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A PROCEDURE FOR ESTIMATING THE SURFACE POTENTIAL OF CHARGED OR NEUTRAL MEMBRANES WITH 8-ANILINO-1-NAPHTHALENESULPHONATE PROBE

ADEQUACY OF THE GOUY-CHAPMAN MODEL

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Using the fluorescent anion 8-anilino-1-naphthalenesulphonate (ANS) for determining the membrane surface potential necessitates that the intrinsic affinity constant K_i for the ANS sites be known. Two methods are presented which do not rely on a determination of K_i at high ionic strength. They are respectively applied to neutral membranes (egg phosphatidylcholine liposomes) and highly charged natural ones (horse bean microsomes and liposomes from their phospholipids). The value of K_i appears to be insensitive to the level of occupancy of the sites, the KCl concentration and the pH in large ranges. Furthermore, the classical Gouy-Chapman model seems to describe correctly the whole set of data, provided apparent mean molecular areas larger than the published crystallographic ones are admitted.

Introduction

This paper focuses on two points: the estimation of the surface potential of membranes from ANS fluorescence measurements and the description of the results with the Gouy-Chapman model. Two different classical situations were examined, corresponding to highly charged natural membranes and neutral ones. The adsorption of the univalent anion ANS on biological membranes may be described by first-order saturation kinetics [1–6].

$$\overline{ANS} = \frac{n \cdot [ANS]_\infty}{K + [ANS]_\infty} \quad (1)$$

where $\overline{ANS}$ is the amount of adsorbed ions, n is the number of membrane sites, K is the apparent dissociation constant and $[ANS]_\infty$ is the bulk concentration of the free ions at infinite distance from the membrane. It is now well established that the value of the apparent dissociation constant is dependent on the surface electrostatic potential, ψ_0. The membrane sites experience the local ionic concentration

$$[ANS]_0 = [ANS]_\infty \exp(e\psi_0/kT) \quad (2)$$

where e is the elementary charge, and k and T have their usual meaning.

The charge brought by ANS is negligible compared to the intrinsic charge of the highly charged natural membrane [4,5]. In this case, the titration of the membrane by ANS in a given ionic condition can be described by Eqns. 1 and 2 with constant ψ_0. From Eqns. 1 and 2, it can be shown that the ratio $(K)_2/(K)_1$ of the apparent dissociation constant values determined for two different

Abbreviations: ANS, 8-anilino-1-naphthalenesulphonate; Hepes,4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PC, phosphatidylcholine; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl.

ionic conditions is given by:

$$(\psi_0)_1 - (\psi_0)_2 = \frac{kT}{e} \ln \frac{(K)_2}{(K)_1} \quad (3)$$

This relation has been used for estimating the effects on ψ_0 of the changes of the net surface charge [7–9].

If an ionic condition may be found for which $\psi_0 = 0$, then K is the intrinsic dissociation constant, K_i, and Eqn. 3 becomes:

$$\psi_0 = \frac{kT}{e} \ln \frac{K_i}{K} \quad (4)$$

This method for the estimation of ψ_0 is very simple but its validity is critically dependent on the accuracy of the estimation of K_i. It has been used [2,5,10–12] to estimate the surface potential with the anionic fluorescent probe, ANS, assuming that K_i is directly measurable in the presence of a high concentration of KCl (e.g., 1.6 M) which is supposed to screen ψ_0 totally. The results we present here suggest that this assumption may be not valid. We propose a method which does not rely on it.

The Gouy-Chapman model conveys the basic concepts for the electrostatical interactions at the surface of the membranes. In the special case of 1 : 1 electrolytes, the surface charge density σ is predicted to be related to ψ_0 by:

$$\psi_0 = \frac{2kT}{e} \operatorname{arc\,sinh}\left(\frac{\sigma}{B\sqrt{C}} \right) \quad (5)$$

where C is the bulk concentration of the salt, $B = \sqrt{8\varepsilon RT}$, R and T have their usual meaning and ε is the dielectric constant of the medium. The effect of the ionic strength on ε is taken into account [13].

In the case of neutral membranes, the surface charge is brought by ANS itself so that ψ_0 is not constant during the titration by the probe:

$$\sigma = \frac{\overline{ANS}}{A} \quad (6)$$

where A is the area of the membrane. It follows that K is not constant for a given ionic condition, and the above procedure is not usable. One method consists in determining by an iterative procedure the values of K_i and A which simultaneously satisfy Eqns. 1, 2, 5 and 6 for the whole set of titration steps [10]. We present here a non-iterative method for calculating ψ_0 which does not imply the estimation of K_i and A.

Materials and Methods

The preparation of the microsomes from roots of horse bean (*Vicia faba* L., var. *minor*) was described in a preceding paper [14]. Lipids were prepared according to the method of Bligh and Dyer [15]. Phospholipids were purified by preparative thin-layer chromatography [16]. They were then converted to their potassium salts by repeated washes with 0.1 M HCl followed by 0.001 M EDTA and 1 M KCl at pH 9 [17]. They were identified by thin-layer chromatography [18,19] and assayed for phosphorus [20]. Egg phosphatidylcholine was extracted according to the procedure of Bergelson [21] and purified by column chromatography on silicic acid. Lipomes were prepared as follows: chloroformic solutions of phospholipids were dried under nitrogen and dispersed in the appropriate aqueous solution (final concentration 0.03 mM) and sonicated during 15 min. The method for the fluorescence measurement was described elsewhere [14,22]. The value of the quantum yield of ANS bound on PC liposomes was 0.33. The value for horse bean liposomes was not determined.

Results

The effect of the ionic strength on the affinity of the sites for ANS was studied by using this probe for titrating the bilayers obtained from the phospholipids of the microsomes. These titrations were performed in various KCl concentrations. The values of the apparent dissociation constant were determined from the slopes of the linear Scatchard plots (Fig. 1). An increase in the ionic strength up to 0.25 M resulted in a steep decrease of K. Thereafter K showed little variation with changes in the KCl concentration. This effect is known to be due to the screening effect of the salt, which attenuates the repulsive interaction of ANS and the net negative surface charge of the membrane [10,23]. The shape of the graph suggests that

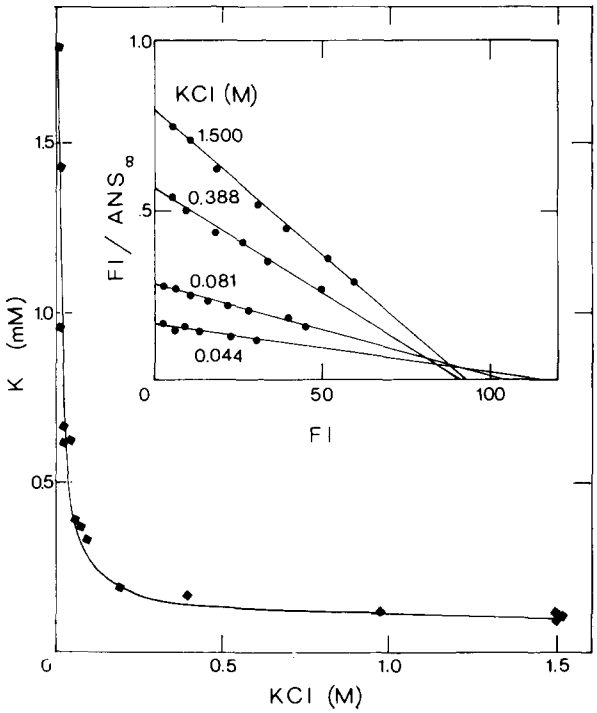

Fig. 1. Effect of the KCl concentration on the apparent dissociation constant of the ANS sites of the horse bean liposomes. The theoretical curve is calculated from Eqn. 4 with $K_i = 73$ μM (Table I) and ψ_0 obtained from Eqn. 5 and $\sigma = 1.6 \cdot 10^{-3}$ charge$\cdot$Å^{-2}. Inset: Scatchard plots for the binding of ANS at different ionic strengths. FI is the specific fluorescence intensity of the ANS bound in arbitrary unit. The medium contained 10 mM Hepes (pH 7.4), 5 mM mercaptoethanol and 0.1 mM EDTA.

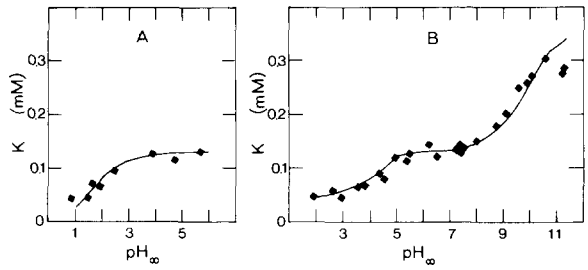

Fig. 2. Effect of the pH on the apparent dissociation constant of the ANS sites. (A) Liposomes from horse bean phospholipids. (B) Horse bean microsomes. The theoretical curves are calculated with the Gouy-Chapman model, taking account for the effect of the pH on the surface charge as described in the text (see Ref. 14). The medium contained 10 mM Hepes, 5 mM mercaptoethanol, 0.1 mM EDTA and 1.5 M KCl. The pH was adjusted with HCl or KOH.

the depolarization is nearly complete in the higher concentration range. In order to check this hypothesis, phospholipid bilayers and microsomes were titrated by ANS in presence of 1.5 M KCl at various pH values. The effect of the pH remains clearly visible at this high ionic strength (Fig. 2). On an a priori ground, the effect of the pH on the affinity of membranes for ANS may be considered as being mediated by the net surface charge, via the acido-basic equilibria, and/or by conformational changes of the adsorption sites. Such conformational changes would probably result in modifications of the quantum yield. The quantum yield of ANS adsorbed on microsomal membranes was determined [22] at various pH values in presence of 1.5 M KCl. It was found to be virtually independent of the pH in the range studied (pH 2.7 to 7.4). The mean value and the 95% confidence limits of seven determinations were 0.388 ± 0.034. Similar results have been published for various membranes [2,3]. Furthermore, we have shown that K_i is insensitive to the pH [14].

Therefore, it appears that the effect of the pH on the affinity for ANS determined in 1.5 M KCl may not be easily explained by conformational changes and that it is worthwhile to examine the hypothesis of a residual surface potential at high ionic strength.

The persistence of a surface potential on natural membranes at high KCl concentration is at variance with the assumption of a complete depolarization made by several authors [5,11,12], who extrapolated this conclusion from titration of neutral PC bilayers by ANS [2,10]. In order to analyze the discrepancy between these works and ours, we performed such titrations of PC bilayers (Fig. 3). The double reciprocal plot and the Scatchard plot bent at high values of ANS binding. The linear Hill transformation suggested the existence of an anticooperative binding (Hill coefficient 0.85).

Analysis of the experimental data

In the case of neutral PC membranes, the surface charge is brought on by ANS itself and can be estimated from measurements of the fluorescence intensity and quantum yield provided the membrane area is known. Considering the molecular area obtained from diffraction studies, it is possi-

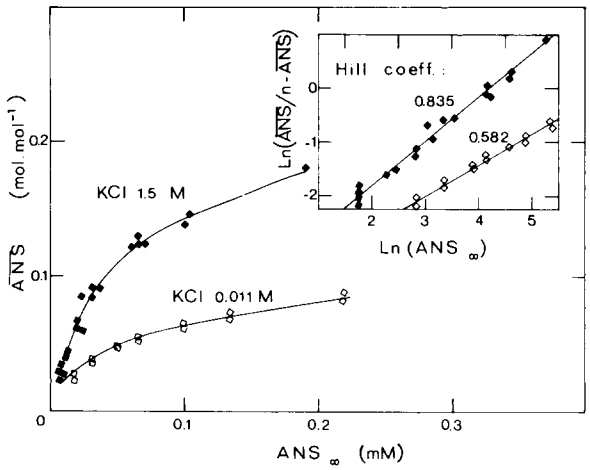

Fig. 3. Binding of ANS on the liposomes of egg phosphatidylcholine. The amount of binding is calculated from the fluorescence intensity and the quantum yield (0.33). The theoretical curves are calculated from the classical Gouy-Chapman model (Eqn. 5) by an iterative procedure [10]. The number of sites used is 0.25 mol per mol phospholipid, the molecular area is 80 Å^2 and the intrinsic dissociation constant is 44 μM. Insert: Hill plot of the data.

ble to determine the value of the intrinsic affinity constant for ANS which allows the direct fitting of the experimental data to the Gouy-Chapman model. This procedure led Haynes [10] to claim that this model was not directly usable, due to discreteness effects. Incidentally, the fitting of the binding data with an empirically modified model led to the conclusion that the surface potential is negligible in 1.6 M KCl.

In the case of the highly charged natural membranes, the surface charge may not be generally calculated. It is necessary to know K_i in order to calculate ψ_0 from Eqn. 4. In this context, the accuracy of the estimation of K_i from the apparent constant K at high ionic strength depends crucially on the assumption of a complete surface depolarization. For highly charged membranes, the charges carried by ANS are negligible as compared to the ones borne by the membrane itself. This has been experimentally shown by electrophoretic measurements [4]. Thus, the observed linearity of the double-reciprocal plot [5] only indicates the absence of interaction between the adsorbed probe molecules and not a zero value of the surface potential.

We have used two calculation procedures for estimating K_i which do not rely on the above assumption nor on experimental measurements in extreme ionic conditions. The only assumptions are (i) the validity of the Gouy-Chapman model, and (ii) the insensitivity of the membrane area to the ionic strength. They will be discussed later.

Natural membranes

The procedure for natural membranes is as follows. By solving Eqn. 5 for σ and replacing ψ_0 by Eqn. 4, one obtains:

$$\frac{\sigma}{\sqrt{8RT}} = \sqrt{\varepsilon}\,\sqrt{[\mathrm{KCl}]}\,\sinh\left(\tfrac{1}{2}\ln\frac{K_i}{K}\right) \tag{7}$$

This relation is written for the two ionic conditions $[\mathrm{KCl}]_1$ and $[\mathrm{KCl}]_2$ and the two graphically determined corresponding values of K. Eliminating the left hand terms between the two sister-relations and rearranging gives:

$$K_i = \frac{(K)_1\sqrt{r((K)_2/(K)_1)} - (K)_2}{\sqrt{r((K)_2/(K)_1)} - 1} \tag{8}$$

where r is the ratio $\varepsilon_1 \cdot [\mathrm{KCl}]_1/\varepsilon_2 \cdot [\mathrm{KCl}]_2$.

Neutral membranes

In the case of neutral PC bilayers, the graphic determination of the apparent dissociation constant is not possible due to inconstancy of surface charge. From the two experiments 1 and 2 with $[\mathrm{KCl}]_1$ and $[\mathrm{KCl}]_2$, one arbitrarily chooses two bulk concentrations $[\mathrm{ANS}]_1$ and $[\mathrm{ANS}]_2$ which give the same binding (i.e., the same surface charge density), and thus correspond to the same surface concentration $[\mathrm{ANS}]_0$. For each ionic condition $[\mathrm{KCl}]_j$, ψ_0 is expressed as:

$$\psi_{0j} = \frac{kT}{e}\ln\left(\frac{[\mathrm{ANS}]_0}{[\mathrm{ANS}]_j}\right) \tag{9}$$

Solving Eqn. 5 for σ, replacing ψ_0 by Eqn. 9 and rearranging gives:

$$[\mathrm{ANS}]_0 = \frac{[\mathrm{ANS}]_1\sqrt{r([\mathrm{ANS}]_2/[\mathrm{ANS}]_1)}}{\sqrt{r([\mathrm{ANS}]_2/[\mathrm{ANS}]_1)} - 1} \tag{10}$$

The value of $[\mathrm{ANS}]_0$ obtained from this relation gives ψ_0 via Eqn. 9. It can be also used together

with the measured amount of bound ANS and the number of sites ($n = 0.25$ mol per mol PC, from Ref. 2) to calculate K_i from Eqn. 1 applied to surface conditions.

The practical procedure is as follows: the two experimental binding curves are linearized with the Hill transformation (Fig. 3, insert) in order to obtain two empirical fitting curves. For each experimental point in one ionic condition, the related theoretical point which corresponds to the same binding in the other ionic condition is calculated from the appropriate fitting curve. The successive estimations of K_i are calculated as indicated above from the pairs $[ANS]_1$, $[ANS]_2$ obtained in that manner.

Application

The mean estimations of K_i are given in Table I. The relatively small dispersion of the individual values suggest that K_i is not affected by the level of occupancy of the sites by ANS. In the case of microsomes, the data used for estimating the mean K_i were obtained at various pH values in the range 2–11.5 [14]. In the case of horse bean bilayers, the calculations were performed with the experimental points of Fig. 4, in which the concentration of KCl ranges from 5 mM to 1.5 M. Thus, it appears that K_i is insensitive to the pH as well as to the ionic strength.

The surface potentials are shown on Fig. 4 (horse bean bilayers) and Fig. 5 (PC bilayers).

The whole set of data in Figs. 1–5 was simulated with the help of the Gouy-Chapman model by using the K_i values in Table I and considering the molecular area as a semiadjustable parameter. The surface charge of the horse bean bilayers was estimated from the known phospholipidic composition and the pK values of the ionic groups [14,17], with a mean molecular area of 120 Å^2. This allowed us to calculate the curve $\psi_0 = f(\mathrm{KCl})$ (Fig. 4) by using the Gouy-Chapman model (Eqn. 5), without relying upon the ANS-binding data.

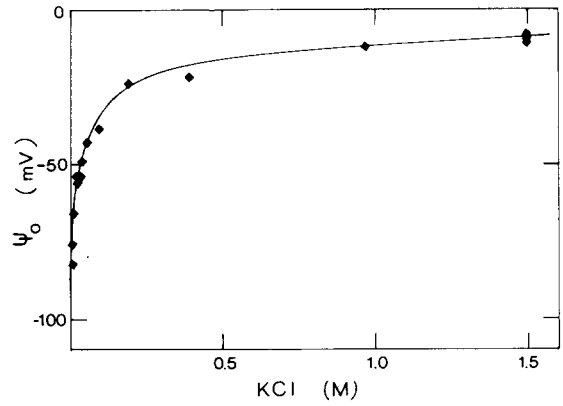

Fig. 4. Effect of the KCl concentration on the surface potential of liposomes from horse bean phospholipids. The values are calculated from the experimental determinations of K (Fig. 1) and from $K_i = 73$ μM (Table I) using the Gouy-Chapman model (Eqn. 5), with $\sigma = 1.6 \cdot 10^{-3}$ charge·Å^{-2}. This value was estimated from the known phospholipid composition [17], using 120 Å^2 per molecule (0.19 charge per molecule). For the composition of the medium, see Fig. 1.

TABLE I

APPARENT AND INTRINSIC AFFINITIES FOR ANS, AND SURFACE POTENTIALS ESTIMATED FROM Eqn. 4

The dissociation constants K_i and K are expressed as μM, the surface potential ψ_0 as mV and the surface charge density σ as 10^{-3} elementary charge·Å^{-2}. The pH is 7.4.

Material	Intrinsic value K_i	1.5 M KCl		0.15 M KCl				
		Apparent value K	Calculated from K_i ψ_0	Apparent value K	Calculated from K_i		Calculated from K in 1.5 M KCl	
					ψ_0	σ	ψ_0	σ
Horse bean								
Bilayers	73 ± 2	105	−9.3	210	−27.1	−1.6	−16.6	−0.9
Microsomes [a]	83 ± 8	120	−9.5	260	−29.2	−1.7	−17.8	−1.0
PC bilayers	44 ± 6	66 [b]	−10.4	–	–	–	–	–

[a] From Ref. 14.

[b] Value at half-saturation of sites.

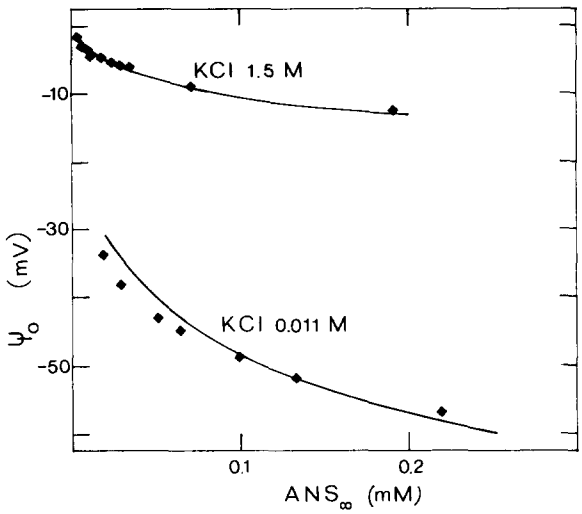

Fig. 5. Surface potential of neutral egg PC liposomes due to the ANS binding. The potential is estimated from Eqns. 9 and 10 applied to the results of Fig. 3. The theoretical curve is calculated by an iterative procedure (see Ref. 10) using the classical Gouy-Chapman model, with the parameters given in Fig. 3.

The curve $K = f(\text{KCl})$ was then calculated from Eqn. 4 by using $K_i = 73$ μM (Table I). The procedure for the calculation of the curve $\psi_0 = f(\text{pH})$ for the microsomes was described in a preceding paper [14]. The curve $K = f$ (pH) for this material was obtained from Eqn. 4. In the case of the neutral PC bilayers, the surface potential due to the bound ANS was calculated by an iterative procedure [10], using Eqn. 5 and Eqn. 1 applied to the surface conditions, $n = 0.25$ mol · mol^{-1} [2], $K_i = 44$ μM (Table I) and a molecular area of 80 Å^2.

Discussion

The first important point is that for each material a unique value of K_i may be used to fit correctly the experimental results under various ionic conditions. This observation supports a posteriori the assumption of the validity of the Gouy-Chapman model with a constant membrane area for estimating K_i. The second important point is that a residual potential is still present in 1.5 M KCl, as attested by the fact that the theoretical model correctly predicts the kinetic of the 6-fold variation of K under the effect of the pH at this high ionic strength (Fig. 2). The residual potential is small at pH 7.4 (approx. 10 mV). Nevertheless, the use of the value of K measured in 1.5 M KCl instead of K_i would lead to an approximately 2-fold underestimation of σ in 0.15 M KCl (Table I).

In summary, the Gouy-Chapman model seems to describe correctly the electrostatical interactions of ANS and the microsomes, the liposomes obtained from their phospholipids and the liposomes from egg PC, in a large variety of ionic conditions. It is worthwhile to note here that this conclusion was obtained by using relatively large molecular areas (e.g., 80 Å^2 for egg PC).

An opposite conclusion was reached by Haynes using a crystallographic value (60 Å^2) for egg PC [10]. The discrepancy between the predictions of the model and the observed results at low ionic strength and high charge density was attributed to discreteness effect. However, as pointed out by McLaughlin [24], the theoretical analysis of this effect predicts that it becomes important in the opposite condition, when the Debye length, X^{-1}, diminishes below the charge separation [25].

In our case, the surface charge density of horse bean liposomes ($1.6 \cdot 10^{-3}$ elementary charges · Å^{-2}) would correspond to a 27 Å spacing in a hexagonal array. The ratio b/χ^{-1} would be 0.3 in 0.001 M KCl and 11 in 1.5 M KCl. Nevertheless, the smeared charge model correctly describes the results in the full range of KCl concentrations. It must be pointed out that in the case of the microsomes and the liposomes obtained from their phospholipids, the microscopic potential at the ANS-binding site appears to be the same as the macroscopic surface potential estimated for a homogeneous mixture of five different ionic groups borne by the lipids and at least two others borne by the protein [14].

The non-linearized approach of the discreteness effect [26] predicts that for all phenomena involving the space average surface potential (as opposed to those involving localized structures), the smeared charge theory gives an accurate description, provided that the distance to the membrane is greater than the width of the adsorption layer.

Thus we are led to hypothesize that the potential determined from the ANS binding corresponds (i) to the average surface potential, (ii)

outside the first hydration layer. The mean molecular areas used in our simulations as in others are relatively large as compared to the ones obtained from crystallographic data. For instance, the value we were led to use (80 $Å^2$ for egg PC) was precisely the same that the one retained for describing the surface potential of this material from the binding of the cationic ESR probe TEMPO [27]. The published crystallographic values for egg PC vary from 60 $Å^2$ [28] to 70 $Å^2$ [29,30] or even 76 $Å^2$ for fully hydrated bilayers [31]. We have not found such data for the mean molecular area of phospholipids in complex nutural mixtures from plants. But as they are highly insaturated and charged [17], their mean molecular area is probably quite high.

Using 60 $Å^2$ for egg PC, Haynes [10] showed that the Gouy-Chapman model would led to an overestimation of the bilayer surface polarization. This apparent inadequacy of the model led him to introduce an empirical correction for the surface charge density related to the ionic strength.

We were faced with the same situation, but we showed that the Gouy-Chapman model may be retained provided that relatively large molecular areas were admitted. The necessity of introducing such a formal reduction of the surface density in the model may be due to the fact that the potential is probed at some distance from the surface. The experimental study of this hypothesis will be presented in a following paper.

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