J Solid State Electrochem (2009) 13:1111–1114
DOI 10.1007/s10008-008-0770-5

ORIGINAL PAPER

Study of the temporal distribution of the adhesion-spreading events of liposomes on a mercury electrode

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Received: 20 October 2008 / Revised: 9 December 2008 / Accepted: 9 December 2008 / Published online: 9 January 2009

Abstract The formal analysis of the mechanism of adhesion spreading of liposomes at mercury electrodes shares several characteristics with the mechanism of metal nucleation at electrodes. It is shown that the description of the temporal distribution of the adhesion-spreading events is similar to that of the temporal distribution of metal clusters. Both processes are stochastic in nature and can be described by the Poisson distribution. Using this approach, a previously proposed model for the overall adhesion-spreading mechanism, considering the formation of active sites on the liposome and the actual attachment of the liposomes to the mercury surface, is validated.

Keywords Liposomes · Chronoamperometry · Adhesion spreading · Mercury electrode · DMPC · Metal nucleation

Dedicated to the 85th birthday of John O'M. Bockris.

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Introduction

Liposomes are closed lipid vesicles that have caught the attention in the latest years due to their unique properties and potential fields of application. They can be used to mimic real cell membranes for developing drug delivery systems and for several other applications [1, 2]. In previous work, we have demonstrated that monitoring the adhesion and spreading of single liposomes on a mercury electrode using high-resolution equipment provides information about elastic, dynamic, and structural properties of the liposomes [3–7]. The single adhesion-spreading events are recorded as capacitive spikes using chronoamperometry. Analyzing each single peak, it is possible to get insight into the adhesion spreading of all single adhering liposomes. Most importantly, a three-step kinetic model can be proposed and the respective time constants can be estimated, describing with high accuracy what happens when a liposome touches the mercury electrode. Although other models have been proposed (e.g., the model proposed by Žutić et al. [8] and the model developed by Lipkowski and coworkers for the adhesion and spreading of liposomes on gold [9, 10]), the three-step adhesion mechanism model has been proven to be consistent with most experimental results obtained on mercury surfaces [5, 6, 11].

Moreover, we have demonstrated that the overall process of adhesion and spreading follows a mixed mechanism in which both the mass transport of the liposomes from the suspension to the mercury surface and the actual kinetics of adhesion spreading, act together to define the total number of adhesion events that will be recorded [12]. In the light of experimental results, which show that the kinetic control is more evident at the first stages of the process, while mass transport (diffusion) controls it at longer times, it has been proposed that the kinetic control arises from a very weak

(although fast) equilibrium in which active "docking" sites appear and disappear constantly on the liposome surface. Based on well-known properties of liposomes [13], we proposed that those active sites are hydrophobic defects on the liposome surface acting as attractive centers. The defects acting as "nucleation" sites should appear and disappear constantly. Therefore, we assume that they are represented by lecithin molecules turning their lipophilic ends to the outer part of the membrane in a process similar to the activation leading to the flip-flop translocation of lipids across the membrane mid-plane. Electric fields may indeed induce the formation of similar defects on lipid bilayer membranes close to a charged surface, as has been reported by others [14].

The aforementioned mechanism (reversible formation of active sites followed by the "docking" or "nucleation" of the liposome on the mercury surface, eventually leading to the adhesion and spreading of the vesicles on the electrode) resembles that of metal nucleation at electrodes proposed by Milchev [15, 16] (reversible formation of active sites at the electrode surface followed by the actual nucleation of a metal cluster). This latter process has been widely studied, and it has been shown that the number of nuclei that are formed on an electrode at certain conditions, i.e., the temporal distribution of the formed clusters, follows a Poisson distribution, meaning that the metal nucleation is a stochastic process [15]. In this work, we show that the same approach can be used to study the adhesion and spreading of liposomes on mercury electrodes. Therefore, the extensive knowledge of metal nucleation at electrodes may be applied to study the adhesion and spreading of vesicles on hydrophobic surfaces.

Experimental

High-purity 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC; Lipoid GmbH, Ludwigshafen, Germany) was used without further purification. KCl (Suprapur®; Merck, Darmstadt, Germany) and Millipore water were used for all solutions and liposome suspensions. Before measuring, the suspensions were deaerated for 20 min with high-purity nitrogen. Electrochemical measurements were performed with an AUTOLAB PGSTAT 12 (Eco Chemie, Utrecht, The Netherlands) with an ACD164 modulus interfaced to a P4 PC in conjunction with an electrode stand VA 663 (Metrohm, Herisau, Switzerland). A multimode mercury electrode was used as working electrode, a platinum rod served as auxiliary electrode and an Ag|AgCl (3 M KCl, E=0.208 V vs. SHE) electrode was used as reference electrode. The surface area of the mercury drop was 0.48 mm^2, as determined by weighting 50 drops. For the determination of the distribution of obtained adhesion-spreading signals, 50 measurements (within 1.5 s and with sampling each 50 μs) were performed at a potential of −0.9 V vs. Ag|AgCl, at which the detection of adhesion-spreading events is improved [4, 12]. The temperature was fixed at 25 °C in order to get DMPC liposomes in the liquid crystalline phase. The program "Signal Counter" was used to determine the number of obtained peaks at different elapsed times.

Giant unilamellar vesicles (GUV) were prepared according to Moscho et al. [17] DMPC (1.5 mg) were dissolved in 1.1 mL of a 1:10 methanol/chloroform mixture. Then, 30 mL of 0.1 M KCl solution were added carefully by pouring along the flask walls, following which, the organic solvent was rapidly removed with the help of a rotary evaporator (Laborota 4000, Heidolph, Nürnberg, Germany) using a Rotavac control pump (Heidolph) at 40 °C and a final pressure of 10 mbar. The rotation speed was 30 rpm. A clear suspension of GUVs (0.05 g L^{-1}) is obtained. The lamellarity of the vesicles was proven by comparing the size distribution of the chronoamperometric spikes obtained with that measured using light scattering. As stated in a previous publication, the size distribution of the chronoamperometric signals depends on the lamellarity of the vesicles attaching [3–5]. From the size distribution and the concentration of lecithin, the concentration of liposomes was estimated to be $C^*_{lip} = 8.03 \times 10^7$ liposomes per milliliter.

Results

Figure 1 shows the distribution of the number of signals obtained at t=100 ms compared with the Poisson distribution given by:

$$P_m = \frac{N^m \exp(-N)}{m!} \quad (1)$$

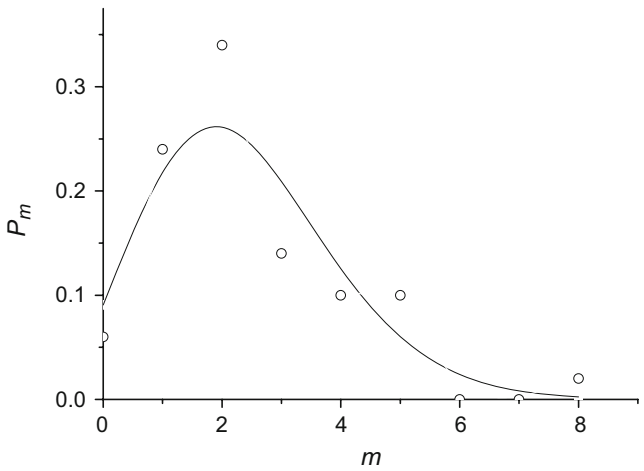

Fig. 1 *Circles* Experimental distribution of the number of liposome adhesion-spreading signals obtained after 100 ms at −0.9 V vs. Ag|AgCl (average=2.4) in a suspension containing 8.03×10^7 GUVs mL^{-1}). *Line* Expected Poisson distribution for the given average

where N is the average number of signals obtained in a certain time period (from 0 to 100 ms in this case), and P_m is the probability to form exactly m nuclei (attached liposomes) within the same time interval.

The good correlation between the experimental data and Eq. 1 implies that the attachment of liposomes to the electrode surface can be considered as a time-dependent flux of independent random events, just as the formation of metal nuclei at electrodes. The number of obtained signals follows the same kind of distribution at all time intervals studied.

A frequently studied stochastic quantity is the probability $P_{\geq 1}$, that is, the probability of forming at least one nucleus. It can be expressed by:

$$P_{\geq 1} = 1 - P_0 = 1 - \exp(-N) \tag{2}$$

which can be differentiated to:

$$\mathrm{d}P_{\geq 1} = \exp(-N)\frac{\mathrm{dN}}{\mathrm{dt}}\mathrm{dt} \tag{3}$$

and the average time of expectation to get one nuclei will be given by:

$$\bar{t}_1 = \int_0^\infty t\mathrm{d}P_{\geq 1}. \tag{4}$$

Equations 2 and 3 provide information about the most important parameters to study the kinetics of the nucleation process: the rate of nuclei formation, dN/dt, and the average number of nuclei, N.

For the case of liposome adhesion and spreading on a mercury electrode, it has been reported before [12] that the average number of attached liposomes as a function of time, i.e. of nuclei, can be approached, considering a mixed transport-kinetics mechanism, by:

$$N(t) = A_{\mathrm{SMDE}}C^*_{\mathrm{lip}}\sqrt{D}\left(A_1\mathrm{erf}\left(\sqrt{-k_{01}t}\right)e^{-k_{01}t} + A_2\mathrm{erf}\left(\sqrt{-k_{02}t}\right)e^{-k_{02}t} + A_3\sqrt{t}\right) \tag{5}$$

where A_{SMDE} is the area of the electrode, C^*_{lip} is the bulk concentration of the liposomes (calculated from the amount of lipid and the average size of the vesicles), D is the average diffusion coefficient, and A_1, A_2, A_3, k_{01}, and k_{02} are constants that can be determined experimentally by analyzing the single adhesion-spreading signals.

From the reported values (see [12]) of A_1, A_2, A_3, k_{01}, and k_{02} at the given experimental conditions (k_{01}=9.3×10^7 s^{-1}, k_{02}=9.55 s^{-1}, A_1=−1.01i×10^{-11}, A_2=0.31i, and A_3=1.07), the calculated average time of expectation for the formation of one nuclei in the case reported here equals $t_1 = 0.049$ s.

The accuracy of Eq. 5 when describing the average number of obtained adhesion-spreading signals can be tested comparing the experimental and predicted values of $P_{\geq 1}$ as a function of time. Experimentally, the determination of $P_{\geq 1}$ is easier and more accurate than the determination of the actual average number of attached liposomes (nuclei) N, as, in the latter case, it is necessary to be sure that every signal recorded corresponds to one, and only one, adhesion-spreading event, and this, of course, can never be guaranteed. In the case of the determination of $P_{\geq 1}$, it does not matter if the recorded signal arises from one or more adhesion events. Important is that *at least one* adhesion-spreading event is taking place.

Comparing the experimental values of $P_{\geq 1}$ at different time intervals with those obtained by combining Eqs. 2 and 5, the curve shown in Fig. 2 is obtained. It is clearly seen that both curves are almost identical. The figure shows also the calculated value of $P_{\geq 1}$ from the experimental average N values and Eq. 2. This curve corresponds also almost perfectly to the other two, suggesting again that the number of recorded adhesion signals follows a Poisson distribution. At times longer than those shown in the figure, all curves converge to $P_{\geq 1}$=1.

These observations support the hypothesis that the mixed transport-kinetic model developed in [12] and described by Eq. 5 describes the overall adhesion-spreading process of liposomes. Furthermore, it shows that the profound knowledge of the kinetics of metal nucleation at electrodes can be used to study the adhesion spreading of vesicles at mercury electrodes and probably also at other hydrophobic surfaces.

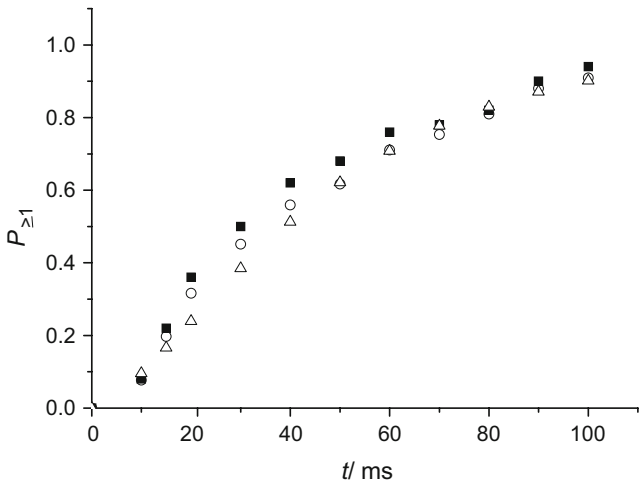

Fig. 2 *Filled squares* Experimental determined values of $P_{\geq 1}$ as a function of time. *Triangles*: $P_{\geq 1}$ as a function of time calculated combining the theoretical model predicting the average number of events and the Poisson distribution (Eqs. 5 and 2). *Circles* Values for $P_{\geq 1}$ calculated from the experimental values of N using the Poisson distribution

Conclusions

The use of chronoamperometry to study the adhesion and spreading of liposomes at mercury electrodes has been shown to have a large potential to be used in order to get information about several properties of the liposomes, as has been illustrated in previous publications [4–6]. In this work, it is shown that the process, although different in nature, follows a mechanism that shares several formal characteristics with the mechanism of metal nucleation at electrodes. Those similarities include the reaction pathway (a reversible activation step followed by an irreversible reaction) as well as the description of the temporal distribution of the studied events (stochastic events following the Poisson distribution). Therefore, available knowledge on metal nucleation can be used to study the adhesion and spreading of liposomes at the mercury electrode and, probably, to other hydrophobic surfaces. It is very important to think about the possible origin of the stochastic nature of the nucleation-like features of liposome adhesion: Based on previous results and on well-studied properties of membranes close to electrode surfaces [12, 14], we believe that the appearance of "inverted" lecithin molecules in the liposome membrane—similar to those found on the activated state of the flip-flop process—is the stochastic process that provokes a docking of the liposomes on mercury. Only when a lecithin molecule is positioned with its lipophilic tail towards the mercury surface can an irreversible binding occur and that will be the nucleus for the attachment.

Acknowledgments V. A. H. acknowledges provision of a DAAD-Conacyt (Deutscher Akademischer Austauschdienst–Consejo Nacional de Ciencia y Tecnología, Germany–México) scholarship. F. S. acknowledges financial support by *Phospholipid Forschungszentrum e.V.* The authors gladly acknowledge provision of high-purity DMPC samples by Lipoid GmbH, Ludwigshafen, Germany.

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