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UN-STIRRED LAYERS AT THE BIOMEMBRANE-AQUEOUS SOLUTION INTERFACE EVIDENCED BY ION TRANSPORT MEASUREMENTS THROUGH PORE-FORMING PROTEINS

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A novel experiment is described, which highlights the presence of un-stirred layers around the artificial biomembranes. A theoretical description is provided, that is able to quantify the thickness of the un-stirred layers.

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1. Introduction

In order to pay for the energy price needed to translocate ions or charged molecules from a high dielectric constant medium (usually water) through the hydrophobic core of the biomembrane, Nature came to devise highly evolved protein supra-structures, with roles not only for transport but ion selectivity as well [1-3]. Ion channels are made up of aminoacids, which first ought to be encoded linearly by genes in an optimal fashion (*i.e.*, sequence is important) and than fold tri-dimensionally in a functionally relevant architecture for cells. The movement of ions (Na^+, K^+, Ca^{2+} and Cl^-) through these channels carries the electrical charge that produces most of the electrical properties of cells and tissues [4-6]. That is, electrical signals encoded by ion transport through such channels help cells communicate to one another via action potentials, coordinate muscle contraction, and regulate salt composition in various organs. Many of these ion transport processes and molecular events which take place the biomembrane level are, however, modulated to a certain extent by the local ion concentration around the biomembrane. When the studied system is considered to be close to equilibrium, the local ion concentration can be relatively easily tackled within the Debye-Huckel theory [7]. In the case of neutral biomembranes and when transport is involved, the diffusion equation needed to predict with accuracy quantitative aspects of ion permeation makes use of one crucial assumption: the permeating ions are thought to be uniformly distributed throughout the intra- and extracellular media. Under such conditions, these solutions are said to be 'well-stirred'. It is however well known that this assumption can break down in certain biological systems and the resulting scenario is of a relatively narrow region in the vicinity of the biomembrane within which a concentration gradient of the permeating ions does exist. This physical domain is known as the 'un-stirred layer' and its very existence can greatly influence a large pleiade of biological processes. That is, un-stirred layers on each side of the membrane will give rise to diffusion potentials which can perturb electrogenic phenomena around biomembranes, play an essential role for the transport processes, they are a source of inaccurate Michaelis constant in membrane transport and can serve as regulatory mechanisms for some relevant biological processes (absorption of small molecules) [8-11]. Over the past decades there were many theoretical and experimental attempts aimed at describing and measuring the thickness of un-stirred layers. From the experimental view-point, one of the simplest, yet powerful approaches toward describing physical properties of un-stirred layers was to employ micro-pH electrodes placed in the very vicinity of an artificial membrane surrounded by buffer solution, which gave a fairly straightforward description of hydrogen ion concentration profile within the un-stirred layer [10]. In this paper we describe a novel experiment which we believe highlights the presence of un-stirred layers around

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artificial biomembranes and provides a theoretical description of our data in this respect, enabling us to quantify the thickness of the un-stirred layers. The core of our work was to measure the reaction rate of a certain analyte (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) with an engineered thiol within a nanopore, when other molecules (threo-1,4-dimercapto-2,3-butandiol) (DTT) known to break apart DTNB and render them non-reactive with the thiol, were allowed to pass through the nanopore from the opposite direction [12]. Having diffused through the nanopore, some of the DTNB molecules will eventually react irreversibly with the engineered thiol; when DTT molecules, coming from the opposite direction will bump into the DTNB-thiol complex, they will break the covalent bond which led to the formation of the DTNB-thiol complex. Since the formation of such a DTNB-thiol complex is stochastic and markovian in nature, from the statistical analysis of time intervals in-between DTNB-thiol complex formation, the reaction rate of the complex formation can be inferred [12-14]. As expected, the measured reaction rate of the DTNB-thiol complex depends linearly on the DNTB concentration; on the other hand, at least from a theoretical view-point, we expected that the above-mentioned reaction rate would not depend upon the DTT concentration. This last presumption was contradicted by our experiments, which have shown that at a constant concentration of DTNB on one side of the biomembrane, further increase in the concentration of DTT on the opposite side led to a decrease of the reaction rate for the formation of the DTNB-thiol complex. We interpreted this result as a direct consequence of local DTNB concentration alteration by the incoming DTT molecules which, in their way down the concentration gradient, got to interact irreversibly with DTNB molecules, on the same side they were initially added to.

2. Materials and methods

As a working model for a nanopore we used the α-hemolysin channel. Current recordings through α-hemolysin channels were carried out by using folded bilayer membranes obtained as previously described [12]. Briefly, a 25-μm thick Teflon septum was clamped between two Teflon chambers each of 1 ml volume. A bilayer was formed on an aperture of 100-μm diameter in the septum that had been pretreated with 10% (v/v) hexadecane (Sigma-Aldrich) in highly purified n-pentane (Sigma-Aldrich). Both chambers contained 2M KCl, 30 mM MOPS, 100 mM EDTA, titrated to pH 8.5 with aqueous KOH. Initially, the level of electrolyte was set just below the aperture and 1% (w/v) 1,2-diphytanoyl-*sn* glycerophosphocholine (Avanti Polar Lipids Inc.) in pentane (6 μL) was spread on the surface of each chamber. After about 2 minutes, during which the solvent evaporated, the electrolyte level in the chambers was raised above the aperture. The formation of a bilayer was monitored by observing the increase in membrane capacitance to a value of approximately 150 pF. In order to suppress spurious electrical interference, the bilayer chamber was properly grounded and enclosed into a home-made Faraday cage. α-Hemolysin protein was added from a stock solution, in the *cis* chamber only, connected to the ground and mechanical stirring was initiated to ensure proper concentration homogenization. All experiments were performed at a room temperature of ~ 25 ^{o}C. The electrical connection between the bilayer chamber and the rest of analog devices was made via Ag/AgCl electrodes. Electrical currents from the bilayer chamber were detected and amplified with an integrating headstage Axopatch 200B amplifier (Axon Instruments, Foster City, USA), set on the voltage-clamp mode. The electrical current was filtered with a low-pass Bessel filter (80 dB/decade) with a corner frequency of 1 kHz and then digitized with a DigiData 1320 A/D converter (Axon Instruments) at a sampling frequency of 5 kHz. Event data files used to determine reaction rates were constructed by manual inspection of the experimental traces with Clampfit 9.0 software (Axon Instruments). The processed data were plotted using Origin 7.0 (Microcal Software).

3. Results and discussion

In order to visualize how diffusional un-stirred layers may arise in real life, we used the mono-dimensional mathematical treatment of the diffusion equation, when the initial concentration source resembled a Heaviside function.

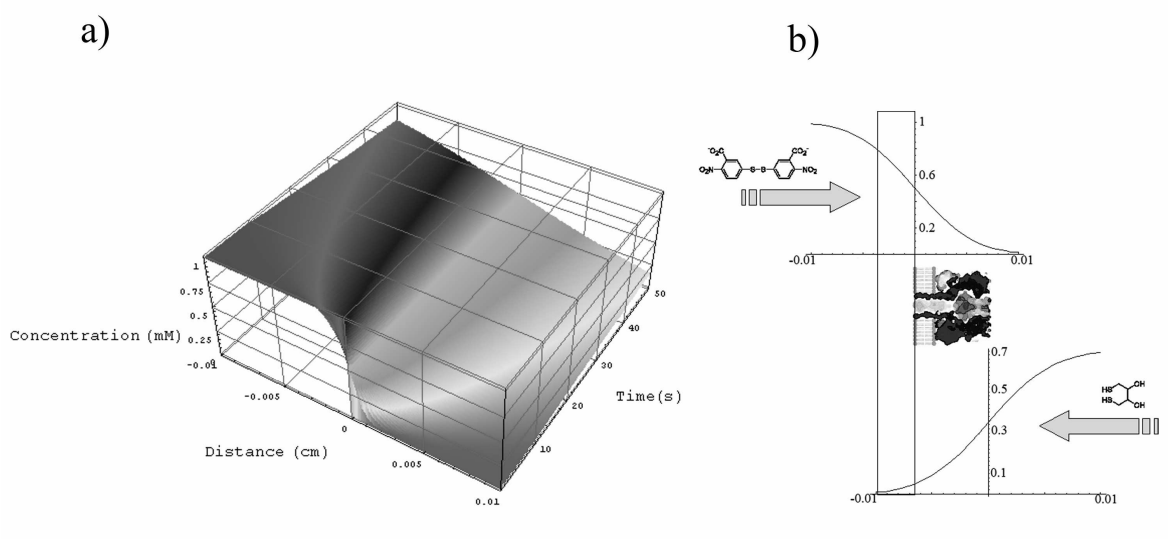

Fig. 1. (a) Spatio-temporal dependence of concentration profile between two media, over which an initial concentration gradient of (1:0) [mM] was set. Before the diffusion process was set to take place, solution was considered well-stirred; the diffusion coefficient was thought to be the same in both media and set to 10^{-6} cm^2s^{-1}. (b) Snap-shot concentration profile showing how DTNB (left) and DTT (right) molecules distribute themselves in the vicinity of the boundary which separates media between which they diffuse, two seconds after diffusion processes have started. At the initial time, both DTNB (present only on the left-handed semi-space) and DTT (present only on the right-handed semi-space) were 'well stirred'. The space - unit in this figure is 'cm', and the diffusion coefficient was set to 10^{-6} cm^2s^{-1}.

From Fig. 1 (a) it is clear that although solution in both compartments were initially 'well stirred', as time passes by, a build up of a limited domain around the boundary plane separating the two compartments appears, where the local concentration of the diffusing molecules differs from that on the bulk solution. One of the brilliant applications of stochastic sensing devised recently (12) makes possible the estimation of reaction rate of a specific analyte (DTNB) with an engineered cysteine. However, since that type of reaction is a 'second-order' one, in order to expand the knowledge inferred from experimental it is of utmost importance to know accurately the value of the local concentration of DTNB at the reaction site. Initially, the concentration of DTT molecules in the compartment where DTNB was present was kept at zero, not to allow the irreversible reaction between the two reactants to take place and jeopardize our experimental strategy conceived as to monitor the interaction between DTNB and cysteine (12). From Fig 1 (b) it is clear however, that during the course of time, DTT molecules do invade the other side of the reaction space, separated by an impermeable biomembrane, via the α-hemolysin channel. Although in our rationale presented in this figure we referred to relative concentration values, by close inspection one may come to realize that local concentration of DTT building up on the other side of the membrane will affect the local concentration of DTNB molecules on the same side, by interacting irreversibly with them. Needless to say that the concentration profile of DTT molecules will change continuously in time, so that the above-mentioned effect will be a time-dependent one, as well. The immediate consequence of this string of phenomena is the continuous alteration of the local concentration of DTNB molecules inside the lumen of the α-hemolysin channel, despite the fact that bulk value of DTNB concentration may remain relatively un-changed. In Fig. 2 we present experimental traces which show indirectly molecular events taking place inside the channel, in the experimental configuration described before (12). The electrical current measured through the nanopore in the absence of any molecular interaction is represented by the state '2'; when one DTNB molecule does react irreversibly with the engineered cysteine inside the nanopore, the conductance of the nanopore changes accordingly and the reduction in electrical current is evidenced by the state '1'. Left alone, no further visible changes will occur in our system; as soon as we add DTT molecules from the opposite side (*cis*), they will diffuse into the lumen of the channel and one DTT molecule eventually displaces the DTNB-bound molecule.

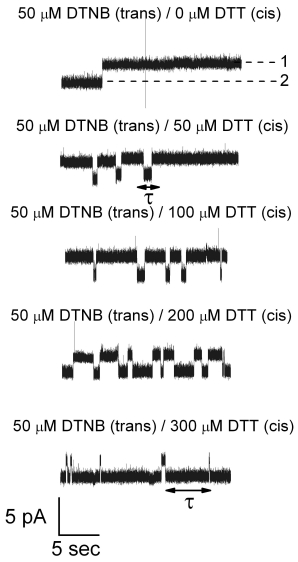

Fig. 2. Experimental traces showing the effect of increasing concentration values of DTT on the reaction rate of DTNB molecules with the engineered cysteine at the position 117 inside the α–hemolysin channel (see text).

Accordingly, the conductance of the channel will return to its initial state ('2'). From the statistical analysis of stochastic time intervals in-between two consecutive binding events of DTNB molecules (τ), one is able to infer the reaction rate of the DTNB molecule with the cysteine inside the channel. It is important to notice that larger concentrations of DTT molecules on the *cis* side do increase the time intervals in-between two consecutive binding events of DTNB molecules (τ) – compare Fig. 2, lower trace with (50 μM DTNB *trans*/300 μM DTT *cis*) with second trace from above (50 μM DTNB *trans*/50 μM DTT *cis*). On a simple, still accurate interpretation, this will translate as a decrease of the reaction rate between DTNB and the engineered cysteine. The possible reason for this happening is a local decrease of DTNB concentration, close to the reaction site, due to the decrease of local DTNB concentration in the *trans* side. This later effect would be caused by an enhanced depletion of DTNB molecules in the *trans* side prompted by the ongoing diffusional DTT un-stirred layers occurring in this region. To provide a mechanistic description of these phenomena and in order to tentatively estimate the thickness of the DTT-altered DTNB diffusional un-stirred layer on the *trans* side of the biomembrane, we made use of a simplified model, shown in Fig. 3. That is, once un-stirred layers set in, we consider that during the observation time no further changes in the DTNB concentration C(x,t) (continuous line) will occur (cvasi-stationary state).

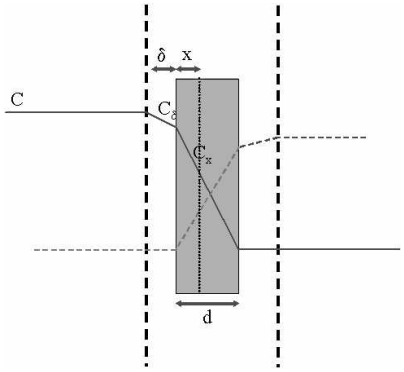

Fig. 3. The simplified diffusion model used in order to facilitate understanding of how membrane diffusional un-stirred layers are set when two different reactants, coming from opposite directions, do deplete each other in the vicinity of the membrane.

Moreover, we regarded changes in concentrations vs. distance as being linear over the nano-channel and un-stirred layers, neglected other sources of transport (convection), assumed that outside un-stirred layers the concentration of the solute is constant and uniform and assumed no accumulation of reactive molecules (DTNB) on the other side of the nano-channel. Under these simplifying assumptions, we measured and compared reaction rates between DTNB molecules and the cysteine inside the nanopore, when DTT molecules are present on the *cis* side on a relatively low and high concentration, respectively. When concentration of DTT on the *cis* side is relatively low (up to 50 μM) we assumed that no visible changes occurred in the DTNB concentration close to the membrane and on the *trans* side, fact substantiated by our previous experiments (12). In that case and under the simplifying assumptions described above, we considered that during the time of observation DTNB was 'well-stirred' in the vicinity of the membrane and the concentration drop of DTNB along the nano-channel axis as linear. The ratio of DTNB reaction rates with the thiol at the distance ('x') within the nano-channel calculated for the two extreme cases (i.e., [DTT] very small and [DTT] large enough so that local alteration of DTNB on the *trans* side will ensue) is given by:

$$\frac{Rate_{DTNB}(DTT<<)}{Rate_{DTNB}(DTT=100\mu M)}=\frac{C\left(1-\frac{x}{d}\right)}{C_x} \tag{1}$$

In formula (1), $Rate_{DTNB}(DTT<<)$ (~ 0.58 s^{-1}) and $Rate_{DTNB}(DTT=100\mu M)$ (~ 0.29 s^{-1}) stand for the experimentally inferred DTNB-cysteine reaction rates measured at low (<50 μM) and respectively high (100 μM) DTT concentrations on the *cis* side, after ~ 15 minutes from the beginning of the molecular diffusion process (12). The DTNB concentration in the bulk solution from the *trans* side taken into account in formula (1) is denoted by C (C = 100 μM). When concentration of DTT on the *cis* side is relatively high (up to 100 μM), the concentration profile of the DTNB molecules within the diffusion space would be as shown in Fig. 3 (continuous line). Dotted concentration profile from Fig. 3 represents variation of DTT concentration; note that through identical mechanisms as described herein, an un-stirred DTT layer will be generated on the *cis* vicinity of the biomembrane, as well. By knowing two crucial parameters resulting from X-ray crystallography, namely the relative distance where the reaction site is located ('x' – 50 Å) and the full-length of the α-hemolysin channel ('d' - 100 Å) and following simple calculation using formula (1), we were able to find an estimation for the local concentration value (C_x) of DTNB molecules at the interacting site ('x'), of ~ 25 μM. Note that for the case when the local concentration of DTNB molecules would not be perturbed via DTT-related diffusion processes, as it is the case when the concentration of DTT is <50 μM, the local concentration of DTNB at the site of interaction is ~ 50 μM. Via similar calculations, we arrived at a value of δ ~ 20 nm for the diffusional un-stirred layer of DTNB molecules on the *trans* side of the biomembrane, when the concentration of DTT molecules on the *cis* side was 100 μM and after ~15 minutes from the beginning of the diffusion process. We point again that this perturbation on the concentration of DTNB molecules close to the biomembrane is caused mainly through chemical neutralization of DTNB by DTT molecules diffusing from the *cis* side of the bilayer. It is obvious that through mechanisms described above, the thickness of this un-stirred layer will change in time and with the concentration of DTT molecules on the *cis* side. Despite the relative simplicity of our rationale toward the description of diffusional un-stirred layers, we were able to provide a novel description of such phenomena based on experimental results stemming from single-molecule experiments.

4. Conclusions

When proper conditions are put in place for the diffusion of some solute over a neutral biomembrane, one of the interesting phenomena taking place at the biomembrane-aqueous solution interface is the creation of the so-called diffusional un-stirred layers. Put simply, within the un-stirred layers the concentration of the soluble solute is no longer equal to that on the bulk, but varies

following a certain analytical distribution. The most striking consequence of such un-stirred layers being present adjacent to the biomembrane is a local alteration on the concentration of the analyte soluble through the biomembrane. This in turn may affect transport phenomena of the soluble solute through the biomembrane and chemical reactivity of certain surface-located functional groups. In this work we present novel evidence which strongly supports the very existence of un-stirred layers, gathered via electrical measurements through a single pore-forming protein embedded into an artificial biomembrane. Through our measurements we may be even able to quantify the magnitude of concentration drop of the studied solute at the biomembrane-aqueous solution interface.

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