Supporting Information

Multiple pH Waves Generated Electrochemically and Propagated from an Electrode Surface

Ilya Sterin, Anna Tverdokhlebova, Evgeny Katz, Oleh Smutok

Department of Chemistry and Biomolecular Science, Clarkson University, Potsdam, NY 13699-5810, USA.
* Corresponding authors: E-mails: osmutok@clarkson.edu (OS); ekatz@clarkson.edu (EK)
§ The authors contributed equally

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Chemicals

Sodium ascorbate (Sigma Aldrich), sodium phosphate dibasic heptahydrate (Thermo Fisher Scientific), sodium phosphate monobasic anhydrous (Thermo Fisher Scientific), sodium sulfate (Thermo Fisher Scientific), hydrogen peroxide (Thermo Fisher Scientific), hydrochloric acid (Thermo Fisher Scientific), sodium hydroxide (Thermo Fisher Scientific), dimethyl sulfoxide (Thermo Fisher Scientific), and ethyl alcohol anhydrous (Pharmco Aaper) were used as received without further purification. The pH-sensitive fluorescent dyes were synthesized according to the procedures reported in literature: 3,4'−dihydroxy−3',5'−bis−(dimethylaminomethyl)flavone (FAM345) and rhodamine−6−aniline (R6H). All experiments were carried out using ultrapure water (18.2 MΩ⋅cm; Barnstead NANOpure Diamond).

Instrumentation

Fluorescent images were obtained with Leica TCS SP5 II Tandem Scanning Confocal and Multiphoton Microscope. Processing of images for pH estimation was done with Adobe Photoshop software. Fluorescence measurements were performed in 96-well microplate using a SpectraMax MiniMax 300 Imaging Cytometer (Molecular devices) and SoftMax Pro 6 software. A Mettler Toledo S20 SevenEasy pH meter was used to measure pH of the solutions. Cyclic voltammetry and constant-potential electrolysis experiments were performed using an electrochemical workstation (ECO Chemie Autolab PASTAT 10) and GPES 4.9 (General Purpose Electrochemical System) software. The potentials were measured using a BASi Ag|AgCl|KCl, 3 M, reference electrode.

Electrochemical experiments

Optimal solution for the system was composed of 12.5 µM FAM345 dye, 15 µM R6H dye, 6 mM sodium ascorbate, 4 mM H₂O₂, O₂ in equilibrium with air, 100 mM Na₂SO₄ in 2 mM phosphate buffer, pH 7.0. Petri dish was used to improvise an electrochemical cell. A part of the Petri dish was separated with a plasticine barrier to form the electrochemical cell (see Figure SI4). A graphite flat electrode (GFE), 3 cm² geometrical surface area, was used as the working electrode. A GFE electrode was polished with 1500 Grit sandpaper (3 M Wettodry), sonicated in ethanol for 3 minutes, and finally polished with a KimTech kimwipe (Kimberly-Clark Professional) to prepare the electrode surface for experiments. A similar electrode was used as a counter electrode. The potentials were measured using a BASi Ag|AgCl|KCl, 3 M, reference electrode. Potential scan rate for all experiments was 10 mV/s. Confocal fluorescence measurements were performed during the constant-potential electrolysis (see Figure SI3 for the experimental setup). Potential switch programs used to achieve triple pH waves are shown in Table 1.
Table 1. The optimized experimental conditions for creation of triple pH waves

<table>
<thead>
<tr>
<th>Triple wave type</th>
<th>Potential applied, V</th>
<th>Time interval, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic-basic-acidic</td>
<td>+0.6</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>-0.8</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>+0.6</td>
<td>10</td>
</tr>
<tr>
<td>Basic-acidic-basic</td>
<td>-0.8</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>+0.6</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>-0.8</td>
<td>15</td>
</tr>
</tbody>
</table>

**Dependence of dye’s fluorescence on bulk pH**

To check the dependence of dyes’ fluorescence intensity on the solution pH, 0.5 mL solutions were prepared in 2 mM phosphate buffer, which contained 12.5 µM FAM345 or/and 15 µM R6H, 4 mM H₂O₂, O₂ in equilibrium with air, 6 mM sodium ascorbate, 100 mM Na₂SO₄. Then, fluorescence was assessed on the confocal microscope (CFM), and on the Imaging Cytometer (microplate reader).

For FAM345 pH was varied between 7 and 11. For R6H pH was varied between 3 and 7. The pH step in both experiments was 1 pH unit.

1) Settings on microplate reader:
   - FAM345: λₑₓ 405 nm, emission recorded between 470 and 550 nm. Emission maximum is observed @ 530 nm.
   - R6H: λₑₓ 540 nm, emission recorded between 565 and 605 nm. Emission maximum is observed @ 575 nm.
2) Settings on confocal microscope:
   - FAM345: λₑₓ 405 nm, emission recorded between 470 and 610 nm, gain 920.
   - R6H: λₑₓ 561 nm, emission recorded between 570 and 630 nm, gain 790.
   - Magnification 10×

**Processing of confocal fluorescent microscope images for the local (interfacial) pH estimation**

The brightness of an image was assessed as a mean gray value, that was calculated using an analysis tool in Adobe Photoshop (namely, selecting Image → Analysis → Record Measurements). For evaluation of pH distribution, a band perpendicular to the electrode surface was selected, which was then divided in equal parts, and in each part the mean gray value was calculated. The cut-offs were set by visual perception using software of confocal microscope which automatically showed the layer thickness.
Concentration dependence of the pH layer formation
The experiments were performed with different concentrations of \( \text{H}_2\text{O}_2 \) and ascorbate (note that the \( \text{O}_2 \) concentration was always the same under equilibrium with air), then always resulting in the formation of layers with different pH values. The results reported in the paper were performed using experimentally optimized concentrations of the reactants which allowed the best visualization of the pH layers in the presence of fluorescent dyes. The optimized solution composition was: \( \text{O}_2 \) (in equilibrium with air), 4 mM \( \text{H}_2\text{O}_2 \), 6 mM sodium ascorbate, 12.5 \( \mu \text{M} \) FAM345, 15 \( \mu \text{M} \) R6H, and 100 mM \( \text{Na}_2\text{SO}_4 \) in 2 mM phosphate buffer, pH 7.0.

Justification of the use of \( \text{H}_2\text{O}_2 \) as an additional oxidizer along with \( \text{O}_2 \)
In some recent publications were used only \( \text{O}_2 \) as the redox species which reduction accompanied with \( \text{H}^+ \) consumption resulted in pH increase (see for example refs. 4, 19 in the paper). This was sufficient for formation of single pH layers with the increased pH values. When the present project was started, we have tried to use \( \text{O}_2 \) as the reducible species, following our previous results. However, soon we found experimentally that formation of multiple pH layers requires higher concentration of redox species. Since it was not easy to increase \( \text{O}_2 \) concentration in the solution (otherwise we had to use a closed electrochemical cell which was not easy to accommodate in the optical system of the confocal microscope), we decided to add another reductant, which was \( \text{H}_2\text{O}_2 \). Notably, \( \text{H}_2\text{O}_2 \) is an intermediate product of the \( \text{O}_2 \) electrochemical reduction, thus, its presence does not change too much the mechanism of the process. The use of \( \text{H}_2\text{O}_2 \) allowed easy variation and optimization of its concentration. Actually, the experiments could be performed using only \( \text{H}_2\text{O}_2 \) without oxygen, however, this would require a closed electrochemical cell with removed \( \text{O}_2 \), thus complicating the experimental setup.

References
Figure SI1. (A) Electrochemical reduction of O$_2$ and H$_2$O$_2$ resulting in H$^+$ ion consumption and local (interfacial) pH increase. (B) Electrochemical oxidation of ascorbate (Asc) producing dehydroascorbate (DHAsc) resulting in H$^+$ ion release and local (interfacial) pH decrease.
Figure SI2. Cyclic voltammograms of solutions with different compositions: (a) the background solution composed of 2 mM phosphate buffer, pH 7.0, under Ar (oxygen was removed by Ar bubbling through the solution at least 40 min); (b) the background solution composed of 2 mM phosphate buffer, pH 7.0, under air (oxygen was present in the solution under equilibrium with air); (c) 4 mM H$_2$O$_2$ was added to the background solution in the presence of O$_2$; (d) 6 mM sodium ascorbate was added to the background solution in the presence of O$_2$; (e) 6 mM sodium ascorbate and 4 mM H$_2$O$_2$ were added to the background solution in the presence of O$_2$. Potential scan rate 10 mV/s.

Comments:

a) All cyclic voltammetry experiments were performed with 12.5 µM FAM345 dye and 15 µM R6G dye added to the background solution to exclude the possibility of their participation in the redox reactions. Note that the cyclic voltammogram of the background solution in the absence of O$_2$ (under Ar) doesn’t show any waves which may originate from the fluorescent dyes present in the solution (curve a).

b) Note that the anodic peaks at ca. 0.35 V (curves d and e) corresponding to electrochemical ascorbate oxidation are not affected by the presence of H$_2$O$_2$, thus showing no chemical ascorbate oxidation by H$_2$O$_2$.

c) Note significant increase of the cathodic current at ca. -0.5 V in the presence of H$_2$O$_2$ compared with the solution containing O$_2$ only. Thus, the reduction of H$_2$O$_2$ significantly contributes to the local pH increase.
Figure SI3. The experimental setup including the confocal fluorescent microscope and the electrochemical instrument.
Figure SI4. The experimental setup; close view on the electrochemical cell.
Figure SI5. pH-Dependent fluorescent dyes used in the study.
Figure SI6. Colored (green) images: Confocal fluorescent microscopy images of FAM345 dye obtained at different bulk pH values. Solution composition: 12.5 µM FAM345 dye, 6 mM sodium ascorbate, 4 mM H₂O₂, O₂ in equilibrium with air, 100 mM Na₂SO₄ in 2 mM phosphate buffer pH 7÷11. The plot shows dependence of the FAM345 fluorescence intensity on the bulk pH values (based on images’ brightness).

How the images were processed: Confocal images of FAM345 dye taken at different pH values were processed through Adobe Photoshop software to relate color intensity with pH. The brightness of an image was assessed as a mean gray value, that was calculated using a software analysis tool (namely, selecting Image → Analysis → Record Measurements). This calibration was later used to quantify pH in images obtained from electrochemical experiments.
Figure SI7. Dependence of FAM345 fluorescence intensity on the bulk pH values (calibration based on fluorescence intensity). The fluorescence was measured in the bulk solution with a fluorimeter. The dependence of FAM345 dye fluorescence on different bulk pH values was confirmed with two instruments: confocal microscope (see Figure SI6) and microplate fluorescence reader (imaging cytometer) (the present calibration plot).
Figure SI8. Colored (pink) images: Confocal fluorescent microscopy images of R6H dye obtained at different bulk pH values. Solution composition: 15 µM R6H dye, 6 mM sodium ascorbate, 4 mM H₂O₂, O₂ in equilibrium with air, 100 mM Na₂SO₄ in 2 mM phosphate buffer pH 3÷7. The plot shows dependence of the R6H fluorescence intensity on the bulk pH values (based on images’ brightness).

How the images were processed: Confocal images of R6H dye taken at different pH values were processed through Adobe Photoshop software to relate color intensity with pH. The brightness of an image was assessed as a mean gray value, that was calculated using a software analysis tool (namely, selecting Image → Analysis → Record Measurements). This calibration was later used to quantify pH in images obtained from electrochemical experiment.
Figure S19. Dependence of R6H fluorescence intensity on the bulk pH values (calibration based on fluorescence intensity). The fluorescence was measured in the bulk solution with a fluorimeter. The dependence of R6H dye fluorescence on different bulk pH values was confirmed with two instruments: confocal microscope (see Figure S18) and microplate fluorescence reader (imaging cytometer) (the present calibration plot).
Figure SI10. Effect of buffer capacity on the propagation of local pH front. Phosphate buffer, pH 7.0, concentrations: (A) 1 mM, (B) 2 mM, (C) 4 mM, (D) 6 mM, (E) 8 mM, (F) 10 mM. The electrolysis corresponding to O$_2$ electrochemical reduction (O$_2$ was dissolved in equilibrium with air) was performed at -0.8 V (vs. Ag/AgCl reference). The green fluorescence was produced by the FAM345 dye (10 µM). The confocal microscope images were taken 50 s after application of -0.8 V potential. Note significant decrease of the pH change propagation upon the buffer concentration increase.
Figure S11. pH-Distribution within the solution layers (single layer formation). (A) A confocal fluorescence image of a basic layer produced electrochemically upon O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} reduction at the potential -0.8 V. (B) pH-Distribution within the layer shown in (A). (C) A confocal fluorescence image of an acidic layer produced electrochemically upon ascorbate oxidation at the potential +0.6 V. (D) pH-Distribution within the layer shown in (C). The solution composition: O\textsubscript{2} (in equilibrium with air), 4 mM H\textsubscript{2}O\textsubscript{2}, 6 mM sodium ascorbate, 12.5 µM FAM345, 15 µM R6H, and 100 mM Na\textsubscript{2}SO\textsubscript{4} in 2 mM phosphate buffer, pH 7.0. The images were taken 70 s after the potential application.
**Figure SI12.** pH-Waves (basic-acidic-basic) propagating from the electrode surface upon reversible switching of the potential applied on the electrode. The experiment is similar to one shown in Figure 2 in the paper, but with the potential switch in a different order. An animated image showing transformations of the layers with different pH values is available in the Supporting Information (see Image 2). (A) Confocal fluorescence images of the interface obtained at different time intervals: (a) 0 s, (b) 20 s, (c) 40 s, (d) 60 s, (e) 80 s, (f) 95 s. The thickness of the solution layers with different pH values is shown in the images. (B) Changes in the thickness of the solution layers with different pH values in time. Potential -0.8 V was applied at time 0 s, then switched to +0.6 V at time 65 s, and then returned back to -0.8 V at time 80 s. The potential of -0.8 V resulted in reduction of O$_2$ and H$_2$O$_2$ leading to the formation of a basic layer (increased pH) at the electrode layer. The potential of +0.6 V resulted in ascorbate oxidation leading to the formation of an acidic layer (decreased pH) at the electrode surface. The potential of -0.8 V reapplied on the electrode resulted in the formation of another basic layer (increased pH) at the electrode surface. The solution composition: O$_2$ (in equilibrium with air), 4 mM H$_2$O$_2$, 6 mM sodium ascorbate, 12.5 μM FAM345, 15 μM R6H, and 100 mM Na$_2$SO$_4$ in 2 mM phosphate buffer, pH 7.0.
Figure S13. The computer-processed images shown in Figures 2A,d and 3C in the paper. These example images have artificially increased contrast of green and pink colors corresponding to the layers with different pH values. The processing was done in a software called "Gimp" (GNU Image Manipulation Program). Actions on the tool bar were: Colors → Hue-Chroma → in the open window set Chroma bar on 75. Note that the artificial computer-processed image has slightly different numbers for the layer thickness, compared to the original image produced by the confocal microscope. Note that all other confocal fluorescent images shown in the paper and in ESI were not computer-processed and appear as obtained in the experiments.
Figure SI14. Confocal fluorescent images obtained with different concentrations of the redox species. The solution composition: O₂ (in equilibrium with air), 1 mM sodium ascorbate, 12.5 µM FAM345, 15 µM R6H, and 100 mM Na₂SO₄ in 2 mM phosphate buffer, pH 7.0. Potential program: -0.8 V (formation of basic layer) followed by 0.6 V (formation of acidic layer). The images were taken at 210 s and 250 s (A and B, respectively).

Comments:
As it can be seen, 1 mM ascorbate is not enough for clear visualization of the acidic layer that follows the basic layer. This required the use of higher ascorbate concentration, but such high concentration impaired visualization of basic layer (not shown here). The pH-layer formation was improved upon using experimentally optimized concentrations of ascorbate and H₂O₂ (see all other fluorescent images in the paper and SI).