisms of action that have been proposed for different agents under such conditions can account for their actual activity in bacteria [6]. For example, the frequently used membrane model containing phosphatidylcholine may be an experimentally convenient choice, but it does not reflect the natural composition of most bacterial membranes and it elicits responses to AMPs that differ from those of bacterial lipids [5]. Furthermore, even if the proper membrane mimetic system is chosen, results obtained with lipid-only systems cannot be extrapolated in a straightforward manner to explain the effects observed in live bacteria [6,7], since the contribution of membrane proteins and other cell envelope components must be considered as well [8,9].

Therefore, in recent years, some techniques frequently used to study model membranes began to be applied to bacteria, among them zeta potential (ZP) measurements [6,10,11]. The ZP, also known as *electrokinetic potential* [12], is determined by the net electrical charge of surface-exposed molecules, and it is an indirect method to estimate the surface potential of bacteria. This physical characteristic is key to maintaining optimal cell function. Moreover, it plays a dominant role in the adhesion of bacteria to substrate surfaces and in their interaction with environmental factors. While the direct determination of the surface potential is difficult, ZP measurements are easier and provide information about interfacial charges. Given the simplicity and reproducibility of this technique to ascertain the surface charge of particles, it is increasingly used to assess and compare the nature of surface interactions between colloidal particles across many disciplines and fields of application [13].

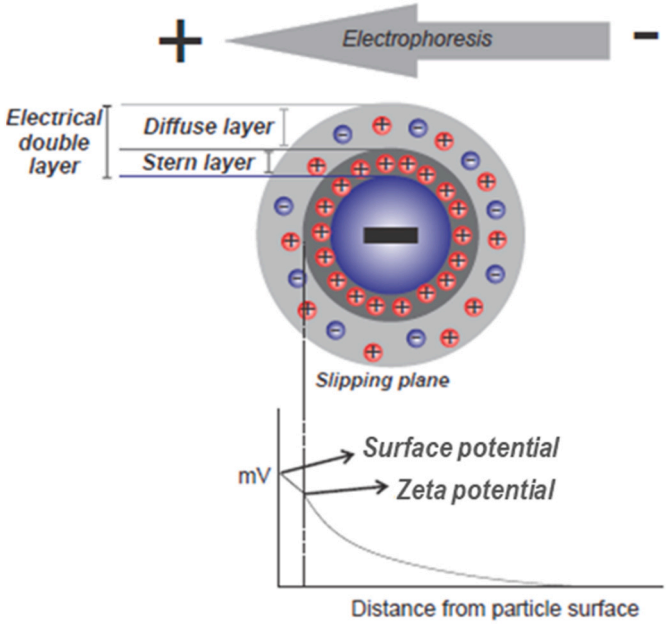

Fig. 1. Schematic representation of the electrical double layer (EDL) and position of the slipping plane. The EDL comprises the diffuse layer and the Stern layer, which surround a particle suspended in an aqueous medium. The zeta potential is the electrical potential at the slipping plane. Modified from [12].

Unfortunately, as the ZP emerged from the realms of physical colloid chemistry, its measurements in bacteria have proved challenging in practice. Furthermore, despite the increasing use of this technique in bacterial systems (Table 1), simple and concise sources on this subject are scarce in the scientific literature. Considering all these facts, this review aims to discuss the full potential of the ZP technique applied to bacteria. First, we will address the basic concepts of ZP. Then, we will detail the applications of ZP measurements to bacteria. Finally, we will argue its usefulness in characterizing the mechanisms of action of novel antimicrobial compounds.

2. Basic principles of zeta potential

As mentioned above, the ZP has emerged from physical colloid chemistry. In aqueous media, colloidal dispersions carry an electric charge, which develops at the particle surface and affects the distribution of ions in the surrounding area. Charged particles thus induce an increase in the concentration of counter ions (*i.e.*, ions with a charge opposite to that of the particle) close to the particle surface. This phenomenon results in the formation of the electrical double layer (EDL). In the inner region, also called the *Stern layer*, counter ions are strongly bound, whereas in the outer region, also called the *diffuse* or *Gouy–Chapman layer*, they are less firmly associated with the particle. Within the EDL, there is a notional boundary (at the limits of the Stern and diffuse layers) in which the ions and the particle form a stable entity. When the particle moves (*e.g.*, owing to an external electric field), the ions within that boundary move with it. Conversely, ions beyond the boundary (surface of hydrodynamic shear or *slipping plane*) stay in the bulk media. The potential at this boundary is the ZP (Fig. 1) [12,40–42].

A key parameter for accurately calculating the ZP is $\kappa\alpha$, the ratio of the particle radius (α) to the thickness of the EDL. This thickness is represented by the Debye length (κ^{-1}), which can be determined by the composition, concentration, and temperature of the electrolyte solution in which the particle is suspended [42]. The Debye length is given by Eq. (1):

Table 2
Henry's function as a function of κα.

κα	$f(\kappa\alpha)$
0	1.000
1	1.025
10	1.260
100	1.460

$$\kappa^{-1} = \left(\frac{\varepsilon_{rs}\varepsilon_0 kT}{2N_A\, e^2 I}\right)^{0.5} \quad (1)$$

where ε_{rs} is the dielectric constant (or relative permittivity) of the electrolyte solution, ε_0 is the electric permittivity of vacuum, k is the Boltzmann constant, T is the thermodynamic temperature (in Kelvin), N_A is the Avogadro constant, e is the elementary charge, and I is the ionic strength of the electrolyte.

The use of ZP in applied science is hindered by the fact that these values cannot be measured directly, and must instead be calculated from electrokinetic measurements. Particle electrophoresis or microelectrophoresis is the most widely studied technique, and it has practical relevance to ZP determination in colloidal suspensions.

Electrophoresis is the movement of charged colloidal particles or polyelectrolytes immersed in a liquid under the influence of an external electric field [43]. The electrophoretic velocity (Eq. (2)), v_e (m s^{-1}), is the velocity of the particle during electrophoresis and is given by [43]

$$v_e = \frac{\varepsilon_{rs}\varepsilon_0 ZP}{\eta} E \quad (2)$$

where η is the viscosity and E is electric field strength. From the v_e, it is possible to obtain the electrophoretic mobility, μ_e ($m^2\, V^{-1}\, s^{-1}$), which is the magnitude of the velocity divided by the magnitude of the electric field strength. The mobility is considered positive if the particles move towards lower potential (negative electrode) and negative in the opposite case, and it is given by Eq. (3):

$$\mu_e = \frac{\varepsilon_{rs}\varepsilon_0 ZP}{\eta} \quad (3)$$

Finally, the ZP is obtained from the μ_e by using the Helmholtz–Smoluchowski (HS) model, which is generally considered accurate for *large* colloidal particles (*i.e.*, when the EDL may be considered *thin* in comparison to the size of the particle; $\kappa\alpha \gg 1$) in solutions of moderate to high ionic strength. Eq. (4) is the commonly employed HS equation:

$$ZP = \frac{\mu_e \eta}{\varepsilon_{rs}\varepsilon_0} \quad (4)$$

Conversely, *small* particles suspended in low ionic strength media tend to have a *thick* EDL relative to the particle size (*i.e.*, $\kappa\alpha \ll 1$). Under this condition, the Hückel–Onsager (HO) equation (Eq. (5)) is suggested to obtain the ZP:

$$ZP = \frac{3\mu_e \eta}{2\varepsilon_{rs}\varepsilon_0} \quad (5)$$

Comparing Eqs. (5) and (4), the ZP value increases 1.5-fold when it is calculated based on the HO model, rather than the HS model, and the μ_e is the same. Therefore, selecting the incorrect model can introduce an error of 50% in this calculation. Henry thus developed Eq. (6) to relate both approaches by introducing Henry's function $f(\kappa\alpha)$ (dimensionless) [44]:

$$ZP = \frac{3\mu_e \eta}{2\varepsilon_{rs}\varepsilon_0 f(\kappa\alpha)} \quad (6)$$

Henry's function is a sigmoid curve whose values vary from 1.0 to 1.5. These extremes correspond to the HO and HS approximations, respectively [43,45]. Using Henry's equation (Eq. (6)) to merge the HS and HO models is thus a convenient way to convert μ_e measurements into ZP for any κα value (Table 2).

As we pointed out above, microelectrophoresis is routinely used to measure the μ_e in order to calculate the ZP of colloidal dispersions [12,42,46]. A voltage is applied between two electrodes in a cell containing the suspension. Charged particles are then attracted to the opposite charge electrode and the μ_e is measured [12]. Several microelectrophoresis instruments have been designed that share some common features, including an observation chamber located between the two electrodes, a microscope for direct observation of particle movement, and a device to deliver the sample [47].

Although these methods have proved effective, they have some disadvantages as well. For example, tracking of individual particles over time can be laborious and time consuming [47]. For this reason, simpler methods have been developed to assess charge properties. Among them, light scattering is the most common technique used to determine the μ_e of particles [12].

The typical optical configuration used in these experiments is shown in Fig. 2. Briefly, a laser beam is split in two. One of these beams serves as a reference, whereas the other is directed at the sample. As particles are mobile, light is scattered, and its frequency is different from the original laser frequency. This phenomenon is known as the *Doppler shift* [13], which is proportional to the v_e and is determined by combining or *optically mixing* the scattered light with the reference beam. The μ_e value measured is finally converted into the ZP, usually through Henry's equation (Eq. (6)), depending on the specification of the instrument. The optical configuration described imposes restrictions on the turbidity of the sample: if its concentration is too high, the laser beam will become attenuated by the particles, thus reducing the scattered light detected [12].

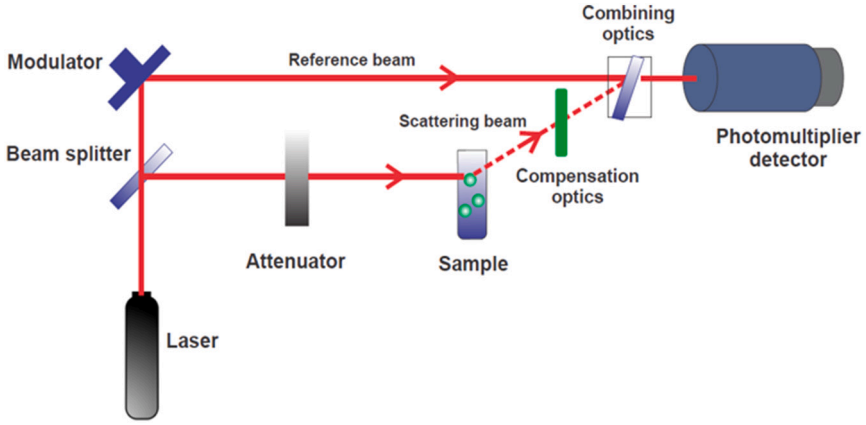

Fig. 2. Schematic of the typical optical configuration of an instrument used in laser Doppler electrophoresis. Modified from [12,13].

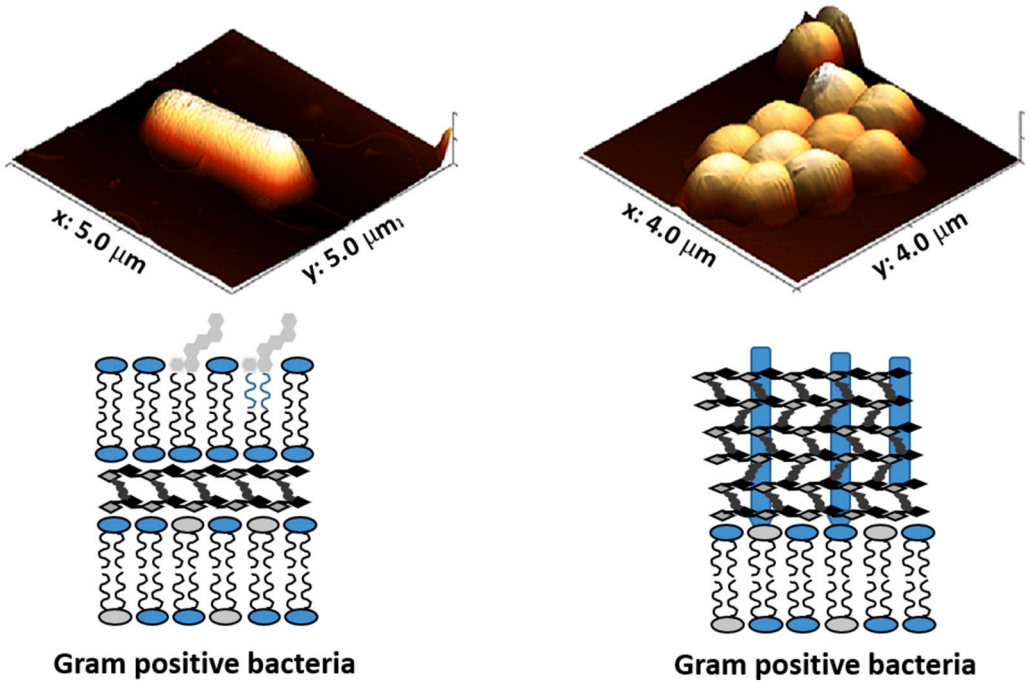

Fig. 3. Complexity of the cell surface of gram-negative and gram-positive bacteria. Schematics and atomic force microscopy (AFM) images reproduced from Ferreyra Maillard et al. [10].

3. Zeta potential in bacteria

Extrapolation of the previous concepts to bacterial cultures need be especially careful because the surface of a bacterial cell is much more complex (Fig. 3), diverse, and dynamic than the surface of an inert particle in a colloidal suspension [48]. Furthermore, bacterial cell surfaces do not fit the definition of a two-dimensional surface, as most of colloidal particles do, because the structures that protrude from them must also be considered [49].

As bacteria are unicellular organisms and lack intracellular membranous compartments, the interface formed between the outer cell envelope and the extracellular environment plays a key role in their overall physiology. This outer cell surface mediates exchange and adhesive processes, influences interactions with immunological factors, and participates in cell growth and division [47]. In this way, surface physicochemical parameters, such as the electrostatic charge, are then extremely important, as they influence overall polarity to confer and maintain the degree of surface hydrophilicity necessary for optimal cell function. In particular, the net cell surface charge can be assessed based on the ZP [47]. However, as we pointed out above, the structure of the bacterial cell surface is complex, and ZP values in bacteria should therefore be interpreted as a result of multiple factors: the molecules comprising the outer cell envelope, the tridimensional structure of the bacterial cell surface, its protrusions (such as pilli, flagella or lipopolysaccharide), the culture media, and its pH and ionic strength, among others [48,50,51].

In this regard, acidic and basic functional groups, such as carboxylic, phosphoric, hydroxyl, and amine groups, are found at the bacterial surface, and charges thus develop as a result of dissociation or protonation of these groups. Consequently, the net charge depends on the pH, but also on other environmental properties, such as the local hydrophobicity and the electric potential [49]. In this sense, should be pointed that besides the complex structure of the bacterial cell wall, in some bacteria the entire cell surface is covered by extracellular polysaccharides in the form of capsular material, these structures can also especially influence the zeta potential [52,53].

Most bacteria exhibit a negative ZP when the pH is higher than 2, which is most likely linked to the predominance of negatively charged functional groups associated with peptidoglycan, teichoic acid, and teichuronic acid on the surface of gram-positive bacteria and with lipopolysaccharide (LPS), phospholipids, and proteins on the surface of gram-negative bacteria [54,55]. However, there are some exceptions, like *Synechococcus* sp. and *Planktothrix* sp. These cyanobacteria are capable of developing a positive surface ZP in alkaline pH conditions, which allows them to attract the bicarbonate anions required for photosynthetic activity [56].

In a recent study, Polaczyk et al. [45] tested alternative models, including the HS, HO, Henry, modified Booth, and O'Brien–Hunter models, to calculate the ZP from the μ_e in cultures of different microorganisms, especially bacteria and bacterial spores, but also viruses and parasites, among others. After comparing the results, they concluded that the HS model was the most appropriate one, a somewhat expected finding, as bacteria are large in comparison with the EDL.

Bacteria not only differ significantly in their structure from inert particles, but they are also living systems. For example, bacterial growth is known to influence the conformation and composition of outer membrane lipids and the LPS, which are likely to alter the composition of the external leaflet of the outer membrane [17]. Furthermore, it has been established that the surface density, average chain length, and chemical composition of membrane components change when bacteria enter nongrowing physiology (*i.e.*, stationary phase) [57]. Consistent with this finding, many studies have shown that surface charges of bacterial cells, and therefore the ZP value, vary with their physiological state or nutrient exposure [18,56–60]. In a similar line of research, although there is a strong consensus that ZP reflects the metabolic state of bacteria, there seems to be no general trend that reflects how and up to what extent incubation time or growth phase affect the μ_e and thus the ZP of cells [63]. For example, it has been reported that ZP values become less negative as *E. coli* cultures in exponential growth enter the stationary phase [57]. Conversely, the ZP became more negative as *S. aureus* cultures aged [64]. Likewise, a significant increase in negative charges was found on the surface of *Mycobacterium smegmatis,* at physiological pH, when cells entered the exponential growth; however, the highest values (*i.e.*, the most negative) were observed when cells were in

the stationary phase [18]. In another study, carried out in *E. coli*, *P. putida*, *Alcaligenes* sp., and *Alcaligenes faecalis*, the μ_e increased until it reached its maximum value early in the stationary phase and then gradually decreased [63]. Altogether, these data suggest that changes in the ZP as a function of the metabolic and physiological state are strain dependent.

This relation between the ZP and the physiological state of bacteria has been conveniently employed to characterize damage on the bacterial structure as a result of different environmental stressors, such as temperature changes and ethanol addition. Several studies have shown that conserved ZP values correlate with preserved structures of surface macromolecules and with the physiological state of cells [39,65]. Based on these findings, we highlight the value of ZP measurements to explore the effect of some antimicrobial compounds on the bacterial surface, as we will discuss later in this review.

Another important consideration at this point is that bacterial populations are not homogeneous. In fact, it is known that several strains display distinct subpopulations even in pure cultures. Subpopulations within one culture can differ in cell surface charge, among other features [21,66]. This heterogeneity in surface charge has been evidenced by performing ZP measurements, which may display either an extremely wide distribution or more than one distinct Gaussian distributions [67]. In particular, ZP heterogeneity is a common trait among cultures of *E. faecalis* strains. Interestingly, Van Merode et al. [21] showed a link between ZP heterogeneity and the ability of *E. faecalis* to form biofilms. The same group demonstrated later that heterogeneity in the apparent ZP of *E. faecalis* was related to the presence or absence of the endocarditis and biofilm-associated pili (Ebp) [68]. This finding is worthy of note, as it highlights that the ZP is determined by the charges on the cell surface and also by the expression of surface structures that influence the width of the double layer and, therefore, the smoothness of the particle [68]. Likewise, studies with isolates of *E. coli* showed that different cell surface morphology, LPS structure, and flagellar, fimbrial, and curli proteins also affect the ZP [69].

The dependence of ZP on surface structure of bacteria has been exploited as a characterization tool. Sonohara et al. [70] have shown the differences in electrokinetic properties between *S. aureus* (gram-positive species) and *E. coli* (gram-negative species). Furthermore, they claimed that μ_e measurements could be used to assess dissimilarities in surface structure between gram-positive and gram-negative bacteria. Variations in the ZP were correlated with the different chemical composition (types of functional groups) of cell envelops, as gram-negative bacteria exhibited a more negative potential than gram-positive bacteria. This finding is explained by the additional layer of negatively charged LPS in gram-negative bacteria, which is lacking in gram-positive species [26,30,32,33,58]. However, it should be pointed out that this difference in ZP has usually been found between *E. coli* and *S. aureus*, but the available evidence is not enough to claim that ZP values systematically differ between gram-negative and gram-positive bacteria. On the contrary, a comprehensive study of ZP dependence on pH carried out in almost a hundred different bacterial strains showed no systematic variation between gram-positive and gram-negative strains [71]. Furthermore, the ZP can vary between strains of a single species [72].

In addition, the possibility of using ZP measurements as a diagnostic tool for strains resistant to colistin, a polypeptide antibiotic, has been recently evaluated. In most colistin-resistant strains, 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (PEtN), or galactosamine moieties are enzymatically added to the lipid A or the LPS core [73,74]. These modifications decrease the net negative charge of phosphate residues, thus leading to a reduction in colistin affinity. This decrease in net negative charge on the bacterial surface can be detected by ZP measurements, as it has been successfully proved in *mcr-1*-positive isolates of colistin-resistant *E. coli* from several sources. Furthermore, it has been previously demonstrated that the phosphoethanolamine transferase enzyme family requires zinc for MCR activity [16], and removing zinc from culture media by adding the chelator EDTA reverted colistin-resistant *E. coli* isolates to a susceptible phenotype. Interestingly, ZP measurements were successfully applied to follow this reversion [14–16]. A similar behavior was observed in *A. baumanii* as well [17]. Likewise, the ZP technique was useful for assessing cell surface charge and thus differentiate the virulent *M. tuberculosis* H37Rv strain from various nonvirulent mycobacterial strains [18]. Taken together, these results strongly support the feasibility of using ZP measurements for diagnostic purposes.

This technique has also been extended to biofilm research. Biofilms are social communities of bacteria that involve several interactions in three-dimensional multicellular structures [75,76]. They are important for survival in their natural environments and protect them from the immune system and antibiotics. Consequently, treatment of biofilm-associated infections has become an important part of antimicrobial chemotherapy, as these pathogens are not affected by therapeutic doses of conventional antibiotics [77,78].

The surface charge is one of the major determinants of whether a bacterium colonizes a surface to establish a biofilm or not [79]. The initial interactions between the bacterial cell and the surface depend largely on their respective surface properties. Therefore, the ZP is a key factor in biofilm formation [80]. Using *S. aureus* and *P. aeruginosa* as model microorganisms, Kumar et al. [20] showed that the ZP of bacteria lowered and their surface hydrophobicity increased under stress induced by a subinhibitory concentration of the antibiotic norfloxacin. These phenomena reduce the physicochemical free energy barrier that bacteria have to overcome in order to approach the substrate surface, thus enhancing biofilm formation. Another study in *S. aureus* showed that biofilm-detached cells are less negatively charged than their planktonic counterparts, probably due to the upregulation of the cationic staphylococcal poly-*N*-acetylglucosamine surface polysaccharide. The negative charge was lower in biofilm-detached cells than in free-living bacteria, which may therefore result in a decrease in repulsive electrostatic forces between cells and negatively charged abiotic surfaces. This finding may explain why they exhibit higher rates of adhesion to abiotic surfaces than their planktonic counterparts [19]. These are just a few examples among many others in the literature, which altogether highlight the potential of the ZP technique to study biofilms.

4. Zeta potential measurements as a tool to characterize novel antimicrobial agents

In the development of new antimicrobial agents, targeting the bacterial surface seems to be of paramount interest because surface-acting agents have been found to display a remarkable bactericidal effect and have simultaneously revealed a lower tendency to trigger resistance than other compounds [81,82]. As we pointed out above, changes in the bacterial surface play a significant role in maintaining cellular function and also provide useful information about cell surface structure [83,84]. In this section, we thus focus on three key aspects to address the feasibility of applying ZP measurements to studies of antimicrobial compounds. First, the ZP as a reporter for bacteria-compound interactions. Then, the role of this interaction on the disarrangement of surface charges and the resulting disruption of the barrier function of the cell membrane. Finally, and closely related to the former, the changes in ZP as a sensor of bacterial viability.

4.1. Bacteria-compound interactions assessed by measuring zeta potential

In the interaction between any compound and bacteria, the molecular properties of the bacterial cell surface are of crucial importance. The surface charge is the first variable typically defined for studying the binding process [85]. Taking into account that attachment is the first and most critical step in the mechanism of action of most novel antimicrobial compounds, such as AMPs [1,86] and nanoparticles [31], measuring the ZP is a powerful technique to evaluate their interactions

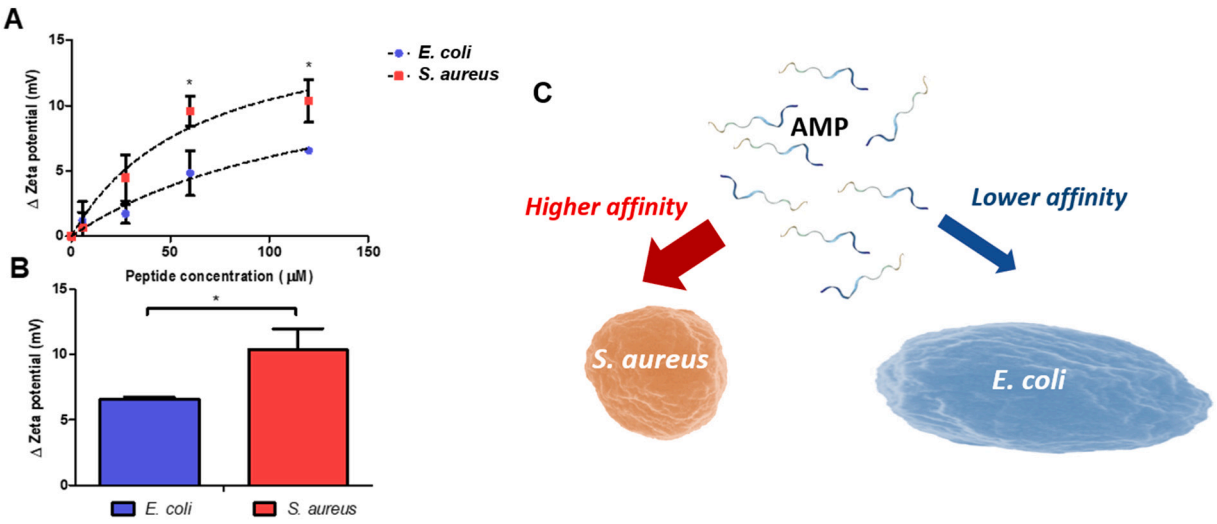

Fig. 4. Effects of the synthetic antimicrobial peptide (AMP) P1 on the zeta potential (ZP) of *S. aureus* and *E. coli*. A) Changes in ZP as a function of P1 concentration. B) Comparison of ZP values obtained in each bacterial system at the highest P1 concentration used. C) Schematic representation of the results inferred from ZP measurements. Modified from Espeche et al. [11].

with bacteria.

In this kind of experiment, the antimicrobial agent accumulates on the bacterial surface in a charge-dependent manner and induces a change in the ZP that can provide information about the bacterial-compound interaction [10,11,35,87]. In an attempt to describe qualitatively and quantitatively how the bacterial surface interacts with different types of interfaces, the classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloid stability has been applied. In this model, the bacterial cell is considered an inert particle whose interaction (V_{TOT}) with a surface is given by Eq. (7). It is the sum of the attractive London – van der Waals interactions (V_A) and electrostatic repulsive interactions (V_R) that result from the overlap of the electrical double layer of the cell with the interacting surface, and are mediated by Coulomb forces [88–92]:

$$V_{TOT} = V_A + V_R \tag{7}$$

This theoretical approach is not new, and it was first employed by Marshall et al. [93] to study the effects of different concentrations of monovalent and divalent electrolytes on the reversible sorption of *Achromobacter R 8*. Moreover, the DLVO theory has been used for exploring different processes, such as bacterial flocculation and wastewater treatment. In this sense, flocculability of *Rhodopseudomonas acidophila* has been shown to depend on both electrolyte concentration and pH [23].

Regarding antimicrobial compounds, Pajerski et al. [31] used this theoretical model to assess the attachment efficiency of gold nanoparticles to gram-positive (*B. subtilis*, *S. carnosus*) and gram-negative (*N. subflava*, *S. maltophilia*) bacteria. They were able to calculate from ZP values and atomic force microscopy (AFM) data the contribution of V_A and V_R, which they added up to obtain V_{TOT}. The authors concluded from these results that the number of gold nanoparticles attached to the bacterial surface correlated with the ZP values of the bacterial strains examined and thus confirmed that this electrical property is a key descriptor responsible for the adsorption process in bacteria-nanoparticle systems [31].

Furthermore, the extended DLVO theory (ExDLVO), which includes Lewis acid-base interactions, proved to be a more suitable model to account for changes in the adhesion of *B. subtilis* to representative soil minerals as a function of ionic strength and pH [94]. Likewise, this theory successfully explained the increase in adhesion of *P. putida* to urethane acrylate nanoparticles with high hydrophobicity and positive charge density on the particle surface. Conversely, a negative charge density on the particle surface hindered bacterial adhesion. Taken together, these results confirm that the ExDLVO model adequately explains the difference in the adhesion of nonionic and ionic nanoparticles to bacterial surfaces [95]. Moreover, bacterial adhesion onto a surface during biofilm formation was also studied from the point of view of the DLVO and ExDLVO models, which were suitable approaches for research on interface phenomena that regulate cell adhesion [96–98].

However, application of the DVLO theory has some limitations. For example, certain solutes can adsorb on the surface and modify surface properties, thus resulting in chemical and electrical heterogeneities [99]. To overcome some of these problems, model systems, even if they are simplifications, can provide additional information to fully understand the interaction between bacteria and antimicrobials. In this respect, measuring changes in ZP, along with other biophysical properties in different membrane models, has been widely employed to estimate physicochemical parameters that can be used to characterize the interaction of potential antimicrobial agents with bacteria, as well as their selectivity [100,101]. Freire et al. [102] have described a mathematical formalism to calculate the partition constants (*Kp*) of AMPs to lipid vesicles from ZP measurements.

The dependence of ZP on the antibacterial agent concentration was described for AMPs in different biomimetic membrane systems, thus allowing apparent dissociation constants (K_{dapp}) to be estimated through nonlinear regression methods. The K_{dapp} values obtained have shown a good correlation with the differential affinity and specificity of these potential antibiotics [1,103]. These physicochemical approaches may help to understand more clearly antimicrobial mechanisms at a molecular level.

Interestingly, this strategy has recently been applied to the direct evaluation of AMP affinity for the bacterial surface. In this regard, using *S. aureus* and *E. coli* as gram-positive and gram-negative models, respectively, the interaction of increasing concentrations of the synthetic AMP P1 with the bacterial envelope was assessed through ZP measurements (Fig. 4). By fitting ZP data, the K_{dapp} for P1 on each bacterium was calculated. The value of this parameter for *S. aureus* was almost half the K_{dapp} for *E. coli*, which may imply that P1 has a higher affinity for the cell envelope of *S. aureus*. These results are consistent with the minimal inhibitory concentration (MIC) previously determined for each bacterial species. However, it should be noted that P1 interacted with different bacterial surfaces. Therefore, the estimated K_{dapp} should only be considered a qualitative approximation to the affinity of P1 for these bacteria, as opposed to the quantitative data that would be

obtained from model membranes [11].

Similarly, the dependence of ZP on the concentration of other antimicrobials, including nanoparticles and natural compounds, was also used to characterize binding of these agents to bacteria and to assess the bacterial membrane as a potential target of their biological activity [37,87,104]. In addition, results from studies of ZP changes as a function of time and dynamic molecular simulations suggest that these approaches may be useful for unraveling the mechanisms of action of certain AMPs, such as melittin [105].

4.2. Zeta potential and permeabilization of bacterial membranes

Besides interacting with the bacterial surface, the aforementioned compounds must be able to successfully induce bacterial death. Several mechanisms have been described [89,106,107], among them membrane permeabilization by most AMPs [108]. In particular, it has been shown that certain AMPs alter membrane permeability as a result of surface charge neutralization [32]. Surface neutralizations of the membrane are thus important for their antimicrobial activity [34] and, what is more, this charge neutralization can be measured with ZP techniques.

Many authors have reported changes in ZP values with the concomitant permeabilization of the bacterial membrane. Recently, Yang et al. [24] used ZP measurements to show that lavender essential oil (LVO) had significantly affected the bacterial membrane by increasing the overall surface charges in carbapenemase-producing *K. pneumoniae* cells. They assumed the bacterial membrane had become more permeable, which they confirmed through a membrane permeabilization assay. From the outer membrane permeability assay, they concluded that cells exposed to LVO had a higher permeability than untreated cells, a condition that significantly prevented bacterial growth. It should be noted that LVO induces cell death *via* oxidative damage inside of the cell once it has disrupted the outer and inner membranes.

Similarly, Ong et al. [25] reported that changes in the ZP of bacteria due to the binding of nanoparticles affect cell surface permeability. A change in ZP modulates bacterial cellular physiology, thus leading to cell death and/or inhibition of growth. According to the mechanism of disinfection with chlorine proposed by Venkobachar et al. [109], ZP, permeability, and oxidative phosphorylation are affected simultaneously by exposure to this chemical agent.

In another study on this topic, Halder et al. [26] found that cetyltrimethylammonium bromide (CTAB) and polymyxin B, which are cationic surface-acting agents, altered the ZP in *E. coli* and *S. aureus*. They also detected an increase in surface permeability (used as a marker for membrane permeability), which suggests a possible correlation between these two parameters. Both of them can eventually be linked to decreased cell viability. However, a different effect was found for ampicillin and other agents that do not target the bacterial surface. It was evident that the drug (regardless of its concentration) neither affected the ZP nor influenced membrane permeability, even if it effectively reduced cell viability. In the same study, heat treatment also altered the ZP and membrane permeability in *E. coli* after a 10-min exposure, but no such changes were observed in *S. aureus*. However, when the exposure time was increased to 30 min, the ZP significantly fell in both *E. coli* and *S. aureus*. The resistance to change observed in *S. aureus* after a brief exposure may be attributed to the thick peptidoglycan layer in gram-positive bacteria.

4.3. Zeta potential and bacterial viability

Many studies have established a relationship between changes in the ZP and bacterial viability. Klodzinska et al. [58] showed in *E. coli* and *S. aureus* that the ZP of dead cells is less negative than the corresponding value of living cells [58]. Consistent with these results, Szumski et al. [110] reported differences in the ZP of live and dead *E. coli* cells at different pH conditions. They found that ZP values became less negative in dead bacteria than in live bacteria when the external pH was 3.5 or higher [111]. Likewise, changes in the ZP towards less negative values have also been reported in heat-killed strains of *P. aeruginosa* and *S. aureus* [110].

Conversely, in the pathogenic bacteria *V. parahaemolyticus*, a gradual decrease in the ZP and a concomitant increase in the percentage of

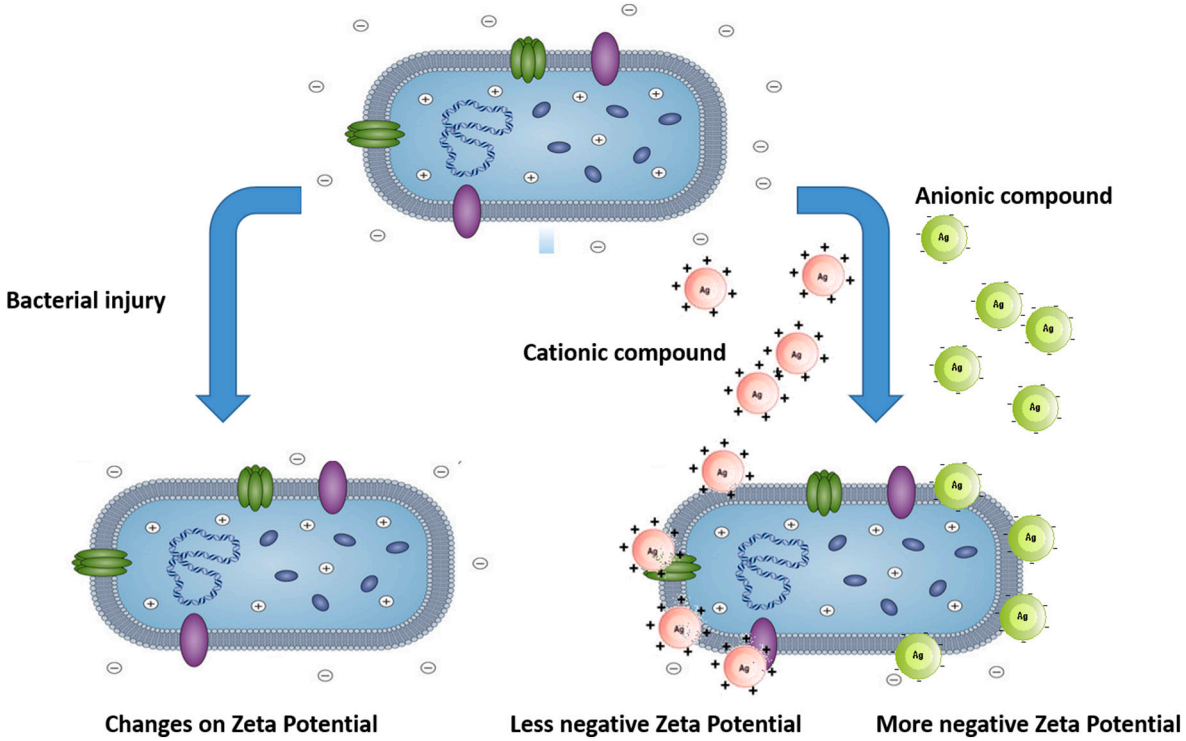

Fig. 5. Schematic representation of the change in ZP of a bacterial cell as a result of bacterial injury or binding of charged compounds to the bacterial surface.

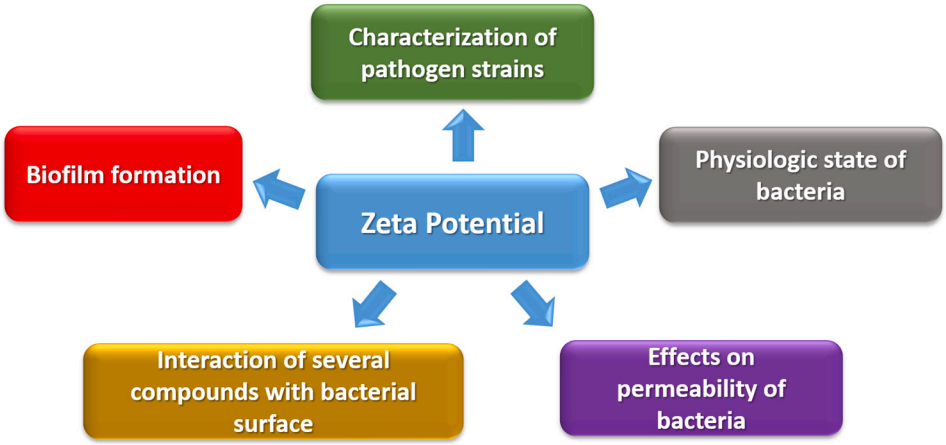

Fig. 6. Applications of zeta potential measurements in bacteria.

sterilization was found with increasing concentrations of aqueous ozone. Furthermore, ozone concentrations below 0.2 mg/mL, which have a low sterilization efficiency, did not lead to variations in the ZP, thus suggesting that aqueous ozone influences the general polarity of the bacterial surface. This phenomenon may in turn determine the integrity of the cell membrane [28].

Recently, Lee et al. described in *P. aeruginosa*, *E. coli*, and *S. aureus* a similar reduction in ZP values after UV irradiation and a concomitant improvement in germicidal efficacy. In agreement with previously cited studies, they have postulated that the decrease in viability, among other factors, may explain the change in the ZP, as a lower metabolism tends to lead to lower ZP values [29].

Although the previous results suggest that ZP values tend to decrease when bacterial viability is compromised, the opposite behavior was observed in two species of cyanobacteria as the pH changed from 3 to 8: less negative values were found in metabolically active bacteria than in dead cells. However, in these particular microorganisms, this effect may result from the concentration of protons outside the plasma membrane, thus giving rise to a less negative net charge at the cell wall [56].

As it is feasible to use ZP measurements for sensing the physiological state of the bacteria, this technique has been employed to characterize different antimicrobial compounds. With such purpose, it should be pointed out that when bacteria are exposed to these agents, the resulting change in ZP may be ascribed to two different phenomena: bacterial injury or antimicrobial binding to the bacterial surface. A combination of both mechanisms should also be considered.

The results of different experiments with positively and negatively charged nanoparticles support these assumptions. Incubation of *B. subtilis* and *E. coli* with positively charged ZnO nanoparticles increased the negative ZP of the bacterial cells to values close to neutrality (*i.e.*, it reduced the negative value of the bacterial surface) [30]. In contrast, the ZP shifted to more negative values when *E. coli* and *S. aureus* cells were treated with negatively charged silver nanoparticles (AgNPs) [10]. In both studies, the changes in the ZP correlate with the antibacterial effect of these nanoparticles, which can be explained by their accumulation on the bacterial surface as the first step to induce bacterial injury. Although the ZP displayed different behaviors depending on the compound tested, in both cases ZP measurements were very useful to assess the antimicrobial effect of nanoparticles.

Similarly, Ong et al. [25] studied the interaction of *S. epidermidis* with negatively charged aqueous and ethanolic extracts of propolis. When the concentration of each compound was increased, a reduction in the viability of *S. epidermidis* was observed, as well as a concomitant decrease in the ZP. In contrast, when these cells were treated with positively charged chitosan-propolis nanoparticles (CPNP), which induce a more marked reduction in viability, the ZP also showed a more considerable change, but shifted to less negative values. Despite the fact that each compound induces a differential change in ZP (*i.e.*, propolis extracts lower the membrane potential of bacteria, whereas the cationic CPNP have the opposite effect), the authors claim that both agents (regardless of the direction of change) affect cellular physiology, thus leading to cell death and/or inhibition of growth. In this sense, the more pronounced changes in ZP observed with CPNP correlate with a higher antimicrobial effect. These different findings on ZP changes as a result of bacterial injury and/or the binding of charged compounds are summarized in Fig. 5.

In another study, Halder et al. [26] assessed the effect of different cationic compounds on *E. coli* and *S. aureus*. When they exposed cells to CTAB concentrations of 30 μg/mL or higher, they measured more negative ZP values in both bacteria. The magnitude of this shift was greater at higher concentrations of CTAB, and the change in ZP was time dependent. A different effect was observed with polymyxin B, which only induced changes in the magnitude of ZP in *E. coli* cells. This finding showed a good correlation with the reduction in bacterial viability. The fact that polymyxin B did not significantly affect the ZP nor the viability of *S. aureus* is consistent with the mechanism of action of this antimicrobial, which attaches to cells by binding to the lipid A. This receptor is only present on the outer membrane of gram-negative bacteria.

As regards AMPs, a relationship has been found between the decrease in bacterial viability and the neutralization of surface charge. When *E. coli* was incubated with BP100 and pepR, an increase in the ZP towards neutral values and a concomitant decrease in viable bacteria were observed [32]. Similarly, when $rBPI_{21}$ was added to *E. coli* and *S. aureus*, a concentration-dependent increase in ZP was observed. Although electroneutrality was achieved in both microorganisms, different concentrations of peptides were required [33].

The same bacterial species were used to assess the effect on the ZP of essential oil components at concentrations equal to the MIC. After exposure to all the molecules tested, the ZP values of *E. coli* cells became less negative than those measured in *S. aureus*. Among compounds that induced the biggest changes in ZP were those with lower MIC values [36]. The same behavior was observed when the interaction between the *Schinus areira* essential oil and *S. aureus* was studied: the addition of essential oil was able to reduce the ZP value of bacteria. Interestingly, a *threshold concentration* close to the bactericidal concentration was found, thus suggesting that the changes in the bacterial envelope are irreversible at that point [37].

Overall, even if the behavior of the ZP seems to depend on the bacterial species and on the compound studied, a relation between this parameter and bacterial viability has consistently been established. These studies thus show that ZP measurements are a very useful tool for unraveling the antimicrobial effect of each compound tested directly in bacteria.

5. Final remarks

Despite the complexity of studying surface charges in living organisms, numerous studies support the proposed use of the ZP technique for exploring surface processes in bacteria. Throughout this review, several applications of ZP measurements in bacterial systems were discussed (Fig. 6), which range from sensing differences in the physiological state of bacteria to diagnosing resistance to some antimicrobial drugs. In particular, this technique has an enormous potential for assessing the interactions of many novel antimicrobial compounds with bacteria, and it provides information about their effects on cells, such as increasing membrane permeability or reducing viability. In addition, it can help to deepen our understanding of the mechanisms of action of these compounds at the molecular level. These advantages, together with advanced instruments to measure ZP in an easy, fast, and economic way, underline that it is feasible to directly apply this technique to bacterial systems.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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