Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 407–415
Published online 14 January 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1005

JOURNAL OF
PEPTIDE SCIENCE

Review

Interaction of peptides with biomembranes assessed by potential-sensitive fluorescent probes‡

PEDRO M. MATOS, SÓNIA GONÇALVES and NUNO C. SANTOS*
Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal

Received 30 June 2007; Revised 31 October 2007; Accepted 16 November 2007

Abstract: Peptide–membrane interaction is an important step to be evaluated in a study of the activity and mode of action of several bioactive peptides. A variety of methods are available; however, few of them satisfy the criteria of being sensitive, biocompatible, versatile, easy to perform, and allowing real-time monitoring as the use of potential-sensitive fluorescent probes. Here we review methods for detecting the effects of membrane-active peptides, even those that are not intrinsically fluorescent, on the different types of membrane potentials, with a special emphasis on studies conducted with living cells. FPE is a probe sensitive to surface potential and detects electrostatic interactions at the water-lipid interface. Di-8-ANEPPS is sensitive to dipole potential and detects membrane incorporations. Transmembrane potential changes reveal major membrane destabilizations, such as in pore formation. The combination of the information obtained from the three potential variations can lead to a more elucidative picture of the mechanisms of the interaction of relevant peptides with biomembranes. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: biomembranes; bioactive peptides; fluorescent probes; surface potential; dipole potential; transmembrane potential

INTRODUCTION

The cell membrane is the interface between the cytoplasm and the outer environment, where all the exchanges and interactions take place, allowing cells to obtain nutrients and energy, export, attach, communicate and sense, with the aid of a myriad membrane-associated proteins. The numerous roles of membranes are indicative of its extraordinary functional flexibility.

Interaction of peptides with lipid bilayers, either cell membranes or artificial vesicles, is an expanding research field, as peptides are a very versatile and heterogeneous group of molecules. Depending on the peptide sequence, charge, hydrophobicity and secondary structure, it can be prone to interact with lipid bilayers by simple electrostatic surface interaction, incorporation due to the hydrophobic effect, translocation or pore formation. All of this brings consequences for membrane structure, namely on lipid organization and packaging, induction of events such as fusion, aggregation or content leakage.

Measuring these types of interactions can help to unveil the mode of action of these membrane-active peptides. These studies are particularly important in drug research, where interaction with membrane lipids can determine the drug effects, delivery, and bioavailability [1,2]. Even for peptides whose targets are membrane proteins, the membrane can act as a catalyst in the reaction, the so-called 'membrane catalysis model', as it is much more probable that the peptide establishes contact first with the membrane and not directly with the receptor [3]. Studies on peptides can also serve as a simple model to understand interactions at higher levels of complexity, such as the interaction of proteins with the membrane [4].

Fluorescence methods are very sensitive, relatively simple to perform and can provide reliable results. In the specific case of the use of simple mimetic systems of biological membranes (lipid vesicles) and if the peptide have intrinsic fluorescent amino acid residues, especially tryptophan, the peptide–membrane binding can be followed by fluorescence spectroscopy due to microenvironment induced changes on quantum yields, spectral changes, fluorescence anisotropy or fluorescence lifetimes of these residues [5]. However, this technique is not suitable for peptides lacking fluorescent aminoacid residues and/or if we want to study the interaction with biomembranes, where other proteic components are already present. In order to face these limitations, alternative strategies were developed to indirectly measure peptide interactions with membranes. This can be achieved by monitoring the effects of these interactions on the different types of membrane potentials, using membrane incorporated fluorescent

* Correspondence to: Nuno C. Santos, Unidade de Biomembranas, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal; e-mail: nsantos@fm.ul.pt
‡ This article is part of the Special Issue of the Journal of Peptide Science entitled "2nd workshop on biophysics of membrane-active peptides".

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

BIOGRAPHIES

Pedro Miguel Matos was born in Batalha, Portugal, in 1985. He graduated in Biochemistry, in 2007, at the Faculty of Sciences, University of Lisbon. His present research comprises the application of biophysical approaches such as fluorescence spectroscopy to understand the action of HIV fusion inhibitor peptides at the cell membrane level. He is heading towards his doctoral studies, focused on viral entry mechanisms, in the Institute of Molecular Medicine (Lisbon, Portugal).

Sónia Gonçalves was born in Caracas, Venezuela, in 1972. She graduated in Chemistry at the Central University of Venezuela, in 1998, and obtained her Ph.D in Physical Chemistry from the same University, in 2002. She worked as researcher at the Laser Spectroscopy Laboratory (Caracas, Venezuela). Currently, she is working as Assistant Researcher in the Biophysics area, with medical applications, at the Faculty of Medicine, University of Lisbon (Portugal). Her research interests mainly comprise the resolution of problems related with the interaction of proteins with membranes by the application of spectroscopic techniques, such as fluorescence, light scattering, and reflectometric interference (biochip technology).

Nuno C. Santos was born in Lisbon, Portugal, in 1972. Lipid membranes have been the common element of his research specialization since 1994. After a majoring in Biochemistry, Nuno C. Santos conducted his Ph.D. research work in the Technical University of Lisbon (Portugal) and in the University of California at Santa Barbara (USA). He completed his Ph.D. in 1999, and joined the Faculty of Medicine of the University of Lisbon as Assistant Professor. He is now also Principle Investigator at the Institute of Molecular Medicine (Lisbon, Portugal). His research is focused on the following: (i) structural characterization of biomolecules and interaction with lipid membranes, using fluorescence spectroscopy, light scattering and AFM; (ii) viral fusion and assembly; (iii) LPS-binding protein-derived drugs; and (iv) nanomedicine.

probes. This is a minimally invasive method and avoids the necessity of peptide derivatization, which modifies its native structure and can potentially lead to biased results.

In this article, we will focus on methods for measuring the interaction of peptides with membrane-model systems and cell membranes based on the use of fluorescent dyes that are sensitive to different types of membrane potential, namely, surface, dipole and transmembrane potentials.

SURFACE POTENTIAL

The surface potential arises from the net excess charges that accumulate in the outer surface of the membrane, in contact with the external medium (Figure 1). It is therefore the potential difference between the membrane surface and the bulk medium. The sources of these charges are mainly charged lipid head-groups and ions electrostatically interacting with them [6–8]. Any interaction of a charged entity with the membrane surface is likely to alter the surface potential and hence it is susceptible to be detected by a fluorescent probe.

Fluorescein phosphatidylethanolamine (FPE) is a probe sensitive to surface potential (Figure 2) [9]. It was first used to measure pH changes in the internal compartment of lipid vesicles due to the variation of its quantum yield with the protonation state of the fluorescein (increased fluorescence with increased deprotonation) [10]. Later it was described more generically for the measure of pH at the water/lipid interface [11], a property that permits, for instance, the assessment of a proton pump activity [12,13]. During these experiments it was realized that changes in the medium ionic strength could compromise the pH measurements, which led to studies concerning the

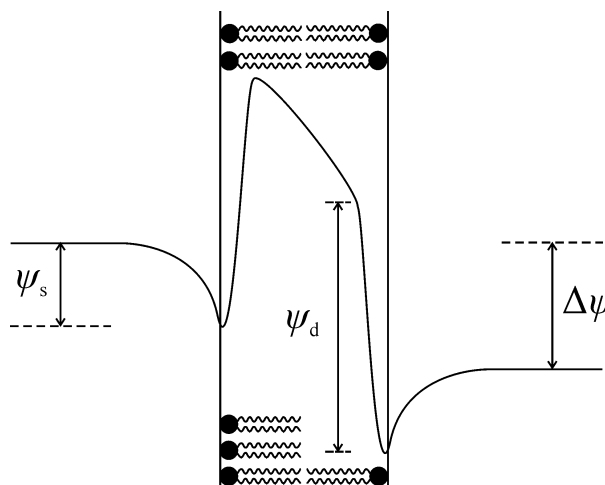

Figure 1 Electrical potential profile across a phospholipid bilayer. The surface potential (ψ_s) arises from charge accumulation at the membrane surface, the dipole potential (ψ_d) is a consequence of the alignment and distribution of lipid and water dipoles, and the difference in charge concentration in the two bulk mediums separated by the membrane constitutes the transmembrane potential ($\Delta\psi$).

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

DOI: 10.1002/psc

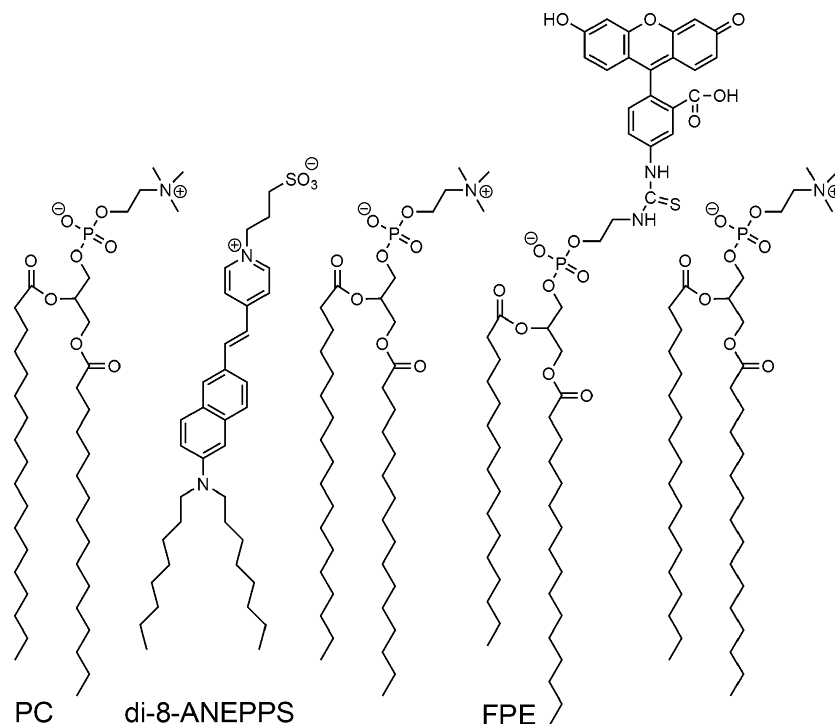

Figure 2 Structures of the membrane potential probes FPE and di-8-ANEPPS, incorporated in a phosphatidylcholine membrane leaflet.

effect of peptides in lipid membrane surface potentials [9].

When incorporated into lipid membranes, the fluorescein moiety of this probe stands precisely in the water–lipid interface, and hence it has the ability to detect pH or charge distribution changes at the membrane surface [9]. FPE behavior can be explained by the Handersen–Hasselbach equation, taking into account the surface potential [14]:

$$\log\left(\frac{c_{\mathrm{F}}}{c_{\mathrm{HF}}}\right) = \mathrm{pH} - \left(\mathrm{p}K - \frac{F\psi_{\mathrm{s}}}{RT\ln 10}\right) \qquad (1)$$

where c_{F} and c_{HF} are the concentrations of the dissociated and protonated forms, respectively, of the fluorophore located at the membrane surface, and the expression $\mathrm{p}K - F\psi_{\mathrm{s}}/(RT\ln 10)$ is the apparent pK, where ψ_{s} is the surface potential. It can be easily understood that for the fluorescein moiety of FPE, standing at the membrane surface, changes in the surface potential at constant bulk phase pH, will affect its apparent pK and consequently, its protonation state, resulting in changes in the quantum yield [9,15]. The simplest test to verify the correct incorporation and responsiveness of FPE in vesicles or cells is the addition of calcium ions to the medium, which will lead to an increased surface potential and consequently decreased fluorescein apparent pK, increased deprotonation, and increased fluorescence intensity [9]. A possible interference factor is the adsorption of proteins, which may change the dielectric constant in the vicinity of the fluorescent probe, so that fluorescence changes may not only reflect surface potential alterations.

The first experiments that used FPE to assess peptide–membrane interactions were done with phospholipid vesicles and involved the study of the peptides pyrularia thionin (PT), CTX (*Naja naja kaouthia* cardiotoxin), p25 (leader peptide of subunit IV of cytochrome *c* oxidase) and melittin, known to be membrane-active [9]. FPE was characterized in terms of its fluorescence spectra in phospholipid vesicles and pH titrations were done to determine the pK_{app} in different ionic conditions. The correct incorporation of FPE in the outer leaflet of the vesicles was tested. PT and CTX were shown to interact with phosphatidylcholine/phosphatidylserine (PC/PS) phospholipid vesicles, as addition of each peptide caused an increase in the fluorescence of membrane-bound FPE. Melittin and p25 had a slightly different behavior: they also caused an increase in the fluorescence intensity upon addition (increase in electropositivity of the membrane surface) but followed by a slow decrease until saturation (Figure 3). This biphasic interaction was more clearly observed using a stopped-flow rapid mixing technique, as it allowed resolving of the initial fluorescence increase in the millisecond time-scale. The final decrease can be explained by the insertion of regions of the peptides, or even its translocation, placing the positive charges away from the membrane surface, no longer affecting its potential. Melittin has a short hydrophobic *N*-terminal helix bearing two positive charges (in a total of six) that can insert into the

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

DOI: 10.1002/psc

lipid bilayer [16]. This is in agreement with the results mentioned above [9], because the extent of the signal decrease following the insertion event is approximately 1/3 of the initial change, as stated by the original authors. For the case of p25, it has been reported that the *N*-terminal region of the peptide, containing positive charged aminoacid residues, inserts into the lipid bilayer with its axis perpendicular to the membrane surface [17]. Pore-formation and peptide translocation, which can also explain the decreased number of positive charges in contact with the surface upon peptide-membrane interaction, have actually been reported for melittin [18], and can be advanced as a hypothesis for the case of p25. Titrating FPE-labeled PC/PS vesicles with p25 held a hyperbolic binding curve with an approximate half saturation point of 4 μM.

A more systematic study of the interaction of p25 with membrane vesicles was done later [19]. Relying on the stopped-flow technique, fluorescence changes were recorded over time and the dependence of fluorescence change with the p25 concentration was found to be described by a cooperative model (sigmoidal curve). It was the first time that a cooperative behavior was identified in a signal-sequence peptide and denotes the complexity of peptide–membrane interactions.

FPE labeling of living cells is also possible and was done, for the first time, with erythrocytes and lymphocytes [15]. Systematic studies for B-lymphocytes were carried out with BSA. Serial additions of this protein caused a decrease in fluorescence intensity, as expected, considering BSA negative net charge at the pH of the experiment (pH 7.5). Changes on fluorescence upon BSA addition were plotted and analyzed according to a single binding site model, resulting in a K_d of 2.85 ± 0.48 μM (Figure 4). The choice of this specific fitting model can be controversial. However this does not imply that there is only one possible molecular explanation for the process. The majority of the peptide–membrane interactions actually follow a simple water–membrane partition [5] but this cannot be quantified directly by these methods. For assessing the response of FPE to protein–ligand interactions at the membrane level, assays were done using MHC class II monoclonal antibodies and a fluorescence decrease was observed. By the opposite, testing with an antibody that lacks binding site in B-lymphocyte membranes, GaM-IgG, showed no alterations in fluorescence. For studying peptide interactions with FPE-labeled erythrocyte membrane, melittin, poly-L-lysine, and BSA were chosen. The first two macromolecules, being positive at the pH of the experiments, should increase membrane-bound FPE fluorescence. However, the opposite was observed instead. Only when FPE-labeled erythrocytes were previously treated with neuraminidase, the expected fluorescence increases were observed. It seems that for the specific case of erythrocytes, the abundant negatively charged sialic acid residues of membrane glycoproteins affects the response of FPE. It can be speculated that binding of melittin or poly-L-lysine to these moieties may alter its organization and disposition in such a way that their influence on the microenvironment on the close vicinity of the membrane surface would change the FPE response, explaining the decrease in fluorescence.

HIV-1 infection of T-lymphocytes involves the interaction of its membrane glycoprotein gp120 with CD4 receptors and co-receptors. When that happens, gp41 is exposed and its fusion peptide domain is inserted in the target cell membrane [20]. The interaction of this 16 residues gp41 fusion peptide ($gp41_{FP}$) with

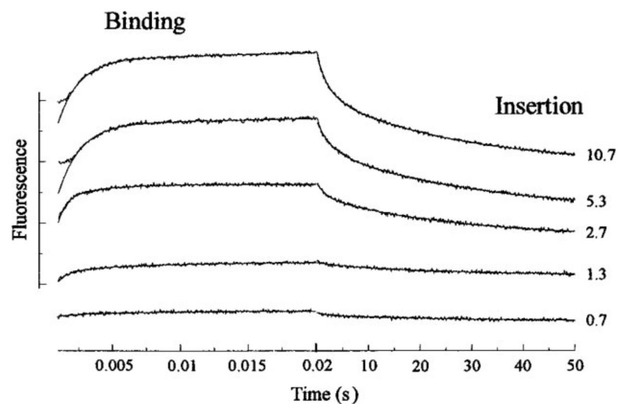

Figure 3 Time-course variation of FPE-labeled PC vesicles fluorescence during mixing with p25 at the concentrations (μM) indicated in each trace. Traces were offset for clarity and for each one, two experimental traces were combined covering time periods with different scales. Reprinted with permission from [19]. Copyright 1996 American Chemical Society.

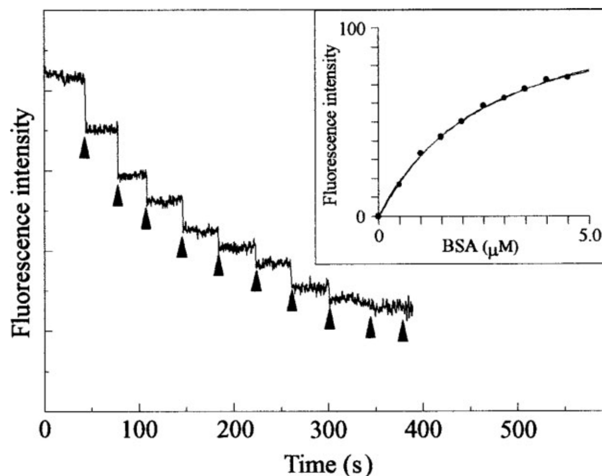

Figure 4 Effect of serial additions of BSA on the fluorescence of FPE-labeled B-lymphocytes. Fluorescence decreases over time as BSA has a net negative charge at pH of the assay (7.5). The inset represents the absolute fluorescence changes as a function of BSA concentration, in which solid lines represent the fittings to a single binding site and a cooperative model [15]. Reproduced with permission of the Company of Biologists.

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

DOI: 10.1002/psc

T-lymphocyte membranes was assessed using FPE [21]. Assays with FPE-labeled PC vesicles show increased fluorescence in the presence of this peptide and a cooperative behavior on the variation of fluorescence with peptide concentration. These results were confirmed using Jurkat T-lymphocytes. Moreover, the effects of interleukin 8 (IL-8), heparitinase, heparin, and heparan sulfate were also investigated. The presence of IL-8 or heparan sulfate in soluble forms, or treatment of the cells with heparitinase, significantly decreased the interaction between the peptide and the cell membrane. Heparin apparently affects the interactions in a much lower extent. These results indicate that $gp41_{FP}$ interacts specifically with heparan sulfate in the cell surface, revealing that the latter may have an important role in the virus attachment to the cell and subsequent membrane fusion.

FPE was also used to study the interaction between fibronectin, an extracellular matrix protein, and the osteoblast membranes [22]. The variation profile of the fluorescence against fibronectin concentration followed a single binding site hyperbolic model, yielding an affinity constant of 120 nM. Treating osteoblasts with heparitinase reduced the binding capacity of fibronectin to almost 50%. In the presence of the RGD peptide, which sequence is involved in the binding of fibronectin to integrin α5b1, the binding capacity is also reduced, but at a lesser extent. Cell-surface proteoglycans are then an essential way by which fibronectin binds to the osteoblast membrane and not only to the integrins.

The versatility of FPE extends also to prokaryotic cells. A successful labeling of *Escherichia coli* and *Helicobacter pylori* was reported, after inactivation with heat or UV light [23]. Although requiring some optimizations, the authors managed to obtain an adequate response with calcium ions and poly-L-lysine. The interaction of the VacA (*H. pylori* vacuolating toxin) p37 subunit with *H. pylori* membrane was studied and a K_d value of 1.7 nM was obtained by a cooperative binding model. The interaction of this peptide with FPE-labeled PC/PS vesicles (model system of eukaryotic cell membranes) was also studied [23], showing a much lower affinity. This indicates that the preferential interaction of p37 with the bacterial membrane can be due to its particular lipid composition or to the existence of a specific receptor.

DIPOLE POTENTIAL

The dipole potential of lipid membranes, or alterations to it, can also reveal interactions at the membrane level. This type of potential originates from the alignment of dipolar residues of lipids (polar headgroups and glycerol–ester regions) and oriented water molecules hydrating the outer surface of the membrane (Figure 1) [24,25]. Various methods are described to measure dipole potentials in planar lipid bilayers and lipid vesicles [26]. In terms of lipid vesicles and cells, the easier to use, the most biocompatible and allowing real-time monitoring and imaging is the method using voltage-sensitive fluorescent dyes. Such dyes are di-8-ANEPPS (4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium) (Figure 2) [27] and RH421 (N-(4-sulfobutyl)-4-[4-[4-(dipentylamino)phenyl]butadienyl]-pyridinium) [28]. Beside the aliphatic chains, these probes possess a conjugated structure (the chromophore region), therefore providing an extensive degree of electron delocalization, a reason for its fluorescence and relatively high stokes shift. They incorporate in the outer leaflet of the membranes and the chromophore group stays near the lipid head-group region, sensing the local electric field derived from the dipoles [27]. The mechanism by which di-8-ANEPPS, and RH421 and other aminostyry1pyridinium dyes operate in order to sense dipole potential is believed to be electrochromic [29]. Absorption and emission peaks shift in response to a nearby electrical field that differentially interacts with the ground-state and excited-state dipole moments of the chromophore [30,31]. Therefore these kinds of probes suggest a dual wavelength ratiometric measurements strategy, similar to what is done with some ion indicators, such as for Ca^{2+} [32]. This approach has several advantages because the signal is independent of probe or cell concentration and avoids photobleaching artifacts [27].

Emphasis will be put in di-8-ANEPPS, the most used dye for dipole potential measurement. Although the vast majority of the work with di-8-ANEPPS relies on the excitation spectrum shift to measure dipole potential, only recently a systematic study was done to compare excitation and emission ratiometric fluorescence methods for this dye, concluding that only the former can be used [33]. Membrane fluidity is another aspect for concern and has been addressed in previous papers. Gross *et al.* [27] states that membrane microviscosity does not significantly influence the fluorescence excitation ratio and Clarke and Kane [34] suggested that in order to eliminate the possible effects of membrane fluidity, fluorescence should be detected at the red-edge of the emission spectra, at 670 nm. It is also relevant to refer that the use of a fluorescent probe is not the most adequate method to measure absolute values of dipole potential, mainly because a rigorous calibration method has not been established yet. An equation for converting fluorescence excitation ratio to voltage is available [35], but is based on potential values calculated on previous and distinct papers, by different methods. However, in the presented cases, the use of absolute values of dipole potential is not relevant.

The influence of dipole potential on p25 interaction with lipid vesicles was studied with di-8-ANEPPS along

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

DOI: 10.1002/psc

with the above-mentioned FPE measurements for surface potential [36]. In the presence of p25, the dipole potential of di-8-ANEPPS labeled PC vesicles decreased, as indicated by the decrease of the ratio of the fluorescence intensities at two excitation wavelengths, 460 and 520 nm, for the same emission wavelength of 580 nm, with the increase of peptide concentration. The dipole potential of PC vesicles was also manipulated adding phloretin and 6-ketocholestanol (6-KC), previously known to decrease and increase the dipole potential, respectively [27,37]. The results showed that the larger the initial dipole potential, the larger is its decrease caused by the interaction of the peptide with the membrane (Figure 5). Kinetic studies using stopped-flow technique and the comparison with the kinetic results obtained from FPE experiments also led to the conclusion that the decrease in dipole potential occurs when the peptide inserts into the membrane rather than during the initial binding event.

A similar study was done for assessing the interaction between the fusion peptide from simian immunodeficiency virus (SIV) gp32 with phosphatidylcholine/phosphatidylethanolamine (PC/PE) vesicles [38]. Addition of the SIV fusion peptide to the vesicles suspension caused a decrease in the dipole potential, and the larger the initial dipole potential, the larger the magnitude of the decrease, as for p25. The influence of the magnitude of the dipole potential in the peptide-dependent membrane fusion was assessed by a fluorescence resonance energy transfer (FRET) based lipid-mixing fusion assay. It was clearly demonstrated that increasing dipole potential with 6-KC enhanced the fusion process and the opposite was true for phloretin. The HIV-1 gp41 fusion peptide interaction with PC/PE vesicles was also studies with FPE and di-8-ANEPPS [39]. Apart from demonstrating once again that 6-KC and, at a lesser extent, cholesterol increases membrane dipole potential, the authors related the K_d derived from FPE kinetic studies with the dipole potential of vesicles with different cholesterol/6-KC compositions. The fusion peptide had a higher affinity for membranes with higher dipole potential. As a high-dipole potential enhances fusion and cholesterol has an increased proportion on SIV and HIV membrane [40,41], the results of these two studies shed some light on the importance of membrane lipid composition for the infectivity of SIV/HIV.

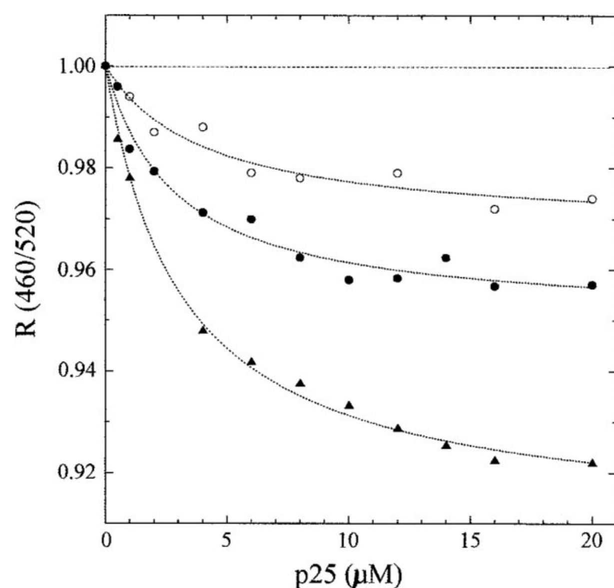

Figure 5 Variation of the ratio of the di-8-ANEPPS fluorescence intensities at two excitation wavelengths, 460 and 520 nm, R(460/520), as a function of p25 concentration. The emission wavelength was kept at 580 nm. Each trace refers to a different vesicle composition: ●, pure PC vesicles; ○, phloretin-enriched PC vesicles; ▲, 6-KC enriched PC vesicles [36]. Reproduced with permission of the Biophysical Society.

The modulation of membrane dipole potential can bring new possibilities in the drug delivery research area. With this in mind two different studies are presented involving the drugs saquinavir and bacitracin.

Saquinavir is a peptidomimetic inhibitor of HIV protease and its interaction with Caco-2 cell membrane was studied [42]. The human intestinal epithelial cell line Caco-2 is often used to assess intestinal permeability of drugs [43]. The shift in the excitation spectra of di-8-ANEPPS, indicative of alterations in the dipole potential, revealed that saquinavir decreases this potential when binding to Caco-2 cell membranes and, at a lower extent, to PC/PS vesicles. Analyzing the dependence of the ratio with the saquinavir concentration, it was shown that for Caco-2 cells the data followed a cooperative binding profile, in contrast with the results obtained for vesicles, which followed a hyperbolic single binding site model. This is indicative that a receptor-mediated process may take place besides the membrane binding *per se*, as it is known to be a substrate of the P-glycoprotein, the product of *MDR1* gene [44]. Membrane dipole potentials were modulated by 6-KC or phloretin, yielding a larger decrease of the fluorescence ratio for 6-KC, both for Caco-2 and lipid vesicles. Moreover, the binding capacity of saquinavir for cholesterol depleted cell membranes was also assessed and found to be decreased relative to normal ones. It is shown that increasing membrane dipole potential enhances saquinavir binding to Caco-2 cells. Such dipole potential modulation can be a strategy to increase intestinal absorption.

The interaction of bacitracin with PC vesicles was studied in the context of enhancing the transdermal delivery of this cyclic peptide antibiotic [45]. The red shift in the excitation spectra of di-8-ANEPPS labeled vesicles showed that bacitracin binding decreases the membrane dipole potential. With 6-KC enriched vesicles, the dipole potential decreased at a higher extent in the presence of bacitracin. The opposite

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

DOI: 10.1002/psc

effect was observed for phloretin. The variation of the surface potential over time was also measured using FPE, upon adding bacitracin to PC vesicles suspension. Those enriched with 6-KC yielded an increased signal. All these studies were done to help to interpret the initial experiment in which FITC-labeled bacitracin penetrated more deeply in epidermis pretreated with 6-KC-enriched PC vesicles, as visualized by confocal microscopy. The more efficient binding of bacitracin to 6-KC enriched membranes suggests a new and more efficient way of delivering this peptide for the treatment of skin infections.

Although di-8-ANEPPS has been the most used probe for measuring dipole potential, recently developed probes based on the 3-hydroxyflavone fluorescent moiety promise better detection [46]. The probes F4N1 and BPPZ were synthesized and tested for their ability to sense dipole potential. These probes have the particularity of undergoing excited-state intramolecular proton-transfer (ESIPT), yielding two tautomeric forms, revealed by the presence of two emission peaks in the fluorescence spectrum. The presence of a nearby electrical field not only originates a shift in the absorption and emission bands, but also alters the intensity ratio of the two emission peaks. These two types of ratios, using two wavelengths at each side of the excitation band and using the two emission peaks, correlated well with the typical excitation ratio used for di-8-ANEPPS. The fact that emission ratios can be used facilitates fluorescence microscopy imaging of the dipole potential in membranes, as for a single excitation wavelength the emission of the probes can be collected on two separate detectors at the same time. This is a very useful approach for visualizing dipole potential distribution in membranes of living cells [47].

TRANSMEMBRANE POTENTIAL

The last member of the membrane potential club is the transmembrane potential. As it is the most well-known and well-studied type of potential, just a contextualization in this case will be given. In simple terms, it is defined as the potential difference between the intra and extracellular aqueous phases (Figure 1). This is a consequence of the selective nature of the membrane, that allows the flux of specific charged solutes (e.g. ions), creating a concentration gradient.

Measuring the transmembrane potential in a context of a peptide–membrane interaction is especially important for antimicrobial peptides, which usually form pores or channels in membranes, enabling the dissipation of an established potential, or for cell-penetrating peptides, whose mode of action may depend on this potential [48,49]. In fact, membrane potential tests are routinely used to assay the membrane potential alterations that occur when these types of peptides are added to a vesicle or cell suspension. There are several fluorescent dyes available for the detection of transmembrane potential alterations, from slow response carbocyanines (DiI, DiS, and DiO derivatives) and oxonols (DiBAC and DiSBAC) to fast response styryl dyes (ANEP and RH dyes) [50].

The majority of the studies test the ability of a certain peptide to alter the transmembrane potential in vesicles or even bacteria, using slow-response dyes. In vesicles, a transmembrane potential is achieved by adding valinomycin to vesicles prepared in a KCl buffer and suspended in NaCl buffer. Some peptides are able to dissipate the potential [51–53] while others simply do not [54], depending on the degree of membrane disruption. Moreover, monitoring transmembrane potential of bacteria when they are exposed to antimicrobial peptides often reveals membrane depolarization [55–57].

CONCLUDING REMARKS

There is no doubt that when certain peptides have the ability to interact with membranes they alter some of their properties, and membranes potentials are no exception. For the three types of membrane potential, there are different types of fluorescent dyes capable of measuring each of them. This is an advantage, as it permits the establishment of similar protocols, based on the same concept of fluorescent dye detection, in order to build a combined study, with readily comparable results. Such combined studies, involving the different membrane potentials, permit a wider vision of what is happening at the membrane level when certain peptides are present.

Alterations in surface potential sensed by FPE can reveal the very first electrostatic binding events at the membrane surface and even peptide insertion or translocation. Di-8-ANEPPS is the most used probe for measuring dipole potential and allows the monitoring of insertion events, as these tend to change the disposition of the molecular dipoles of membrane phospholipids. It has the advantage of being sensitive to uncharged molecules. A study of the effect of transmembrane potential is important to find out if a more extensive disruption is made on the membrane by the peptide, such as pore or channel formation.

These tools, although available for a while, can be more extensively explored, especially in the context of *in vivo* environments, in conjunction also with fluorescence imaging and real-time observation of the interaction events.

Acknowledgments

This work was partially supported by the Fundação para a Ciência e Tecnologia (FCT) of the Portuguese Ministry of Science, Technology and Higher Education (MCTES).

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

REFERENCES

1. Burton PS, Conradi RA, Ho NF, Hilgers AR, Borchardt RT. How structural features influence the biomembrane permeability of peptides. *J. Pharm. Sci.* 1996; **85**: 1336–1340.
2. Fahr A, van Hoogevest P, May S, Bergstrand N, Leigh MLS. Transfer of lipophilic drugs between liposomal membranes and biological interfaces: consequences for drug delivery. *Eur. J. Pharm. Sci.* 2005; **26**: 251–265.
3. Castanho MA, Fernandes MX. Lipid membrane-induced optimization for ligand-receptor docking: recent tools and insights for the "membrane catalysis" model. *Eur. Biophys. J.* 2006; **35**: 92–103.
4. Killian JA, Nyholm TK. Peptides in lipid bilayers: the power of simple models. *Curr. Opin. Struct. Biol.* 2006; **16**: 473–479.
5. Santos NC, Prieto M, Castanho MA. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. *Biochim. Biophys. Acta* 2003; **1612**: 123–135.
6. McLaughlin S. The electrostatic properties of membranes. *Annu. Rev. Biophys. Biophys. Chem.* 1989; **18**: 113–136.
7. Langner M, Kubica K. The electrostatics of lipid surfaces. *Chem. Phys. Lipids* 1999; **101**: 3–35.
8. Cevc G. Membrane electrostatics. *Biochim. Biophys. Acta* 1990; **1031**: 311–382.
9. Wall J, Golding CA, Van Veen M, O'Shea P. The use of fluorescein phosphatidylethanolamine (FPE) as a real-time probe for peptide-membrane interactions. *Mol. Membr. Biol.* 1995; **12**: 183–192.
10. Thelen M, Petrone G, O'Shea PS, Azzi A. The use of fluorescein-dipalmitoylphosphatidylethanolamine for measuring pH-changes in the internal compartment of phospholipid vesicles. *Biochim. Biophys. Acta* 1984; **766**: 161–168.
11. Soucaille P, Prats M, Tocanne JF, Teissie J. Use of a fluorescein derivative of phosphatidylethanolamine as a pH probe at water/lipid interfaces. *Biochim. Biophys. Acta* 1988; **939**: 289–294.
12. Yuanbo C, Fan Z, Jiachang Y. Detecting proton flux across chromatophores driven by F_0F_1-ATPase using *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethano lamine, triethylammonium salt. *Anal. Biochem.* 2005; **344**: 102–107.
13. Thelen M, O'Shea PS, Petrone G, Azzi A. Proton translocation by a native and subunit III-depleted cytochrome c oxidase reconstituted into phospholipid vesicles. Use of fluorescein-phosphatidylethanolamine as an intravesicular pH indicator. *J. Biol. Chem.* 1985; **260**: 3626–3631.
14. O'Shea P. Intermolecular interactions with/within cell membranes and the trinity of membrane potentials: kinetics and imaging. *Biochem. Soc. Trans.* 2003; **31**: 990–996.
15. Wall J, Ayoub F, O'Shea P. Interactions of macromolecules with the mammalian cell surface. *J. Cell Sci.* 1995; **108**: 2673–2682.
16. Bechinger B. Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. *J. Membr. Biol.* 1997; **156**: 197–211.
17. Chupin V, Leenhouts JM, de Kroon AI, de Kruijff B. Cardiolipin modulates the secondary structure of the presequence peptide of cytochrome oxidase subunit IV: a 2D 1H-NMR study. *FEBS Lett.* 1995; **373**: 239–244.
18. Matsuzaki K, Yoneyama S, Miyajima K. Pore formation and translocation of melittin. *Biophys. J.* 1997; **73**: 831–838.
19. Golding C, Senior S, Wilson MT, O'Shea P. Time resolution of binding and membrane insertion of a mitochondrial signal peptide: correlation with structural changes and evidence for cooperativity. *Biochemistry* 1996; **35**: 10931–10937.
20. Chan DC, Kim PS. HIV entry and its inhibition. *Cell* 1998; **93**: 681–684.
21. Cladera J, Martin I, O'Shea P. The fusion domain of HIV gp41 interacts specifically with heparan sulfate on the T-lymphocyte cell surface. *EMBO J.* 2001; **20**: 19–26.
22. Sim B, Cladera J, O'Shea P. Fibronectin interactions with osteoblasts: identification of a non-integrin-mediated binding mechanism using a real-time fluorescence binding assay. *J. Biomed. Mater. Res. A* 2004; **68**: 352–359.
23. Fitchen N, O'Shea P, Williams P, Hardie KR. Electrostatic sensor for identifying interactions between peptides and bacterial membranes. *Mol. Immunol.* 2003; **40**: 407–411.
24. Gawrisch K, Ruston D, Zimmerberg J, Parsegian VA, Rand RP, Fuller N. Membrane dipole potentials, hydration forces, and the ordering of water at membrane surfaces. *Biophys. J.* 1992; **61**: 1213–1223.
25. Zheng C, Vanderkooi G. Molecular origin of the internal dipole potential in lipid bilayers: calculation of the electrostatic potential. *Biophys. J.* 1992; **63**: 935–941.
26. Clarke RJ. The dipole potential of phospholipid membranes and methods for its detection. *Adv. Colloid Interface Sci.* 2001; **89–90**: 263–281.
27. Gross E, Bedlack RS, Loew LM. Dual-wavelength ratiometric fluorescence measurement of the membrane dipole potential. *Biophys. J.* 1994; **67**: 208–216.
28. Zouni A, Clarke RJ, Holzwarth JF. Kinetics of the solubilization of styryl dye aggregates by lipid vesicles. *J. Phys. Chem.* 1994; **98**: 1732–1738.
29. Fluhler E, Burnham VG, Loew LM. Spectra, membrane binding, and potentiometric responses of new charge shift probes. *Biochemistry* 1985; **24**: 5749–5755.
30. Loew LM, Bonneville GW, Surow J. Charge shift optical probes of membrane potential. theory. *Biochemistry* 1978; **17**: 4065–4071.
31. Bublitz GU, Boxer SG. Stark spectroscopy: applications in chemistry, biology, and materials science. *Annu. Rev. Phys. Chem.* 1997; **48**: 213–242.
32. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 1985; **260**: 3440–3450.
33. Vitha MF, Clarke RJ. Comparison of excitation and emission ratiometric fluorescence methods for quantifying the membrane dipole potential. *Biochim. Biophys. Acta* 2007; **1768**: 107–114.
34. Clarke RJ, Kane DJ. Optical detection of membrane dipole potential: avoidance of fluidity and dye-induced effects. *Biochim. Biophys. Acta* 1997; **1323**: 223–239.
35. Clarke RJ. Effect of lipid structure on the dipole potential of phosphatidylcholine bilayers. *Biochim. Biophys. Acta* 1997; **1327**: 269–278.
36. Cladera J, O'Shea P. Intramembrane molecular dipoles affect the membrane insertion and folding of a model amphiphilic peptide. *Biophys. J.* 1998; **74**: 2434–2442.
37. Franklin JC, Cafiso DS. Internal electrostatic potentials in bilayers: measuring and controlling dipole potentials in lipid vesicles. *Biophys. J.* 1993; **65**: 289–299.
38. Cladera J, Martin I, Ruysschaert JM, O'Shea P. Characterization of the sequence of interactions of the fusion domain of the simian immunodeficiency virus with membranes. Role of the membrane dipole potential. *J. Biol. Chem.* 1999; **274**: 29951–29959.
39. Buzon V, Cladera J. Effect of cholesterol on the interaction of the HIV GP41 fusion peptide with model membranes. Importance of the membrane dipole potential. *Biochemistry* 2006; **45**: 15768–15775.
40. Aloia RC, Jensen FC, Curtain CC, Mobley PW, Gordon LM. Lipid composition and fluidity of the human immunodeficiency virus. *Proc. Natl. Acad. Sci. U.S.A.* 1988; **85**: 900–904.
41. Aloia RC, Tian H, Jensen FC. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. *Proc. Natl. Acad. Sci. U.S.A.* 1993; **90**: 5181–5185.
42. Asawakarn T, Cladera J, O'Shea P. Effects of the membrane dipole potential on the interaction of saquinavir with phospholipid membranes and plasma membrane receptors of Caco-2 cells. *J. Biol. Chem.* 2001; **276**: 38457–38463.
43. Shah P, Jogani V, Bagchi T, Misra A. Role of Caco-2 cell monolayers in prediction of intestinal drug absorption. *Biotechnol. Prog.* 2006; **22**: 186–198.
44. Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, Pastan I, Dey S. HIV-1 protease

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

DOI: 10.1002/psc

inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* 1998; **37**: 3594–3601.

45. Cladera J, O'Shea P, Hadgraft J, Valenta C. Influence of molecular dipoles on human skin permeability: Use of 6-ketocholestanol to enhance the transdermal delivery of bacitracin. *J. Pharm. Sci.* 2003; **92**: 1018–1027.
46. Klymchenko AS, Duportail G, Mely Y, Demchenko AP. Ultrasensitive two-color fluorescence probes for dipole potential in phospholipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* 2003; **100**: 11219–11224.
47. Shynkar VV, Klymchenko AS, Duportail G, Demchenko AP, Mely Y. Two-color fluorescent probes for imaging the dipole potential of cell plasma membranes. *Biochim. Biophys. Acta* 2005; **1712**: 128–136.
48. Deshayes S, Plenat T, Charnet P, Divita G, Molle G, Heitz F. Formation of transmembrane ionic channels of primary amphipathic cell-penetrating peptides. Consequences on the mechanism of cell penetration. *Biochim. Biophys. Acta* 2006; **1758**: 1846–1851.
49. Terrone D, Sang SL, Roudaia L, Silvius JR. Penetrating and related cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. *Biochemistry* 2003; **42**: 13787–13799.
50. Haugland RP. Probes for membrane potential. *The Handbook – A Guide to Fluorescent Probes and Labelling Technologies*. Invitrogen Molecular Probes: Eugene, OR, 2005; 973–986.
51. Moll GN, Brul S, Konings WN, Driessen AJ. Comparison of the membrane interaction and permeabilization by the designed peptide Ac-MB21-NH2 and truncated dermaseptin S3. *Biochemistry* 2000; **39**: 11907–11912.
52. Rintoul MR, de Arcuri BF, Morero RD. Effects of the antibiotic peptide microcin J25 on liposomes: role of acyl chain length and negatively charged phospholipid. *Biochim. Biophys. Acta* 2000; **1509**: 65–72.
53. Ibrahim HR, Sugimoto Y, Aoki T. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochim. Biophys. Acta* 2000; **1523**: 196–205.
54. Tiriveedhi V, Butko P. A fluorescence spectroscopy study on the interactions of the TAT-PTD peptide with model lipid membranes. *Biochemistry* 2007; **46**: 3888–3895.
55. Zhu WL, Lan H, Park IS, Kim JI, Jin HZ, Hahm KS, Shin SY. Design and mechanism of action of a novel bacteria-selective antimicrobial peptide from the cell-penetrating peptide Pep-1. *Biochem. Biophys. Res. Commun.* 2006; **349**: 769–774.
56. Nuding S, Fellermann K, Wehkamp J, Mueller HA, Stange EF. A flow cytometric assay to monitor antimicrobial activity of defensins and cationic tissue extracts. *J. Microbiol. Methods* 2006; **65**: 335–345.
57. Yang ST, Shin SY, Kim JI. Interaction mode of a symmetric Trp-rich undeca peptide PST11-RK with lipid bilayers. *FEBS Lett.* 2007; **581**: 157–163.

Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.