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# pH fluctuations in unilamellar vesicles

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pH fluctuations in small unilamellar vesicles (SUV) are theoretically estimated. We determine that these fluctuations are dependent on macroscopic variables such as pH, $pK_a$, buffer group concentration and surface electrical potential. Based on a previously reported definition of buffer electrical capacitance (Procopio and Fornés, *Phys. Rev. E*, 1995, **51**, 829) an equation is derived which relates the pH fluctuation, the buffering power and the SUV size. We also derived an equation for the mean square charge thermal fluctuation using the fluctuation–dissipation theorem which coincides with that independently obtained by Kirkwood and Shumaker (*Proc. Natl. Acad. Sci.*, 1952, **38**, 863) using mechanical statistical methods. From our results it is inferred that measurement of pH in small systems has to be performed near the pK of the buffer groups in order that the fluctuational errors be minimized. We show that pH fluctuations diminish with increasing the size of the SUV and the predicted pH fluctuations decrease as the surface potential becomes less negative as a consequence of decreasing density of charged groups in the inner vesicular surface. It is predicted that measurable effects will appear on the fluorescence detection due to protonic fluctuations close to the pH sensing region of the probes.

## I. Introduction

Artificial unilamellar vesicles (UV) constitute models in which many transport problems have been studied in recent years.[1] The so-called small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) have been extensively employed to obtain information concerning the passage of different compounds through the cell membrane, such as fatty acids (FA) and to serve as vehicles for transport of pharmaceutically relevant substances and, more recently, for genetic materials.

One of the recent problems studied with the help of vesicular systems is the translocation of fatty acids across the bilayer matrix. Fluorescent probes (FL) have been trapped inside SUV and LUV serving as indicators of pH changes related to FA translocation across the vesicular wall. Half-times of vesicular acidification as low as 25 ms have been measured,[2] indicating a fast passage of FA across the bilayer vesicular wall. On the other hand, natural vesicular or vacuolar systems found in the cytoplasm resemble both in size and constitution the artificial vesicles and have important functions in metabolism allowing extension of conclusions obtained in artificial vesicular systems to biology.[1] Many studies using pH sensitive FL have detected intravesicular changes of pH due to FA entry.[3]

Vesicles in the range 25 to 100 nm, which are produced artificially or found naturally in the cell, are sufficiently small to provide a microscopic enclosure of near molecular dimensions. In such systems the time averaged concentrations of some chemical species and the corresponding intravesicular absolute numbers of ions or molecules may be exceedingly small, in effect often below 1 unit per vesicle. We explore, in this study, the consequences and implications of this for the proton behaviour as well as the meaning of pH in enclosures of near molecular dimensions.

We take, as an example, a SUV of 25 nm diameter and exclude the significant volume contribution of the bilayer (5 nm thickness) proper, which gives a fluid volume of 883 $nm^3$. Using the classical definition of pH, at pH 7.4, we obtain an average of about $5 \times 10^{-5}$ free protons inside the vesicular sac, what means that in a time average a vesicle contains a free proton only 1/20 000 of the time. Equivalently, in an ensemble of 20 000 vesicles only one has a free proton at a given instant. The laws of statistical mechanics establish the equivalence of the two descriptions. In this context the fluctuational means in this work are estimated in the frequency space in accordance to the fluctuation–dissipation theorem. A highly schematic rendering of the system being discussed is given in Fig. 1, drawn in approximate scale.

In this way it is more appropriate to define the *probability* that a given SUV contains 1 or 2 free protons at a given instant rather than simply defining an intravesicular pH.

Even inside natural vesicles having larger diameters such as 50 or 100 nm we still find that the calculated number of free protons defined by a physiological value of pH is well under unity. Interestingly enough many such natural systems have transporters in their membranes, such as proton pumps, which are necessarily exposed to a coarse grained proton concentration.

A poorly known issue is the typical proton residence time in either free or bound states. This certainly depends on the particular characteristics of the proton donator/acceptor as well as on the buffering properties of the medium. What makes this problem the more interesting is the fact that the interchange between free and bound proton states in a microscopic enclosure can supposedly be identified and measured by molecular machines utilizing or producing proton gradients across the vesicular membrane.[1–6] Such transporters are, in principle, capable of sensing the intense electrical field associated with the proton vicinity and to react appropriately. Also, the transporter itself might change substantially the proton "concentration" in its vicinity. pH sensitive fluorescent probes are also sufficiently fast so as to detect these pH fluctuational changes.



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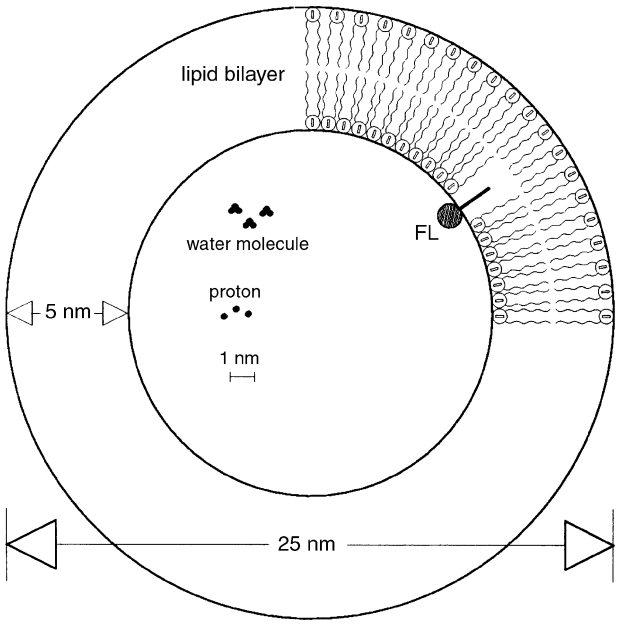


**Fig. 1** Schematic depiction of the system, consisting of a SUV and one membrane bound probe, drawn on approximate scale.

Reasoning from a purely physicochemical standpoint the addition or removal of one proton in a 25 nm diameter vesicle should respectively decrease or increase the pH by 4.3 units with respect to an average 7.4 value. Therefore, in such a small system, each protonation/deprotonation event should implicate a profound change in the physicochemical properties of the vesicular microambient.

### I.1. pH sensitivity of the fluorescence response

The proton translocation time across a distance equivalent to a vesicular radius is given by Einstein's well known diffusion equation:

$$t = \frac{\langle x^2 \rangle}{2D} \tag{1}$$

where $x$ is the travel distance and $D$ the diffusion coefficient. Making $x = 8$ nm and $D = 9.31 \times 10^{-9}$ m$^2$ s$^{-1}$ in eqn. (1) we obtain that the average proton translocation time is $t = 3.44 \times 10^{-9}$ s. This time is fast relative to the mean free proton dwell time which suggests that the proton can access essentially all the vesicular fluid during its "free" existence.

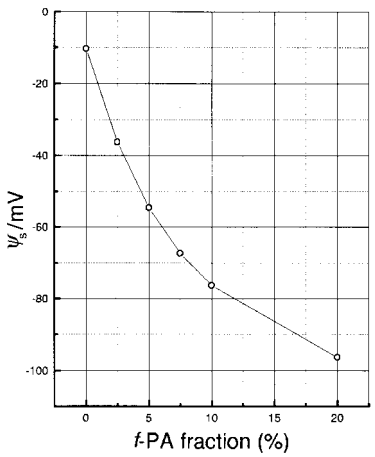


**Fig. 2** Surface potential, $\psi_s$, *versus* the fraction, $f$, of PL headgroups which are dissociable (data from Kraayenhof *et al.*[8]).

According to Gutman and Nachliel[7] the average lifetime of a free proton is given by

$$\tau = \frac{1}{k_a \times [A^-]} \tag{2}$$

where $k_a = (1\text{–}3) \times 10^{10}$ M$^{-1}$ s$^{-1}$ is the rate of association with the acceptor, $A^-$. In a solution containing 1 mM buffer we obtain $\tau = 30$ ns, which is comparable with the proton translocation time calculated from eqn. (1).

This establishes the radius of action of a pH sensitive probe as the whole vesicular sac and means that one fluorescent probe can react to the proton activity of the whole content of a SUV, during its response time. It is then apparent that there is a close matching between proton travel time and response time, which allows us to describe the internal vesicular surface and intravesicular fluid as a proton exchange system, governed by its own characteristics and isolated from external medium by the vesicular membrane, during the brief response time of the FL.

In order to sense a fluctuation of pH in a microscopic enclosure, a pH probe must be both small and fast enough. Biological ion channels, enzymatic molecules, pumps and other molecular machines constitute systems which are, in principle, capable of sensing such fluctuations. pH sensitive FL, constitute, on the other hand, ideal sensors of pH fluctuations, since their response time to pH changes is measured in the nanosecond time frame.

## II. General model

The model we consider consists of one pH sensitive FL, fixed at the internal surface of a SUV (Fig. 1). Phospholipid (PL) charged headgroups lining the inner bilayer leaflet are considered to be the sole source and sink of protons to the microambient sensed by the fluorophore. The vesicular sap is considered, in a first approach, to have a negligible buffering power, so that only the exchange of protons between the FL molecule and the charged PL groups is considered and the only buffering power is that provided by the PL headgroups. This setting is experimentally achievable, provided the vesicles are formed with no added buffers.

For calculating the buffer concentration, [Buffer group], we use data from Kraayenhof *et al.*,[8] Fig. 2, where the surface potential, $\psi_s$, is plotted as a function of the fraction, $f$, of PL headgroups which are dissociable. The corresponding buffer concentration will be:

$$[\text{Buffer group}] = \frac{N_{PL}}{N_A V_V} \tag{3}$$

where $V_V$ is the vesicular volume, $N_A$ is Avogadro constant and $N_{PL}$ is the total number of PL headgroups in the internal vesicular leaflet given by:

$$N_{PL} = \frac{A}{A_{PL}} f \tag{4}$$

where $A$ is the internal vesicular area and $A_{PL}$ is the phospholipid headgroup area ($\simeq 70$ Å$^2$).[9]

## III. Electrical properties of the system

### III.1. Ionizable groups

The vesicular surface is assumed to contain acidic ionizable groups at a density $1/S$, where $S$ is the surface area per acidic group. A fraction $\alpha$ (the degree of dissociation) of these groups will be dissociated so that the surface charge density is:

$$\sigma = -\frac{e_0 \alpha}{S} \tag{5}$$

and which will depend on the *dissociation constant*, $K_a$, for the surface ionizable groups of the lipid corresponding to the reaction:

$$A \rightleftharpoons AH^- + H^+ \tag{6}$$

whose equilibrium constant is given by:

$$K_a = \frac{[H^+]_s[A^-]}{[AH]} = [H^+]_s \frac{\alpha}{1-\alpha} \tag{7}$$

where $[H^+]_s$ is the hydrogen ion concentration at the surface of the lipid. This concentration is related to the one in the bulk solution, $[H^+]$, through the Boltzmann equilibrium condition,[9]

$$[H^+]_s = [H^+]e^{-y_s} = 10^{-pH}e^{-y_s} \tag{8}$$

or

$$pH_s = pH + 0.434y_s \tag{9}$$

where we have used $pH = -\log_{10}[H^+]$ and $y_s = (e_0/kT)\psi_s$ is the uniform reduced surface potential. The SI system of units was employed throughout, $e_0$ is the electron charge ($e_0 = 1.602 \times 10^{-19}$ C), $k$ is the Boltzmann's constant ($k = 1.281 \times 10^{-23}$ J K$^{-1}$) and $T$ is the absolute temperature. Substitution of eqn. (8) into eqn. (7) yields:

$$K_a = 10^{-pH}e^{-y_s}\frac{\alpha}{1-\alpha} \tag{10}$$

or, in terms of $pK_a = -\log_{10} K_a$

$$pK_a = pH + 0.434y_s - \log_{10}\frac{\alpha}{1-\alpha} \tag{11}$$

which is a well-known equation used in protein titration (see, *e.g.*, Tanford[10]).

### III.2. Electrical and polarization shift in the fluorescence spectrum

The electrical potential at the surface of a charged membrane or interface can be measured by a fluorescent probe sufficiently small as to be close to the surface. The interfacial ionization equilibria have been analysed in detail by Fernández and Fromherz,[11] followed by Kraayenhof *et al.*,[8] Thelen *et al.*,[12] Miyazaki *et al.*[13] and others. The $pK_a$ of any protonable probe group at a membrane interface, $pK_a^{int}$, will generally differ from the intrinsic value, $pK_a^0$, in bulk water due to thermodynamic differences in the ionization equilibria at the two locations. In general, the interfacial $pK_a^{int}$ is given by:[8,13]

$$pH - pH_s = pK_a^{int} - pK_a^0 = \Delta pK_a^{el} \pm |\Delta pK_a^{pol}| \tag{12}$$

where $\Delta pK_a^{el}$, is the *electrostatic shift* which is a function of the surface potential and is given by:

$$\Delta pK_a^{el} = -y_s/\ln 10 \tag{13}$$

and $\Delta pK_a^{pol}$ is the polarity-induced shift which has been attributed to the hydration of the amphiphilic probe at the interface,[9] and takes the positive sign for dissociation of a molecular acid ($HA \rightleftharpoons H^+ + A^-$) and the negative sign for the dissociation of a cationic acid ($HB^+ \rightleftharpoons H^+ + B$).

### III.3. Buffer capacity

The *buffering power*, $\beta$ of a solution is:[14,15]

$$\beta = \frac{dB}{dpH} \tag{14}$$

where $dB$ is the amount of base added to the solution, and dpH is the change in pH of the solution due to that base addition. The addition of acid to the solution is equivalent to a negative addition of base, -d$B$. The units of $\beta$ are mM per pH unit. In a closed system the total buffer concentration remains constant and the buffering power of a weak acid is given by:[15]

$$\beta = \frac{2.303[A_T]K_a a_H}{(K_a + a_H)^2} \tag{15}$$

where $[A_T]$ is the total concentration of weak acid and $a_H$ is the proton activity.

Substituting in eqn. (15), $a_H = [H^+]_s$, $\alpha = [A^-]/[A_T]$, $K_a = [H^+]_s\{\alpha/(1-\alpha)\}$, we obtain:

$$\beta = 2.3[A^-](1-\alpha) = 2.3[A_T]\alpha(1-\alpha) \tag{16}$$

Neglecting, in a first approach, the contribution of free buffers and considering only the PL headgroups buffers, we have $[A_T] = [\text{buffer group}]$, where [buffer group] stands for the concentration of protonable phopholipid headgroups, namely:

$$\beta = 2.3[\text{buffer group}]\alpha(1-\alpha) \tag{17}$$

From eqn. (11) we obtain:

$$\alpha = [1 + e^{-y_s}10^{(pK_a - pH)}]^{-1} \tag{18}$$

Substituting in eqn. (15), we obtain:

$$\beta = 2.303\frac{[\text{buffer group}]}{2 + e^{y_s}10^{(pH - pK_a)} + e^{-y_s}10^{(pK_a - pH)}} \tag{19}$$

or

$$\beta = 2.3\frac{[\text{buffer group}]}{2 + e^{y_s}\dfrac{K_a}{[H^+]} + e^{-y_s}\dfrac{[H^+]}{K_a}} \tag{20}$$

In general, for a number $>1$ of buffer groups, we will have:

$$\beta = 2.3\sum_i \frac{[\text{buffer group}]_i}{2 + e^{y_s}10^{(pH - pK_{ai})} + e^{-y_s}10^{(pK_{ai} - pH)}} \tag{21}$$

or

$$\beta = 2.3\sum_i \frac{[\text{buffer group}]_i}{2 + e^{y_s}\dfrac{K_{ai}}{[H^+]} + e^{-y_s}\dfrac{[H^+]}{K_{ai}}} \tag{22}$$

The total number of bound protons, $\bar{\nu}$ summed over all types of groups $i$ is:

$$\bar{\nu} = \sum \nu_i(1-\alpha_i) \tag{23}$$

where $\nu_i$ is the number of groups of type $i$ in the lipid.

$$\nu_i = N_A V_V[\text{buffer group}]_i \tag{24}$$

We can write eqn. (23) as:

$$\bar{\nu} = \sum_i \frac{\nu_i}{1 + e^{y_s}10^{(pH - pK_{ai})}} \tag{25}$$

or

$$\bar{\nu} = \sum_i \frac{\nu_i}{1 + e^{y_s}\dfrac{K_{ai}}{[H^+]}} \tag{26}$$

Any one of eqns. (25) and (26) describes the titration curve of the phospholipid headgroups with all nonelectrostatic effects on the dissociation described by $K_{ai}$.

In order to calculate the charge fluctuation associated with the proton we use the electrical equivalent of the buffer capacitance as defined by Procopio and Fornés,[16] namely:

$$C_{buffer} = \frac{\beta V_V}{\ln 10}\frac{F^2}{RT} \tag{27}$$

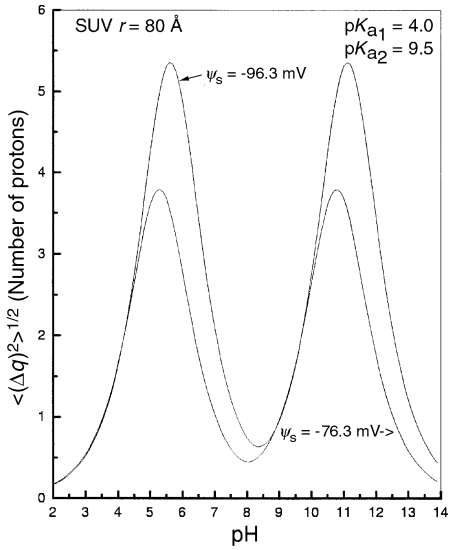


**Fig. 3** Average theoretically estimated protonic charge fluctuation, inside a hypothetical 8 nm internal radius SUV bearing phosphatidic acid groups on its internal surface for two surface potentials; representation of eqn. (29).

The magnitude of the proton related mean square charge thermal fluctuation is given by:[17,18]

$$\langle(\Delta q)^2\rangle = kTC_{\text{buffer}} \tag{28}$$

After substituting eqn. (27) in eqn. (28) using eqns. (21) and (22), we obtain:

$$\langle(\Delta q)^2\rangle = e_0^2 \sum_i \frac{\nu_i}{2 + e^{y_s}10^{(\text{pH}-\text{p}K_{ai})} + e^{-y_s}10^{(\text{p}K_{ai}-\text{pH})}}$$

or

$$\langle(\Delta q)^2\rangle = e_0^2 \sum_i \frac{\nu_i}{2 + e^{y_s}\dfrac{K_{ai}}{[\text{H}^+]} + e^{-y_s}\dfrac{[\text{H}^+]}{K_{ai}}} \tag{30}$$

Eqn. (30) with $y_s = 0$, coincides with that given by Kirkwood and Shumaker,[19] obtained independently using mechanical statistical methods, when calculating the forces between protein molecules in solution arising from fluctuations in proton charge and configuration.

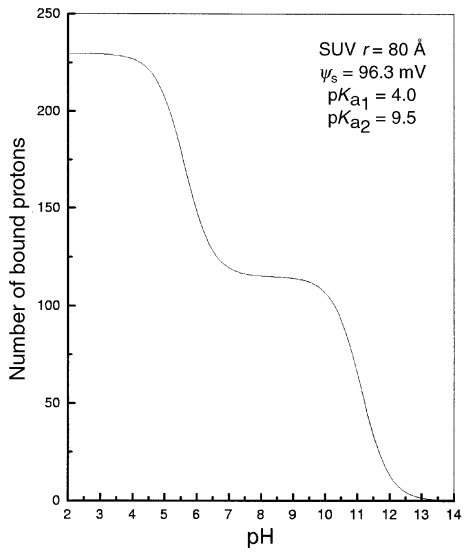


**Fig. 4** Titration curve, representation of eqn. (23).

From eqns. (20), (30) and (24), we obtain a relation between the mean square of the proton charge fluctuation, $\langle(\Delta q)^2\rangle$, and the buffering power $\beta$, namely:

$$\beta = \frac{2.3}{e_0^2 N_{\text{A}} V_{\text{V}}} \langle(\Delta q)^2\rangle \tag{31}$$

Fig. 3 depicts the average theoretically estimated protonic charge fluctuations, inside a hypothetical 8 nm internal radius SUV bearing phosphatidic acid groups on its internal surface for two surface potentials.

From eqns. (27) and (31), we obtain the relation between $C_{\text{buffer}}$ and the mean square of the proton charge fluctuation, $\langle(\Delta q)^2\rangle$, namely:

$$C_{\text{buffer}} = \frac{\langle(\Delta q)^2\rangle}{kT} \tag{32}$$

Differentiation of eqn. (25) with respect to pH, gives:

$$\frac{\partial\bar{\nu}}{\partial\text{pH}} = -\frac{2.303}{e_0^2} \langle(\Delta q)^2\rangle \tag{33}$$

*i.e.*, the slope of the titration curve, Fig. 4, at any pH gives the charge fluctuation at that pH. Eqn. (33) was also obtained by Timasheff,[20] for the titration of proteins.

## IV. pH fluctuations

The mean square of the fluctuating voltage will be:[17,18]

$$\langle(\delta V)^2\rangle = \frac{kT}{C_{\text{buffer}}} \tag{34}$$

Correspondingly, the fluctuation in pH is given by:[16] (29)

$$\langle(\delta\text{pH})^2\rangle^{1/2} = -\frac{e_0}{2.3kT} \langle(\delta V)^2\rangle^{1/2}$$

$$= \frac{e_0}{2.3(kTC_{\text{buffer}})^{1/2}} \tag{35}$$

or

$$\langle(\delta\text{pH})^2\rangle^{1/2} = \frac{e_0}{2.3\langle(\Delta q)^2\rangle^{1/2}} \tag{36}$$

or

$$\langle(\delta\text{pH})^2\rangle^{1/2} = \beta^{-1/2}(2.3N_{\text{A}} V_{\text{V}})^{-1/2} \tag{37}$$

A consequence of eqn. (37) is that the relation between the pH fluctuations for two small systems with identical volumes and different buffering powers, $\beta_1$ and $\beta_2$, is given by:

$$\frac{\langle(\delta\text{pH}_1)^2\rangle^{1/2}}{\langle(\delta\text{pH}_2)^2\rangle^{1/2}} = \sqrt{\frac{\beta_2}{\beta_1}} \tag{38}$$

In case that the only source of buffer groups are on the surface, as is our case, eqn. (36) gives the pH fluctuations on the surface of the buffering system produced by the protonic fluctuations. In order to relate surface pH fluctuations with those in the bulk phase we make use of eqns. (9) and (35), namely:

$$\delta\text{pH}_{\text{s}} = \delta\text{pH} + 0.434 \frac{e_0}{kT} \delta\psi_{\text{s}} \tag{39}$$

as, in this case, the variation of surface potential is caused by variation in pH, we obtain,

$$\delta\psi_{\text{s}} = -2.3 \frac{kT}{e_0} \delta\text{pH}_{\text{s}} \tag{40}$$

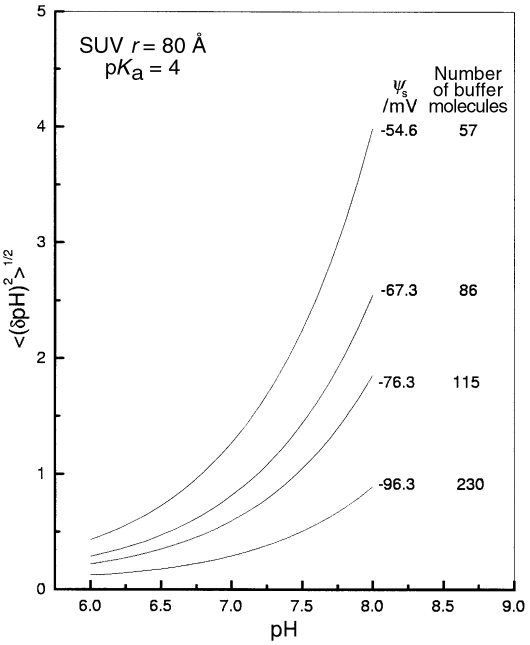


**Fig. 5** Average pH fluctuation as a function of the pH, inside a hypothetical 8 nm internal radius SUV with buffer groups having $pK_a = 4$.

From eqns. (39) and (40) and considering the statistical mean of the fluctuations, we obtain

$$\langle(\delta pH)^2\rangle^{1/2} = 2\langle(\delta pH_s)^2\rangle^{1/2} = 2\,\frac{e_0}{2.3(kTC_{buffer})^{1/2}} \quad (41)$$

This means that the mean pH fluctuation in the bulk is twice that on the surface.

Fig. 5 depicts the average pH fluctuations, as a function of the pH, inside a hypothetical 8 nm internal radius SUV with buffer groups having a single $pK_a = 4$ and Fig. 6 for SUV containing groups of phosphatidic acid with $pK_a = 4$ and $pK_a = 9.5$.[9] Different surface potentials were studied as determined by the fraction of protonable groups to total PL head-

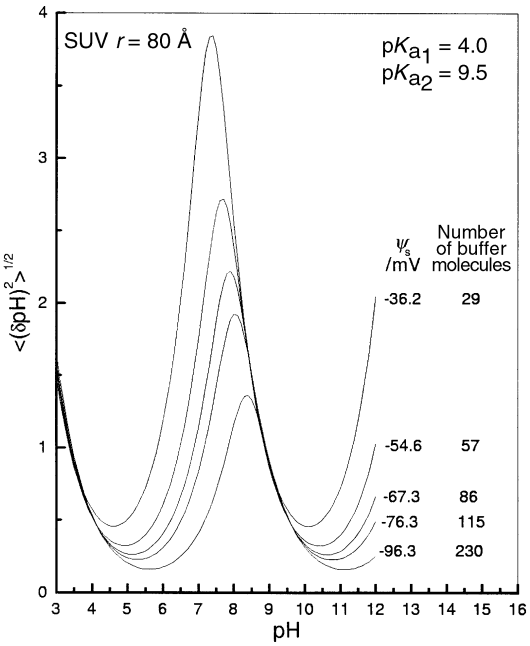


**Fig. 6** Average pH fluctuation as a function of the pH, inside a hypothetical 8 nm internal radius SUV containing groups of phosphatidic acid $pK_a = 4$ and $pK_a = 9.5$.[9]

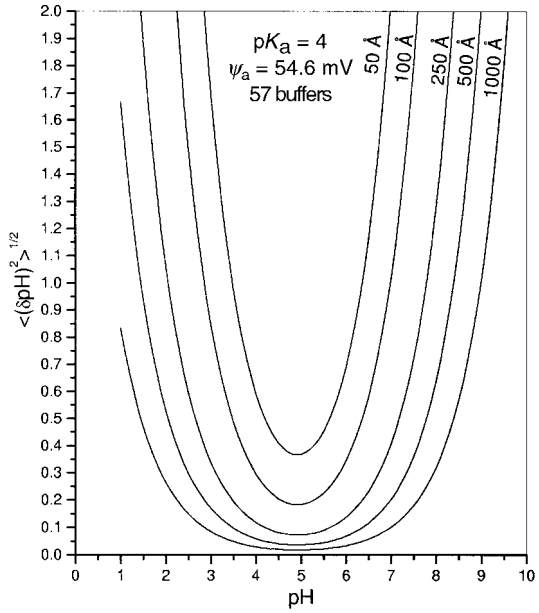


**Fig. 7** Variation of PH fluctuation with vesicular size.

groups according to ref. 8 and considering an average area of 70 Å$^2$ per headgroup.[9]

In Fig. 7 is shown the variation of pH fluctuations with vesicular size, for a given $pK_a = 4$, it is observed that they are almost negligible for vesicules with 1000 Å radius.

## V. Results and discussion

The study of fluctuations in small systems is important not only from an experimental point of view in knowing measurement intervals but also because they provide a deep insight into the occurring mechanisms and processes. Throughout this paper we observe that macroscopic defined variables such as buffering power and capacitance and titration curve can be expressed as functions of protonic charge fluctuations.

The validity of our approach through the electrical equivalent of the buffering capacity is reinforced by the coincidence of the results of eqn. (30) with the one obtained independently by Kirkwood and Schumaker[19] when calculating the forces between protein molecules, due to proton fluctuations.

From the preceding derivations we infer that these fluctuations are dependent on macroscopic variables such as pH, $pK_a$ and buffer concentration. Eqn. (38) gives the relation between the pH fluctuations for two solutions and the corresponding buffering powers and permits us to verify our theoretical predictions of pH noise measurement using a fluorescent probe.

In Fig. 3 the charge fluctuation profile for the same vesicular system is shown. We can observe here also that the maximum in fluctuation is shifted in relation to the corresponding pKs, due to the presence of a surface potential. The number of fluctuating protons increases with the surface potential due to the increase in the number of buffering molecules. The charge fluctuation, as depicted in this figure, is proportional to the derivative of the titration curve Fig. 4, see eqn. (33).

In Fig. 5 is plotted the pH fluctuation, $\langle(\delta pH)^2\rangle^{1/2}$, *versus* pH for given values of the surface potential and number of buffer groups for a lipid with $pK_a = 4$ (first pK of PA as reported in ref. 9) as obtained from eqn. (41). We can observe that pH fluctuations for pH values centered about 7.4 (6.4–8.4) are no larger than 1.5 pH units for surface potentials lower than $-50$ mV.

In Fig. 6 we have the same plot of Fig. 5 including the second $pK_a$ of PA (also data from ref. 9) where we have considered the same number of molecules for each $pK_a$. It can be

observed that there occurs a maximum in the pH fluctuation between the two $pK_a$ values. In the interval $[pK_{a_1}, pK_{a_2}]$ the pH fluctuation can reach several units of pH except for pH surrrounding the corresponding pKs. The shift with respect to the pKs observed in the minima of the curves is due to the surface potential. Also, from Fig. 6, we can infer that the measurement of pH in small systems has to be done near the pK of the buffer groups in order that measurement errors using fluorescent probes be minimized.

Comparing Figs. 3 and 6, we can observe, that the charge fluctuation has a maximum where pH fluctuations are at a minimum. This is a result of the fluctuation–dissipation theorem,[18] where the pH fluctuations can be viewed as a response of the medium to the protonic charge fluctuations of the system and vice versa, namely:

$$\langle(\Delta q)^2\rangle\langle(\delta V)^2\rangle = |kT)^2 \tag{42}$$

or

$$\langle(\Delta q)^2\rangle\langle(\delta \mathrm{pH})^2\rangle = \left(\frac{e_0}{2.3}\right)^2 \tag{43}$$

We can also observe from Figs. 5 and 6 that the pH fluctuations diminish with decreasing surface negativity due to decreasing the number of buffer molecules on the inner vesicular surface.

In Fig. 7 it can be observed that pH fluctuations diminish with increasing the size of the SUVs, this being an expected result in accordance with ref. 16.

From the above results we can predict that there will be measurable effects on the fluorescence detection due to protonic fluctuations close to the pH sensing region of the probes.

Many probes used as pH indicators show a shift in absorption and emission spectra between protonated and deprotonated forms, allowing the spectroscopic measurement of the acid dissociation constant $K_a$ in the ground state. The protonation and deprotonation reactions can also be examined in the excited states of the probe, under the assumption that the acid–base equilibrium may be established during the lifetime of the first singlet excited state, typically in the range of ns. As a consequence, the acid dissociation constants in the excited state can be determined using fluorometric techniques.[21,22] As pointed out by Rettig and Lapouyade,[23] the knowledge of prototropic reactions of chromophores in the ground and excited states results in probes which can monitor the pH of its microscopic surroundings. As a rule, absorption and emission spectra are largely dependent on pH over *circa* 2 pH units around its $pK_a$, and since probes with a wide range of $pK_a$ values are available, detailed monitoring of pH from 0 to 10 are possible. In particular, probes like hydroxycoumarin, pyranine, fluoresceine, seminaphthofluorescein (SNAFLs) and seminaphthorhodafluors (SNARFs) are employed as physiological pH indicators. Szmacinski and Lakowicz[24] indicated that the fluorescence sensor assays are based mainly on intensity measurements, following the intensity changes resulting from the different electronic properties and interactions with the surroundings presented by the protonated and deprotonated forms of the probes. It is suggested by these authors, however, that lifetime-based sensing methods would be developed to be applied mainly to overcome difficulties with intensity measurements as for example in the case of turbid samples, imprecise or dirty optical surfaces or variations in optical alignment of different samples. A use could be made from the fact that many probes display changes in fluorescence lifetime on protonated/deprotonated states. For example, the SNAFL-1 probe has a lifetime of 1.19 ns in the acid form and 3.74 ns in the basic form.[24] A heterogeneous decay of fluorescence in time-resolved experiments performed in a sample in which there is a mixture of two populations of the probe having different lifetimes would then be expected. With the instrumentation presently available, it is possible to measure lifetimes with precision in the picosecond range and also to discriminate the different contributions to a heterogeneous fluorescence decay. Thus, in the context of the present work, and with adequate choice of the probes, it would be possible to detect pH fluctuations within the small internal volumes of vesicles, using fluorometric techniques. Natural candidates for such probes are lipophilic derivatives of hydroxycoumarins and fluorescein. Using probes as described by Fernández and Fromherz[11] and Kraayenhof *et al.*,[8] that monitor precise positions in the neighbourhood of the membrane surface, one could follow pH fluctuations in nanometer space range and picosecond time range.

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