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Review

What can light scattering spectroscopy do for membrane-active peptide studies?‡

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Abstract: Highly charged peptides are important components of the immune system and belong to an important family of antibiotics. Although their therapeutic activity is known, most of the molecular level mechanisms are controversial. A wide variety of different approaches are usually applied to understand their mechanisms, but light scattering techniques are frequently overlooked. Yet, light scattering is a noninvasive technique that allows insights both on the peptide mechanism of action as well as on the development of new antibiotics. Dynamic light scattering (DLS) and static light scattering (SLS) are used to measure the aggregation process of lipid vesicles upon addition of peptides and molecular properties (shape, molecular weight). The high charge of these peptides allows electrostatic attraction toward charged lipid vesicles, which is studied by zeta potential (ζ-potential) measurements. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: dynamic light scattering; static light scattering; zeta-potential; therapeutic peptides

INTRODUCTION

Pathogen resistance to host defense is one of the main problems in health care. Finding new biomolecules with powerful action against those pathogens is an effort of many laboratories around the World [1,2]. Peptides, present in the innate immune system or synthetically designed, are raising interest in academia and industry due to their therapeutic activity [3,4]. Most of the peptides adopt an amphipathic or amphiphilic structure in solution. This ability, combined with a high hydrophobicity and positive/negative net charge, is believed to be the main factor modulating the interaction with membranes [5,6]. Membrane-active peptide, such as antimicrobial peptides (AMPs), cell-penetrating peptides (CPPs), and fusogenic peptides (FP), have their action crucially dependent on these properties.

The interaction of unilamellar lipid vesicles with peptides can be very useful for studying the basic events that contribute to the cell-membrane level action of peptides, since vesicles can be prepared with manipulated compositions to specifically mimetize selected biomembranes. Peptides can interact with vesicles due to electrostatic effects when they are constituted by charged lipids [7–10]. Nevertheless, peptides can also interact with neutral lipids [11–14].

Although frequently overlooked, light scattering spectroscopy is one of the possible noninvasive approaches to study membrane–peptide interactions. Techniques based on light scattering theory have been used to determine the weight-averaged molecular weight (M_w), size [given by the value of the intensity-weighted mean hydrodynamic size (R_H)], and aggregation behavior of peptide in solution, either alone or interacting with other particles, such as lipid vesicles [15,16]. The most frequently used techniques based on light scattering are the so-called intensity or static light scattering (SLS) and dynamic light scattering (DLS). SLS involves the measurement of the time-averaged intensity in the seconds time-scale, while DLS measurements involve light scattering intensity fluctuation on the microsecond time-scale in a small scattering volume, in order to obtain a meaningful autocorrelation function [17–21]. In general, SLS is useful for the determination of the M_w and intermolecular interactions [22]. It is advantageous for studies of proteins or peptide aggregation in solution [23–25]. Peptide aggregation monitored by SLS gives rise to stoichiometry data in direct relation to the calculated M_w. DLS is used to measure particle hydrodynamic diameter and size distribution of molecules or supramolecular aggregates [5,7–10,26,27].

Recently, another technique associated to light scattering phenomena arose: the ζ-potential determination. This experimental approach is based on the measurement of the electrophoretic mobility of the charged particle in the presence of an electrical field, by laser Doppler velocimetry and phase analysis light scattering.

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BIOGRAPHY

Marco M. Domingues was born in Lisbon, Portugal, in 1985. He graduated in Biochemistry at the Faculty of Sciences, University of Lisbon, Portugal, in 2007. He is now a Ph.D student at the Institute of Molecular Medicine, Lisbon, Portugal. His research interests are focused on the application of light scattering and other spectroscopic techniques to the study of the interaction of antimicrobial peptides with biomembranes and biomembrane model systems.

Patrícia S. Santiago was born in Jaboticabal, Brazil, in 1978. She graduated in Chemistry (University of San Paulo, Brazil, 2002), has a Masters degree in Analytical Chemistry (University of San Paulo, Brazil, 2004) and is a Ph.D student at the Institute of Chemistry, University of San Paulo (San Carlo, Brazil) and at the Institute of Molecular Medicine (Lisbon, Portugal). Her research is focused on the interaction of porphyrins with biomembrane model systems, and on the study of the structure and stability of hemoproteins, using spectroscopic techniques (mainly fluorescence spectroscopy, light scattering and SAXS).

Miguel Castanho was born in Santarém, Portugal, in 1967. He graduated in Biochemistry (University of Lisbon, Portugal, 1990), has a Ph.D degree in Molecular Biophysics (Technical University of Lisbon, Portugal, 1993) and habilitation in Physical Biochemistry (University of Lisbon, Portugal, 1999). He became a group leader in the Faculty of Sciences at the University of Lisbon, Portugal, where he started working on the mechanism of action of membrane active peptides at the molecular level. His work includes the development of methodologies aiming at specific functional and structural information, related to cell-penetrating, antimicrobial, viral fusion inhibitor and neuropeptides. Both *in-vitro* and *in-vivo* work is carried out, using mainly optical spectroscopic techniques. Miguel Castanho has recently been appointed full professor (Biochemistry) at the Faculty of Medicine, University of Lisbon, Portugal.

BIOGRAPHY

Nuno C. Santos was born in Lisbon, Portugal, in 1972. Lipid membranes have been the common element of his research since 1994. After a major in Biochemistry, he conducted his Ph.D research work at the Technical University of Lisbon (Portugal) and at the University of California, Santa Barbara (USA). He completed the Ph.D in 1999, and joined the Faculty 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: (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 proteins-derived drugs; and (iv) nanomedicine.

The parameter quantifies the charge of the molecule or supramolecular aggregate at its surface, in contact with an aqueous environment. The ζ-potential is related to the stability of a colloidal system. Particles with high positive or negative charge tend to repulse the particles of the same charge, whereas particles with low positive or negative charge (ζ-potential close to zero) tend to aggregate and cause flocculation due to interparticle interaction.

Thus, light scattering spectroscopy is of potential applicability in membrane-active peptide work: (i) as DLS and SLS techniques are very sensitive to changes in the shape and size, the peptide aggregation or peptide-induced aggregation of lipid vesicles can be easily detected, and (ii) when charged peptides interact with charged vesicles, the electrophoretic mobility of the resulting supramolecular entities is altered, enabling ζ-potential measurements to be used to study the membranes involved in the interaction.

STATIC LIGHT SCATTERING (SLS)

SLS uses the time-averaged intensity of the sample in a long time-scale relative to molecular diffusion (seconds to minutes) instead of the fluctuations of the signal due to molecular dynamics in the scattering volume (microsecond time-range). This technique enables the determination of the M_w (in the typical range of 1 kDa to 20 MDa) and the second Virial coefficient (A_2), trough the Zimm method [28,29]:

$$\frac{KC}{R_\theta} = \frac{1}{M_W P(\theta)} + 2A_2 C \tag{1}$$

where,

$$K = \frac{2\pi^2}{{\lambda_0}^4 N_A}\left(n_0 \frac{dn}{dC}\right)^2 \tag{2}$$

$$R_\theta = \frac{I_A n_0^2}{I_R n_R^2} R_R \tag{3}$$

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$$P(\theta)^{-1} = \left(1 + \frac{q^2 Rg^2}{3}\right) \quad (4)$$

In the previous equations, C is the scattering particle concentration, K the optical constant, A_2 the second Virial coefficient, N_A the Avogadro's number, R_θ the Rayleigh ratio, I_R the reference scattering intensity (usually toluene is used as a standard), I_A the residual intensity of the solute (i.e. scattered intensity of the sample after subtracting the scattered intensity of the solvent), n_0 the solvent refractive index, n_R the reference refractive index, λ_0 the vacuum wavelength of the incident light, dn/dC the refractive index increment (relates to how much the refractive index of a solution changes with the concentration of the solute), R_R the Rayleigh ratio of the reference, $P(\theta)$ the intraparticle structure factor (accounts for the interference of light scattered from different point of the same particle), θ the angle at which the intensity is being measured relative to the transmitted beam, R_g is the radius of gyration (shape-independent related to the dimension of the scattering particle) and q is the scattering vector:

$$q = \frac{4\pi n_0}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (5)$$

In the Zimm method, M_w is calculated by extrapolation of the scattered intensity both to zero angle (Eqn (6)) and infinite dilution (Eqn (7)). A_2 describes the interparticle interactions: positive values indicate a tendency toward stable scattering particles (monomers) in solution, while negative values reveal a tendency for aggregation (i.e. solute–solute interactions superimpose to solute–solvent interactions). It is calculated by the slope of Eqn (6).

$$\frac{KC}{R_\theta} = \frac{1}{M_W} + 2A_2C \quad (6)$$

$$\frac{KC}{R_\theta} = \frac{1}{M_W} P(\theta)^{-1} \quad (7)$$

In the simpler Debye method, intensity measurements are conducted at different concentrations but not at different scattering angles, therefore allowing the determination of M_w and A_2, but not of R_g [18,19]. This method applies to conditions where the solvent can be considered ideal.

The scattered intensity is dependent of the shape and dimension of the particle and proportional to the sixth power of the particle radius (i.e. squared volume). Thus, particles with higher R_g values result in increased scattering of light. R_g is also related with the particles shape, although it can be calculated with no prior assumption on shape. Particles with the same M_w but with different shapes scatter differentially the incident light (e.g. the light scattering by a globular particle is more intense than the scattering by an equivalent particle with a cylindrical shape). For more detailed description please refer to Refs 18–21.

DYNAMIC LIGHT SCATTERING (DLS)

DLS is based on the scattering fluctuation on a small volume in the time-scale of the molecular diffusion (typically microseconds), due to Brownian motion of the particles (due to random collision of molecules). The light scattered by particles is shifted according to Doppler effect on their random motion. As the molecules diffuse randomly in the illuminated volume of the sample, they tend to locally cluster and separate from each other randomly. As the frequency shift, through Doppler effect, is negligible for low-velocity molecules, the diffusion coefficient of the particle is obtained from the decay rate (Γ) of the intensity autocorrelation function using the relation $\Gamma = Dq^2$ [17]. Thus, the correlation kinetics depends on the intensity-weighted diffusion coefficient (D), which can be calculated using several methods, such as Cumulants [30,31] or CONTIN [32,33]. Cumulants uses a monoexponential correlogram fit to get information about an average D, while CONTIN uses a multiexponential correlogram fit to assess D distribution in a solution. Using the Stokes–Einstein equation (Eqn (8)), the value of the hydrodynamic radius (R_H) is determined from D (for detailed description see [17]).

$$D = \frac{\kappa T}{6\pi \eta R_H} \quad (8)$$

where η is the dispersant viscosity, κ the Boltzmann constant and T the absolute temperature. Depending on the equipments and the quality of the sample preparation, the range of detection of the R_H varies from 0.6 nm to 6 μm.

ZETA POTENTIAL (ζ-POTENTIAL)

Charged particles, such as proteins and peptides, suspended in a solution attract ions of opposite charge to their surface. These ions are strongly bound, forming a layer covering the particle surface. The layer is commonly called the Stern layer. Another layer beyond the Stern layer is formed where ions diffuse more freely. When the particle travels trough the solution, the ions that are strongly attached move with it. In the diffuse boundary, the ions do not move with the molecule. The potential that exists at this boundary is called the ζ-potential. This potential is calculated by the electrophoretic mobility of the particles in solution, on an electric field, to the electrode of opposite charge. Viscous forces oppose the movement of the particles until equilibrium, where a constant velocity is reached. The electrophoretic mobility can be calculated by laser Doppler velocimetry in Zeta-sizer devices, in which particle velocity is related to the frequency measured by intensity fluctuation of the scattered light. The sensitivity of Doppler effect to the low mobility of larger

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particles is very low, causing difficulties in calculating the electrophoretic mobility. In conventional DLS, the mean square displacement of the particles is measured, rather than the mean displacement itself, which allows the measure of small displacements simply by waiting for an adequate time. This is not a good option in an electrophoresis experiment because the application of an electrical field in one direction can cause electrode polarization. Increasing the electrical field is another inconvenient option because it can create an excessive Joule heating which may damage the sample. Some new devices possess a different method of calculation of the electrophoretic mobility. It is based on phase shift analysis where differences in phases between the unshifted reference beam and the sample-scattered beam are analyzed with higher sensitivity. This phase shift is related to the position of the particle. Hence, the mean phase change with time yields the electrophoretic motion [34,35]. Using the Henry's relation (9) it is possible to calculate the ζ-potential of the particle (for more detailed description, see Refs 36,37):

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta} \quad (9)$$

where z is the ζ-potential, U_E the electrophoretic mobility and *f(ka)* the Henry's function. The value of this function is 1.5 when the particles are suspended in aqueous solutions (Smoluchowski approximation) and 1 for nonaqueous media (Huckel approximation). The ζ-potential can usually be determined accurately for measurements with samples with particle sizes between 5 nm and 10 μm.

APPLICATIONS

Depending on the pH, peptides may acquire positive or negative charge, attending to the pKa of the amino acid residues. The presence of hydrophobic residues facilitates lipid membrane insertion and can be responsible for peptide self-aggregation in solution, which can be measured by the size of the aggregates and the respective M_w, to yield the stoichiometry of the peptide aggregate. Likewise, the stability as well as the aggregation of lipid vesicles in the presence of peptides can be measured by their overall charge and size.

ζ-potential measurement is a useful method to evaluate membrane–peptide interactions. Bacterial membranes are composed of a high proportion of negative lipid, which ensures the selectivity and efficient activity of positively charged antibiotics. In addition to negative lipids, endotoxin or lipopolysaccharide (LPS) is present in the outer leaflet of Gram-negative bacteria's outer membrane and further contributes to an overall negative charge. Due to the high relevance of LPS to human diseases, peptides with positive charge and LPS-binding properties are needed and sought. AMPs, such as the ones based on Limulus anti-LPS factor, have LPS-binding properties reducing their overall charge, measured by ζ-potential technique [38]. This potential reduction ensures the high affinity of those peptides toward LPS moieties.

The design of new peptides as drug carriers through the cell membrane is a possible strategy to an alternative therapeutic role. Their positive charge allows electrostatic interaction with membranes composed of negative lipid and enables ζ-potential measurements to study the effect. Abrunhosa *et al.* studied the effects of antibiotic peptide hybrids of cecropin A and melittin on membranes composed of dimyristoilphosphatidylcholine (DMPC), dimyristoilphosphatidylglycerol (DMPG), or a mixture of them [11]. They showed that the ζ-potential of the lipid vesicles becomes less negative upon increase of peptide/lipid ratio. The highest interaction of one of the two hybrid peptides with negative membranes, evaluated by ζ-potential measurements, is considered as an indication of a higher microbicide activity. Figure 1 shows results of a ζ-potential study of the interaction of poly-L-arginine, a positively charged peptide, with vesicles [8]. This peptide is believed to be a good system for facilitating the transport of drugs trough biological membranes. As shown in Figure 1, ζ-potential measurements at different temperatures, with addition of peptides to mixed lipid vesicles consisting of phosphatidylcholine, cholesterol, and dihexadecylphosphate (PC : Chol : DHP) results in less negative ζ-potential values. At 65°C, where lipids are in the liquid-crystalline phase, the plateau is reached at higher guanidium/phosphate molar ratios. This could be due to partial incorporation of the peptide, resulting in an accurate drug transport across membrane. The same way, Yaroslavov *et al.* studied poly-lysine, a positive polypeptide and showed

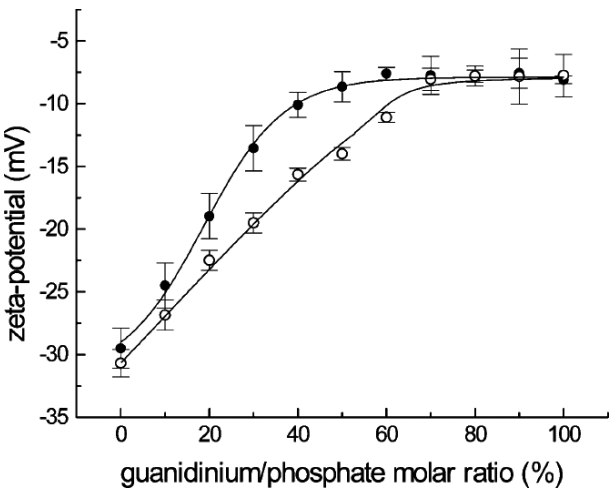

Figure 1 ζ-potential of PC : Chol : DHP lipid vesicles as a function of guanidium/phosphate molar ratios, before (closed symbols) and after (open symbols) incubation at 65°C [8]. Reproduced with permission of American Chemical Society.

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that it can interact with lipid vesicles composed of negative lipids (in this case cardiolipin) resulting in a slightly positive complex [39]. This interaction with negative lipid enables the increase of the permeation of vesicles to Dox (fluorescent antitumor drug) when lipid vesicle charge is close to zero, corresponding to peptide electrostatically adsorbed to negative lipid.

Almost all the cationic peptides induce aggregation of negatively charged lipid vesicles, at least at high enough peptide concentrations. Besides this, many hydrophobic peptides are capable of interacting with neutral charged lipids and inducing their aggregation. These properties can help the design of new peptides with antibiotic action. Size measurements were done in order to evaluate the aggregation process of PC : Chol : DHP lipid vesicles as a consequence of the presence of the poly-L-arginine peptide. In Figure 2, there is an increase of the particle size with peptide concentration, which confirms the aggregation of the lipid vesicles, leading to the formation of large particles (1 μm), prone to sedimentation. This demonstrates an efficient interaction of polyarginine with liposomal membranes [8]. Vagt *et al.* studied by DLS measurements, the effect of the structure of three variants of coiled-coil peptides on their biological activity [9]. Figure 3 shows the strongest membrane binding of variant 1 while the basic peptide has no interaction. The same approach was applied by Epand *et al.* to the study of the interaction of four analogs of the AMP trichogin. One of the peptides, BT, was shown to have the ability to aggregate dioleoylphosphatidylcholine (DOPC) and DOPC : dioleoylphosphatidylethanolamine (DOPE) vesicles, leading to the increase of their hydrodynamic diameter, with a faster aggregation in the presence of DOPE, while other peptide, ST, does not show that capability [40]. This was expected due to the hydrophobic nature of BT, unlike the amphiphilic structure of ST. The hydrophobic properties of BT promote interaction with lipid vesicles, which was not seen for ST. Cummings *et al.* observed by DLS, the size increase of aggregates of palmitoyloleoylphosphatidylglycerol (POPG) vesicles upon addition of cryptdin-4, an AMP found in mice, which causes aggregation and hemi-fusion of negative lipid vesicles [41]. The lipid vesicles aggregation in the presence of cryptdin-4 reaches a limit for very large aggregates, less prone to fusion. Thus, the ability of cryptdin-4 to promote stable fusion of anionic lipid vesicles can create vesicular structures to be used as drug delivery agents.

The fusion process of the vesicles in the presence of peptides can also be evaluated by SLS. Intensity measurements for vesicles dispersion at different pH in the presence of wtfp (wild type fusion peptide of hemagglutinin) and mutfp (sequence of the fusion peptide of hemagglutinin with a mutation at the *N*-terminal glycine residue) are shown in Figure 4 [42]. The average M_w of the scattering particles for wtfp at neutral

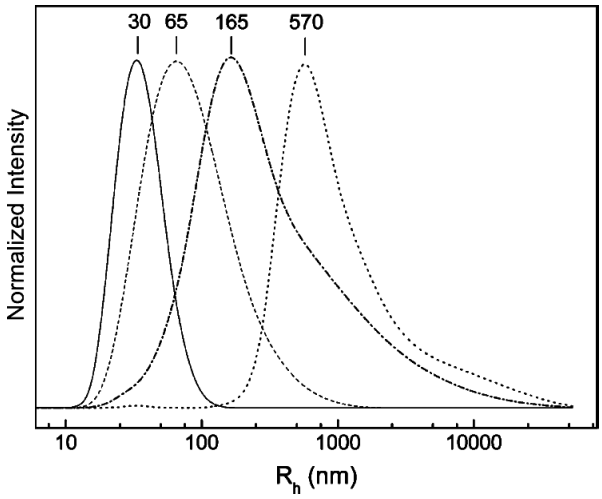

Figure 2 The size distribution of the PC : CHOL : DHP lipid vesicles (solid line) and at various guanidium/phosphate molar ratios: 40% (dashed line), 60% (dash-dotted line) and 80% (dotted line) [8]. The hydrodynamic radii (nm) of the peak values are shown for each distribution curve. Reproduced with permission of American Chemical Society.

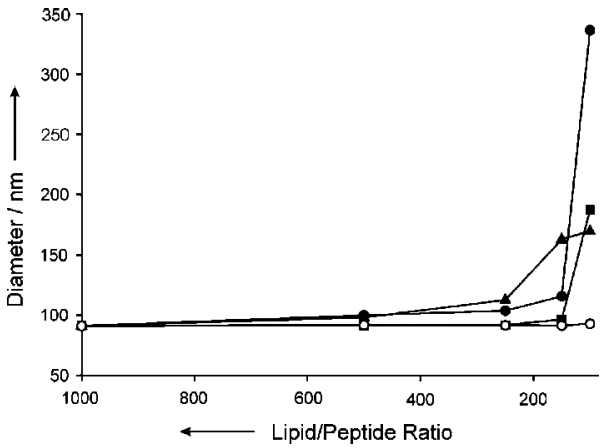

Figure 3 Hydrodynamic diameter of POPC : POPG (1 : 1) large unilamellar vesicles at different lipid/peptide ratios: basic peptide (open circles), variant 1 (closed circles), variant 2 (closed squares) and variant 3 (closed triangles) [9]. Reproduced with permission of Wiley-VCH Verlag GmbH & Co. KGaA.

pH (1.1×10^7 g mol^{-1}) is lower than that is obtained at acidic pH (3.5×10^7 g mol^{-1}). In the presence of mutfp there was no difference on the average M_w calculated at neutral and acidic pH (1.2×10^7 g mol^{-1}). Trivedi *et al.* showed by this methodology that wtfp had more effect on vesicular fusion than mutfp and, consequently, the important role of the *N*-terminal on fusion activity.

PRACTICAL ASPECTS

Independent of the application, some conditions must be fulfilled on light scattering experiments. The light

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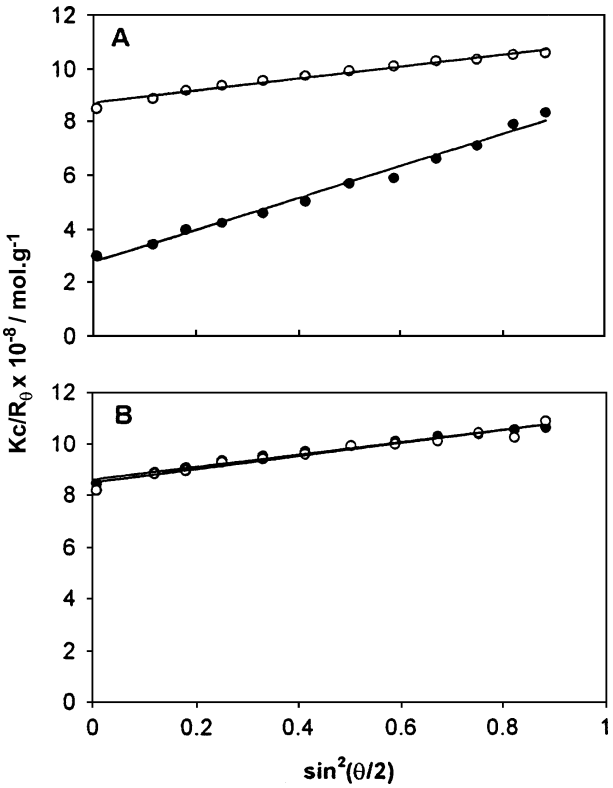

Figure 4 Determination of the average molecular weight, by SLS, for vesicular dispersion in the presence of fusion peptides wtfp (A) and mutfp (B), at acidic (closed symbols) and neutral pH (open symbols) [42]. Reproduced with permission of Elsevier.

source must be monochromatic and continuous, in order to have a signal of the same frequency. Laser is the only practical source of radiation that matches these conditions. Another requirement to do light scattering experiments is related to stray light, as it can be very problematic since uncontrolled light can mix with the light from the scattering volume and generate false signals [21]. The contamination by dust and other large particles can also be a severe problem, since scattering intensity depends on the sixth power of the radius of the particle. Filtration and centrifugation can be useful to overcome this problem [18]. Absorbing samples are a limitation to the light scattering measurements since the efficiency of the autocorrelation can be considerably reduced [21]. The loss of the signal is the major consequence of this limitation since there is attenuation of the incident and scattered light. Increasing the power of the incident light is not recommended since heating problems may occur and cause alterations in the diffusion coefficient. Another consideration must be done relatively to ζ-potential measurements, where changes in the pH should be avoided, due to the alterations in the protonation of peptide amino acid residues. Ionic strength should be kept low (not significantly above the physiologic saline concentration), as high conductivity causes Joule heating of the sample and affects the particle mobility as well as its integrity [36,37]. However, too low a conductivity would also impair the electrophoretic mobility. A minimal salt concentration (e.g. NaCl 0.1 mM) is needed for field stability and double layer definition) [37].

CONCLUSION

Peptides constituted by charged residues and an amphipathic structure can have a strong interaction with membranes. This interaction can be studied by spectroscopic techniques. Since many peptides do not adopt secondary structure, usually due to their short sequence, the interaction with membranes cannot be studied by circular dichroism or FTIR. Several peptides do not have fluorescent amino acid residues on their sequence, impairing the study by fluorescence techniques without the introduction of an external fluorophore. Thus light scattering can be very helpful as an alternative or additional method to identify the interaction between peptides and membranes, without affecting the integrity of the system. The interaction of peptides with membranes may be accompanied by vesicle aggregation, which can be measured by their size and scattering intensity, using DLS or SLS techniques. Charged residues in peptide structure can promote electrostatic interaction with membranes and lead to a variation of their charge. This membrane surface charge alteration in the presence of peptides can be efficiently followed by ζ-potential measurements.

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