

Electrochemical Signals of Mitochondria: A New Probe of Their Membrane Properties**

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Although mitochondria (MI) are intensively studied cell organelles^[1–4] and even the redox communication with electrodes has been addressed,^[5] there is still a demand for techniques to probe their properties under (patho-)physiological conditions. The integrity of the mitochondrial membrane and changes in its composition play a crucial role in preventing or even triggering apoptosis. Here, we report that isolated functionally intact MI interact with the surface of a static mercury electrode in a way which is similar to the adhesion-spreading of liposomes^[6–9] and thrombocytes,^[10] that is, they attach to the hydrophobic mercury surface and disintegrate by forming islands of adsorbed molecules. This attachment is caused by the hydrophobic interaction between mercury and the lipid chains,^[11] a topic with a long history and recently reviewed by Nelson.^[12] This attachment is measurable because of the changes of double-layer capacity, which give rise to defined capacitive signals. The quantitative analysis of these signals allows the determination of the phase-transition temperature of the mitochondrial membrane, the determination of the size of MI, and indicates the physiological status of MI.

Freshly isolated MI dispersed in a physiological KCl solution interact with the Hg surface giving capacitive current spikes which have a positive sign at negative potentials (Figure 1), and a negative sign at positive potentials versus the point of zero charge (pzc). The highest frequency of spikes was observed at -0.9 V. This is very similar to the behavior of lecithin liposomes, indicating that the mitochondrial membrane also disintegrates on Hg and forms an island of adsorbed molecules. Counting the number of current spikes per time and surface area units allows analysis of the macrokinetics, that is, the number of disintegrations as a

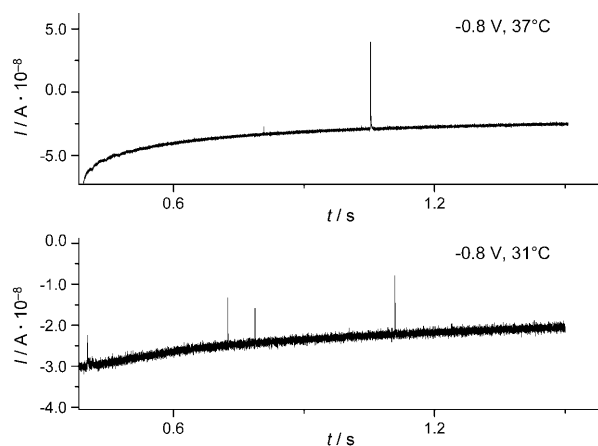


Figure 1. Current-time traces measured in a suspension of mitochondria at two different temperatures.

measure of the rate at which the MI interact with the Hg surface.

For each MI we can also analyze the microkinetics, that is, the rate at which a single MI disintegrates. This can be accessed through integration of the single current spikes to yield charge (Q)-time (t) traces, which mirror the time-resolved disintegration, leading to the displacement of the aqueous side of the electric double layer of the Hg electrode by the adsorbed constituents of the mitochondrial membrane (including cardiolipins; Figure 2). The resulting charge-time trace follows the same equation [Eq. (1)] as the traces reported for liposomes,^[6–9,13]

$$Q(t) = Q_0 + Q_1(1 - \exp(-t/\tau_1)) + Q_2(1 - \exp(-t/\tau_2)) \quad (1)$$

where τ_1 and τ_2 are two time constants, characterizing the rate of adhesion and spreading, respectively, and Q_0 , Q_1 , and Q_2 are constants.

Figure 3 shows typical and reproducible Arrhenius plots of the macrokinetics (J is the peak frequency per unit area) for MI isolated from BRIN-BD11 cells grown under two different conditions, that is, normoglycemic (5.5 mmol L^{-1} glucose) and hyperglycemic (25 mmol L^{-1} glucose) conditions. There is a pronounced break of the straight lines at around 27.5°C . This break is typical for a phase transition, and the phase-transition temperature (PTT) is also in accordance with literature data of submitochondrial particles.^[14] Our results relate to intact MI and reflect the PTT of the intact mitochondrial membrane. The plot also yields the activation energies of the macrokinetics: the MI grown under the two conditions behave differently below the PTT. Essentially the same change of the disintegration kinetics

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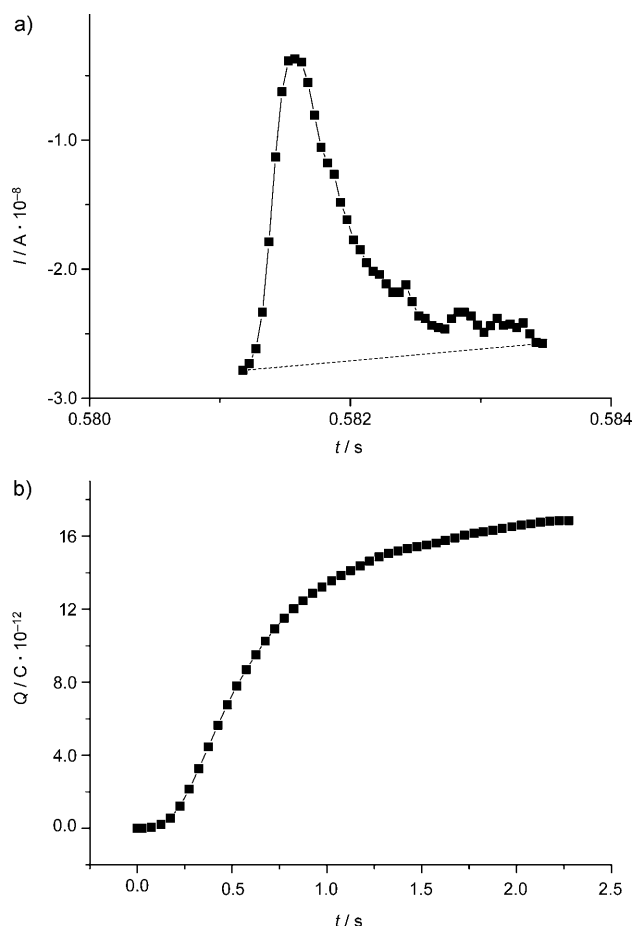


Figure 2. a) A single current (I) spike and b) the same peak after integration to yield a charge (Q)-time (t) trace, which mirrors the time-resolved disintegration, leading to the displacement of the aqueous side of the electric double layer of the Hg electrode by the adsorbed constituents of the mitochondrial membrane.

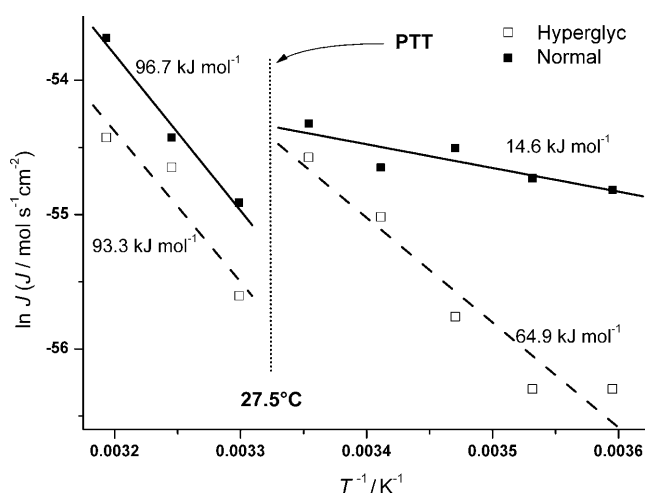


Figure 3. Arrhenius plots of the macrokinetics of the adhesion-spreading of mitochondria on mercury.

has been observed for mitochondria stressed by a palmitinic-acid diet (0.1 mM).

From the absolute charges of a single spike, we can calculate the approximate size of the MI, provided that we know the specific double-layer capacity (dlc) of the mercury electrode covered by the disintegrated MI. Mitochondrial membranes contain lipids (especially cardiolipins) similar to dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC) (at least with respect to the expected dlc of a monolayer on Hg), and we have calibrated our data using the dlc of the Hg|DMPC interface, that is, the charge-density difference between the aqueous electrolyte|Hg interface and the Hg|DMPC interface (at -0.9 V, 25°C , $\Delta q = 0.07 \text{ C m}^{-2}$). Further, we assume that the overall area covered with a monolayer of the mitochondrial membrane is 12 times the surface area (A) of an intact MI; this assumption follows from 1) the surface area of the inner membrane which is five times the surface area of the outer membrane and 2) the surface area of the monolayer which is twice the surface area of the bilayer. Thus, we calculate from the charge data of the single MI their size, assuming a spherical shape. Figure 4

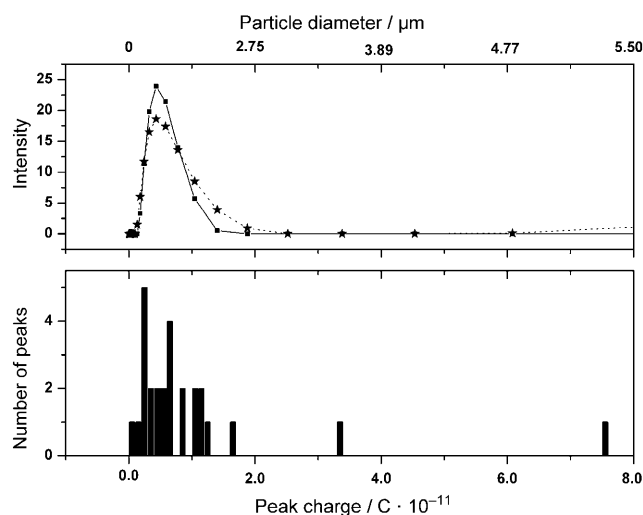


Figure 4. Comparison of the size histograms of mitochondria obtained by light scattering (top panel; curves for two sets of evaluation parameters) and by the new electrochemical approach (bottom panel) under the assumption that the mitochondria disintegrate completely.

compares the calculated data with light-scattering data. The excellent agreement between these independent data strongly backs our assumptions, indicating that the MI are completely disintegrated. The reported results show that we can study the properties of the mitochondrial membrane in fully functional MI in vitro, that is, without destroying the MI *before* analysis, which is normally the case when changes of the cardiolipins need to be assessed.

We have verified the applicability of the electrochemical approach to study MI by concomitant functional analyses using identically prepared MI from rat insulinoma BRIN-BD11 cells, which were exposed to 5.5 or 25 mmol L^{-1} glucose, respectively, for 24 h. Hyperglycemia is implicated in the development of diabetes as it facilitates pancreatic β -cell dysfunction. The underlying mechanisms are closely related to an enhanced metabolism of glucose, leading to

increased generation of superoxide anion radicals within the respiratory chain of MI. This process has been demonstrated to generally occur in situations of increased supply with reducing equivalents.^[15,16] Production of hyperglycemia-induced superoxide is a major aspect of glucose toxicity towards β -cells. Typical indicators for β -cell damage include loss of the membrane potential of MI, release of cytochrome c, increased expression of apoptotic proteins, and increased apoptosis.^[17] We show here, that hyperglycemia leads to increased oxidation of cardiolipin, a major constituent of the inner mitochondrial membrane. Because of its close proximity to the site of production of reactive oxygen species (ROS), especially complexes I and III of the respiratory chain, and its high content of unsaturated fatty acids, cardiolipins represent an early target for ROS. Whereas only $(11.3 \pm 1.4)\%$ of the total cardiolipin (oxidized plus non-oxidized) is oxidized under normoglycemic conditions, this fraction is significantly increased to $(19.1 \pm 3.5)\%$ in response to 25 mmol L^{-1} glucose ($p < 0.05$). Oxidative modification of cardiolipins negatively affects the membrane functions of MI by altering the membrane fluidity, surface charge, and ion permeability.^[18,19] Peroxidized cardiolipin has been shown to induce MI permeability transition (MPT), an effect associated with the release of cytochrome c from MI.^[20,21] We assume that changes in the membrane properties described here, that is, reduction of the membrane fluidity (see Figure 3), are due to the oxidative modification of cardiolipins. In accordance with this finding is the observation that hyperglycemia leads to a significant increase in the amounts of cytosolic cytochrome c in BRIN-BD11 cells exposed to high concentrations of glucose when compared to cells cultured under normoglycemic conditions (Figure 5).

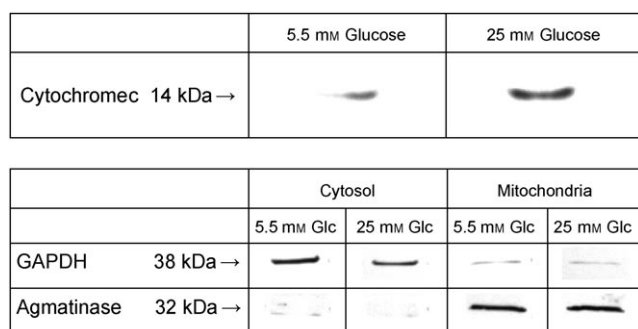


Figure 5. Immunoblots. Top panel: Increased amounts of cytochrome c released to the cytosol in response to the exposure of BRIN-BD11 cells to 25 mmol L^{-1} glucose. Bottom panel: Exclusive detection of GAPDH in the cytosol and of agmatinase in MI indicates the purity of both fractions. An amount of $11.5 \mu\text{g}$ of protein was loaded per lane (kDa is kiloDalton, Glc is glucose).

Our data demonstrate for the first time that electrochemical analyses as performed here are feasible to quantify alterations in physicochemical properties of mitochondrial membranes caused by pathophysiological conditions such as hyperglycemia.

Experimental Section

Rat BRIN-BD11 insulinoma cells^[22] were maintained in Dulbecco's modified Eagles medium (PAA, Linz, Austria) containing 5.5 mmol L^{-1} glucose, 2 mmol L^{-1} glutamine, and 10% (v/v) FBS. Cells were seeded into 75 cm^2 cell culture flasks at a density of 6×10^6 cells/20 mL. After 24 h, the medium was replaced and the cells were cultured in the presence of either 5.5 mmol L^{-1} (normoglycemic) or 25 mmol L^{-1} glucose (hyperglycemic) for further 24 h. MI were prepared using the Qproteome Mitochondria Isolation-Kit (QIAGEN, Hilden, Germany) following the recommended protocol. MI were resuspended in a storage buffer (component of the kit) and used immediately for subsequent analyses.

The oxidation of cardiolipin (CL) was estimated by determining the ratio of $(\text{C18:2})_3$ -monohydroxylinoleic acid-CL (oxidized CL) to $(\text{C18:2})_4$ -CL (nonoxidized CL) by LC-MS/MS as precisely described by Schild and co-workers.^[23]

Immunoblot analyses were performed as described^[24] using polyclonal anti-cytochrome c (Cell Signaling), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ABfrontier), and anti-agmatinase^[25] as primary, and anti-rabbit-horseradish peroxidase (HRP) as secondary antibodies.

Electrochemical measurements were performed within 10 h after isolation of the MI. Before the measurements, the MI were cooled with ice. The MI were dispersed in 20 mL of an isotonic KCl solution (Suprapur). Measurements were performed utilizing an Autolab PGSTAT 12 with an integrated high-performance module ADC 750 (Eco Chemie, Utrecht, Netherlands), interfaced to a PC in conjunction with an electrode stand VA 663 (Metrohm, Herisau, Switzerland). A multimode electrode using a static mercury drop (SMDE, drop size 3) was used as the working electrode, and a Pt rod and an Ag|AgCl (3 M KCl, $E = 0.208 \text{ V}$ vs. the standard hydrogen electrode, SHE) electrode served as auxiliary and reference electrode, respectively. We performed chronoamperometry within 1.5 s by sampling in intervals of $50 \mu\text{s}$ (normal-resolution mode), and within 0.04 s by sampling in intervals of $1.33 \mu\text{s}$ (high-resolution mode), respectively. At least eleven repetitive measurements were performed at each temperature point.

Particle sizes were determined by laser Doppler anemometry measurements with a Zetasizer Nano-ZS (Malvern Instruments, UK).

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