

Experimental Demonstration of Localized Excess Protons at a Water-Membrane Interface

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Abstract

The widespread Mitchellian proton motive force equation has recently been revised with the proton-electrostatics localization hypothesis, which, for the first time, successfully elucidates the 30-year longstanding energetic conundrum of ATP synthesis in alkaliphilic bacteria. To demonstrate the fundamental behavior of localized protons in a pure water-membrane-water system in relation to the newly derived pmf equation, excess protons and excess hydroxyl anions were generated by utilizing an “open-circuit” water-electrolysis system and their distributions were tested using a proton-sensing aluminum membrane. The proton-sensing film placed at the membrane-water interface displayed dramatic localized proton activity while that placed into the bulk water phase showed no excess proton activity during the entire experiment. These observations clearly match with the prediction from the proton-electrostatics localization hypothesis that excess protons do not stay in water bulk phase; they localize at the water-membrane interface in a manner similar to the behavior of excess electrons in a conductor. This finding has significance not only in the science of bioenergetics but also in the fundamental understanding for the importance of water to life in serving as a proton conductor for energy transduction in living organisms.

Keywords: Proton-electrostatic localization; Localized excess protons; Proton capacitor at water-membrane interface; Corrected pmf equation; Bioenergetics; Importance of water to life

Introduction

Peter Mitchell’s work on his chemiosmotic theory [1-3] won the 1978 Nobel prize in chemistry and his bioenergetics (proton motive force) equation since then was introduced into many textbooks [4-6]. One of the forms of the Mitchellian bioenergetics equation is expressed as free energy difference $\Delta\tilde{\mu}H^+$ (also known as the Gibbs energy change (ΔG)) for protons across a biological membrane including a term for the concentration difference and a term for the electrical potential:

$$\Delta\tilde{\mu}H^+ = \Delta G = RT \ln \frac{[C_2]}{[C_1]} + ZF\Delta\psi \quad (1)$$

Where C_1 and C_2 are the proton concentrations on the two sides of the membrane, Z is the charge on a proton (1 for a proton), F is the Faraday’s constant, $\Delta\psi$ electrical potential difference across the membrane, R is the gas constant, and T is the absolute temperature.

This Mitchellian equation (1), sometimes, is written also as the equation for the proton motive force (pmf) Δp that drives the protons through the ATP synthase:

$$pmf (\Delta p) = -\Delta\tilde{\mu}H^+ / F = \Delta\psi - 2.3RT / F \times \Delta pH \quad (2)$$

Where ΔpH is the pH difference between the two bulk aqueous phases separated by the membrane.

From equation (1), one can clearly see that Mitchell treated the protons as solutes such as sugar molecules that are delocalized and can stay everywhere in the bulk aqueous phase. Consequently, the Mitchellian delocalized proton-coupling view is that the ATP synthase is coupled to the redox proton pumps via bulk phase-to-bulk phase proton electrochemical potential gradients generated across the biological membrane; while the membrane is regarded as an insulator between the two bulk phases that plays no role in the lateral transduction of the protons to the ATP synthase.

The chemiosmotic theory was a major milestone in the history of

bioenergetics when the early bioenergeticists including the “metabolic enzymologists” were still using substrate-level phosphorylation as a model to seek some kind of “chemical coupling” for “phosphorylated intermediate” with “energy-rich” squiggle (~) bonds [3,7]. The chemiosmotic theory provided a revolutionary concept that the enigmatic link between electrogenic proton pumps and a proton-translocating ATP synthase is a proton gradient across the membrane [8]. Its revolutionary significance or influence to the field of bioenergetics could be hardly overstated, which is almost something like the Schrodinger equation to the modern quantum mechanics. It generated continuous discussions and sometimes heated debates that energized the entire field of bioenergetics. As a result, biochemists can now understand the biological energy transduction processes far better than before the era of the chemiosmotic theory [8].

However, the question of whether the proton pathway is delocalized throughout the bulk aqueous volume or is localized at its membrane surface has remained open to discussion since it was first raised in 1961 by Williams [9-13]. He [14] rightly pointed out the deficiency of the Mitchellian delocalized proton-coupling view by stating: “If charge is thrown out into the medium, as in osmotic theories, then we face the problem of equilibration of the energy of single cell on its outside with the whole of the volume in which it is suspended, say the Pacific Ocean.”

This statement made by Williams 40 years ago remains as a valid criticism to the Mitchellian chemiosmotic theory even of today.

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Unfortunately, probably because of the “Storm and Stress” period in the history of bioenergetics where the debates among disjunctive factions of bioenergeticists were so fiercely that the opposing parties with such deep emotions apparently lost the ability to objectively consider the other’s points including Williams’ “Pacific Ocean” arguments [15]. Hopefully, our new generation of scientists who are not emotionally attached with any of the opposing factions and bear none of the historical baggage will be able to restore the scientific civility and help move the field forward. Let’s think about a bacterial cell growing in liquid culture medium in a flask. It is known that bacterial cell membrane is energized by pumping protons across the cellular membrane from the inside to the outside of the cell, which creates a proton motive force across the membrane. However, according to the Mitchellean equation (1 or 2) to create a proton motive force across the membrane, the bacterial cell in a liquid culture flask would have to cause a bulk-phase pH change in the entire volume of its liquid culture medium?, which physically is really impossible. From here, we can also understand that there is something really wrong in the Mitchellean equation (1 and 2) since it does not fit with the known physical reality here.

Perhaps, the most well-established scientific observations that showed the failure of the Mitchellean delocalized proton view are in alkalophilic bacteria, such as *Bacillus pseudofirmus* [16-18]. These bacteria keep their internal pH about 2.3 pH units more acidic than the ambient bulk pH 10.5, while $\Delta\psi$ is about 180 mV in the direction from outside across the cellular membrane to the cytoplasm [19-21]. The application of Eq. 2 in this case would yield a pmf (Δp) value so small (44.3 mV at T=298K) that it has remained as a mystery for the last three decades as to how these organisms can synthesize ATP [22-24].

This long-standing unresolved energetic conundrum [25,26] can now be explained by the proton-electrostatics localization hypothesis [27]. Recently, Lee (one of us) has further elaborated his proton-electrostatics localization hypothesis and derived the following new proton motive force (pmf) equation [28]:

$$\text{pmf}(\Delta p) = \Delta\psi + \frac{2.3 RT}{F} \left(\text{pH}_{\text{nb}} + \log_{10} \left[\frac{C}{S} \times \frac{\Delta\psi}{l \times F \left(\prod_{i=1}^n (K_{\text{pi}} \left(\frac{[\text{M}_{\text{PB}}^{i+}]}{[\text{H}_{\text{PB}}^{i+}]} \right) + 1 \right))} + [\text{H}_{\text{PB}}^{i+}] \right] \right) \quad (3)$$

Where $\Delta\psi$ is the electrical potential difference across the membrane; pH_{nb} is pH of the cytoplasmic bulk phase; $[\text{H}_{\text{PB}}^{i+}]$ is the proton concentration in the periplasmic bulk aqueous phase; C/S is the specific membrane capacitance; l is the thickness for localized proton layer; K_{pi} is the equilibrium constant for non-proton cations ($\text{M}_{\text{PB}}^{i+}$) to exchange for localized protons; and $[\text{M}_{\text{PB}}^{i+}]$ is the concentration of non-proton cations in liquid culture medium.

The use of this newly derived equation has recently yielded an overall pmf (Δp) value (215~233 mV) that is 4 times more than that (44.3 mV) calculated from the Mitchellean equation for the alkalophilic bacteria growing at pH 10.5 [28]. This newly calculated value is sufficient to overcome the observed phosphorylation potential ΔG_{p} of -478 mV to synthesize ATP in the bacteria. Therefore, the 30-year-longstanding bioenergetics conundrum in alkalophilic bacteria has now, for the first time, been successfully elucidated as how they are able to synthesize ATP.

The core concept of the proton-electrostatics localization hypothesis is built on the premise that a water body, such as the water within a bacterial cell, can act as a proton conductor in a manner similar to an electric conductor with respect to the electrostatic behavior

and charge conduction. This is consistent with the well-established knowledge that protons can quickly transfer among water molecules by the “hops and turns” mechanism that has been first outlined by Grotthuss two centuries ago [29-31]. By applying Gauss Law equation to a water-membrane-water system, it was found mathematically that the excess protons are localized at the water-membrane interface, forming a proton-capacitor-like structure: the localized excess protons-membrane-anions system [28]. Therefore, we may use the proton capacitor concept to calculate the effective concentration of the localized protons $[\text{H}_{\text{L}}^{+}]^0$ at the membrane-water interface in a pure water-membrane-water system assuming a reasonable thickness (l) for the localized proton layer using the following equation:

$$[\text{H}_{\text{L}}^{+}]^0 = \frac{C}{S} \times \frac{\Delta\psi}{l \times F} = \frac{\Delta\psi \cdot \kappa \cdot \epsilon_0}{d \cdot l \times F} \quad (4)$$

Where C/S is the membrane capacitance per unit surface area; F is the Faraday constant; κ is the dielectric constant of the membrane; ϵ_0 is the electric permittivity; d is the thickness of the membrane; and l is the thickness of the localized proton layer.

This proton-capacitor equation (4) is a key component for the revised pmf equation (3), which has an additional term that accounts for the effect of non-proton cations exchanging with the localized protons. An experimental demonstration of the proton capacitor concept with a pure water-membrane-water system in relation to Equation 4 is fundamentally important to testing the proton-electrostatics localization hypothesis. In this article, we report our recent experimental study in which the distribution of localized excess protons at a water-membrane interface was demonstrated for the first time.

Materials and Methods

Excess protons generation

Two ElectroPrep electrolysis systems (Cat no. 741196) purchased from Harvard Apparatus Inc. were used with one of them as a control. Each of these ElectroPrep electrolysis systems (Figure 1) comprised a cathode chamber, a small Teflon center chamber and an anode chamber. The small Teflon center chamber was inserted to the middle of the inter-chamber wall that separates the cathode and anode water chambers. To test the proton capacitor concept predicted by the proton-electrostatics localization hypothesis, a 25- μm thick aluminum membrane (Al) was sandwiched in between two pieces of impermeable 75- μm thick Teflon (Tf) membrane (all with a diameter of 2.35 cm), forming a Tf-Al-Tf membrane as shown in Figures 1, 2a and 2b.

Bulk-phase water pH measurement

After the Teflon center chamber was placed into the inter-chamber wall, the two compartments of each ElectroPrep electrolysis system were filled with ultrapure MilliQ-deionized water (Millipore, 18.2 $\text{M}\Omega\cdot\text{cm}$ at 22.5°C): 300 ml in the cathode chamber and about 600 ml in the anode chamber rendering an equal water level in both chambers (Figure 1). The bulk phase pH of the deionized water was measured using Inlab pure pro ISM pH probe (Mettler Toledo) in a small beaker at the beginning and in the anode and cathode chambers at the end of each 10-hour experiment.

Detection of localized excess protons with a proton-sensing film

Aluminum membrane (proton-sensing film) purchased from VWR was cut into round disks with a diameter of 2.35 cm. A Teflon

membrane disk (T_f) was sandwiched in between a pair of aluminum (Al) membrane disks with an equal-diameter of 2.35 cm, forming a proton-sensing Al-Tf-Al membrane assembly. This assembly was then fit with the small Teflon center chamber (Figure 2c). In addition, pieces of proton-sensing Al film were placed on the water surfaces (P_S and N_S), and more importantly into the water bulk-water phase (P_B and N_B) near the middle of both the anode and cathode water chambers (Figures 1 and 2a) to track the distribution of the created excess protons.

After the apparatus was set up as shown in (Figure 1), 200 V of electrolysis voltage was applied to the system for 10 hours using a Source Voltage/digital multi-meter system (Keithley instruments series 2400S-903-01 Rev E). The resulting electric current was measured as a function of time using the digital multi-meter interfaced with a PC computer using LabVIEW software. The area under the current versus time curve was integrated using Originpro 8.6 and LabVIEW program (detailed procedure in SI Materials and Methods).

Results and Discussion

Localized excess protons demonstrated with a proton-sensing film

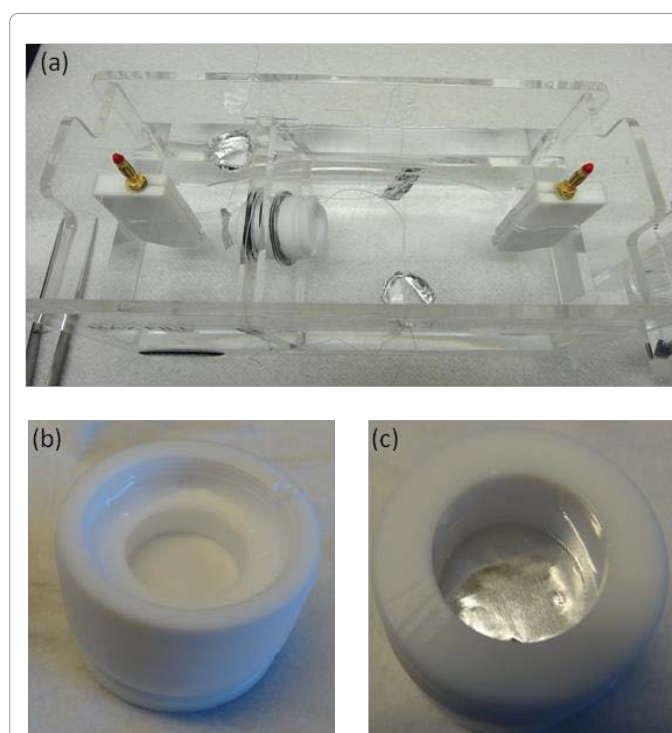
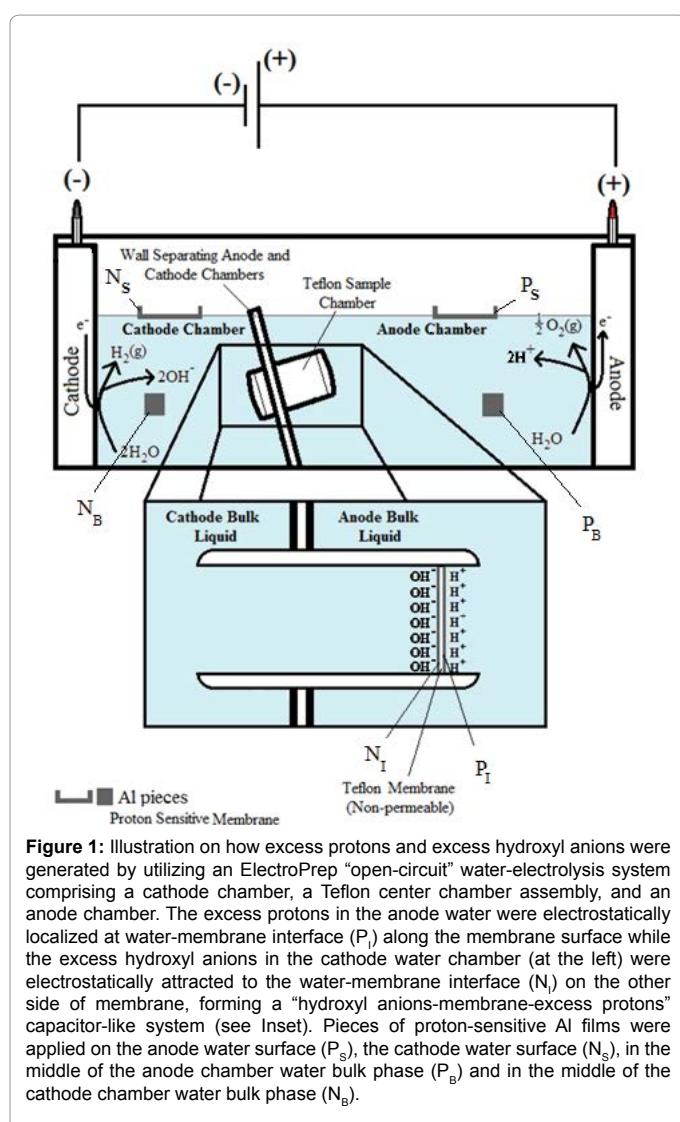


Figure 2: (a) A top view photograph showing the ElectroPrep apparatus. Pieces of proton-sensitive films were applied on the water surface and in the middle (bulk phase) of both the anode and cathode water chambers. Nylon strings were used to anchor the pieces of proton-sensitive films that were suspended in the middle of both the anode and cathode water chambers. (b) Teflon center chamber assembly with a Tf-Al-Tf membrane. (c) Teflon center chamber assembly with a proton-sensing Al-Tf-Al membrane.

During the open-circuit electrolysis of ultrapure water, excess protons were produced in the anode (P) chamber while excess hydroxyl anions were generated in the cathode (N) chamber (Figure 1). According to the proton-electrostatics localization hypothesis, the free excess protons in the anode water body would not stay in the bulk liquid phase; they would localize to the water-membrane (Teflon) interface (the P_I site) in the anode (P) chamber and attract the excess hydroxyl ions of the cathode water body to the N_I site at the other side of the membrane, forming an “excess anions-membrane-excess protons” capacitor-like system (as shown in the inset of Figure 1). According to this prediction, the bulk pH in either the anode water body or the cathode water body would not be affected by the excess protons or the excess hydroxyl ions created by the water electrolysis process. These predicted features were indeed observed in this experimental study. It is known that aluminum surface can begin to be corroded by protons when the effective proton concentration is above 0.1 mM (equivalent to a pH value below 4) [32,33]. This property was therefore employed as a proton-sensing mechanism in combination with the bulk phase pH electrode measurement to determine the distribution of excess protons in the water-membrane-water system (Figure 1). In the first set of experiments (performed in triplicate), small pieces of aluminum film were employed as a proton sensor at a number of locations in both of the water chambers to serve as an indicator for the excess protons. As illustrated in Figures 1 and 2c, a Teflon membrane (Tf) was sandwiched in between two pieces of aluminum film (Al), forming a proton-sensing Al-Tf-Al membrane system that separate the two water bodies: the cathode water body on the left and the anode water body on the right. The result of the “cathode water Al-Tf-Al water anode”

experiment showed that only the proton-sensing film placed at the P_1 site facing the anode liquid showed proton-associated corrosion (see the dark brownish grey on the exposed part of the proton-sensing film in Figure 3) while the proton-sensing film placed in the bulk liquid phase (P_B) of the anode chamber or floated on the top surface (P_S) of the anode water body showed no proton-associated corrosion activity. This is a significant observation since it indicates that excess protons are localized primarily along the water-membrane interface at the P_1 site, but not in the bulk liquid phase (P_B). This observation agrees with the proton-electrostatics localization hypothesis perfectly. Also as expected, all pieces of proton-sensing film placed at the N_1 , N_B , and N_S sites of the cathode liquid showed no-proton-associated corrosion activity as well.

According to the Mitchellian proton delocalized view, the excess protons in a water body would behave like a solute such as a sugar molecule which can stay anywhere in the liquid including its bulk liquid phase. Certain commonly heard arguments in favor of the Mitchellian proton delocalized view even as of today seem still believe that the excess protons would behave like solutes that could delocalize into the bulk liquid phase somehow by “proton solvation” or “electro diffusion”. If that delocalized view is true, it would predict that all the proton-sensing films in the anode water chamber including the one placed in the bulk liquid (P_B) should be able to detect the excess protons. The observation that the proton sensor placed into the anode chamber bulk water phase (P_B) could not detect any excess protons while the proton sensor placed at the P_1 site showed proton-associated aluminum corrosion activity clearly rejects the Mitchellian proton delocalized view.

Result of bulk-phase pH measurements

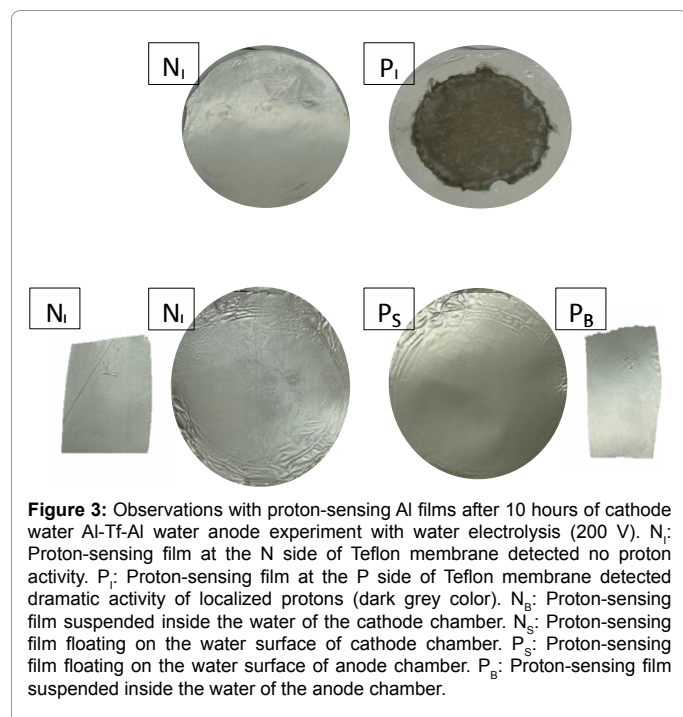
During a 10-hour experiment with 200V-driven water electrolysis, it was noticed, as expected, the formation of small gas bubbles at both the anode and cathode platinum electrodes. This observation is consistent with the well-known water electrolysis process in which

water is electrolytically oxidized to molecular oxygen (gas) producing protons in the anode water compartment while protons are reduced to molecular hydrogen (gas) leaving more hydroxyl anions in the cathode water compartment. If the Mitchellian proton delocalized view is true, it would predict that the production of excess protons in the anode water compartment would result in a lower pH value for the bulk water body while the generation of excess hydroxyl anions in the cathode water body would result in a higher pH in its bulk water body. That is, if the proton delocalized view is true, it would predict a significant bulk-phase pH difference (ΔpH) between the anode and the cathode water bodies. Our experimental result with the bulk-phase pH measurements demonstrated that the Mitchellian proton delocalized view is not true. As shown in Table 1, after the 10-hour experiment with the water Al-Tf-Al (membrane) water system, the measured pH value in the anode bulk water body (5.76 ± 0.09) remained essentially the same as that of the cathode bulk water phase (5.78 ± 0.14). These bulk water phase pH values averaged from 3 replication experiments (each replication experiment with at least 6 reading of pH measurement in each chamber water, $n=3 \times 6=18$) were statistically also the same as those (5.78 ± 0.04 and 5.76 ± 0.02) in the control experiments in absence of the water electrolysis process. This is a significant experimental observation since it confirmed the prediction of the proton-electrostatics localization hypothesis that the excess protons do not stay in the bulk water phase and thus cannot be measured by a pH electrode in the bulk phase.

This observation can also explain why in certain bioenergetic system such as thylakoids where ATP synthesis through photophosphorylation sometimes can occur without measurable ΔpH across the thylakoid membrane between the two bulk aqueous phases [9]. As shown in the present study, although the bulk-phase pH difference (ΔpH) between the anode chamber water and the cathode chamber water is zero, the excess protons were localized at the water-membrane interface as demonstrated by the dramatic proton activity on the proton-sensing film placed at the P_1 site (Figure 3). This indicated that the concentration of localized excess protons was much higher than 0.1 mM.

Furthermore, the measured pH value of 5.76 ± 0.09 in the anode bulk water phase was also consistent with the observation that the piece of proton-sensing film placed in the anode bulk water phase (P_B) showed no sign of proton-associated corrosion activity while the proton-sensing film placed at P_1 site had dramatic proton-associated corrosion (Figure 3). This indicated that the generated excess protons are localized primarily at the water-membrane interface at the P_1 site resulting in a proton surface density that is high enough ($pH < 4$) to cause the aluminum corrosion there.

The pH measurements also showed that the freshly deionized water had an average pH value of 6.89 ± 0.03 before being used in the experiments (Table 1). Since the experiments were conducted with the laboratory ambient air conditions, the gradual dissolution of atmospheric CO_2 into the deionized water during a 10-hour experiment period resulted in water pH change from 6.89 ± 0.03 to 5.68 ± 0.06 , which was observed in the control experiment with the same “cathode water Al-Tf-Al water anode” setup except without turning on the electrolysis voltage (0 V). Therefore, this bulk water pH change had little to do with the 200V-driven water electrolysis process. The same magnitude of bulk water pH change before and after the experiment was observed for the deionized water in both the anode and cathode chambers, which also supports the understanding that this bulk water pH change from the beginning to the end of the experiment was due to the gradual dissolution of atmospheric CO_2 into the deionized water during the 10-hour experiment period. There was no difference between



| Experiments | | pH of cathode water | pH of anode water |
|------------------------------|--------|---------------------|-------------------|
| With (Al-Tf-Al) 200 V | Before | 6.89 ± 0.03 | 6.89 ± 0.03 |
| | After | 5.78 ± 0.14 | 5.76 ± 0.09 |
| With (Tf-Al-Tf) 200 V | Before | 6.71 ± 0.10 | 6.71 ± 0.10 |
| | After | 5.81 ± 0.04 | 5.76 ± 0.03 |
| With (Al-Tf-Al) control (0V) | Before | 6.89 ± 0.03 | 6.89 ± 0.03 |
| | After | 5.68 ± 0.06 | 5.78 ± 0.02 |
| With (Tf-Al-Tf) control (0V) | Before | 6.71 ± 0.10 | 6.71 ± 0.10 |
| | After | 5.76 ± 0.02 | 5.78 ± 0.04 |

Table 1: Averaged pH values measured in bulk water phase before and after 10 hours experiment with cathode water membrane water anode systems.

*The averaged pH values and standard deviation (\pm sign) were calculated from the original data of bulk water phase pH measurements presented in detail in the Support Information document (Tables S1a, S1b, S2a, S2b, S3a and S3b) (Supplementary File).

the bulk-phase pH of anode chamber water (pH 5.76 ± 0.09) and that of the cathode chamber water (5.78 ± 0.14) at the end of the experiment. This result also points to the same underline understanding that the excess protons do not behave like typical solute molecules. Excess protons do not stay in the water bulk phase; they localize at the water-membrane interface at the P_1 site so that they cannot be detected by the bulk-phase pH measurement.

A further set of experiments with the setup of “cathode water Tf-Al-Tf water anode” was also conducted in triplicate. In this set of experiments, Tf-Al-Tf membrane system was used instead of the Al-Tf-Al membrane system. Since the Teflon membrane is chemically inert to protons, the use of the Tf-Al-Tf membrane system eliminated the consumption of excess protons by the aluminum corrosion process at the P_1 site that was demonstrated above. In this set of the experiments, no bulk-phase pH difference (Δ pH) between the anode and cathode water bodies was observed as well. As shown in Table 1, after 10 hours run at 200V with the “cathode water Tf-Al-Tf water anode” system, the measured pH value in the anode bulk water phase (5.76 ± 0.03) was essentially the same as that of the cathode bulk water phase (5.81 ± 0.04). This experimental observation again indicated that the excess protons do not stay in the bulk water phase and thus cannot be measured by the bulk liquid phase pH measurement. Since liquid water is an effective proton conductor as discussed above, the excess protons produced in the anode water compartment electrostatically localize to the water-membrane interface at the P_1 site.

Excess protons assessed with water electrolysis electric current

The proton-charging-up process in this “excess hydroxyl anions Tf-Al-Tf excess protons” capacitor system was monitored by measuring the electric current of the 200V-driven water electrolysis process as a function of time during the entire 10-hour experimental run. The data in the inset of Figure 4 showed that the electric current of the water electrolysis process decreased with time as expected. That is, when the excess protons were generated in the anode water compartment (while the excess hydroxyl anions were generated in the cathode water compartment), this “excess hydroxyl anions Tf-Al-Tf excess protons” capacitor is being charged up by localization of the excess protons at the P_1 site and the excess hydroxyl anions at N_1 site (Figure 1). According to our analysis, this process reached thermodynamic equilibrium after about 1500 seconds (shown in the inset of Figure 4) under this experimental condition where the curve of the water electrolysis current quickly became flat indicating the completion of the water electrolysis-coupled proton-charging-up process.

By calculating the area under the water-electrolysis current curve

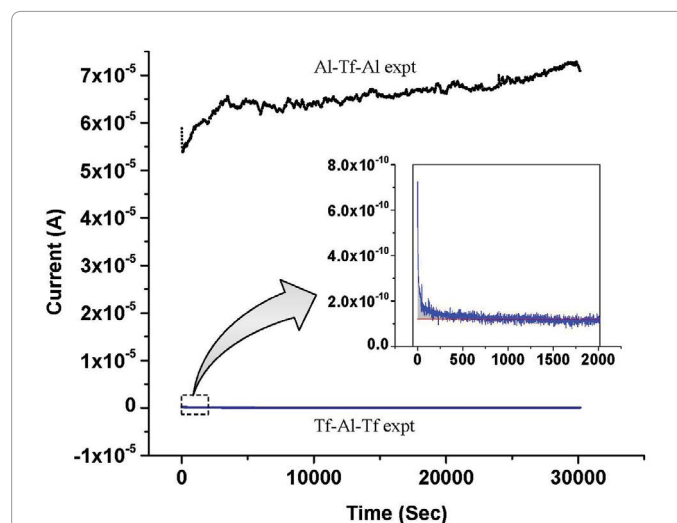


Figure 4: The electric current of water electrolysis measured as a function of time with 200 V during 10 hours experimental run. The black curve shows average of three experiments with “cathode water Al-Tf-Al water anode”. The blue line shows average of three experiments with “cathode water Tf-Al-Tf water anode”; and its initial part within the first 2000 seconds is plotted in an expanded scale showing the integration for the area under the curve (Inset).

above the flat baseline as shown in the inset of Figure 4, the amount of excess protons loaded onto the “excess hydroxyl anions Tf-Al-Tf excess protons” capacitor was estimated to be 2.98×10^{13} moles (Supplementary Table S5a). The area of the Teflon membrane surface exposed to the anode water at the P_1 site was measured to be 2.55 cm². If that amount of excess protons were loaded at the P_1 site onto the Teflon membrane surface exposed to the anode water, the maximal localized excess proton density per unit area was estimated to be 1.19 nanomoles H⁺/m². Although the exact thickness of the localized excess proton layer at the P_1 site is yet to be determined, our recent study [28] indicated that the effective thickness for this type of the electrostatically localized excess proton layer may be about 1 ± 0.5 nm. If that is the case, then the localized excess proton density of 1.19 nanomoles H⁺/m² would translate to a localized excess proton concentration of 1.19 mM H⁺ (equivalent to a localized pH value of 2.92 as calculated in SI) at the P_1 site, which can explain why they can be detected by the proton-sensing Al film there.

The water electrolysis current in the “cathode water Al-Tf-Al water anode” experiment was also monitored. As shown in Figure 4, after about 5000 seconds, the water electrolysis electric current at the steady state of this experiment reached around 6.5×10^{-5} A, which was much bigger than that (below 1×10^{-10} A) of the “cathode water Tf-Al-Tf water anode” experiment. This large water electrolysis electric current can be attributed to the consumption of excess protons by the proton-sensing Al film at the P_1 site. As the proton-sensing film at the P_1 site consumes the excess protons, more excess protons can then be produced at the anode electrode, resulting in a significant water-electrolysis electric current. The high concentration of the electrostatically localized excess protons at the P_1 site thermodynamically drives the aluminum corrosion reaction in which aluminum atoms are oxidized by protons resulting in evolution of molecular hydrogen gas. During the experiment, we indeed noticed the formation of gas bubbles on the aluminum membrane surface at the P_1 site (Figure S2).

By calculating the area under the water-electrolysis current curve from the “cathode water Al-Tf-Al water anode” experiment

and subtracting that of the “cathode water Tf-Al-Tf water anode” experiment, we were able to calculate the amount of excess protons that were generated by the anode and consumed by the proton-sensing film at the P_1 site. As shown in Supplementary Table S5b, during the 10-hr “cathode water Al-Tf-Al water anode” experiment, a total of 2.11×10^{-5} moles of excess protons were generated by the anode platinum electrode. These excess protons were apparently translocated to the proton sensing film surface at the P_1 site and consumed there by the corrosion reaction as shown in Figure 3b. The amount of protons consumed per unit area was calculated to be 8.29×10^{-6} moles per cm^2 .

Conclusion

The experimental results reported above clearly demonstrated that excess protons were localized at the water-membrane interface in the anode water-membrane-water cathode system. The most remarkable evidence for the localized excess protons came from the observation that the proton-sensing film placed at the P_1 site of Teflon membrane showed dramatic excess proton activity (corrosion) while the proton-sensing film placed into the anode chamber water bulk phase (P_B) showed no proton activity during the entire experiment. The density of localized excess protons created in this experiment was estimated to be about 1.19 mM H^+ (pH value of 2.92) at the water-membrane interface (P_1 site), which explains why it can be sensed by the proton-sensing Al membrane. Furthermore, the bulk-phase pH measurements in both anodic and cathodic water chambers also confirmed that excess protons do not stay in the bulk aqueous phase, which clearly rejects the Mitchellian proton delocalized view. These observations clearly match with the predictions from the proton-electrostatics localization hypothesis: excess protons do not stay in the water bulk phase; they localize at the water-membrane interface in a manner similar to the behavior of excess electrons in a conductor. This finding has significance not only in the science of bioenergetics but also in the fundamental understanding for the importance of water to life. It is now quite clear that water serves not only as a solvent and substrate but also as a proton conductor for proton coupling energy transduction in living organisms.

Furthermore, the localized excess protons that have now been demonstrated for the first time through this research may have practical implications as well. For example, the utilization of localized excess protons that can be created in pure water may lead to clean “green chemistry” technologies for industrial applications such as metal acid washing and/or protonation of certain micro/nanometer materials without requiring the usage of conventional acid chemicals such as nitric and sulfuric acids.

Author Contributions

J.W.L. designed research and experiments, analyzed data, and wrote the article; H.A.S. performed experiments, analyzed data and wrote the article under the guidance of J.W.L.

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Supporting Information

Supporting information for this article is available on-line at the journal website. The supporting information describes: (1) Detailed materials and methods; (2) Localized excess protons demonstrated with a proton-sensing

film; (3) Result of bulk-phase pH measurements; (4) Excess protons assessed with water electrolysis electric current; and (5) Experimental calculation of proton density.

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