Charge Reversal in Anionic Liposomes: Experimental Demonstration and Molecular Origin

Alberto Martín-Molina,[1] César Rodríguez-Beas,[1] and Jordi Faraudo[2,*]

[1]Grupo de Física de Fluidos y Biocoloides, Dept. de Física Aplicada, Universidad de Granada, 18071 Granada, Spain
[2]Institut de Ciència de Materials de Barcelona (ICMAB-CSIC), Campus de la UAB, E-08193 Bellaterra, Spain

(Received 14 September 2009; published 23 April 2010)

We present experimental and simulation evidence for a new mechanism of charge reversal operating only for ions capable to penetrate into soft interfaces. It is based on the preferential solvation of counterions by amphiphilic molecules and hydration water rather than by bulk water. This mechanism does not require high surface charge densities and it is not affected by the addition of 1:1 salt. This behavior is opposite to that observed in systems as diverse as microfluidic channels or latex colloids. The robustness of the mechanism to physiological amounts of 1:1 salt suggests a significant impact in processes involving ion-amphiphile interaction in salty water (typical, e.g., of biophysics).

DOI: 10.1103/PhysRevLett.104.168103 PACS numbers: 87.16.dr, 47.57.jd, 87.15.ap

The interaction of interfaces with electrolyte is of key importance in determining physico-chemical properties and functionality of systems as diverse as macromolecules, colloids, membranes or microfluidic devices [1]. The case in which the counterions are multivalent is attracting a great deal of experimental and theoretical interest due to their ability to induce complex and rich new phenomena [2–4]. A paradigmatic example is the charge reversal phenomenon (also known as overcharging or charge inversion) in which counterions are attracted to a charged interface in excess of its own bare charge [2,5]. The driving force for this counterintuitive phenomenon is still subject to debate [5,6], but there is strong evidence supporting the view that ion-ion correlations play a major role [2]. Calculations including ion-ion correlations are consistent with the concentrations of multivalent counterions required to obtain charge inversion in DNA [7], silica nanochannels [8], latex colloids [9] and calcium silicate hydrate nanoparticles [10]. According to the ion-ion correlation mechanism, charge reversal shows two outstanding features. First, it is found only for highly charged surfaces [2,11] and secondly, the effect is strongly weakened after addition of background 1:1 electrolyte [12] (as demonstrated both by experiments [8,13] and computer simulations [14,15]).

A common feature in all these previous experimental examples of charge reversal is that ions are able to accumulate at the solid-liquid interface but being always restricted to be in the water phase (i.e., they are unable to penetrate into the solid phase). The situation is different in the case of interfaces such as those found in Langmuir monolayers, membranes, liposomes and nanoparticles or colloids functionalized with surfactants. Experimental techniques [16–18] reveal that in these cases, small ions are able to penetrate to the nonaqueous side of the interface. As a consequence, the phenomenology of ionic interactions observed in these systems highly depends on structural or chemical details of the system [19–21], so current models of charge reversal (based on general features of ion-ion correlations) cannot be applied [6]. Our objective in this Letter is to identify (by combining electrophoretic measurements and simulations) a different mechanism for charge reversal in these systems (soft interfaces). As we will see, this mechanism is clearly distinguishable from ion-ion correlations since it is robust to physiological concentrations of added background 1:1 electrolyte and it does not require high surface charge.

The experimental systems considered in this work will be liposomes based either on anionic phosphatidylserine (PS^-) or zwitterionic phosphatidylcoline (PC) lipids (see the chemical formula in Fig. 1). Both lipids are extremely important in industrial applications (drug delivery systems, nanoreactors,...) and biophysics [22]. They are the most abundant lipids of the plasma membrane, giving rise to the major contribution to its surface potential [23].

Let us first discuss our results for PS^- liposomes (the preparation of the liposomes is described in the accompanying EPAPS material [24]). The electrophoretic mobility of the liposomes at 25 °C was measured with a *ZetaPALS* apparatus (Brookhaven Instruments, USA) as a function of $La(NO_3)_3$ concentration both in absence of background electrolyte and with 100 mM of added $NaNO_3$. All experiments were made at $p\mathrm{H} = 5.4$ so the PS molecule had a total charge of $-e$ (PS has three charged groups with $p\mathrm{Ka} = 1$, 3.6 and 9.8) and La cations had $+3e$ charge since they are not significantly hydrolyzed (La has a hydrolysis constant K_H of $\log_{10} K_H \approx 8.6$ [25]).

We observe (see Fig. 1) that in both cases electrophoretic mobility reverses its sign at $[La(NO_3)_3] \approx 10^{-4}M$, i.e., charge reversal is observed at low concentrations of trivalent counterions. The effect of the 1:1 salt is typical of an indifferent electrolyte (see curve I of Fig. 6.8 of Ref. [26]): it reduces the absolute value of the mobility leaving the point of zero mobility unaltered. This result is opposite to that obtained for negatively charged latex microspheres [11,13], which are a classical model system for colloidal behavior. Charge reversal is observed at $[La(NO_3)_3]$ between 10–20 mM in absence of background electrolyte. After addition of $[NaNO_3] = 0.1M$, a completely different

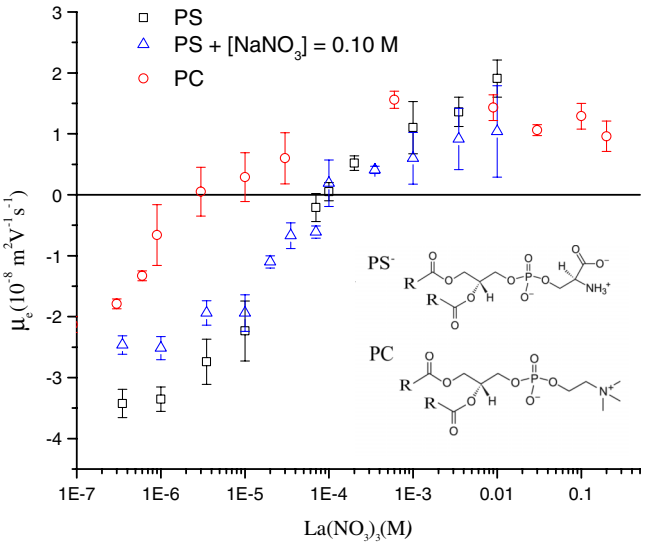

FIG. 1 (color online). Electrophoretic mobility μ_e of PS^- and PC liposomes as a function of $[La(NO_3)_3]$. Squares correspond to PS^- (no background electrolyte), triangles to PS^- with 100 mM of $NaNO_3$ as a background electrolyte and circles correspond to PC (no background electrolyte). Inset: Chemical structures of the PS^- and PC lipids.

behavior is observed. For $[La(NO_3)_3] < 20$ mM, the mobility is higher than that observed in absence of background electrolyte, and strongly decreases (being essentially zero) for larger $La(NO_3)_3$ concentrations (no charge reversal can be identified). This behavior observed for latexes, is typically expected for charge reversal induced by the ion-ion correlation mechanism mentioned above, as demonstrated by Monte Carlo simulations [14,15]. In the ion-ion correlation mechanism, the condensation of trivalent cations at the negative latex interface is weakened by the added 1:1 electrolyte thereby producing a larger mobility. It is clear that in this case, the 1:1 electrolyte plays a very active role not being the simple screening of interfacial charge found in our PS^- liposomes.

It has to be noted that the extremely low value for the charge reversal concentration obtained in Fig. 1 can be attributed to the high valency of La^{3+}. In previous systematic electrophoresis experiments of PS^- considering salts of different divalent cations [27] it was found that the point of zero mobility was very difficult to attain and charge reversal was barely noticeable for concentrations larger than 0.1M (with only slight differences between different cations following the sequence $Ba^{2+} > Ca^{2+} > Mg^{2+}$). Since these experiments were done using background electrolyte ([NaCl] = 100 mM), we have repeated the experiments in absence of added 1:1 salt, obtaining essentially the same results [28].

In order to obtain a molecular picture of the cation-PS^- interaction, we have performed all-atomic molecular dynamics simulations (MD) in the NPT ensemble of a fully hydrated phosphatidylserine (PS^-) bilayer in contact with La^{3+} neutralizing counterions. Our simulation box contains 14 700 atoms from a membrane with 128 PS^- molecules distributed in two leaflets (as in previous simulations, see [29]), 2624 water molecules and enough counterions to ensure charge neutrality (42 La^{3+} and 2 Na^+). As a model for the La^{3+} ion, we employed the force field developed in [30] which reproduces correctly the hydration structure and free energy of the cation. All other technical details concerning molecular models, force fields, algorithms, parameters and equilibration procedure are the same as reported in previous simulations of hydrated PS membranes [29] except otherwise stated. Our production run was of 10 ns preceded by an equilibration run of 4 ns. The simulations were performed using the DLPOLY2 package [31] running in parallel using 64 PowerPC processors during 376 hours.

The main results are as follows. We have obtained a mean area per phospholipid of $a_p = 55.4$ Å^2, in agreement with the results obtained in [29] with Na^+ as the counterion. The La^{3+} cations, uniformly distributed in the initial configuration (before the equilibration run), are observed to condense to the negatively charged bilayer membrane, i.e., all La^{3+} counterions contain at least one oxygen atom from PS molecules in their first coordination shell, implying binding to the bilayer. In Fig. 2 we show the distribution of electronegative atoms (oxygen atoms from lipid molecules and water molecules) around adsorbed La^{3+} ions. Note the nearly isotropic distribution of oxygen atoms from either PS or water molecules around La^{3+}, although there is a slight tendency of finding more lipid oxygens towards the membrane interior and more water oxygens towards the aqueous region.

Integration of the density distributions up to the first minimum gives the average coordination numbers. On average, the first coordination shell of La^{3+} contains 4.95 ± 0.01 oxygen atoms from phospholipids. These oxygen atoms come from 2 or 3 phosphate groups from different PS^- molecules. Also, the first coordination shell of La^{3+} contains an average of 4.17 ± 0.01 oxygen atoms from water molecules (for La^{3+} in bulk TIP3P water the coordination number is 10 [30]). In Figure 3 we show several density profiles averaged over the xy plane as a function of z. The distributions for both La^{3+} cations and oxygen atoms from PS have very similar shapes, with a peak at 2 nm from the center of the bilayer and a thickness of $\sim$1 nm. The cations are typically embedded by oxygen atoms from lipids, as can be seen in the snapshot shown as an inset in Fig. 3. This region containing La^{3+} and lipid headgroups is substantially hydrated, with interfacial water with a density about half the density of bulk water (see Fig. 3). Overall, the results shown in Figs. 2 and 3 (also see additional figures in the EPAPS material [24]) demonstrate that La^{3+} cations are incorporated deep inside the hydrophilic region containing the headgroups and interfacial water. This prediction of MD simulations can be tested by surface sensitive anomalous x-ray experiments, which allow the identification of the environment of ions at

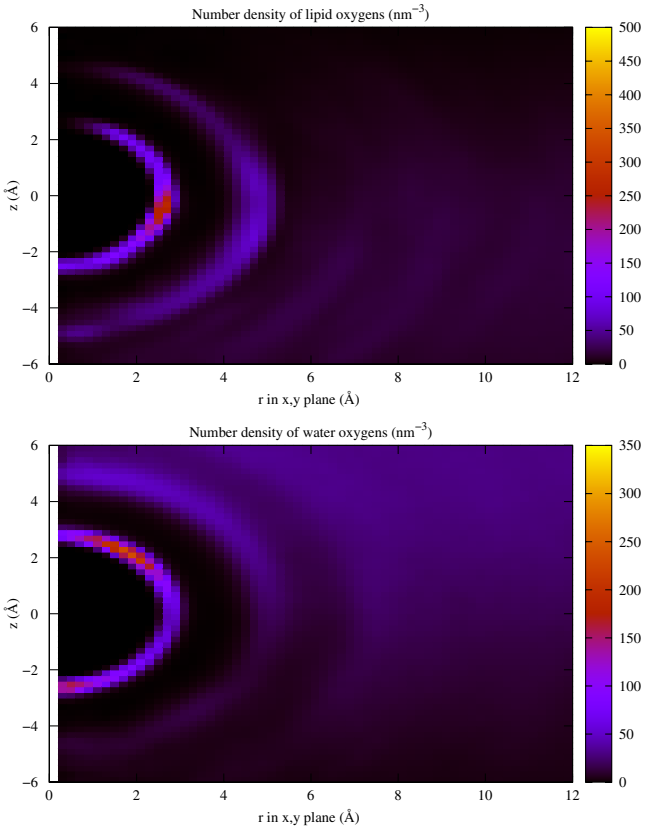

FIG. 2 (color online). Structure of the interface near adsorbed La^{3+} counterions from MD simulations. (a) Particle density (atoms/nm^3) of oxygen atoms from PS^- molecules around adsorbed La^{3+} cations. The cylindrical coordinates r, z centered at the adsorbed ions are defined so that z is negative towards the membrane interior and positive towards the bulk water; (b) Same as (a) but for oxygen atoms from water molecules.

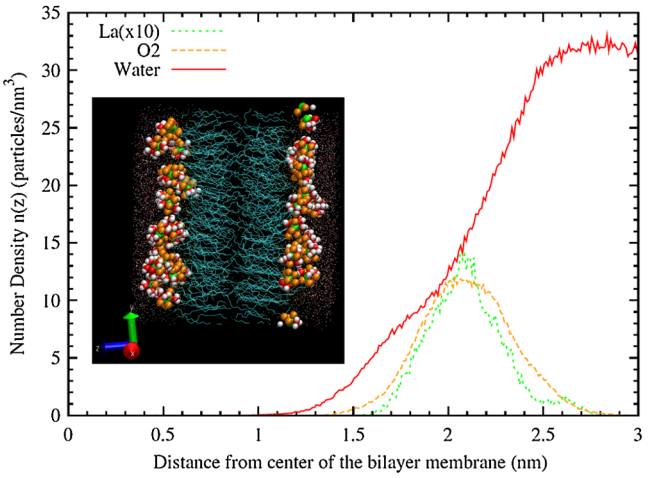

FIG. 3 (color online). Average density profile of different species as a function of the z coordinate (perpendicular to the membrane) obtained from MD simulations. Solid line: water density (molecules/nm^3), dashed line: oxygen atoms of O2 type from PS^- molecules (atoms/nm^3), dotted line: number density of La^{3+} cations (ions/nm^3) multiplied by a factor 10 for clarity. Inset: snapshot from MD simulations showing the oxygen lipids, La ions and hydration water as spheres and the other atoms as lines.

interfaces [32]. Our simulation results can be interpreted in physico-chemical terms by saying that small metal ions are preferentially solvated by oxygen atoms from lipids and interfacial water rather than bulk water, thereby penetrating inside the region containing the interfacial charges. The free energy involved in this preferential solvation can be estimated as follows. At the point of zero electrophoretic mobility, the charge of the liposomes is balanced by multivalent counterions of charge q_c distributed across a layer of thickness δz (see Fig. 3) with a typical concentration $\approx e/(q_c a_p \delta z)$. Outside the membrane, there is an approximately uniform concentration c_0 of counterions (recall that we are considering the zero mobility point), so the free energy difference per adsorbed La^{3+} ion is $\Delta\mu = k_B T \ln[c_0 \delta z a_p q_c/e] \approx -9 k_B T$ (we used $q_c = +3e$, $\delta z = 1$ nm and $c_0 \approx 10^{-4} M = 6 \times 10^{-3}$ ions/nm^3).

In view of our simulation results, we can understand why in our case the addition of 1:1 salt does not change the charge inversion concentration. The competition for binding between different ions is controlled by the respective free energies of interaction. The competition for binding between different ions is controlled by the respective free energies of interaction. The interaction between Na^+ and PS^- has been extensively analyzed by different experimental methods (electrokinetics, NMR,...), giving an association constant of $K = 0.6 M^{-1}$ [27,33] which corresponds to a free energy per ion of $\mu = -k_B T \ln(55.5\,\mathrm{K}) \approx -3.5 k_B T$, much smaller than that of the La^{3+}-PS^- interaction.

In order to corroborate our interpretation for the charge inversion mechanism, it is relevant to consider the interaction of La^{3+} with phosphatidylcholine (PC) lipids whose chemical structure is very similar to the PS^- structure (see Fig. 1) but is zwitterionic instead of anionic, which means that it has no net charge but it has a substantial dipolar moment which generates a significant surface potential in membranes [22]. Calorimetric measurements [34] give a free energy around $\Delta G = -12 k_B T$ for the interaction of La^{3+} with PC, which is even larger than our estimate for the interaction of La^{3+} with PS^-. Consequently, we would expect to observe an inversion of electrophoretic mobility even at smaller La^{3+} concentrations than in the previous PS^- case. This expectation is confirmed by our measurements (see Fig. 1); in this case electrophoretic mobility reverses its sign at the extremely low concentration $[La(NO_3)_3] \approx 3\ \mu M$.

Again, experiments with PC liposomes demonstrate that the effect is strongly dependent on the valency of the ions. Titration calorimetry gives $\Delta G \approx -6.5 k_B T$ for the PC-Ca^{2+} interaction [34], much lower than that obtained for La^{3+}. Interestingly, measurements show that the (favorable) entropic contributions to ΔG are almost equal in Ca^{2+} and La^{3+} being the difference in the enthalpy. Electrophoretic results are also in line with these thermo-

dynamic results. For example, previous results [35] show reversal of electrophoretic mobility of PC for $Ca(NO_3)_2$ concentrations between 0.01 mM–0.1 mM. Our own results [28] give inversion of electrophoretic mobility at $\approx$0.6 mM and 2 mM concentrations of $Ca(NO_3)_2$ and $Mg(NO_3)_2$ respectively.

In conclusion, in this Letter, we provide electrophoresis measurements demonstrating charge reversal in anionic liposomes at low concentrations of trivalent cations. The driving force for this effect is shown to be not related to the surface charge and not weakened by monovalent salt. Hence, we show that charge inversion is also possible in presence of physiological amounts of monovalent salt, usual in biophysical systems and in biotechnological applications [36]. Comparison of our MD simulation results and experiments allow us to identify the underlying mechanism as a preferential solvation of counterions by amphiphilic molecules and hydration water. It can be concluded that, under appropriate conditions, solvent mediated effects (hydration in the case of small metal ions as discussed here or hydrophobic interactions in the case of solutions containing organic ions [37]) are strong enough to govern the electrostatics of soft interfaces.

This work is supported by the Spanish Government Grants No. MAT2009-13155-C04-04, No. FIS2009-13370-C02-02 and CONSOLIDER-NANOSELECT-CSD2007-00041, Catalan Government Grant No. 2009SGR164 and Junta de Andalucía and FEDER Grants No. P07-FQM-02517 and No. P09-FQM-4698. A. M.-M. is supported by the Programa Ramón y Cajal, MEC-Fondo Social Europeo (RYC-2005-000829). C. R.-B. acknowledges receipt of a PhD Grant from CONACYT (Mexico). We acknowledge computer resources and technical assistance provided by the RES-Red Española de Supercomputación (CESVIMA). We also thank C. Calero (ICMAB) and A. Travesset (Iowa State Univ.) for fruitful discussions.

*jfaraudo@icmab.es

[1] *Electrostatic Effects in Soft Matter and Biophysics*, edited by C. Holm, P. Kékicheff, and R. Podgornik, NATO Science Series Vol. 46 (Springer, Berlin, 2001).
[2] A. Y. Grosberg, T. T. Nguyen, and B. I. Shklovskii, Rev. Mod. Phys. **74**, 329 (2002); Y. Levin, Rep. Prog. Phys. **65**, 1577 (2002).
[3] Y. S. Jho *et al.*, Phys. Rev. Lett. **101**, 188101 (2008); H. Boroudjerdi *et al.*, Phys. Rep. **416**, 129 (2005).
[4] H. Greberg and R. Kjellander, J. Chem. Phys. **108**, 2940 (1998).
[5] J. Lyklema, Adv. Colloid Interface Sci. **147–148**, 205 (2009); J. Lyklema, Colloids Surf. A **291**, 3 (2006).
[6] J. Faraudo and A. Travesset, J. Phys. Chem. C **111**, 987 (2007).
[7] K. Besteman, K. van Eijk, and S. Lemay, Nature Phys. **3**, 641 (2007).
[8] F. H. van der Heyden *et al.*, Phys. Rev. Lett. **96**, 224502 (2006).
[9] A. Martín-Molina *et al.*, Colloids Surf. A **319**, 103 (2008).
[10] C. Labbez, B. Jönsson, M. Skarba, M. Borkovec, Langmuir **25**, 7209 (2009).
[11] A. Martín-Molina *et al.*, J. Phys. Chem. B **113**, 6834 (2009).
[12] S. Pianegonda, M. C. Barbosa, and Y. Levin, Europhys. Lett. **71**, 831 (2005).
[13] A. Martín-Molina *et al.*, J. Phys. Condens. Matter **15**, S3475 (2003).
[14] A. Martín-Molina, M. Quesada-Pérez, and R. Hidalgo-Álvarez, Langmuir **21**, 9231 (2005); A. Martín-Molina, M. Quesada-Pérez, and R. Hidalgo-Álvarez, J. Phys. Chem. B **110**, 1326 (2006).
[15] O. Lenz and C. Holm, Eur. Phys. J. E **26**, 191 (2008).
[16] M. Petersheim and J. Sun, Biophys. J. **55**, 631 (1989); J. Bentz *et al.*, Biophys. J. **53**, 593 (1988); M. M. Hammoudah *et al.*, Biochim. Biophys. Acta, Biomembr. **645**, 102 (1981).
[17] M. Sovago, G. W. H. Wurpel, M. Smits, M. Mller, and M. Bonn, J. Am. Chem. Soc. **129**, 11 079 (2007).
[18] T. Ohki, M. Harada, and T. Okada, J. Phys. Chem. B **110**, 15 486 (2006).
[19] S. Kundu and D. Langevin, Colloids Surf. A **325**, 81 (2008); E. Le Calvez *et al.*, Langmuir **17**, 670 (2001).
[20] J. Pitler *et al.*, Phys. Rev. Lett. **97**, 046102 (2006).
[21] J. Faraudo and A. Travesset, Biophys. J. **92**, 2806 (2007).
[22] The lipid library, http://www.lipidlibrary.co.uk.
[23] T. Yeung and S. Grinstein, Immunological Reviews **219**, 17 (2007).
[24] See supplementary material at http://link.aps.org/supplemental/10.1103/PhysRevLett.104.168103.
[25] J. Lyklema and T. Golub, Croat. Chem. Acta **80**, 303 (2007)
[26] R. J. Hunter, *Zeta Potential in Colloid Science* (Academic Press, USA, 1988), 3rd ed..
[27] S. McLaughlin *et al.*, J. Gen. Physiol. **77**, 445 (1981).
[28] A. Martín-Molina *et al.* (to be published).
[29] S. A. Pandit and M. L. Berkowitz, Biophys. J. **82**, 1818 (2002).
[30] F. C. J. M. van Veggel and D. N. Reinhoudt, Chem. Eur. J. **5**, 90 (1999).
[31] W. Smith, Mol. Simul. **32**, 933 (2006).
[32] W. Bu, P. J. Ryan, and D. Vaknin, J. Synchrotron Radiat. **13**, 459 (2006).
[33] R. Kurland *et al.*, Biochim. Biophys. Acta, Biomembr. **551**, 137 (1979); M. Eisenberg *et al.*, Biochemistry **18**, 5213 (1979).
[34] R. Lehrmann and J. Seeling, Biochim. Biophys. Acta, Biomembr. **1189**, 89 (1994).
[35] J. Sabin *et al.*, Langmuir **21**, 10 968 (2005).
[36] Y. Kim and W. Sung, Phys. Rev. Lett. **91**, 118101 (2003).
[37] A. Martín-Molina *et al.*, Soft Matter **5**, 1350 (2009).