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<td>Balakrishnan, Divya; Luxembourg Institute of Science and Technology, Material Research and Technology Lamblin, Guillaume; Luxembourg Institute of Science and Technology, Material Research and Technology Thomann, Jean-Sebastien; Luxembourg Institute of Science and Technology, Materials Research and Technology (MRT) van den Berg, Albert; University of Twente, MESA Research Institute Olthuis, Wouter; Universiteit Twente MESA+ Pascual Garcia, Cesar; Luxembourg Institute of Science and Technology, Materials Research and Technology</td>
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Electrochemical control of pH in nano-litre volumes

Divya Balakrishnan$^{a,b}$, Guillaume Lamblin$^a$, Jean Sebastien Thomann$^a$, Albert van den Berg$^b$, Wouter Olthuis$^b$, César Pascual-García$^{a,*}$

a. Luxembourg Institute of Science and Technology (LIST), 41 Rue du Brill, L-4422 Belvaux, Luxembourg

b. MESA+ Institute, University of Twente, Drienerlolaan 5, 7522 NB Enschede, Netherlands.

ABSTRACT: The electrochemical management of the proton concentration in miniaturised dimensions opens the way to control and parallelise multi-step chemical reactions but still it faces many challenges linked to the efficient proton generation and control of their diffusion. Here we present a device operated electrochemically that demonstrates the control of the pH in a cell of ~ 140 nL. The device comprises a microfluidic reactor integrated with a hydraulic mechanism that allows the exchange of reagents and the isolation of protons to decrease the effect of their diffusion. We monitored the pH with a fluorescence marker and calculated the final value from the redox currents. We demonstrate a large pH amplitude control from neutral pH’s beyond the fluorescence marker range at pH 5. Based on the calculations from the faradaic currents, the minimum pH reached should undergo pH ~ 0.9. The pH contrast between neutral and acid pH cells can be maintained during periods longer than 15 minutes with an appropriate design of a diffusion barrier.

KEYWORDS: pH control, proton generation, microreactor, proton diffusion, pseudocapacitance.
INTRODUCTION

The acidity or proton concentration in aqueous and organic solutions can be used to control a large number of chemical reactions. Some of them, like the assembly of biopolymers [1, 2], the control of enzymatic reactions [3, 4], the poration of cellular membranes [5] or the self-assembly of mesostructures [6] are fundamental for such important and disruptive applications like the in-situ synthesis in microarrays [7-10], the creation of DNA logic devices [10-12], systematic studies of drug delivery or the fabrication of new functional materials for the healthcare [13]. These applications can benefit from multiplexed schemes and the automation of repetitive routines. As it happened with transistors allowing the digital development in the last century, the device miniaturisation and electronic control is the key to achieve the necessary high-throughput to allow a qualitative step forward in the current state of the art of the automatic control of chemical reactions. However, in order to open the door for the implementation of many new applications into practical cases it is necessary to develop the means to automate and miniaturise the control of the pH.

While the effect of the chemical environment on electrodes is widely used for the development of many electrochemical sensors, its reciprocal, namely the use of electrodes to modify the chemical environment is much less studied and quite challenging [14]. In particular, the electrochemical control of pH to implement the automation of chemical reactions has been addressed before typically by the use of proton generation or consumption redox reactions [15-18]. However these approaches lack a good control of the pH, lead to the degradation of electrodes and/or the production of side products like hydrogen or oxygen. Many of these reactions are also
irreversible, blocking the implementation of multi-step processes. In addition, because of the small surface-to-volume ratio these systems usually achieved small pH changes. More recently, the cyclic control of pH in macroscopic volumes of few millilitres was demonstrated through the use of gold nano-composites\textsuperscript{[19]} or the use of hydrogen permeable bifacial working electrodes (WEs)\textsuperscript{[20]}. Yet the use of these configurations for miniaturised volumes requires modifications able to anticipate the fast diffusion between the electrodes and the reduction reactions of the protons at the counter electrode (CE), the protection of the electrodes that undergo several cycles and the possibility to exchange reagents in a multi-step process.

Here we present and demonstrate a design that takes advantage of the large surface area that can be achieved by microfabrication. For the control of the pH we use nano-structured electrodes functionalised with redox active self-assembled monolayers (SAM’s) , which we recently demonstrated to be capable of exchanging protons with small voltage biases in a reversible way for more than hundred cycles\textsuperscript{[21]}. The reactor design is integrated in a microfluidic platform that allows addressing the electrodes from an external control. In addition it comprises optical access to monitor the fluorescence of a pH marker and hydraulic actuation to open and close all the cells that can be miniaturised below nL volumes while enabling the possibility to exchange reagents for sequential steps. The pH changes were tracked by a fluorescence marker down to its minimum range at pH $\sim 5$. From the faradaic currents associated with proton exchange reactions we calculated that the real pH change was from neutral to pH $\sim 0.9$. With our experiments we demonstrated the production of protons and the control of their diffusions between cells of nL volumes. Technologically, the pH changes within the SNARF range are sufficient to unlock many enzymatic reactions\textsuperscript{[3,4,9,12]}, while the more abrupt changes below the SNARF range open the door to improve the yield of organic synthesis of biopolymers like nucleotides or peptides.
The proton diffusion was controlled using a custom designed diffusion barrier that allowed to validate that the pH contrast in the electrochemical cells did not significantly vary during more than 15 minutes. The major technological improvements with respect to other configurations in literature are: the demonstration of the large pH changes confined in nL volumes, the long lasting stability of the set pH and the use of immobilised SAM’s to protect the electrodes from corrosion.

EXPERIMENTAL

A. Design and fabrication of the μ-electrochemical cells.

The general design of the electrochemical micro-reactor is schematised in fig. 1(a). It consists of a two electrode cell where the WE and the CE electrodes are separated by a diffusion barrier. The electrodes are functionalised with organic molecules able to store or release protons at low applied voltage. Shown in Fig. 1(b), the design may also include a pseudo-reference electrode connected to the WE through a diffusion barrier. The electrodes were platinised to obtain a large surface to area ratio, favourable for the impact of proton exchange reactions in the pH.

The oxidation and reduction of organic aromatic molecules like aniline can be used in a reversible manner to exchange protons with the objective of changing the acidity. These reactions happen at low potentials, which makes them suitable for the control of pH in an electrochemical cell where other reagents could be present. Some approaches have used the small pH changes happening in proximity of the electrodes during the oxidation of aniline to control other chemical processes, but the fast diffusion of protons in liquids hampers the use of this method to processes that require long reaction times or large pH amplitudes. In order to overcome, at least partially, these issues electrodes coated with porous materials
immersed in scavenging solutions were used allowing the synthesis of nucleotide polymers [9]. However the remaining proton diffusion and the buffering effect of the electrolyte hampered the efficiency of these reactions that needs to be close to 100% in order to meet the requirements of commercial applications.

Conceptually, the simplest solution to avoid the proton diffusion may be their physical confinement. Blocking the diffusion of reagents in independent cells has been demonstrated to improve the contrast and the yield of multiplexed peptide and nucleotide reactions in a microfluidic configuration very similar to the one we employ [25, 31]. However to confine the electrochemical redox reactions involving protons one must take into account that, while they are produced in the oxidation processes at the WE they are also reduced in the CE into hydrogen gas.

To overcome this issue one solution is to separate physically the electrodes by diffusion barriers. For the ease of implementation and design we have chosen a linear diffusion barrier made with a long channel in form of serpentine characterised by a length $L$, much longer than their width and height. Migration and diffusion, the two mechanisms that bring protons from the WE to the CE, can be considered in this case one dimensional. Migration may be avoided with a moderate ionic strength (see SI). The use of high ionic strength electrolytes (1 M KCl in our case) also decreases the over-potential produced by the current resistivity across the diffusion barrier. The diffusion in one dimension will be ruled by the length of the barrier and the diffusion constant of protons ($D=9 \times 10^{-5} \text{cm}^2/\text{s}$) [32], which provides the system a characteristic diffusion time $\tau$ in which the diffusion barrier is effective:

$$\tau=L^2/\pi^2 D \quad \text{Eq. (1)}^{[27]}$$

Considering the cell of fig. 1 (a) where the of WE and CE are separated by the unidimensional diffusion barrier, redox currents producing only protons and that these will exceed the ones at
neutral conditions (see SI for the limits of this approximation), for times much smaller than $\tau$, the pH can be approximated from the Faradaic redox currents ($I_{\text{redox}}$) or the total exchanged charged ($Q$) as follows:

$$pH = -\log \left[ \frac{I_{\text{redox}}}{F V_{\text{cell}}} \right] = -\log \left[ \frac{Q}{F V_{\text{cell}}} \right] \quad \text{Eq. (2)}$$

were $t$ is the time during which the current has been applied, $F$ is the Faraday constant and $V_{\text{Cell}}$ is the volume of the cell at the WE. The use of surface functionalised electrodes with redox active self-assembled monolayers (SAMs) like in [21], can be advantageous with respect to the use of aniline in solution because it avoids unnecessary diffusion limited mechanisms and the preservation of the electrodes that may suffer the polymerisation at the surface. In a redox active SAM the maximum charge exchange possible is the number of molecules at the surface (the surface functionalisation, $S$) multiplied by the total area of the functionalised electrode ($A_{\text{WE}}$) (and in this case, by the number of electrons exchanged per molecule). Eq. 2 can then be simplified considering also that the volume of the cell is $A_{\text{WE}}$ times its height $h$ to find what the minimum achievable pH is:

$$pH = -\log \frac{S A_{\text{WE}}}{h V_{\text{cell}}} = -\log \frac{S}{h} \quad \text{Eq. (3)}.$$ 

Thus, in order to favour large acidic amplitudes one must decrease as much as possible the height of the cell and increase the surface functionalisation. Nano-structuration of electrodes achieved by methods like platinisation [33] can increase the electrochemical surface area and the effective $S$.

Figure 1 (b) shows the design used in this article for the implementation of the electrochemical cell where the WE was connected with a serpentine barrier to the CE. The design includes also a third cell with a gold pseudo-reference electrode that we used to have a better control of the
redox reactions, and which was also connected by a serpentine diffusion barrier to the working electrode to be protected from the pH variations. The electrodes occupied a total area of 1 cm diameter, in which diffusion barriers longer than 2 cm could be introduced. The width of the diffusion barrier was 100 µm and the height delimited by the dielectric spacer (SU8 epoxy in our case) was 3.5 µm before the platinisation. Different chips were fabricated on top of 100 µm thermally grown oxide. Data in this article come from three of the samples studied. The fabrication steps are schematically detailed in the SI fig S1. The chips were fabricated by performing first the definition of the electrodes by optical lithography, then they were e-beam evaporated using a 3 nm Ti adhesion layer and 50 nm deposition of gold. The spacer barriers were defined using also optical lithography on the epoxy resist (SU-8). WE and CE were platinised following standard recipes[33] cycling them in a solution of 40 mM of chloroplatinic acid and 100 µM of lead acetate between -0.2 and 0.6 V against a Ag/AgCl reference electrode for 4 cycles at a scan-rate of 100 mV/s. After the platinisation the sample was rinsed in water and ethanol, and then cleaned under UV ozone lamp. This provided the electrodes more surface area, and further decreased the effective height of the chip (see more on supplementary information). Finally the functionalisation of electrodes was achieved by immersion of the chip in 0.5 mM 4-amino-thiol-phenol (4ATP) solution in ethanol overnight functionalising all three electrodes. No particular protection was used for the pseudo-reference electrode. The chip was thoroughly rinsed with ethanol and prepared for experiments into the microfluidic platform.

**B. µ-fluidic platform and experimental setup.**

Figure 1 (c) shows the schematic configuration of our microfluidic platform that consists in two frames to hold the chip, which is sealed with an elastic O-ring that allows two positions (pictures shown in SI fig S5). In the opened position the O-ring pushes down the exchangeable chip
allowing a ~ 60 µm gap between the chip and the top optical access to allow the cell filling and
the exchange of fluids. The chip can be pushed up in contact with a glass by a hydraulic actuator
placed in the bottom frame to reproduce the configuration shown in fig. 1 (a) and (c) where the
cells are isolated from the fluidic channel and connected only through diffusion barriers. The
glass may be used as optical access to track fluorescence changes in the cells. This configuration
does not follow the normal common paradigm of “lab on a chip” where normal valves and
actuators are implemented into channels but it can be used in the future to include several cells
allowing the parallelisation of several chemical reactions using a single hydraulic actuation.
However the microfluidic filling of the cell may present some issues due to the small dimensions
of the channel and the large area of the chip \cite{25}. To overcome these problems vacuum was
applied between the chip and the top glass before the filling. The chip is electrically contacted
through springs included in the top frame and connected to a switching box to allow the
electrodes to be grounded or connected to instrumentation. The microfluidic platform was placed
in the experimental set-up shown in fig. 1 (d). The electric signal was controlled by a Solartron
Modulab XM Pstat 1MS/s potentiostat using two and three electrode configurations. The
platform allows also optical inspection through the top glass window closing the cells.

The measurement of pH in nL volumes is a challenge in itself. Electrochemical measurement of
pH requires the integration of reference electrodes that consume valuable footprint, and which
can be difficult to incorporate. Only sensors like nanowires \cite{26,27} would be suitable with the
design of our small cells. Optical methods do not have an impact in the footprint. Plasmonic
devices using the enhancement of fluorophores or Raman signals associated to nanoparticles can
report high intensities \cite{28,29} but they require a long development for standardisation.
Fluorescence sensors are an effective way to monitor the pH \cite{30} if the signal is sufficient and the
fluorophore does not interact with the electrodes. The intensity of a fluorescence peak associated with a mini-band created by the electronic structure of an aromatic component of the molecule changes with the degree of protonation of some of the radicals. Fluorescein is one of the typical dyes with a high fluorescence yield used to measure the pH having one peak that, once calibrated, can report the pH value. The problem with single peak pH reporters is that the peak intensity can also change due to factors like photo-bleaching, aging or simply a change in the optical alignment.

The fluorescence dye carboxy semi-naphthorhodafluors (carboxy SNARFs) instead has two bands at 590 and 650 nm, which ratio can be used to track the pH in a range approximately from 8 to 5 \[^{[19]}\]. The electrolyte filling of the micro-reactor consisted of 1 M KCl, with the addition of 0.5 µM of SNARF. This dye was previously used with the same concentration to measure electro generated protons \[^{[19]}\]. These experiments and also ours (see the description of the experiments of electro-polymerisation in SI) proved that the pH measurements were not affected by interactions of the fluorophore with the electrodes. Figure 1 (e) shows the calibration of the SNARF fluorescence with pH using the relative intensity of the peaks at 530 nm and 580 nm excitation and also the intensity of the 650 nm line normalised to the peak intensity at pH 7.3. The inset shows representative spectra of the fluorescence using the 530 nm excitation (dotted lines). The 590 nm peak is stronger at acidic pHs while the 650 nm is dominant at neutral pH’s. In order to increase the fluorescence signal the light collected by the objective was sent into an optical fibre using collecting lenses, and then sent to a wide range spectrometer FLAME-S-VIS-NIR. With this configuration the illuminated/collected region had a radius of \(~63.5 \mu m\) in which the excitation power (1.3 mW) was distributed and averaged over a large number of molecules (\(~10^7\) molecules) avoiding photo-bleaching and limiting the thermal heating (details in SI). All the
spectra in the article, unless otherwise specified were integrated for 1 s, to allow monitoring real
time the pH changes. Due to the low integration time and the low power used we were forced to
excite at 580 nm close to the maximum of absorption of SNARF (grey line in the inset of figure
1 (e)) in order to maximise the fluorescence efficiency. To avoid the overlap of the signal from
the excitation diode and the stray light, we used a dichroic mirror CROMA-49017 with an
excitation window between 540 and 580 nm and with a low band pass filter at 590 nm for
collection. The inset of figure 1(e) shows also the comparison of the fluorescence spectra with
the 530 nm excitation line and the 580 through the dichroic mirror (solid and dotted lines
respectively). The spectra using both excitations were fit to two-Gaussian peak functions, with
fixed peak centre and width. Even the spectra through the dichroic mirror were cut close to the
centre of the 590 nm peak it was possible to fit them with enough accuracy, and both calibrations
provided 650 nm /590 nm peaks resulting in similar values (solid and open triangles for the
excitation at 530 nm and 580 nm respectively). The only difference we observed was slightly
higher values of the 590 nm peaks for the 580 nm excitation, which can be explained by a closer
resonance of the illumination wavelength with respect to that band. The red dots in figure 1 (e)
intensities of the 650 nm peak normalised to the maximum intensity at pH 7.3, which follow a
similar behaviour of the ratio of the two peaks. Thus the spectrum at pH 7.3 can be used to assess
the initial pH. The normalisation of the peak at 650 nm to the peak intensity at 7.3 provides then
a calibration independent of external factors like the alignment. Since the pH in our device could
go below the pH range of the SNARF in the article we show directly the fluorescence of the
SNARF or the normalised peak intensity instead of the pH calibration.

RESULTS AND DISCUSSION

A. Electrochemical control of pH in confined cells
The electro-polymerisation of 4ATP has been well studied on different articles including our own [21-23]. The resulting molecules are the dimers depicted in the inset of Fig. 2 (a) that support the quasi-reversible redox exchange reactions involving the exchange of 2 protons and 2 electrons. We performed the electro-polymerisation of the 4ATP layers functionalising the platinised electrodes with the cell opened and tracking the pH (details shown in the SI). After the polymerisation the electrolyte of the cell was exchanged to recover neutral pH and equilibrium conditions. The cell was then closed leaving the WE communicated only through diffusion barriers to the counter and the pseudo-reference electrodes, and connected to our potentiostat as shown in figure 2 (b). The cyclic voltammogram of the polymerised 4ATP is shown in Fig 2 (a). The CV potential was narrowed with respect to the polymerisation process between ± 0.75 V, using a scan-rate of 50 mV/s. The characteristic quasi-reversible oxidation and reduction peaks attributed to proton exchange reactions of the polymerised 4ATP were observed at ~ 0.35 and ~ -0.3 V respectively. It is noteworthy highlighting that during the electropolymerisation that we did in the chamber (see SI) there was no oxidation peak at 0.35 V during the first cycle before the electropolymerisation, indicating that only the polymerised molecules lead to reversible proton exchange reactions and that there is little or no contribution from the electrolyte (that contained SNARF). A change of the redox potentials is observed in our platinised electrodes with respect to the reactions on flat gold [21], which is the same as the one observed with the opened cell (see more experimental data from the electropolymerisation in SI), which shows that it is not due to the IR drop at the diffusion barrier, but rather to the different material of the electrode (nanostructured Pt in our case). The oxidation of the first cycle is a particular case because in this cycle the current is limited because at the CE there are no dimers of 4ATP that can reduce. The rest of the cycles in the voltammogram overlap, indicating that the proton
distribution from the equilibrium at the resting point reaches a steady state after the first cycle, with no further contributions from electro-polymerisation and that the number of protons in the system is constant. The reversibility of the CVs is also an indication that the electrodes do not suffer degradation and that the redox reactions are associated to reversible proton exchange reactions. Figures 2 (c) and (d) show the measured current and applied voltage time evolution, respectively. Cycles 2, 5 and 8 are highlighted in red, green and blue respectively and the rise and fall voltage of the cycles are depicted with light and darker colours respectively. The colour code on the cycles is maintained in all the figures including the representation of the fluorescence spectra.

Figures 2 (e), (f) and (g) show the sequence of SNARF fluorescence spectra extracted during the cycles 2, 5 and 8 with a vertical offset corresponding to equally spaced voltage differences during each cycle. The spectra corresponding to the positive and negative sweep rates and are indicated with light and dark tonalities matching the colours of figs. 2 (c) and (d). The left and right panels on each graph figure report the spectra taken at the WE and CE respectively. They were acquired first focusing the objective on top of the WE and then the experiment was repeated collecting the signal from the CE. Several series of 10 CV cycles showed to be equivalent after several experiments, so the spectra from the CE and WE were grouped according the time and voltage with respect to the open circuit before applying the driving voltage. The spectra just above the green dotted line correspond to the ones at the voltage indicated with the green arrow in Fig 2 (a) just before the oxidation process. These spectra show the maximum intensity dominated in all the cycles at the WE only by the peak at 650 nm indicating that the pH in that electrode was close to the initial neutral conditions before the oxidation that were used as reference. The behaviour is opposite at the CE where 650 nm
fluorescence was completely quenched beyond the SNARF range, indicating that from cycle 2 (t = 50 s) the polymerised 4ATP had released the protons to convert the cell environment of the CE into an acidic medium below pH 5 by the release of protons. After the oxidation of the 4ATP at the WE the SNARF fluorescence decreases indicating that the pH becomes lower in this cell and the opposite happens at the CE where the rise in the fluorescence signal is an indication of the increase of the pH because the protons in solution are recovered by the 4ATP. The comparison among the spectra at figs. 2 (e) to (g) show that the overall intensity of the SNARF fluorescence increases from cycle 2 to 8 both in the WE and CE cells. This increase of fluorescence reflects an increase of the average pH during the cycles and thus it is an indication that the number of protons is decreasing in the system. This can be explained because when the electrodes are negatively biased the protons may get reduced into molecular hydrogen H₂, more probably at gaps where 4ATP did not reach to cover the metal, and thus decreasing the total number of protons in the system. However, we did not observed any significant degradation of the 4ATP redox reactions on the CV and we always obtained the quenching of the fluorescence signal. After each 10 cycles of each experiment, the pH and the fluorescence could be recovered for several experiments on both WE and CE by exchanging the electrolyte by a fresh one. Each chip could be used for experiments lasting at least one day.

The SNARF fluorescence could track the pH down to pH 5. Below the final pH can be derived using equation 2 since the reversible electrochemical processes in our system are only associated to the proton exchange reactions. Figure 2 (a) shows the overlap of the 10 different cycles studied in this figure, and the hatched area is the area corresponding to the 4ATP oxidation. The amount of charge associated to these faradaic currents is equivalent to the amount of protons released to the electrolyte and it can be used to calculate the total pH in the cell of the WE (eq.
This was possible in the case of the CV because the shape of the curves allows to distinguish the faradaic currents from the capacitive and resistive ones, but in other experiments the faradaic and non-faradaic currents could not be distinguished. The resulting charge exchanged was ~ 1.7 mC (see calculations in SI). We also consider that the time of each cycle is much smaller than the diffusion time through the barrier connecting the electrodes, and thus the produced protons were distributed only in the cell of the WE. Considering that the initial pH of the electrolyte was ~ 7.4 and that the total volume of the WE cell was ~ 140 nL, the maximum proton molarity was ~ 0.13 M, thus the final pH was ~ 0.9. To calculate the volume of the cell we have considered the initial height of the cell before platinisation, since this material is porous and the decrease in volume is difficult to measure (see details in SI). Thus the pH reported value above is just an upper limit, but this amplitude is at least ~ 5 pH units, which is enough to control many chemical reactions used in purposes like the ones already cited \cite{1-6}.

Figure 2 (h) shows a summary representation of the acid control in the cells. In the middle cartoon the cell status before oxidation is represented with the WE in red for the more basic conditions. In such state the 4ATP layer is reduced and works as a proton reservoir. Meanwhile in the CE the 4ATP is oxidised and the protons are in solution producing an acidic environment (represented in green in the cartoons). The acid concentration in the different cells can be reversed driving the system with voltage or current as represented on the top cartoon, which is the cell status after the oxidation peak. Since the system was driven out of equilibrium starting from a neutral electrolyte, the addition of protons from the 4ATP induces a cell status during the cyclic voltammetry between ~ 0.6 and ~ -0.6 V where both cells are acid and where the protons in the system are shared between both cells and not stored in one of the WE or CE (including the
bottom two spectra below the dotted line in figs 2 e to (g)). This situation is represented by the bottom cartoon in fig 2 (h) with both cells are depicted with the same colour.

To use the pH control in an efficient way it is necessary to keep the pH value constant over time because many chemical reactions are slower than the proton diffusion time. With the cell open, with no current inducing exchange of protons between the electrolyte and the electrodes the pH in the WE evolves to an equilibrium with the rest of the cell losing the control of the acidity in 10 s (see SI fig 7 (d) that reports the pH behaviour during the electropolymerisation with the opened cell). The micro-reactor built-in a 2 cm diffusion barrier where the diffusion time \( \tau \) from the WE to the CE should be \( \sim 75 \) minutes (eq. 1). Figure 3 (a) shows the fluorescence spectra after oxidation at the WE right after imposing open circuit conditions (red) and 15 minutes later (blue) with the cell closed to avoid diffusion. The spectra correspond to the same extremes of the pH shown in figure 2 and they were integrated for 10 s instead of 1, which resulted in a lower noise background spectrum shown in black. Figure 3 (b), shows the corresponding spectra at the CE for the same time lapses. The spectra were vertically shifted (the red and blue lines), other ways they would superimpose almost perfectly. In fig. 3 (a), the difference of the red and blue spectra with the background in black show clearly the existence of the peak at 590 nm, which was cut by the band-pass filter of the dichroic mirror. This maximum of fluorescence is the characteristic shape of SNARF when the pH is below its range in acidic conditions (fig 1 (e) and the comparison with the spectra SI fig S7 (b)). In fig. 3 (b) the fluorescence after current and 15 minutes later are dominated by the 650 nm peak characteristic of the basic/neutral conditions, reproduced in Figure 1 (e) for pH 7.3. There were no significant differences between the spectra after the end of the current control and 15 minutes later with open circuit conditions, indicating
that the pH was constant in the cell during this time, even with the basic conditions where the
SNARF fluorescence is more sensitive to pH variations.

The proton confinement not only improves the time in which the pH is stable, but also the
amplitude of the pH that can be achieved because the produced hydrogen ions are distributed in
smaller volumes. In figure 4, the applied voltage (in black) and the measured current (in red) is
shown as function of time corresponding to the second cycle of CV’s for the open and closed cell
configurations (figs 4 (a) and (b) respectively). The voltage was narrowed between 0.5 and – 0.2
V, which allowed us to control the pH change within the SNARF range before the fluorescence
was completely quenched, but which did not allow to distinguish the faradaic from the non-
faradaic currents to make a pH calculation. Figures 4 (c) and (d) show nevertheless the
corresponding SNARF fluorescence peak intensity collected right at the top of the WE for the
open and closed configurations respectively. The existence of redox reactions can be noticed by
the non-linear behaviour of the currents and the changes of pH sensed by the SNARF
fluorescence. As observed during the polymerisation experiments (see SI), the pH changes can
also be detected with the cell opened even if the WE and CE were in contact through the
electrolyte because the detection was close to the electrode and the focus depth of the
microscope is sufficiently small (1 µm). However the amplitude of this pH change is limited to ~
10 % of the maximum peak fluorescence (fig. 4 (c)) associated to a ΔpH of less than ~ 0.4 units
(fig. 1(e)). This change was even possible due to the small height of the channel compared to the
large area of the electrodes, but also because the focus of the microscope was on top of the
surface of the electrodes. On the other hand, within the same voltage range the fluorescence
quenching with the cell closed could be observed to decrease down to ~ 60 % of the original
maximum peak fluorescence (fig. 4 (d), associated to a ΔpH of ~1.4 units. The increase of the
amplitude change in the peak fluorescence is a proof of the increase of the pH change with the closed cell configuration.

**B. Control of the pH with a 2 electrode configuration.**

The implementation of reference electrodes in microfluidic devices is a cumbersome task. Equation 2 describes the pH in the cell as a function of the redox (Faradaic) currents that can be implemented with a two electrode configuration like the one shown in the inset of fig. 5 (a). Yet eq. 2 does not consider the kinetics of the system that is influenced by both the electrochemical Faradaic and the capacitive non-Faradaic components. In addition to the double layer capacitance, the diamine functionalities contribute to the capacitance by storing energy on the redox reactions that exchange electrons (pseudo-capacitance) \(^{34-37}\). The pseudocapacitance may significantly increase the overall capacitance of the amidized large surface area electrodes and influence the charging and discharging behaviour \(^{29}\). Figure 5 (a) shows applied square current pulses (in black) of ± 4 mA of amplitude, 2 s of excitation time and 10 s of resting time and the corresponding voltage response (in red). They were applied after 5 s of resting time with the system in equilibrium (open circuit and fresh electrolyte). During the application of the current, the voltage rises non-linearly due to the contribution of the IR drop produced by redox currents and the capacitance of the system followed by a decrease similar to discharge, but with no current flowing from the electrodes to the potentiostat. The normalised peak fluorescence acquired during the current excitations is shown in fig 5 (b). Since the cell is very small, the diffusion of protons is much faster than the integration time of each spectrum so the fluorescence indicates the pH variations in the cells that depends on the changes of the number of reduced or oxidized groups. A delay with respect to the applied current is observed that follows the
charging/discharging evolution of the voltage. To interpret this delay and the fact that the pH continues to lower even if there are no currents in the circuit, we take into consideration that the electron transfer rate of the redox reactions, which we measured in our previous article, is $\sim 0.6$ s $^{[21]}$, which is in the order of the on-time of the current pulse. Thus not all the molecules react during the large current pulse imposed and the built up potential, which is discharged through the redox reactions that we observe with a delay. This process can be described as a combination of a pseudo-capacitance that stores charge originated from the electron transfer between the metal and the benzenoid diamine molecules and a battery-like behaviour that yields a flat discharge plateau$^{[38]}$. Because of the discharging the system does not yield as a good capacitor, but the device shows the capability to study the electrochemical process with the advantage of being able to follow the proton exchange reactions with the pH probes.

Also noticeable is the difference of the pH amplitude between the first pulse and the others. As seen in fig. 2 (a), during the first cycle the oxidation reactions are limited because on the counter electrode all the amines are reduced, so they cannot accept protons to balance the oxidation reactions. After the second negative pulse, the system achieves a steady state, where the more basic and more acidic environments alternate between the oxidation and reduction of the working and counter electrodes respectively as explained in fig 2 (h). This dependence on the polymerised 4ATP dimers is another evidence that the hydrolysis or other components in the electrolyte are not responsible for the pH changes.

We repeated these experiments with current control on the same chip for nine times with different current amplitudes and duty cycles, with always the same behaviour. Complete quenching of the fluorescence could be achieve at large currents (see SI). No signs of fatigue or degradation of the electrodes were observed, which indicates that regardless the high voltages
measured between the electrodes the polymerised molecules were preserved. Neither did we observed any bubbles on the electrodes, thus we also exclude the electrolysis of water, probably due to the protecting effect of the 4ATP monolayer.

CONCLUSIONS

We have presented the design of an electrochemical device to control the pH in miniaturised systems. The pH is changed using proton exchange reactions, which are produced by quasi-reversible redox states of polymerised 4ATP molecules immobilised on top of nanostructured electrodes. Compared to other methods, this enhances the reversibility of the reactions with respect to other more corrosive processes \cite{15-18}, prevents modification of the electrodes by further polymerisations \cite{7-10}, and avoids diffusion limited mechanisms \cite{6,8,10,13}. It also allows the possibility to isolate the electrodes in the future to avoid interferences (using for example with proton membranes), although we have not observed any interference from the components of the electrolyte that we have used. Using a cell of a few micrometres high allowed a large impact on the pH of the occurring redox reactions. We were able to track the pH changes in a range down to the quenching of our fluorescence marker at 5 and we calculated that the pH in the cell could reach extremely low values down to ~ 0.9.

The effect of the proton diffusion is controlled by a diffusion barrier that can be tailored to maintain a stable pH in a cell for a time depending on the requirements of the chemical control needed. The comparison of the pH changes between the open and closed cell configurations showed that the confinement of protons increases the amplitude of the pH variations and provides a stable pH for a relatively long time. In our case we proved that the pH did not change during 15 minutes using a linear diffusion barrier of 2 cm inserted between electrodes that were
separated by only 2 mm. The open cell configuration is analogue to the one used in previous articles where they used the pH changes of aniline to control chemical reactions \cite{6-8,10,13,24}. The chemical yield in these applications could improve with a closed cell configuration by benefiting of a higher swing in pH and longer pH stability.

The integrated design of the electrochemical cell with microfluidics using a hydraulic actuation allows the exchange of reagents that we used to regenerate the cell for each different experiment by exchanging the liquid with neutral pH. This configuration is compatible with miniaturisation. Indeed the pH amplitude swing depends only on the height of the cell, and not on its lateral dimensions. The biggest constrain of the design we present relies on the relation of the length of the diffusion barrier and the time in which the pH is needed to be kept constant, according to eq. 1. To alleviate this issue in the future the diffusion barrier could be substituted by porous materials. Nevertheless the 1D design makes it easy to predict the diffusion time and to tailor the diffusion barrier depending of the chemical reactions that need to be controlled. The microfluidic configuration that we have shown can support different cells in the same channel, thus combined with the miniaturisation of the cells it can be used to multiplex different chemical reactions. Since the cell is compatible with organic electrolytes this device can be used in the future for applications like peptide or nucleotide synthesis that can benefit from the combined effect of the improved pH control and the decrease of importance of the role of the diffusion of reagents \cite{24,25}.

The integration of reference electrodes in miniaturised devices is a challenging task. The pH in our case can be conveniently controlled with current using a two electrode configuration, though for the control of the charging/discharging behaviour of the redox reactions the kinetics of the pseudocapacitance associated to the amine redox sites should be taken into account. The pH
variations observed during the resting time after the current pulses are to our knowledge the first
direct observation of the pseudo-capacitance discharge. The redox mechanism for the
capacitance characteristics of the amino-functionalized sites still deserve a deeper investigation,
however we show that the configuration of our reactor can be used to track the redox reactions
and study the effect of the pseudocapacitance on electrodes employed in other applications like
batteries or supercapacitors[34-37].

In summary, we have presented a device aiming for the future miniaturisation and multiplex of
chemical reactions with a large electrochemical control of the pH.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest to publish this investigation.
FIGURES

Figure 1: (a) Schematic representation of a simple electrochemical cell able to control the pH in miniaturised environments. (b) Design of the microfluidic cell including a pseudo-reference electrode and SEM picture of a small region representative of the nanostructured platinised electrodes at the WE and CE (scale bar is 100 nm). (c) Schematic representation of the µ-fluidic platform holding the exchangeable chip holding the cells schematised in (b) (See fabrication process of the chip in S.I. fig S1). (d) Schematic representation of the experimental setup used to control and monitor the pH changes in the microfluidic platform. (e) Calibration of SNARF fluorescence. The relative intensity of the 655 nm and 580 nm peaks are shown with closed and open black triangles for the excitation lines of 530 and 580 nm respectively as a function of the pH. The normalised intensity of the 650 nm peak as function of the pH is also shown in red crossed circles. The inset shows the absorption peak at pH 7.3 (grey solid line) and the fluorescence spectra at representative pH’s (7.3, 6 and 4) using the excitation lines at 532 nm and 580 nm (solid and dotted black lines respectively).
Figure 2 (a) CV of 10 cycles corresponding to the reversible proton exchange reactions of polymerised 4ATP done in a closed cell configuration. The inset shows schematically the 4ATP dimers that produce the oxidation (top to bottom dimer) and reduction (bottom to top dimer) reactions involving the exchange of two protons and two electrons (b) Schematic representation of the chip connections to the potentiostat. (c) and (d) current and voltage vs time during the CV as a function of time, respectively. Cycles 2, 5 and 8 are highlighted in red, green and blue colour respectively and the rise and fall voltage branches of the cycles are depicted with light and darker colours respectively. The colour code on the cycles is maintained on (e), (f) and (g) where the sequence of SNARF fluorescence spectra acquired at the WE and
CE (left and right panels on each) are shown. A vertical offset is introduced for each spectrum corresponding to the acquisition at equal voltage differences. The colour changes from light to dark tonalities indicate the voltage sweep is reversed. The arrow in (a) indicates the voltage before the oxidation that corresponds to the spectra just above the green dotted line in (e) to (g). The sequence progresses clockwise. (h) shows a schematic representation of the acid. In the middle cartoon the state before oxidation is shown and the WE is basic (represented with red colour) and the CE is acid (represented by green). After the oxidation (top cartoon) at the WE the pH becomes acid and opposite in the CE. Since the system is brought from neutral conditions to acid conditions there is a state around 0.6 V where both cells are acid (Bottom cartoon).

**Figure 3 (a)** background spectrum (black) and SNARF fluorescence spectra right after an oxidation at the WE (red) and 15 minutes later of open circuit conditions (blue). (b) Equivalent
spectra obtained at the CE. Spectra were vertically shifted to avoid the overlap of the red and blue spectra. Spectra in (a) are shown with a 8X amplification of the signal respect to (b).

Figure 4 (a) and (b) Voltage bias (in black) and current response (in red) corresponding to the second cycle of a CV narrowed between 0.5 and – 0.2 V as function of time with the cell opened and close respectively. (c) and (d) corresponding SNARF peak fluorescence intensity at the WE. Vertical scales are common through all the figure.
**Figure 5 (a)** Current bias (in black) and voltage response (in red) corresponding to square pulses of 0.4 mA of amplitude 2 s of excitation time followed by 10 s of resting time. **(b)** Normalised peak fluorescence at the WE during the current application.

**ASSOCIATED CONTENT**

**Supporting Information.**

Supporting information includes notes regarding migration and diffusion of protons, considerations of the approximation of equations 2 and 3, details of the platinisation of the electrodes, details of the resealable microfluidic platform, details of SNARF fluorescence, details of the electropolymerisation in the microfluidic platform, calculation of the minimum pH, and quenching of fluorescence with two electrode configuration.

**AUTHOR INFORMATION**

**Corresponding Author**

Corresponding author: cesar.pascual@list.lu

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**Notes**

* The decrease of the overall acidity observed during the CV with the closed cell configuration (see the increase of fluorescence from fig 2 (e) to (g)) is opposite to what we observed during the
polymerisation experiments shown in SI with the cell opened where the overall acidity increased due to electro-polymerisation process (see the SI).

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ABBREVIATIONS

WE Working electrode
CE Counter electrode
τ : characteristic diffusion time for a diffusion barrier
L : Length of a Diffusion barrier
D : Proton diffusion time
I : current
t : time
F : Faraday constant
V_{cell} : Volume of electrochemical cell
Q : Exchanged charge
SAM : Self assembled monolayer.
A_{WE} : Area of the working electrode
S : Surface functionalization
h : height of the cell.
4ATP 4-amino-thiol-phenol
SNARF carboxy semi-naphthorhodafluors
REFERENCES


BRIEFS (Word Style “BH_Briefs”). If you are submitting your paper to a journal that requires a brief, provide a one-sentence synopsis for inclusion in the Table of Contents.

SYNOPSIS (Word Style “SN_Synopsis_TOC”). If you are submitting your paper to a journal that requires a synopsis, see the journal’s Instructions for Authors for details.
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