ACIDITY CONTROL IN MINIATURISED VOLUMES

ENGINEERED MICROREACTORS FOR
HIGHTHROUGHPUT CHEMICAL REACTIONS

DIVYA BALAKRISHNAN

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Abstract

The miniaturised regulation of acidity has the potential to increase the speed of the chemical reactions that are used on the synthesis of different polymers including the DNA and the proteins, through the control of their structure and introducing a multiplexed manipulation in several addressable regions can increase the throughput for combinatorial chemistry. The electrochemical means to control the acidity would offer a compact, low cost technology, with the integration of microfluidics and electronics that could function as a micro-total-analysis system for on-demand assembly or manipulation of biopolymers. Here the challenge lies on the miniaturised production and the confinement of the protons with a design that allows multiplexed processes. In this thesis, we present our research advances towards this end. First we study the molecule 4 Aminothiophenol (4ATP) that when polymerised can reversibly generate the protons through electrochemical redox reactions. The different methods to polymerise 4ATP are investigated. The electrochemical behaviour of the different methods with respect to their charge transfer capacitance and reversibility of the redox reactions are measured and compared. Then we show the design of the microfluidic platform that integrates microfluidic and electrical connections. The platform can hold an exchangeable chip that contains the electrochemical reactor. The working model and the demonstration of miniaturised acidity control in one microreactor chip (~ 100 nL volume) mounted on the platform is shown where the acid is generated reversibly through the generation of protons from the polymerised 4ATP molecules. The stability of the generated acid to confine the protons in the cell is also investigated. The multiplexed control of acidity is studied by the second design that contains further miniaturised four electrochemical reactors on a chip with each cell holding a volume of ~ 3 nL. The demonstration of multiplexed control is shown on the platform with two of the electrochemical reactors that could be driven to maintain contrast acidity conditions.

Contents

Abstract.....	<i>i</i>
Contents.....	<i>ii</i>
1. Introduction.....	1
1.1. Introduction.....	2
1.2. Outline.....	4
1.3. References.....	5
2. Influence of polymerisation on the reversibility of low-energy proton exchange reactions by Para-aminothiophenol.....	7
2.1. Introduction.....	9
2.2. Results and discussion.....	11
2.2.1. Polymerisation of 4ATP layers.....	11
2.2.2. XPS analysis.....	12
2.2.3. Cyclic voltammetry evolution.....	16
2.3. Conclusion.....	20
2.4. Methods.....	21
2.5. References.....	24
2.6. Supplementary information.....	27
3. Redox active polymer working as a pH actuator on a re-sealable microfluidic platform.....	31
3.1. Introduction.....	33
3.2. Materials and methods.....	35
3.2.1. Study of the redox coatings.....	35
3.2.2. Implementation in a microfluidic platform.....	36
3.2.3. Electropolymerisation and pH control on the platform.....	39
3.3. Results.....	39
3.3.1. Polymerisation and reversibility of redox reactions of ATP.....	39
3.3.2. Modelling the pH actuation in the cell.....	41

3.3.3.	pH control experiments on the re-sealable platform..	42
3.4	References.....	45
4.	Electrochemical control of pH in nano-litre volumes.....	49
4.1.	Introduction.....	51
4.2.	Experimental.....	52
4.2.1.	Design and fabrication of the μ -electrochemical cell...	52
4.2.2.	μ -fluidic platform and experimental setup.....	55
4.3.	Results and discussion.....	57
4.3.1.	Electrochemical control of pH in confined volumes....	57
4.3.2.	Control of the pH with a 2 electrode configuration....	63
4.4.	Conclusions.....	64
4.5.	References	66
4.6.	Supplementary information	68
5.	Miniaturized control of acidity for multiplexing chemical reactions.....	79
5.1.	Introduction.....	81
5.2.	Results and discussion.....	82
5.2.1.	Design of the chip and description of the setup.....	82
5.2.2.	Control of the acidity in one microreactor.....	84
5.2.3.	Retention time and quantitative control of the acidity range.....	86
5.2.4.	Reversibility tested over 100 cycles.....	88
5.2.5.	Multiplexed control of acidity in two cells with different phase.....	90
5.3.	Conclusions.....	91
5.4.	Experimental section.....	92
5.5.	References.....	93
5.6.	Supporting Information.....	95
6.	Conclusions and future perspectives.....	99
6.1.	Conclusions.....	100
6.2.	Future Perspectives.....	102
6.3.	References.....	103
	Publication list.....	105
	Samenvatting.....	103
	Contributions.....	109
	Acknowledgements.....	111

1

Introduction

A brief introduction of the topic along with the framework of the thesis is presented. The significance of acidity control with the advances in the management of combinatorial diversity of bio-polymer molecules and the objectives of the thesis are described. The overview of the contents of each chapter addressed in this thesis is also presented.

1.1 | Introduction

This thesis belongs to a project aiming to improve the control of the synthesis and detection of bio-polymer assays, in which I contributed to the development of a microfluidic device to control the acidity in miniaturised features (in the scale of tenths or hundreds of microns) with the final goal to increase the throughput and the speed of the production of bio-polymer libraries.

Bio-polymers including nucleotides, peptides and saccharides have a large combinatorial diversity even though they are made by the combination of few monomers (four nucleotides, twenty three amino acids and four different monosaccharides), which provides them with wide range of properties used in different applications such as medicine (disease diagnostics¹, personalised medicine²), pharmaceutical industry (drug discovery³), defence (control of warfare agents⁴) etc. One of the most important breakthroughs to control the assembly of these molecules was introduced by Merrifield through solid phase synthesis⁵. It was a big step forward because it allowed to increase the control of reagents through binding of the molecule to an insoluble substrate that enabled the molecules to be manipulated during the synthesis cycle facilitating the exchange of reagents. Solid phase synthesis starts with a growing molecule linked to a solid substrate which is initially protected by a labile group to avoid multiple reactions. The labile groups can be removed by a deprotection step by changing some of the conditions in the environment like the temperature, acidity or light etc. More radicals can be added to the growing molecule by adding more protected monomers in sequence which allow to cycle the process to create a polymer with an accurate control. Over the years, commercial automated peptide synthesisers using one-pot coupling and deprotection methodology were developed to produce large quantities of peptides⁶.

To increase the availability of large combinatorial diversity, the process of solid phase synthesis was integrated with microfluidics to increase the number of spots where independent chemical synthesis can be carried out. The miniaturization of the spots decreased the consumption of reagents and reaction times allowing the production of cost-effective microarrays, which are molecular libraries where each spot holds one of the chemical species. There are different approaches for the fabrication of microarrays depending on the various methods used to decrease the size of the spots and the control of the deprotection steps. The first method of microarray fabrication involves microfluidic dispensing, whether by direct allotting of the reagents in each spot⁷ and flushing the acid entirely for deprotection in all the spots or by the confinement of these reagents in micro-

particles and inkjet printing⁸. The limitation of these methods is that they are sequential so the time to fabricate a microarray increases with the number of spots limiting the total combinatorial throughput. Using a new scheme in the fabrication of these microarrays that is based on the introduction of optically directed synthesis⁹, it was possible to introduce a parallel process that would not escalate with the number of spots of the microarray. The monomers use a photo labile protecting group that can be removed with the illumination of light. Each of the individual spots can be addressed by illuminating the light through an optical mask where the sequence of the next monomer is encoded into the transparent and opaque regions which allows to implement the addition of one kind of monomer to the growing sequence in different spots simultaneously. The process can be automated with the use of digital mirror devices that substituted the mechanical masks making the process faster and more cost effective¹⁰. The performance of fabrication of microarrays with optical synthesis depended largely on the yield of the deprotection and coupling steps of the monomers protected with optical labile groups. In order to use a well-known chemistry, another method that used monomers linked to the acid labile t- BOC group. Here the deprotection was carried out by the acid generated upon the illumination of the spot¹¹. The drawback of these techniques is that they require customized setups that are complex and difficult to integrate with some of the new generation in-situ analyses that promise to improve the limits of detection.

An alternative approach that had the potential to simplify the integration of chemistry and instrumentation was introduced with the use of electrochemical methods to generate the acid¹². This method used an array of microelectrodes that generated the acid with the electro-oxidation of organic molecules that was introduced in the electrolyte. They demonstrated the production of nucleotides through solid phase synthesis by using acid labile groups. The challenge of the electro-generated acid is that the chemical reactions occur much slower than the diffusion of the generated acid close to the electrode. In the original method by Southern and Egeland¹², the confinement was attempted by alternating cathode and anode connections of the microelectrode array with extra electrodes to create electric field intended to repel the electro-generated protons. To take advantage of the generated acid, the substrate with the synthesis was brought in close proximity to the electrodes. But the screening of the electrical field by the ions in the electrolyte made the proton confinement not enough to generate biopolymers with enough yield and length. An improvement to confine the protons was made by using a porous substrate on the electrodes to limit their diffusion¹³. They tried to have a better screening of the electrical field using scavenging solutions that consume protons. The drawback was that the scavenging solution was distributed in the electrolyte and they reduced the overall concentration of the protons thus producing less acidity changes. These devices that were intended to control the

acidity to produce large combinations of polymers, in reality could produce molecules only with few monomers due to the low yield and the control of the acidity achieved by these methods was not enough to compete with those using deprotection of optical labile groups.

To progress in our control of biopolymers, the production of reliable, compact and cost effective systems, ideally a system that has the potential to adapt and support different chemical manipulations would democratise the access to produce assays of different polymers including nucleotides and peptides. Electrochemical methods still have room to explore for the better control of the acidity, that would allow the integration of electronics and microfluidics enabling the control to multiplex and automate several chemical processes. The challenge that remains to use this method for driving chemical reactions is the miniaturisation of the control of acidity. The underlying concept is the confinement of protons, but this faces many additional challenges. This includes the generation of enough protons to provide large acidity range to control the different chemical reactions. Then the generated protons should be retained during the time necessary to allow the chemical reactions to take place with sufficient yield per reaction, to produce polymers with enough quality. Another problem lies in the nature of the electrochemically driven generation of the acid, which is based on the oxidation of a molecule that releases the protons. To maintain the electrical circuit it is necessary to have the same number of reduction reactions in the counter electrode to equilibrate the charge balance. Unfortunately, protons can reduce in the cathode at very low energies to produce hydrogen gas, and this process would annihilate the generated acid, posing further challenges towards the design of a miniaturized electrochemical reactor. Since biopolymers are based on the addition of many monomers that require different cycles of acidic and basic conditions, another property of the acidity control for their synthesis requires a reversible acid generation process. To combine the steps where the protons are confined in the reactor to deprotect the acid labile groups, with the ones for the addition of monomers into the reactor, it is necessary to design a system that can be opened and closed. In open position it can facilitate the exchange of reagents (new monomers) and in closed position it confines the acidity. In this thesis, we present a new design that addresses the above challenges and provides an innovative route to improve the manipulation of biopolymer assays.

1.2 | Outline

In this thesis we present a microfluidic platform that holds a microreactor chip capable of controlling the acidity in miniaturized volumes. Chapter 2 discusses about the generation of the acid through a redox active molecule. The

electrochemical behaviour and redox reversibility of the molecules were studied with respect to their different polymerisation methods. Chapter 3 shows the design of the microfluidic platform with a specialised microreactor chip able to control and measure the acidity changes. The working model and fabrication of the microreactor chip along with the working of the platform is explained. Chapter 4 explains the electrochemical actuation method to control the acidity in miniaturized volumes. The stability of the achieved pH over time and the two electrode configuration of pH control is demonstrated. Chapter 5 presents a second design of the microreactor with further miniaturisation and capable of multiplexed acidity control. Quantitative pH control and the reversibility of the actuation reactions are shown. Chapter 6 describes a summary of the thesis along with the perspectives opened by the miniaturisation of the acidity control.

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2

Study of molecules for the generation of the acid

Acidity can be controlled by electrochemical methods that offers the possibility to integrate microfluidics and electrical connections. The first step towards this objective is the generation of acid. For combinatorial chemistry applications we require the system to produce acid at low operating voltages as higher voltages could hamper the synthesis reactions or create side products. The system should provide large acidity range and reversible control. In literature different electrochemical methods to control the acidity have been reported like the electrolysis of water, redox active molecules on the electrolyte, conductive polymer coatings of electrodes, but they are limited by the higher operating voltages and non-reversibility, lack control of the acidity, degradation of electrodes respectively. We choose para-aminothiophenol (4ATP) to generate the acid as it fulfils the necessary conditions listed above. This molecule forms self-assembled monolayers on Au and Pt surfaces that eases the fabrication process. They can be polymerised using different methods to form different structures of redox active states that exchanges protons at low voltages (around 0.2 V). In this chapter we discuss about the properties of this molecule with respect to the different polymerisation methods that focusses on the electrochemical behaviour and the reversibility of the redox reactions that relate to the performance of acidity control in the system.

Influence of polymerisation on the reversibility of low-energy proton exchange reactions by Para-aminothiophenol

Abstract

Reversibility of redox processes is an important function to achieve dynamic control of acidity. We used Para aminothiophenol - a redox active molecule since its properties were suited for our applications. In this chapter, we present the electrochemical behaviour and redox reversibility of para-aminothiophenol (PATP) after different polymerisation methods. We used electrochemical, photopolymerisation and plasma polymerisation methods to induce reversible redox states. The chemical stoichiometry and surface coverage of PATP in the polymerized layers were analysed and compared for all the different polymerisation methods to estimate the good control of acidity well suited for our application.

*This chapter is based on the publication [D. Balakrishnan](#), G. Lamblin, J.S. Thomann, J. Guillot, D. Duday, A. van den Berg, W. Olthuis, C. Pascual-García. Influence of polymerization on the reversibility of low-energy proton exchange reactions by Para-Aminothiophenol. *Sci Rep.* **7**: 15401 (2017).

2.1 | Introduction

Surfaces with active reversible redox states are key elements in the fabrication of sensors, batteries, supercapacitors or chemical switches¹. Organic materials using proton exchange reactions, have an additional interest due to the promise of a greener approach and better efficiencies than alkaline metals^{2,3}. Aminothiophenol (ATP) is one simple molecule that is able to form self-assembled monolayers (SAMs) on noble metals, which can be used to modify the physico-chemical surface properties or to initiate the growth of well-ordered molecular layers, maintaining a good electrical conductivity. The mercapto group and benzene ring of ATP act as the linker and separator respectively. The amino group can exchange protons with the electrolyte or be used as a target for chemical bonds. In the para aminothiophenol (PATP) the mercapto and amino groups are opposite to each other providing higher conductivity, due to the more extensive electronic conjugation with respect to other isomeric states⁴. Polymerisation of PATP adsorbed layers on noble metals leads to different reversible redox states corresponding to different molecular coupling of two PATP molecules. The redox reactions occur at low potential, between 0 and 200 mV, and involve the exchange of two protons and two electrons. These polymerised forms of PATP have an important role for many of its applications because they support stable states with redox reversibility in contrast to the monomeric state⁵.

PATP has been used for the design of chemical sensors and switches or synthesis of conductive composites in combination with metallic nanoparticles and carbon materials⁶. PATP is one of the most important pH molecular reporters thanks to the interplay of the amine group with protons⁷. In acidic solution, PATP retains its aromatic state and in alkaline or neutral solution, it dimerizes into quinonoidic state 4,4'- dimercaptobenzene (DMAB). When chemisorbed to nanoparticles using SERS measurements the intensity of double bonds between nitrogen molecules in DMAB are detected, reporting pH between 3 and 7 in miniaturized environments^{6,7}. The modification of the pH by PATP would be the counterpart concept of this application by inducing proton release using an applied current. The pH variation by this process is limited by the reduced surface area of the electrodes compared to the total volume in solution. Using a nanocomposite coating it was possible to change the pH in a small volume and reversibly activate, a pH dependent DNzyme⁸ which opens the door to new applications like the control of the chemical environment by electronics or DNA computation^{9,10}. One of the drawbacks of the electrochemical generation of protons is its limited reversibility.

The photo-detection and electro-production of protons are almost equivalent processes that show very different reversibility. The susceptibility to electrical degradation of the PATP layers depends on the polymerised state induced. In literature, two main routes of polymerisation of PATP have been described to different extents: electro-polymerisation (e-poly) and photo-polymerisation. E-poly layers are achieved by applying a potential on the PATP SAMs. Hayes and Shannon reported the first study of the e-poly of PATP in acidic solution and explained the oxidation of PATP to form 2-(4-mercaptophenylamino) benzoquinone which is bound to the surface through the sulphur, resulting in a head to tail polymerisation¹¹ confirmed by other authors^{4,12,13}. Photo-polymerisation is induced by illuminating UV light on the PATP SAMs. Different studies suggested that UV-polymerisation (UV-poly) of PATP favours head to head polymerisation and results in different redox active species (DMAB) similar to those described in pH sensors^{14–16}. Polymerised states can also be achieved by plasma treatment: direct energy transfer of plasma can produce damages, but plasma can also create intermediate species (radicals, ions, electrons, photons) that permit the polymerisation of PATP. Plasma treatments of aniline at low pressure, in presence of O₂ are known to lead to polymerisation, etching or oxidation depending on the plasma parameters as discussed in other papers^{17,18}. Recent works have shown that plasma polymerisation can also be a dominant process at atmospheric pressure by choosing the proper electrode configuration¹⁹ or the electrical signal^{20,21}. Polyaniline nanofibers or nanoparticles were recently obtained with an atmospheric pressure plasma process¹⁹.

Regarding the importance of reversibility of the redox states in polymerised layers, to the best of our knowledge, no comparison of the efficiency of the redox states has been reported. In this paper we report the reversibility of polymerised states of PATP induced by electrochemical, UV and plasma methods. Although plasma-polymerisation has been used for polyaniline, plasma had never been used to treat aniline-like SAMs, even if plasma polymerisation holds advantages in the treatment of large samples. Our main goal is to investigate the efficiency of the polymerisation processes to provide reversible redox states. X-ray photoelectron spectroscopy is used to characterize the chemical configuration of the polymerised states and the surface coverage, while the efficiency of redox reactions was accessed using CV to measure the charge transfer (CT), film capacitance and reaction rates.

2.2 | Results and Discussions

2.2.1 | Polymerisation of 4ATP layers

Figure 1 shows a representation of the PATP chemisorbed layers (Fig. 1(a)) along with the three main reactions generally recognised in reversible proton exchange⁵ (Fig. 1(b)). Figure 1(c) shows one representative cyclic voltammogram corresponding to the electropolymerisation of one of the PATP modified Au electrodes. The current was acquired driving the potential between -0.2 and 0.7 V against a Ag/AgCl reference electrode for 6 cycles with a rate (v) of 100 mV/s.

Figure 1. Schematic representation of the PATP monolayers adsorbed on gold (a) and most common products of polymerisation (b) (in a structural formula representation). Representative cyclic voltammograms corresponding to the electropolymerisation of PATP (c) with the first and subsequent cycles (in red and black respectively) and UV (d) and plasma polymerised (e) samples respectively.

In cycle 1 (in red) a large irreversible oxidative wave is observed at 0.5 V with a full width half maximum (FWHM) of the peak of 0.2 V. According to literature this peak can be attributed to the oxidation of PATP and the consecutive polymerisation that occurs when an oxidised molecule couples to an adjacent one to yield a head to tail dimer (Fig. 1(b-ii))¹¹. An oxidation-reduction couple at 0.17 and -0.03 V with respective FWHMs of 0.2 and 0.1 V respectively were observed after cycle 2 (in black) and attains a steady state within the subsequent cycles.

According to literature, these peaks are attributed to the oxidation and reduction of 4-mercapto-N-phenylquinone diimine and 4-mercapto-aminodiphenylamine respectively, which involve the exchange of two protons and two electrons. Below 0.1 V an increase of the negative current that decreases on successive cycles is observed. In literature was attributed to the reduction of thiols and to side reactions^{11,12}. UV polymerised samples were obtained by illuminating the functionalised samples with a 365 nm lamp under 0.1 M phosphate buffer. Figure 1(d) shows a representative cyclic voltammogram from one of the UV-polymerised samples. The CV shows the first 5 cycles ($v = 0.1$ V/s). The potential range was narrowed between -0.1 and 0.4 V to avoid further electropolymerisation, although a small rise of the current above 0.3 V can be attributed to electropolymerisation of unpolymerised molecules. The quasi-reversible oxidation and reduction peaks corresponding to the proton exchange reactions of the amine groups were observed at 0.1 and 0.06 V respectively. While the UV polymerisation has not been studied in literature as much as electropolymerisation, several papers suggest that aniline of thiol aniline in neutral solution undergo head to head polymerisations as schematically shown in Fig. 1(b)-(iii–iv) also exchanging two protons and electrons per reaction¹⁵. Plasma samples were obtained using an open plasma reactor with a direct plane-to-plane configuration²². Figure 1(e) shows the first five cycles of a representative cyclic voltammogram. The reversible redox peaks corresponding to the amine oxidation/reduction were observed at 0.16 and 0.08 V respectively. Also a small contribution of electropolymerisation was observed on the first cycles. The most probable polymerisation reactions during the plasma treatments are H abstraction on the benzene rings leading to a C-C bonding between benzene rings from adjacent PATP molecules as proposed in¹⁸, or H abstraction on the NH_2 groups leading to $\text{N}=\text{N}$ bonds between PATP adjacent molecules. Plasma can also lead to oxidation of benzene rings ($-\text{OH}$ addition) or NH_2 groups and to some thiol or C- NH_2 bond breaking leading to a partial etching of the SAM. Here the conditions were optimized to favour the plasma polymerisation processes instead of plasma oxidation and plasma etching processes by exploring the plasma conditions which provided the greater CT for redox reactions.

2.2.2 | XPS analysis

XPS can provide insights of the chemical composition of thin layers. Figure 2(a) shows the survey spectra for the PATP and Au reference samples, as well as electro, UV and Plasma polymerised samples (grey, green, black, red and blue respectively, colour convention is maintained herein). The table shows the extracted analysis. XPS can provide insights of the chemical composition of thin layers. Figure 2(a) shows the survey spectra for the PATP and Au reference samples, as well as electro, UV and Plasma polymerised samples (grey, green, black, red and blue respectively, colour convention is maintained herein). The

Figure 2. (a) XPS survey spectra from Plasma, UV, e-poly samples (blue, red and black respectively) and the PATP and Au samples (grey and green respectively). (b) Elemental composition (in atomic %) for each sample (bold) and percent of excess of each element with respect to the PATP stoichiometry considering S content as reference (italic numbers).

table shows the extracted elemental composition in atomic percentage (in bold). The Au reference samples exhibit C and O contamination (24 and 1% respectively) due to exposure to air. The reference unpolymerised PATP sample exhibited also N and S present in PATP molecule. In order to study the extrinsic chemical elements in the functionalised layers, we separated the Au contribution originated in the substrate, and calculated the theoretical percentage for each element using the S contribution as a reference and the PATP stoichiometry. The excess is reported in the table in Fig. 2 in italic numbers. Thus 20% of the C detected in the PATP reference sample is attributed to contamination, which is similar to the amount found in the Au reference. In addition some extra presence of O (5%) is attributed to oxidation of PATP. The e-poly samples showed an increase in the C and O in excess (39% and 18% respectively). This increase of contaminants and in particular the high increase of oxygen can be attributed to the head to tail polymerisation characteristic of electropolymerisation, which leaves unprotected thiols that may oxidise and attract carbon to the surface when exposed to air. In UV-poly samples there is a comparative decrease of O respect to e-poly samples even if remains significantly higher than in the reference sample (10% of the total content), probably due to the contribution of oxidative species formed in solution during UV-poly. The plasma-poly samples, exhibited a decrease of the organic content and an increase of the signal from the Au substrate, the highest C excess (55.1%), a small increase of the N signal as well as a moderate oxidation respect

to the PATP reference. Our interpretation is that plasma-poly samples suffered some etching during the plasma treatment that could lead to an increase of the contamination since the uncovered Au surface has a very high surface energy and oxidation of the layers due to the radicals formed in the plasma. The fine structure of XPS peaks can also provide insights to the chemical bonds to clarify the nature of the adsorbed and polymerised layers. Figure 3 shows the narrow scans for C 1 s, N 1 s and S 2p of the polymerised and PATP reference samples, including the peak analysis of the fine structure of the bonds. The C 1 s core level spectrum (Fig. 3(a)), shows four peaks that have been assigned as follows: C–(C, H) at 284.5 eV, which is the main contribution as expected from aromatic compounds, C–(O, N) located at 286 eV, C = O at binding energy 287.6 eV and O = C–O centred on 288.8 eV. As expected, the four samples have very similar spectra as the C bonds are the same.

The study of the N 1 s narrow scans (Fig. 3(b)) indicated the effectiveness of the polymerisation processes. The spectrum from the PATP reference presents only two contributions at 399.0 eV (~90% of the total nitrogen contribution) corresponding to primary amino groups (–NH₂) and at 401.4 eV belonging to protonated or hydrogen-bonded amino groups (NH₃⁺, H...NH₂)^{23–25}. It was already shown that amino groups are the dominating species for aromatic molecules whereas it can be the opposite for aliphatic amines^{23,24}. Instead, polymerised samples presented four contributions, previously observed on polyaniline films^{26–29}. The main line at 399.0 eV is characteristic of quinoid di-imine nitrogen groups (–N =). The second main component at 399.8 eV is ascribed to benzenoid diamide nitrogen compounds (–NH–). The peak at 401.2 eV can be attributed to positively charged nitrogen species such as protonated amine (–NH⁺) or to the oxidised amine (–NO). The contribution at 402.9 eV is attributed to the oxidised amine (–NH⁺ =). The main signature of the polymerisation is the splitting of the primary amine groups into the quinoid di-imine and the benzenoid diamide groups.

Figure 3(c) shows the S 2p spectra. The line at 161.9 eV is attributed to thiol or thiolate strongly bonded to the gold surface^{23,30–33}. The second doublet centred at 163.1 eV was previously ascribed to unbounded or non-chemisorbed thiol, thiolate or disulphide for high coverage^{30,32,33}. Two additional components are detected at 165.0 and 167.6 eV, attributed to the presence of oxidised sulphur species SO₂[–] and SO₃[–] (sulfinic and sulfonic) respectively^{23,30,33}. Most of the S signal from the reference PATP sample is associated with bound thiol. The component associated to the line at 163.1 eV may be due to excess of PATP adsorbed on top of the first monolayer. An increase of the unbounded thiol or thiolate contribution was observed in this batch of UV polymerised samples. We assigned it to the presence of more than one monolayer, which is in agreement

Figure 3. Core level spectra of the XPS analyses for the C, N and S contributions of the polymerised and PATP samples.

with further data in the article (Figs 4 and 5). The peak at 168.5 eV is observed only for the polymerized species and we attribute it to the oxidized sulphur species originated in the unbound -SH produced also by head to tail polymerisation.

Figure 4. XPS inelastic scattering normalised by the Au4f signal of the polymerised and reference samples.

The higher content of oxidised thiols in the case of e-poly is consistent with the interpretation of head to tail polymerisation as main component in this method. The background due to inelastic scattering of photoelectrons can provide

information about the layer thickness. Figure 4 shows the XPS inelastic electron scattering spectra from the polymerised and reference samples normalised to the Au 4f zero loss peak. The inset shows a blow up of the background displaying a ranking of Au substrate, plasma poly, unpolymerised PATP, e-poly and UV-poly from thinner layer to thicker layer. The signal in Au can be attributed entirely to contamination. Plasma-poly layers are thinner than the original PATP, which indicates a reduction of the layer coverage attributed to the etching effect of the plasma. The resulting larger layer thickness of the e-poly samples observed with respect to the reference PATP layers can be attributed to absorption of contaminants by the isolated thiols resulting of the head to tail polymerisation. Finally the thicker layer of PATP signals the presence of unbound thiols (Fig. 3(c)), which is consistent with the electrochemical data here after. This effect was particular of this batch of UV polymerised samples used for XPS and CV.

2.2.3 | Cyclic voltammetry evolution

The reversibility of the redox reactions, attributed to the proton exchange reactions of the polymerised PATP was studied with CVs for over 50 cycles (Fig. 5). e-poly, UV and plasma polymerised samples are presented with black, red and blue colours respectively. Figure 5(a–c) show the comparison of the evolution of the cyclic voltammograms for representative curves from one of the samples for each polymerisation at cycles 2, 25 and 50. The CVs are dominated by redox peaks at ~ 0.15 and ~ 0.08 V and according to the literature discussed above, in the case of electro and UV-polymerised samples are due to the reactions presented schematically shown in Fig. 1(b). E-poly samples displayed higher oxidative and background current densities than the others but decreased until cycle 50 where the redox currents of the three samples tend to converge. UV-poly samples exhibited lower redox currents than the e-poly and a considerable background current. An extra reduction peak around 0 V was observed in particular during the first cycles, which did not appear in other sets of UV-poly samples. We attribute this peak to the effect of the unbound PATP that we observed in some samples. The oxidative peaks displayed narrower FWHMs and the effect of unbound PATP was not distinguished from other samples. Plasma-poly samples were characterised by lower background currents while the redox currents were similar to the ones observed with UV-poly. The existence of the reversible wave is an indication of the effective plasma polymerisation, regardless the significant etching effect. To observe better the evolution of the redox reactions we calculated the CT from the area of the redox peaks for consecutive cycles (Fig. 5(d)). The CTs corresponding to oxidation and reduction (CTox and CTred) had different behaviours, while CTred remained constant within the standard deviation, CTox that initially was three times higher decreased over the cycles until converging with CTred on the 50th cycle. The surface coverage corresponding to the observed

Figure 5. (a–c) CV after e- (black), UV (red)- and Plasma (blue) polymerisation methods for the cycles 2, 25 and 50 after polymerisation (first cycle was avoided to discard open circuit and unbalance of chemical equilibrium effects) (d) Charge transfer for the different polymerisation and for the oxidative and reduction peaks as a function of cycles. (e) FWHM of the oxidation peaks as a function of cycles, (f) Overpotential between oxidative and reduction peaks as the function of cycles.

charge transfer in Fig. 4(d) calculated from CT_{ox} for the e-poly samples is $2.7 \cdot 10^{-11} \text{ mol}/\text{cm}^2$ in cycle 1 and lowers to $1.12 \cdot 10^{-11} \text{ mol}/\text{cm}^2$ in cycle 50 ($S = \text{CT}/nF$, where n is 2 due to the number of electrons for each reaction in our case and F is the Faraday constant). The degradation of CT_{ox}'s may be due to imperfect recovery of protons by the amine groups or by the degradation of the molecule (e.g. loss of aromaticity in the benzene ring that would increase the resistivity of the molecular system). In the UV and plasma-poly samples the CT_{ox}'s were constant while the CT_{red}'s that were initially slightly higher than the oxidation, tend to decrease with the following cycles reaching values below CT_{ox}. A possible reason

for this behaviour in UV and Plasma poly films is the contribution from residual e-poly occurring at the highest biases of the CV. This residual e-poly that occurs mainly in the first cycles increases the number of reversible states, which are then observed first in the following reduction peak within the same cycle. The corresponding surface coverage in UV-poly samples ranges between $1.97 \cdot 10^{-11}$ and $1.02 \cdot 10^{-11}$ mol/cm² for cycle 1 and 50 respectively while for the UV poly sample ranges between $1.87 \cdot 10^{-11}$ and 1.08 mol/cm² for cycle 1 and 50 respectively. Plasma-poly CT's showed similar absolute values, which is remarkable considering the decrease of the organic content due to etching. At cycle 50 the CT of all the polymerisation methods converged to similar values.

The shape of redox peaks is associated mainly with the homogeneity of the layers although factors such as neighbour interactions, SAM organisation or substrate crystallinity may increase this value¹. To understand the impact of the polymerisation method on the reversibility, we analysed only the oxidative peaks that exhibited lower FWHM and seemed to be less affected by the unbound thiols (Fig. 5(e)). The theoretical width associated with an ideal film in the case of no interactions is $353RT/nF$. (being R the ideal gas constant, T the temperature, F the Faraday constant and n the number of electrons associated with each reaction (2 in our case)). This number is ~ 45 mV at room temperature. Our values are much higher and the difference FWHM cannot be fit with the simple non interaction model of a single molecule. We observed a large difference between UV and e-poly samples that decreased from 41 mV on the first cycles to 26 mV at the 50th cycle. Since the oxidised and reduced species of the proton exchanged reactions are not charged, and the low total surface density attributed to the dimerized molecules, the molecule to molecule interactions should not have a large effect³⁴. Therefore the large FWHM may be attributed mainly to contributions from different redox reactions originated in different dimers, like the ones presented in Fig. 1(b), rather than to factors like neighbour interactions or disorder. Consequently e-poly samples would be more heterogeneous, possibly having more contributions from redox reactions, than the UV poly. We also observed the increase of the FWHM of the plasma polymerised samples. The origin can be in the activation of some extra redox couples, as we have seen that residual electropolymerisation occurred during the cycles. The FWHM of plasma and e-poly samples coincide practically after the 20th cycle.

In redox active SAMs the difference between the redox peaks ($E_{ox}-E_{red}$) increases with the distance of the redox group to the electrode¹. Also here to understand the impact of polymerisation we took into account only the difference of E_{ox} with E_{red} from the peak we attribute to bound thiols (Fig. 5(f)). The larger peak separation observed for e-poly samples with respect to UV poly can be attributed to the effect of the larger distance of the redox groups in head to tail

polymerisation (associated to e-poly) and head to head dimers (dominant in UV poly). In addition the UV dimers have a double tunnelling connection to the electrode through the two thiol bonds that would increase the conductance thus decreasing the peak separation. Plasma poly samples initially have higher Eox-Ered, but their values decrease until they become below the UV poly. Eox-Ered of both e-poly and plasma poly tend to decrease for consecutive cycles which would indicate that the more reversible species are the ones that have less resistance between the electrode and the redox amine. The UV-Plasma evolution is almost constant, which is consistent with the CT behaviour observed. The global picture supports the idea that head to head species are more stable than the head to tail.

The double layer capacitance Cdl normalised to the sample surface (Fig. 6(a)) is derived from the charging current observed in the CV's ($C_{dl} = i_{ch}/v$). Plasma-poly samples had lower values than the e-poly and UV-poly samples which followed similar trends. This behaviour was already noticed on the background currents described in Fig. 5(a–c). The etching occurred in the plasma treatment can explain the lower layer capacitance of the layers. As seen by XPS inelastic scattering background (Fig. 3) the thickness of the plasma-poly sample is below the PATP reference, considered as one monolayer. Therefore it is very likely that the plasma-poly sample has open pockets that become contaminated due to the high surface energy of Au, that decrease the total effective area of the layer and are reflected in a lower Cdl. The tendency of Cdl to decrease in all the layers could also be interpreted as a decrease of the active area for the successive cycles due to the degradation of the conductivity of the layers.

Figure 6(b) shows the electron transfer rate (k_s) for the oxidation potential of the different polymerised methods calculated from the maximum oxidative current divided by the charge transferred¹. This transfer rate can only be considered as an average transfer rate of the layer since the parameters of the Gaussian functions we used to fit are not directly linked to a model from a known redox molecule³⁴. However, the fact that k_s remains nearly constant, indicates that for e-poly and UV poly the nature of the redox species did not change significantly during the 50 cycles (since k_s depends on the chemical redox species). The differences and the trends are consistent with what was observed for the FWHM of the redox peaks, where plasma polymerised samples become similar to the electropolymerised after the first 10 cycles. The faster exchange rate of UV-poly samples is also consistent with the interpretation of a lower impedance of head to head dimers, due to the parallel thiol connection of the redox groups with the electrodes respect to the linear impedance in head to tail polymerisation.

Figure 6. (a) Double layer capacitance of the polymerised films. (b) Electron transfer rate by the three different cycles as the function of cycles.

2.3 | Conclusions

In this article we have used electro, UV and plasma polymerisation to induce quasi-reversible redox states. The results are interpreted in terms of polymerised dimers with reversible proton exchange reactions. In literature there is a well-established description that electropolymerisation produces head to tail dimers, while UV polymerisation can have different contributions of head to head dimers⁵. Plasma polymerisation of PATP SAMs has not been used to the date, and the chemical nature is not yet mentioned in literature. We have seen that all the polymerisation processes increase the contamination and oxidation of the layers as seen by XPS measurements, which can be due to the single thiols left by the head to tail polymerisation (observed in electropolymerisation), to the degradation of the molecules (by reactive species in plasma or UV polymerisation) or to the etching effect (mainly occurring by the effect of the plasma). The comparison of the N1s core spectra from the PATP reference samples and the polymerised ones provided a signature of their polymerisation observed by the splitting of the main amine peak ($-\text{NH}_2$) into benzenoic diamide ($-\text{NH}-$) and diimine nitrogen ($-\text{N}=\text{N}-$). The study of the S 2p core spectra showed that in electropolymerised samples the amount of thiols was bigger for electropolymerised samples, attributed to the oxidation of the single thiols. The presence of unbound thiols was also detected in some samples, which was consistent with an observation increase in the layer

thickness and the shape detected by the XPS inelastic scattering background. However in the CV's the effect of unbound thiols decreased over consecutive cycles.

Initially electropolymerisation provided the most effective technique to produce redox exchange dimers, but after 50 cycles they degraded to the same CT value achieved by other polymerisation methods. The shape of the CV curves also indicated differences in the nature of the layers. The large FWHM that we observe in all the cases is interpreted in terms of sample heterogeneity, in particular electro and plasma polymerised layers provided the higher FWHM than UV. Even if head to tail and head to head polymerisations may be predominant for electro and UV polymerisation respectively, the contribution of other species cannot be discarded. UV and plasma polymerisation initially had a much lower activation but the electronic structure of the dimers produced by these methods seemed to have advantages as they did not seem to degrade over large cycles (in other experiments not shown they were stable up to 100 cycles). The behaviour of the UV polymerised dimers were the closest to the ideal redox active SAM with lower FWHM, Eox-Ered and higher Ks. Its structure with two thiols in parallel seems to have advantages with respect to the linear structure of the head to tail polymerisations, and these layers suffer less degradation. The characteristics of the CVs from plasma polymerised layers are more similar to electropolymerised layers in the FWHM and Ks, but Eox-Ered was similar to the UV polymerised samples after 30 cycles, and they also exhibited similar stability.

To produce alternatives to e-poly polymerisation, an ideal redox surface should have maximum possible surface coverage possible, a fast Ks and maximum stability. From our experiments the method that would produce a molecule approaching this characteristics would be the UV functionalised layers, but they suffer limitations in the achievable surface coverage. Plasma polymerisation provided an interesting alternative since it produced a significant surface coverage of stable active redox couples considering the inactive areas left by the etching. In principle, this method can still be optimised and these etched areas offer the possibility of a second functionalisation thus further experiments of characterisation and optimisation of these layers could be of interest for improved applications.

2.4 | Methods

Chemicals

4 Aminothiolphenol, Absolute Ethanol and Phosphate buffer solution (pH 7.2) were purchased from Sigma Aldrich. Millipore filtered water was used to dilute the electrolyte solution and for rinsing purposes. Electrode preparation. Si substrate with 50 nm of oxide layer were evaporated with Au (30 nm)/Ti (5 nm)

using an e-beam evaporator and then diced into pieces of about $>1\text{ cm}^2$ obtaining samples with low roughness ($\sim 2\text{ nm}$). The evaporation was carried out under a vacuum less than $1\text{e-}7\text{ mbar}$ with an evaporation rate of $0.9\text{ \AA/s}$ and the samples were placed at a distance of 90 cm from the source. We observed that the sample reproducibility was improved by immediately cleaning them under UV ozone chamber for 30 minutes. The cleaned electrode was then immersed in ethanol solution containing 50 mM of PATP for 24 hours.

Electro-polymerization

We used a Faraday cage to carry on our experiments, a Pt wire as counter electrode, cleaned with sand paper and ethanol, and a calomel reference electrode. For electropolymerization the standard parameters were: scan range 0.7 to -0.2 V , scan rate -100 mV/s , cycles $1 + 3$. We used 10 mM of Phosphate buffer solution as the standard electrolyte for the results presented in the article, which gave the highest CT results. However we also explored electropolymerisation in acid and basic solutions (see SI Fig. 1). All the solutions were purged with N_2 during 5 minutes. Samples could be then studied or rinsed with ethanol and placed in Buffer solution for XPS studies.

Photo-polymerization

Following different references^{13–15} we followed photo-polymerisation of PATP in liquid. The functionalized PATP Au electrodes were immersed in neutral phosphate buffer solution (0.1 M) for about 30 minutes, under UV light of 365 nm wavelength and power 0.9 mW/cm^2 . Also in this case we explored acidic and basic buffers obtaining also less CT than the case of neutral buffers (see SI Fig. 1).

Plasma polymerisation

Plasma polymerisation was performed in air at atmospheric pressure with a plane-to-plane direct dielectric barrier discharge (DBD) configuration with different conditions of flux, power, and number of cycles. The DBD configuration used in this study was already described elsewhere²². The discharge was produced between two plane parallel high voltage electrodes ($15 \times 300\text{ mm}^2$ each) covered by an alumina dielectric barrier and moving stage as the grounded electrode. The gas gap between the high voltage electrodes and the substrate was maintained at 1 mm . During the deposition process, the plasma was ignited using a Corona generator from SOFTAL electronic GmbH, generating a 10 KHz sinusoidal signal. Plasma treatments were carried out using a modulated sinusoidal electrical excitation with successive ON-time ($250\text{ }\mu\text{s}$) and OFF-time ($350\text{ }\mu\text{s}$) pulses. We explored Ar and N_2 plasma using different cycles of our mildest possible conditions

with a standard pulsed AC generator (mean power 1-2 W, power density 25–50 mW/cm²), voltage 0.4–0.6 KV, frequency 10 KHz, with successive 250 μ s plasma ON duration and 350 μ s plasma OFF duration, sample displacement rate 20 mm/s, plasma treatment length 2 cm and gas flow 20 L/min on a blanket plasma reactor. We obtained the highest charge transfer rate for the process with two runs using Ar plasma. As optimisation of plasma treatment is not the focus of the article, here we have presented only the results of our best plasma treatment. To reduce the variability between experimental conditions in a non-dedicated plasma reactor, after optimization of the conditions, we polymerised the batch of samples here reported on the XPS and CV analyses.

XPS Measurements

Experiments were carried on polymerised and reference samples using two samples for each method and performing the analyses on two separated points on each of the samples. XPS measurements were carried out with a Kratos Axis Ultra DLD spectrometer using a monochromatic Al K α radiation (primary energy $E = 1486.6$ eV) operating at 300 W. High resolution spectra (20 eV pass energy) were acquired for the quantification and peak fit. Electron binding energies were calibrated using the Au 4f_{7/2} transition at 84.0 eV. The quantification were obtained after the removal of a Shirley type background. The O 1 s, N 1 s, C 1 s and S 2p peak fit was performed with a symmetric Voigt function with a Lorentzian-Gaussian ratio of 30–70. The same full width at half-maximum (FWHM) for both components, a spin-orbit splitting of 1.2 eV and a branching ratio of 2 were used for the S 2p_{3/2} and S 2p_{1/2} contributions. The thickness and coverage values of the films were determined by using the Quases software [<http://quases.com>] on large Au 4 f spectra acquired with 160 eV pass energy. The fits were analysed using the least number of peaks to optimise the standard deviation of the spectra.

Electrochemistry

We extracted data from 4 samples for electropolymerisation 3 samples for UV polymerisation and 5 samples for plasma polymerisation. All the samples were evaporated with Au, functionalized with 4ATP and polymerized on the same day to avoid variability of the experimental conditions. The electrochemical experiments were carried out in a three electrode cell configuration with 4ATP functionalized Au working electrode, Pt wire counter electrode and KCl saturated Ag/AgCl reference electrode working in a Faraday cage. The electrolyte was phosphate buffer solution of 10 mM concentration. Before each experiment, the electrolyte was purged with N₂ for about 10 minutes.

We used a Princeton applied research Versastat MC, to record cyclic voltammetry measurements of the electro and UV polymerised samples and a Solartron XM PSTAT 1 MS/s for the plasma polymerised samples. The scan rate (v) of 100 mV/s provided the best signal to noise ratio while assuring the complete polymerisation of the film (data presented in SI Fig. 2) so unless indicated other ways, all the results reported correspond to that rate. All the reported currents were normalised using the area of the samples immersed in liquid. The CVs were studied for over 50 cycles to extract the data about CT, sample homogeneity, layer capacitance and reaction rates. The results reported in Fig. 5 correspond to the same batch of samples reported on the XPS measurements. The CV curves were fitted using the second derivative to determine the inflection points to calculate the redox peaks and determine the points at which the capacitance currents were taken.

2.5 | References

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2.6 | Supplementary information

UV wavelength – 365nm, Power = 0.88mW/cm², scan rate – 0.1V/s,
PBS buffer – pH 7.4

UV wavelength – 365nm, Power = 0.88mW/cm², scan rate = 0.1V/s,
PBS buffer – pH 5.2

UV wavelength – 365nm, Power = 0.88mW/cm², scan rate = 0.1V/s,
PBS buffer – pH 9.0

SI Figure 1 UV polymerisation of different samples at neutral (samples A, B) acid (samples C, D) and basic (samples E, F) buffer conditions. Below the surface coverage determine from Oxidation reactions is expressed in red and blue for the first and 50th cycle respectively.

SI Figure 2 (a) cyclic voltammetry measurements carried on both Electro and UV polymerised samples for different scan rates ranging from 0.02V/s to 0.1V/s. the inset graph shows the oxidation peak charge density against the scan rate for Electro and UV polymerised samples. (b) Surface coverage vs scan rate derived from oxidation reactions of different e-poly and UV poly samples (cycle 1 is first cycle after electropolymerisation).

SI Figure 3 Raw data corresponding to the different electro-polymerised samples used to calculate the data in fig 5 a-f and 6 (cycle 1 is first cycle after electropolymerisation).

SI Figure 4 Raw data corresponding to the different UV polymerised samples used to calculate the data in fig 5 a-f and 6

SI Figure 5 Raw data corresponding to the different Plasma polymerised samples used to calculate the data in fig 5 a-f and 6

3

Design and working of the μ -fluidic platform

We aim to control the acidity in miniaturized volumes by the generation of acid through the redox reactions of 4 Aminothiophenol. To demonstrate the acidity changes, an electrochemical cell was designed where the electrodes were functionalized with 4 Aminothiophenol. The electrochemical cell chip was mounted on a specialized microfluidic platform that integrates electrical and microfluidic connections. In this chapter we present the design and working model of the electrochemical cell along with the design of the microfluidic platform. The detailed steps of the fabrication of the chip and the experimental settings were explained. We show a demonstration of the acidity changes in aqueous electrolyte on the microfluidic platform.

Redox active polymer working as a pH actuator on a re-sealable microfluidic platform

Abstract

Here we review our recent works aiming the control of proton concentration combining redox self-assembled monolayers with a modified platform. We used, Aminothiolphenol, to obtain coatings with quasi-reversible redox states able to exchange protons with the electrolyte at low voltages using different polymerization methods. We achieved the control of proton concentration over the liquid with an efficient design of the microfluidic device to control the diffusion of protons and providing long lasting stability to carry control of chemical reactions during several tenths of minutes. The experiments were carried out in aqueous environment using a pH fluorescence marker to track the pH that allowed us to monitor the proton concentration down to pH 5, where the fluorescence molecule lost its sensitivity, while calculations indicate that the pH can be below 1.

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3.1 | Introduction

Redox active self-assembled monolayers (SAMs) are materials capable of exchanging electrons and ions between electrodes and electrolytes that can facilitate the design of devices like sensors, batteries, or chemical switches [1]. In these devices the changes in the liquid make the chemical modifications on the electrodes to provide them some functionality. In other cases, the electrodes can be conceived as actuators to change the chemical environment [2]. The acidity is one of the main factors of the chemical environment facilitating the control of reactions like the synthesis of polymers [3], the activation of enzymes [4] or physical properties like the swelling of polymer hydrogels [5]. The efficiency to control other chemical processes through the electrochemical generated acid will depend on the proton concentration range achievable, the number of reversible cycles that can be repeated and that the applied voltages to generate or uptake protons do not interfere with the other chemical the reactions that want to be controlled. Nevertheless, the control of the proton concentration from electrodes is quite challenging mainly because it is proportional to the surface to volume ratio of the electrodes, which is unfavorable in normal conditions leading to very small changes of acidity.

Different methods to achieve control of the acidity have been explored in literature. The simplest one in water is electrolysis [6], which separates the water molecules into H^+ and OH^- ions at voltages over 1.23 V (against normal H^+ reference electrode). Though this method is easy to implement, the disadvantage is that it is an irreversible reaction and that the high potentials could trigger some other reactions with the chemicals in solution. To reduce the energy of the reactions and achieve some quasi-reversibility the electrode surfaces can be coated with redox active conducting polymers like PEDOT or polypyrrole [7, 8]. The disadvantage is that polymers coatings tend to degrade with repeating reactions through excess of crosslinking showing poor reversibility. The impact of the redox reactions to change the pH can also be very low due to the unfavorable surface to volume ratio. An alternative approach can be using redox active molecules like hydroquinone and benzoquinone in the electrolyte that exchange protons [9]. The molecules in contact with the electrode can be oxidized or reduced to change the pH. Using this approach the pH change close to the electrodes was used to control different chemical reactions but the protons diffuse rapidly, so that the pH control was not uniform over a large volume. Also, though the working potentials were lower because it avoided the potential drop across several monomer bonds, the problems of electrode degradation and low pH changes can also be a problem in this case. To increase the surface area and the impact of the redox reactions, Willner and his group reported the electrochemical change of pH in a confined volume using a three dimensional layer of Au nanoparticle composites. The

composites were electrochemically deposited by crosslinking the thiol functionalized Au nanoparticles with an electrode surface functionalized with SAMs of Aminothiophenol [10]. The system showed reversible reactions for several cycles with a pH range of ± 1 pH units. A different approach was adopted by Wang et. al. to achieve large pH ranges. Instead of changing the electrodes they constructed a device separated by an interfacial Pd thin film that allowed reduction in one side and oxidation in the other, obtaining opposite changes of pH in each of the cells [11]. This device achieved a large useful pH range between 5 and 11.

The advantages of SAMs to change the acidity is that they can be easily used to obtain Highly ordered layers with low redox voltages that can exchange protons in aqueous or organic environments. Amino thiol phenol (ATP) is an organic molecule able to form SAMs in noble metals thanks to the affinity of its mercapto group and the interactions among the benzene rings [12, 13]. The dissociation constant (pK_a) of the amino-group and its interaction with protons has been used to fabricate plasmonic pH sensors associating the molecule to metal nanoparticles to enhance the vibronic signatures that reveal the protonation state of the molecule [14, 15]. ATP can also provide reversible redox active reactions in its polymerized state at low voltages to exchange protons. Though in normal SAMs electrodes the surface volume is very unfavorable within the correct device it can be used to control the pH.

In this paper we review our latest works studying pH modifications using ATP. We studied different methods of polymerization of ATP including the well studied electrochemical polymerization and less common ones like photopolymerization and plasma polymerization attending to the charge transfer, which is the main factor from the SAM's that can influence the pH change. Then we combined the most efficient method with a device that addresses the challenge miniaturizing the pH control through the design of a microfluidic platform. The difference with previous works is that in our case we took care that device could be compatible with miniaturization and the use of small volumes. In this paper we include details of the model and calculations to put an emphasis on the rules we used to obtain a design of a platform that allowed the use of SAMS to change the pH in more than 6 units in reduced volumes avoiding the degradation of the electrodes. We provide additional details of the fabrication process that we followed to produce chips that are integrated on the platform. Finally, we provide the conclusions and perspectives of such device combined with the improvements of new polymerization methods.

3.2 | Materials and Methods

3.2.1 | Study of the redox coatings

Sample preparation to study redox reactions of ATP

Silicon with 50nm thermal grown oxide layer was cleaved to obtain 2 x 1 cm² samples that were evaporated with Au 30 nm with an adhesion layer of Ti 5 nm using an e-beam evaporator. We achieved low roughness (< 2 nm) using an evaporation rate of 0.9 Å/s at a pressure of 10⁻⁹ mbar. Substrates were then cleaned under a UV ozone lamp for 30 minutes. 0.5 mM concentration of 4 Aminothiophenol solution in 30 ml of absolute was prepared in a beaker. After the completion of UV ozone cleaning, the samples were placed on the thiol solution beaker and covered with aluminium foil. The beaker was placed under a fume hood for overnight functionalization.

Methods of Polymerization

Three different methods of polymerization of ATP were performed, Electropolymerization, Photopolymerization and Plasmapolymerization. Electropolymerization was carried out by cyclic voltammetry using a Princeton applied research Versastat MC potentiostat. The experimental set-up used was a three electrode electrochemical cell with the ATP functionalized Au as working electrode, platinum wire as counter electrode and a calomel reference electrode. Everything was mounted inside a Faraday cage with the beaker with the electrolyte mounted on top of a stage to adjust the height of the exposed working electrode that was fixed to a stand in the Faraday cage. The electrolyte used had 1 M concentration of phosphate buffer solution purged with N₂ for five minutes before performing the experiment. The CV cycles were programmed in the potentiostat with a voltage range between -0.2 to 0.7 V, at 100 mV/s for 5 cycles.

For photopolymerization or UltraViolet polymerization (UV), the substrate functionalized with ATP was placed in a beaker containing 1 M concentration of phosphate buffer solution. The beaker was placed under a UltraViolet lamp of power 0.9 mW/cm² and wavelength of 365 nm for 30 minutes. Finally, for plasma polymerization, the ATP functionalized substrate was placed on a plane-to-plane direct dielectric barrier discharge plasma generator at atmospheric pressure. Argon plasma with a voltage of 0.4 - 0.6 kV, frequency 10 kHz and a gas flow of 20 L/min for two runs is exposed on the substrate.

Electrochemical performance of the polymerized layers

The charge transfer for each polymerization method was calculated from CV measurements in the same setup and experimental conditions described for the electro polymerization of ATP. The experiments were recorded for the voltage range of -0.2 to 0.7 V at 100 mV/s scan rate. The reversibility of the redox reactions

were tested by repeating the cycles for up to 50 times.

3.2.2 | Implementation in a microfluidic platform

Fabrication of the electrochemical cell chip for miniaturized control of pH

Figure 1 Schematic representation of the electrochemical cell (a) for the oxidation reaction increasing proton concentration in the WE and (b) for the reduction reaction to recover the more basic conditions. (c) Fabrication of the electrochemical cell: a 3 X 3 cm² SiO₂/Si substrate (i) is spin coated with optical resist (ii) the first layer of optical lithography (Layer M1) is defined with laser lithography (iii) and Ti/Au electrodes are e-beam evaporated (iv), second layer of SU8 optical resist is spin coated (v) and optical lithographed (layer M2) (vi) (d), Representation of the Latinization of WE and CE (f) representation of the ATP functionalization (g) Picture of the fabricated electrochemical chip (g) Schematic representation of the microfluidic platform with the optical setup.

The electrochemical cell chip was fabricated on a 3 X 3 cm² Si Substrate with 50 nm oxide layer using optical lithography (fig. 1). The cell chip consisted on a platinized working electrode (WE) which was connected to a platinized counter electrode (CE) and Au pseudo reference electrode (RE) each by diffusion barriers (fig. 1(a and b)). The dimensions of the cell are area of WE $A_{we} \sim 0.05 \text{ dm}^2$, total area of the cell $A_{cell} \sim 0.1 \text{ dm}^2$, length L_{ch} and width W_{ch} of the diffusion barrier $\sim 2.6 \text{ cm}$ and $\sim 100 \mu\text{m}$ respectively, the height of the cell $h = 3.5 \mu\text{m}$ and the volume of the cell $V_{cell} \sim 120 \text{ nL}$. The complete fabrication process (fig 1(c-e)) we used goes as follows; Optical lithography was performed using a maskless laser writer MLA 150 from Heidelberg instruments. Two designs were made using Klayout CAD

design program [16]. The first design (M1) included the design of the electrodes with the contact pads. For the M1 design, the substrate was spincoated with LOR 3A (300nm) and S1813 (1.3 μ m) photoresist. Using the laser writer the substrates were exposed with the design M1 with an exposure dose of 120 mJ/cm². Then the samples were developed in MF319 solution for 50 seconds and blow dried with N₂. The photolithographed samples were placed on an e-beam evaporator and 5 nm Titanium and 50 nm of Gold were evaporated. The Lift-off process was carried out in a beaker containing 50 ml of acetone. After 15-20 minutes the photo resist S-1813 dissolves leaving the patterned electrodes. The sample was rinsed with distilled water and blow dried with nitrogen. The evaporated sample was again spin coated with SU8 2005 epoxy resist at 5500 rpm and baked at 95 °C for 1 minutes to obtain a uniform layer of 3.5 μ m thickness. The second design (M2) had the definition of the cell volumes with the diffusion barriers along with the opening for contact pads and electrodes. The substrate was then loaded into the laser writer for the second lithography of design M2 with an exposure dose of 1000mJ/cm². The sample was then baked before developing at 95° for 3 minutes and then inserted into MICRO-CHEM SU8 developer solution for 1 minute and dried with nitrogen.

Platinisation of Electrodes

The experimental set-up (fig 1 (d)) was as follows: beaker 1 contained 40 mM of chloroplatinic acid and 100 μ M lead acetate solution in distilled water, beaker 2 contained 1 M of phosphate buffer solution. A salt bridge was made with a filter paper strip that was immersed in potassium chloride solution, after removing the excess of electrolyte it was placed between the two beakers. The salt bridge was immersed in both liquids of the beakers. Using a clamp, the working electrode contact pad of the sample was connected to the potentiostat. The sample was immersed in Beaker 1 covering the electrode to be platinized. We used a Ag/AgCl reference electrode immersed in beaker 1. The Pt counter electrode was immersed in beaker 2. A cyclic voltammogram experiment was created using the potentiostat between the voltage range -0.2 V to 0.6 V at a scan rate of 100 mV/s for 4 cycles. After the completion of the 4 cycles, the sample was removed from the beaker and washed with distilled water, ethanol and blow dried with nitrogen. The counter electrode was also platinized following the same procedure. Then sample was cleaned with ethanol, water and placed under UV lamp for 30minutes. Finally the sample (fig 1(c)) was functionalized with ATP by following the same procedure as explained in previous section.

Description of the microfluidic platform

The μ -fluidic platform to hold the chip consisted on two parts with top and the bottom frames (in yellow and grey respectively in (fig. 2 (a-e)). The top frame included a glass window that provided optical access, the μ -fluidic inlet and outlet

Figure 2 (a) Pictorial representation of the set-up of the microfluidic platform with valves for fluidic transport and pneumatic actuation, (b) opened platform showing the top frame in yellow, bottom frame in grey holding the chip in green. (c) shows the underlying view of the top frame containing the glass and the O-ring. (d) Cross-section view of the chip mounted on the platform along X axis showing the inlet and outlet for microfluidic transport (e) The same along Y-axis showing the springs of the electrical contacts on the top to connect the chip. (f) Schematic representation of the microfluidic platform with the optical setup.

for the flow of liquids and the electrical contacts. The contacts to the chip were made through springs and they were connected to a switching box allowing the electrodes to be grounded or connected to the potentiostat. The bottom frame included the sample stage with a pneumatic actuator (in blue) using compressed air to lift the chip after the liquid was inserted in the channel, leaving in these way the cells connected only through the electrolyte in the diffusion barriers. The two parts were sealed with an elastic O-ring (fig.2 (c)) and closed by screws. The chip on the stage (in blue) could be moved up (cell closed) or down (cell open) by opening the valve of the compressed air (V1). In open position, the O-ring pushed down the chip allowing a 60 μm gap between the chip and the glass window on the top frame. This position will allow the filling and exchange of liquids in the cell. In closed position the chip was pushed up in contact with the glass window on the top frame and the cells are isolated from μ -fluidic channel and are only connected through diffusion barriers. A syringe with the electrolyte was connected to the μ -fluidic inlet tube (V2) and an empty syringe is connected the μ -fluidic outlet tube (V3). A vacuum pump was connected to the other inlet of T-valve V3, and to ensure the complete filling vacuum was done before inserting the electrolyte.

Optical set-up

In Figure 2 (f), the optical set-up was built above the platform for optical inspection through the glass window on the top frame. pH change inside the electrochemical cell was detected using fluorescence dye carboxy semi-naphthorhodafluors (carboxy SNARFs). These molecules are excited using a 565 nm diode source through a dichoric mirror CROMA-49017 with an excitation window between 540 nm and 580 nm and with a low band pass filter at 590nm for collection. The excited area was limited by the focusing/collective objective to a field of view smaller than the WE. In order to increase the fluorescence signal all the light collected by the objective was sent to a wide range spectrometer FLAME-S-VIS-NIR. SNARF fluorescence has two peaks at 590 and 650 nm. The peak at 590 nm is dominant for acidic pH and 650 nm peak is dominant for neutral pH, and the ratio between the two peaks can be used to track the pH in the range from 8 to 5 [17]. The peak at 590 nm is much weaker than the one at 650 nm, and the signal at high proton concentrations became very weak. For this reason we used the ratio of the two peaks to assess the initial pH and we followed the total pH using the 565 nm excitation. More details about the calibration of SNAR are reported in the supplementary information. The spectra were integrated for 10 seconds.

3.2.3 | Electropolymerization and pH control on the platform

The electropolymerisation was performed with the cell in its opened position. For these experiments and all the others in the platform we used a Solartron Modulab XM Pstat 1mS/s potentiostat using three electrode configuration. A cyclic voltammogram was programmed in the potentiostat between of -0.9 V to 0.9 V using a scan rate of 50 mV/s for 4 cycles.

Before the pH control experiments, the cell was flushed exchanging the electrolyte between the two syringes in the circuit. The cell was then closed using the valve V1, leaving the WE communicate to the RE and CE only through diffusion barriers. A cyclic voltammetry experiment is pre-programmed in the potentiostat for a voltage range of 0.75 V to - 0.75 V at a scan rate of 50 mV/s. The fluorescence acquisition was started with before the voltage scan, focusing on the signal from the WE.

3.3 | Results

3.3.1 | Polymerization and reversibility of redox reactions of ATP

Figure 3 (a-i) shows the structural representation of the self-assembled ATP adsorbed on Au, which can be polymerized in different ways (fig. 3ii-iv) depending on the polymerization method. We discussed the different possibilities of

Figure 3 (a) Structural representation of (i) Amino thiophenol, (ii) electropolymerized, (iii-iv) optical and plasma polymerized SAMs on Au electrodes. (b) Cyclic voltammograms of the polymerized layers where black, red and blue correspond to electrochemical, optical and plasma methods respectively. (c) Charge transfer for oxidation and reduction reactions of ATP for different polymerization methods is plotted against the number of cycles. (Figure adapted from our previous publication in 18).

polymerization in our previous article [18]. All the three methods provided reversible proton exchange reactions where 2 protons and 2 electrons are exchanged from the molecule to the electrolyte. Figure 2(b) shows representative cyclic voltammograms (CV) of the polymerized samples electropolymerized in black, UV polymerized in red and plasma polymerized in blue. Redox active behavior was observed in all the cases with an oxidation and reduction potential around ~ 0.2 V and ~ 0 V respectively. The total charge exchange (Q) of these peaks could be calculated from the area withstanding below the redox peaks:

$$Q = \int_{V_1}^{V_2} I \cdot dV - (V_2 - V_1) \left\{ I_1 + \frac{1}{2} (I_2 - I_1) \right\} \quad \text{Eq. (1)}$$

To study the reversibility of redox reactions of the different polymerization types we repeated 50 CV cycles. In Fig 3 (c) the charge transfer for the oxidation and reduction peaks are plotted against the number of cycles. We observed that while electropolymerization initially showed a higher charge transfer respect to the other methods and afterwards it degraded to approximately the same value of the other polymerization methods. In the other hand, the charge transfer of the

UV and plasma polymerization was nearly constant and shows less degradation with successive cycles. The advantage of electropolymerization is a higher charge transfer at the beginning, however, UV or plasma polymerizations are more suitable for delivering large batches of samples. If the application intends many cycles, they do not have much difference of charge transfer after 50 cycles. More details of the characterization and performance of the polymerized layers can be found in our previous work [18].

3.3.2| Modelling the pH actuation in the cell

To model the pH changes in the cell shown in Figure 1 (a) due to proton exchange reactions like the ones described above, we assume that the charge transfer from the electropolymerized layers corresponds only to proton exchange reactions and that the initial proton concentration in the WE electrode is negligible, which can occur in water if the total change of pH is several units. We also assume that the protons are confined (and thus they cannot be reduced at the CE), which occurs if the diffusion time across the barriers is much longer than the CV cycles.

The cell in figure 1(a) is a 3 electrode electrochemical cell with WE, CE and RE separated by diffusion barriers. To provide an example of how the cell can control the pH we will assume that the electrode materials are the ATP coated flat electrodes with same surface coverage as described above. With an applied voltage, the ATP molecules exchange protons with the solution thereby changing the pH from higher (neutral) (Fig 1(b)) to lower (acidic) (fig 1(a)) and vice versa. The diffusion barrier separates the electrodes into different cells connected by a narrow long channel. In order to avoid diffusion of protons, the length of the barrier was designed extensively longer than the width and height of the barrier and this increase the diffusion time of protons. A barrier length of 2.6 cm is chosen and for this length, the diffusion time (t) needed for each proton to reach the CE was calculated using the proton diffusion coefficient $D = 9 \times 10^{-5} \text{ cm}^2/\text{s}$ [19].

$$t = \frac{(l_{\text{ch}})^2}{D} = 130 \text{ minutes} \quad \text{Eq. (2)}$$

We assume at this point that the total charge involved in the redox process is the one derived from the peak where the limits are indicated in the cyclic voltammogram data of the electropolymerization process in Fig 3 (b) and the surface coverage (S) of the ATP molecules is calculated.

$$S = \frac{Q}{A \cdot F} = \frac{4.671 \times 10^{-6} \text{ C cm}^{-2}}{96485.33 \text{ C mol}^{-1}} = 4.84 \times 10^{-11} \text{ mol/cm}^2 \quad \text{Eq. (3)}$$

where the area chosen was the footprint of the WE of the chips in in figure 1 and F is the Faraday constant. Another factor that contributes to the loss of protons is

migration across the barrier. High concentration of electrolyte (1M KCL) is used to avoid migration and to decrease the over potential across the barrier. After a complete CV oxidation, the maximum number of protons (N) that can be in solution are the ones corresponding to the surface area of the electrodes. The minimum possible pH will correspond to the distributions of these protons in the volume of the WE, which can be calculated as follows:

$$\text{pH} = -\log \left[\frac{N}{V_{\text{we}}} \right] = -\log \left[\frac{S \cdot A_{\text{we}}}{h \cdot A_{\text{we}}} \right] = -\log \left[\frac{S}{h} \right] = -\log \left[\frac{4.84 \times 10^{-11} \text{ mol cm}^{-2}}{3.5 \mu\text{m}} \right] = 3.9 \quad \text{Eq. (4)}$$

where A_{we} and V_{we} corresponds to Area and volume of the working electrode respectively and h is the height of the cell. Again for an estimation of the possible pH with our fabrication parameters we have used the dimensions from the chips in figure 1 and the data from the electropolymerization in figure 3 (b). In order to increase the impact of the electrodes in the pH we increased the surface area by platinizing, leading to an effective surface charge normalized to the footprint, which was much higher.

3.3.3 | pH control experiments on the re-sealable platform

Figure 4 CV of ATP corresponding to the electropolymerization on the chip as a function of voltage (a) and time (b). (c) Sequence of SNARF fluorescence spectra during the first cycle of polymerization. (d) Peak intensity of the fluorescence normalised to its maximum value at neutral pH as a function of time during the polymerization cycles.

The reversible states in our chip were achieved through an electropolymerization in the platform. Figure 4 shows the cyclic voltammogram of the electropolymerization process on the chip as a function of voltage (a) and time

Figure 5 (a) Voltage, (b) current and (c) the intensity of SNARF fluorescence represented as a function of time. (d) The inset above shows the zoomed view of the plot for 1.5 cycle.

(b), with reversible oxidation and reduction peaks around 0.2 V vs the cell gold pseudo reference electrode. During the first rise of current (in black) an increase of the slope after 0.6 V can be observed that corresponds to the polymerization. This process is similar to what we observed in our previous work [18], although some changes were observed in the value of the voltages because of the pseudoreference electrode and the materials used in the counter-electrode. For each cycle the current density increases. We attribute this effect to additional polymerization of ATP to form redox active molecules, which were not able to

polymerize in the first oxidation.

The pH changes which were simultaneously monitored by measuring the fluorescence at the WE through the SNARF fluorescence marker dissolved in the electrolyte. We used SNARF molecule to monitor the pH behavior (details of the SNARF fluorescence behavior and calibration is included in supporting information). Figure 4 (c) shows fluorescence during the first rise in black. SNARF has two peaks at 590 and 655 nm which are dominant at acidic and basic pH respectively, however at acidic pH's the overall intensity is much weaker. For this reason we could use the relative intensity of the peaks to verify the initial neutral pH, and then, followed the pH with the intensity of the 655 nm peak. In Figure 4(c) the first four spectra from the bottom correspond to the initial state with neutral pH. In the next spectrum, the 590 peak is more dominant showing that the pH had decreased. This corresponds to the polymerization and the subsequent oxidation. When the acidity increases the spectra are quenched, indicating that the fluorescence marker is beyond its acidity range ($\sim$ pH 5). The spectra is recovered after 60s, in the last spectrum shown after the reduction, which recovers protons into the coating.

In figure 4 (d) the intensity of the 655 nm is shown versus time for all the cycles. This switch of pH from higher to lower is repeated with successive cycles. The changes in acidity proved that the redox reactions involved the exchange of protons. These pH changes were observed because the cell was still only 60 μ m, but the pH increased with time, as it can be observed by the raise of the fluorescence occurring after the last cycle in blue. After the polymerization, the cell was closed to observe the pH changes with confined protons. Figure 5 shows the CV of 8 cycles representing the proton exchange reactions of ATP in a closed cell. (a) The applied voltage and (b) the measured current with respect to time are plotted. The corresponding SNARF fluorescence spectra is extracted and plotted against time in (c). For each cycle the fluorescence intensity shifts from maximum to minimum and this process is repeated for successive cycles showing reversible pH changes. Using the pH calibration curve of the SNARF fluorescence marker [SI] the maximum and minimum intensity corresponds to pH 7.2 and 5 respectively. The minimum pH achieved is beyond the detection limit of the SNARF fluorescence marker. Using the CV plot the lowest pH (using Equation 1) was calculated as 0.9. A more detailed information about the SNARF fluorescence marker and CV experiments were reported in our previous publication [20].

In summary, we have presented a molecule, ATP that traditionally was used for sensors thanks to its ability to exchange protons, but that in a smart microfluidic device, we have been able to use it to change the pH in nanoliter volumes. We have reviewed the routes to obtain reversible redox states by

polymerizations, and the efficiency of the redox active reactions achieved. A miniaturized electrochemical cell of few 100 nL was modelled to control the pH. The detailed steps involved in the fabrication process and the design of the platform are explained.

We have presented how to control critical parameters involved in the design of the cell like diffusion time of protons through the barrier, dimensions of the cell and the estimation of minimum pH that could be achieved with flat electrodes. We have demonstrated a setup involving our platform to monitor the pH changes in these volumes, in the electropolymerization and in the pH control experiments. The cell that we have presented is compatible with miniaturization. Equation 4 shows that the minimum pH does not depend on the area of the electrodes, thus different designs could be implemented depending only on the effective surface coverage of ATP and the height of the cell in its closed position. This opens the way to new designs that could include further nanostructuration of the electrodes, other than platinisation to control the effective surface area in a rational way. The single channel architecture of our cell is also compatible with the inclusion of several cells, which would allow multiplexing of pH control in a single device. For these novel implementations, the presented methods of polymerization provide flexibility for mass production outside the chamber, and post modification of the electrodes for example ion permeable layers.

In conclusion, we believe that our platform can open the way to the control of chemical reactions through the systematic control of proton concentration, allowing miniaturization and multiplexing.

3.4 | References

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4

Demonstration of acidity control

In this chapter we demonstrate the control of acidity in miniaturized volumes (nL) on the microfluidic platform. In addition to the experimental details, the stability of the pH actuation to maintain the attained acidity levels in the electrode cells was tested. Acidity was also controlled by the two electrode configuration of the electrochemical cell eliminating the use of reference electrode.

Electrochemical control of pH in nano-litre volumes

Abstract

Electrochemical management of the proton concentration in miniaturised dimensions opens the way to manipulate chemical reactions. Here we address the challenges linked to the efficient proton generation and control of their diffusion by an efficient design of an electrochemical cell. We present a device operated electrochemically that demonstrates the control of the pH in a cell of ~ 120 nL. The device comprises a microfluidic reactor integrated with a pneumatic 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.

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4.1 | 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 milliliters was demonstrated through the use of gold nano-composites [19] or the use of hydrogen permeable bifacial working electrodes (WEs) [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

[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 pneumatic 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 [3,4,9,12], while the more abrupt changes below the SNARF range open the door to improve the yield of organic synthesis [1,2,6-10]. 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.

4.2 | Experimental

4.2.1 | 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 [21-23]. Some approaches have used the small pH changes happening in proximity of the electrodes during the oxidation of aniline to control other chemical processes [6-8, 10,13, 24], 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.

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).

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 ($D=9 \cdot 10^{-5} \text{cm}^2/\text{s}$) [32], which provides the system a characteristic diffusion time τ 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 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 τ , the pH can be approximated from the Faradaic redox currents (I_{redox}) or the total exchanged charged (Q) as follows:

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

where t is the time during which the current has been applied, F is the Faraday constant and V_{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_{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_{WE} times its height h to find what the minimum achievable pH is:

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

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.

4.2.2. | 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 $\sim 60\ \mu\text{m}$ 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 pneumatic 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 pneumatic 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 [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 [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 [28,29] but they require a long

development for standardisation. Fluorescence sensors are an effective way to monitor the pH ^[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 μ M 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 fiber 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 $\sim 63.5 \mu\text{m}$ in which the excitation power (1.3 mW) was distributed and averaged over a large number of molecules ($\sim 10^7$ 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.

4.3 | Results and Discussion

4.3.1. | 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 quasireversible 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

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 color respectively and the rise and fall voltage branches of the cycles are depicted with light and darker colors respectively. The color 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 color 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 color) 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).

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 colors respectively. The color code on the cycles is maintained in all the figures including the representation of the fluorescence spectra.

Figures 2 (e-g) shows 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 colors 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 H_2 , 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. 2). 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 [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

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).

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

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.

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 color.

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 τ from the WE to the CE should be ~ 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 ($1\text{ }\mu\text{m}$). However the amplitude of this pH change is limited to $\sim 10\%$ 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 $\sim 60\%$ 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.

4.3.2. | Control of the pH with a 2 electrode configuration.

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.

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 ~ 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 achieved 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 observe any bubbles on the electrodes, thus we also exclude the electrolysis of water, probably due to the protecting effect of the 4ATP monolayer.

4.4 | 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 [15-18], prevents modification of the electrodes by further polymerisations [7-10], and avoids diffusion limited mechanisms [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 [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 pneumatic 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 [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 pseudo

capacitance 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 pseudo capacitance on electrodes employed in other applications like batteries or super capacitors [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.

4.5 | References

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4.5 | Supplementary information

A. Notes regarding migration and diffusion of protons.

Protons under an electrical field migrate towards the anode. This drift can decrease the time for which the diffusion barrier is efficient. However, in our microreactor the electric field is rapidly screened by the other ions in solution. In an electrolyte, the screening is characterised by the Debye length l_d as:

$$l_d = \sqrt{\frac{\epsilon_r \epsilon_0 RT}{2F^2 C_0}} \quad \text{Eq. S 1 [SI 1]}$$

where ϵ_0 is the permittivity of free space, ϵ_r the dielectric constant of the medium, R the gas constant, T the absolute temperature in kelvin, F the Faraday constant, and C_0 the molar concentration of the electrolyte. At room temperature (25 °C), l_d is:

$$l_d(\text{nm}) = \frac{0.304}{\sqrt{I(M)}} \quad \text{Eq. S 2}$$

where $I(M)$ is the ionic strength expressed in molar. Thus in our case with ionic strength 1M the screening occurs in fractions of nanometers and thus the ion migration is negligible respect to diffusion.

B. Considerations of the approximation of equations 2 and 3.

Equations in the article 2 and 3 calculate the pH considering only the protons generated from the polymerised 4ATP redox reactions taking place in the cell. This approximation neglects the existing protons at neutral conditions and becomes more accurate as the number of electrochemically generated electrons increases: at pH 6, the error of disregarding the initial proton concentration at neutral pH is less than 0.05 and at pH 5 is completely negligible.

C. Details of the fabrication and platinisation of the electrodes

Figure S1, shows the sequence of microfabrication steps for the implementation of the chip that consist of: 1 Spin-coating of optical resist, 2 Optical lithography, 3 e-beam Au evaporation, 4 Lift-off, 5 Spin-coating of epoxy barriers, 6 Optical lithography, 7 Platinisation of electrodes, 8 Functionalisation of electrodes with 4-ATP

Figure S1: Fabrication steps of the electrochemical cell

In order to achieve a higher effective surface functionalisation we platinised our electrodes. Figure S2 shows scanning electron microscope (SEM) images of representative areas in the WE.

Figure S2: Scanning Electron Microscope images of details the platinised WE

After platinisation we cleaned our samples for at least 15 minutes under a UV lamp ozone cleaner. The electrodes were probed with Ferri/ferrocyanide (0.01 M $\text{Fe}^{2+}/\text{Fe}^{3+}$ (CN)₆ and 0.1 M KCl) redox before and after platinisation and after Ozone treatment (fig. S3).

Figure S3: Cyclic voltagrams of the electrodes before and after platinisation (black and red respectively) and after UV ozone cleaning.

Figure S4 shows details of the electrodes of one of the samples used in the article. The images were taken using one of the samples after the experiments. The platinisation of the electrodes reduced the space between the electrodes and sealing glass (see fig. 1 (a)). However, in the article, to calculate the final pH with eq. 2 we used the maximum possible volume using the height of the SU8 spacer used in the cell.

Figure S4: Scanning Electron Microscope images of the details of the WE (a) and (b), CE (c) and RE (d) electrodes to at the exit with the diffusion barriers. The images were taken from one of the chips after the experiments.

D. Details of the resealable microfluidic platform.

Pictures of the microfluidic platform described in figure 1 (c) are shown in fig. S5. The outlet could be connected to a membrane vacuum pump (min vac. 1 mbar) or to a recovery deposit using a two way valve. After vacuum the filling was done using syringe pumps. Efficient filling of the chamber could be achieved with 5ml of electrolyte. In order to avoid bubbles the electrolyte was pumped using the vacuum from the chamber into the syringe.

Figure S5: Resealable microfluidic platform.

E. Details of SNARF fluorescence calibration.

The fluorescence dye carboxy semi-naphthorhodafluors (carboxy SNARFs) was used in our article to track the pH. It was excited using a cool-LED source at 565 nm diode source and the spectra through the dichoric mirror is shown in Fig S6 - green. The intensity of the absorbance peak at pH 7.3 is shown in grey and the fluorescence intensity at 532 nm excitation for pH 7.3 and pH 5 are shown in black.

Figure S6: The spectrum shows the intensity of the incident light from the excitation source through dichoric mirror (green), the absorbance spectra at pH 7.3 (grey) and the fluorensce spectra of the excitation 532nm at pH 7.3 and 5 .

Figure S7: SNARF fluorescene spectra using (a) 532nm excitation and (b) 580nm excitation sources at different pHs.

Figure S7 shows SNARF fluorescence spectra at different pH's exhibiting two bands at 590 and 650 nm. In order to increase the fluorescence efficiency we excited close to the maximum of absorption using a 580 nm diode source. For this reason we had to use a Dichroic with a bandpass filter at 590nm to cut the stray light. The spectra in figure S7 (a) were fitted using two gaussian peaks. The derived peak intensity from the Gaussian fits provided a calibration of the pH. The spectra in fig. S7 (b) were fitted using the parameters of the peak center and peak

width from fig. S7 (a), and the peak height provided a second calibration. The comparison of both data is shown in Figure S8, which also shows the peak intensity of the 650 nm peak normalised to the maximum intensity at pH 7.3. Since the shape of the peak at neutral pH could be univocally determined, the intensity of the 650 nm provides also a valid calibration. Due to overall decreasing efficiency of the SNARF fluorescence and the low pass filter and the required short integration times to measure the changes of pH during the applied bias, practically, for most of the spectra we could only follow the pH by the quenching of the 650 nm line.

Figure S8: Relative intensity of SNARF fluorescence at different pH's for 532nm and 580nm excitation sources. The plot on the right shows the 650nm peak intensity against different pHs.

F. Calculations of Temperature changes and number of SNARF molecules

Figure S9 show details of the experimental set-up. The illuminated area was defined with an aperture to fit the collection area which was determined by the combination of the collecting lenses and the microscope objective. In our case the collecting lenses had a diameter of $\frac{1}{4}$ of inch, while we used a long working distance objective of 50X magnification and 3 μm focus depth. The radius of the illuminated area is $(6.35 \text{ mm}/2) / 50\text{X} = 63.5 \mu\text{m}$, and thus the total volume of excitation was 3.8×10^{-11} liters.

Figure S9: Schematic representation of the microfluidic platform with the optical setup and the inset shows the enlarged image of the sample with the parameters used for the below calculation

The temperature change (ΔT) due to the heating of sample with the source was calculated using the below equation S3,

$$P = \frac{\kappa A (\Delta T)}{d} \quad \text{Eq. S3}$$

Where P is the incident power (1.3 mW in our case), κ is the thermal conductivity of Silicon (1.3 W/cmC°), A is the illuminated area defined above by the combination of the collection lenses and microscope objective ($1.26 \cdot 10^{-4} \text{ cm}^2$), and d is the height of the silicon sample through which the heat is being dissipated ($775 \text{ } \mu\text{m}$). The estimation for the maximum ΔT assuming that there were no other dissipation channels is with this equation 0.61 C° .

The number of SNARF molecules N , is calculated as,

$$N = M \cdot N_A \cdot V \quad \text{Eq. S4}$$

Where N_A is the Avogadro number ($6.023 \cdot 10^{23}$), M molar concentration of SNARF (in our case $0.5 \mu\text{M}$) and V is the illuminated volume calculated above. Thus the number of molecules of SNARF is $\sim 10^7$.

G. Details of the electropolymerisation in the microfluidic platform

The electropolymerisation of 4ATP on the chip electrodes was performed with the open position of the cells, thus the platinised WE was connected through the electrolyte to the platinised CE and the Au pseudo-reference electrode through a channel of $\sim 60 \text{ } \mu\text{m}$. fig. S10 (a) shows the cyclic voltammetry (CV) cycles one to 4 in black, red, green and blue respectively executed between -0.9 and 0.9 V at a rate of 50 mV/s. The current time evolution is shown in fig as S 10 (b). The most

prominent aspect of the CV in the first cycle is the rise of the current slope observed next to 0.5 V.

Figure S10 (a) CV of 4ATP corresponding to the electropolymerisation on chip in the microfluidic platform in the opened configuration. (b) Current and time evolution of the electro-polymerisation. Cycle 1 to 4 (c) Sequence of SNARF fluorescence spectra during the first cycle of polymerisation. (d) Peak intensity of the fluorescence normalised to its maximum value at neutral pH as a function of time during the polymerisation cycles.

This contrasts to the behaviour of 4ATP polymerisation performed against AgCl reference electrode where the polymerisation was completed in a voltage bias range between 0.3 and 0.75 V [21]. This difference can be explained by the different nanostructured material respect to the Au flat samples. The overpotential varied amongst the different chips that we studied (see SI) due to the variability between platinisation processes, and it was difficult to predict. In this sample, the appearance of the oxidation peak from cycle 2 onwards was at ~ 0.2 V, indicating the formation of the pseudo reversible states allowing proton exchange reactions analogue to the ones found in the electropolymerisation of 4ATP on gold samples [21]. The reduction peaks at ~ -0.6 V were less evident but still well noticed in the representation of fig. S10 (b) on top of the large capacitive currents. In cycles 2 to 5 the amplitude of the CV currents also increased, probably due to residual polymerisation.

During the polymerisation process the SNARF fluorescence tracked the pH on top of the WE. SI fig. 7(c) shows the spectra obtained during the first cycle (shown in black) presented with a vertical offset corresponding to time lapses of 6 s for each spectrum. The first two spectra from the bottom were obtained with no applied bias (open circuit, in pink (Fig S10 (d))), during the first 10 s. Since the electrolyte was fresh, the fluorescence was dominated by the peak at 650 nm that corresponds to the initial neutral pH conditions. After the application of a bias voltage, the pH remained constant until the beginning of the oxidation process at 0.5 V, which reflects on a decrease of the fluorescence intensity indicating that the acidity is increasing. On spectrum corresponding to $t = 30$ s (sixth spectrum from the bottom) it can be observed that the SNARF peak band at 550 nm, which is cut by the fluorescence filter at its peak, is almost double than the intensity at 560, which corresponds to a pH around ~ 5.5 [19]. Further oxidation of the 4ATP producing more increase of the acidity was reflected by the complete quenching of the fluorescence. In the last spectrum of SI fig.10 (c) corresponding to $t = 60$ s, a recovery of the fluorescence signal dominated again by the 650 nm peak indicates that the pH is increasing back as result of some reduction reactions. However the weaker intensity of the peak fluorescence respect to the one at $t = 0$ s together with a small asymmetry observed towards shorter wavelengths indicating a contribution from the 550 nm peak, indicates that the initial neutral pH was not recovered. Although the cut of the fluorescence filter does not allow quantification of the pH we can deduce that the maximum peak fluorescence observed at the initial conditions correspond to neutral pH and when the pH was completely quenched the SNARF probe is over-ranged thus pH is below 5.25. Hence, during the polymerisation at least a change of 2 pH units was observed. It was also verified that in that range or the SNARF sensitivity the peak intensity could be used to track the pH changes.

SI fig. 10 (d) shows the peak intensity of the SNARF fluorescence associated to the pH changes as the function of time. The comparison with the associated currents in fig. 10 (b) shows that the reductions and increments of pH observed correspond to oxidation and reduction processes at the functionalised electrodes respectively, verifying that the SNARF was not interacting with the bias but only with the protons in solution. The pH change was observed at the top of the WE and it can be explained due to the small focus depth of the microscope ($\sim 1 \mu\text{m}$) and the large amplitude to the small volume of liquid even with the open cell since the separation between the chip and the upstanding glass is only $60 \mu\text{m}$. After the first cycle (fig. 10 (d) red circles, green face down and blue face up triangles corresponding to cycles 2, 3 and 4 respectively) the maximum peak fluorescence decreases 20%, indicating that the solution has increased its acidity due to the extra protons produced during the polymerisation. After the end of the applied voltage bias the fluorescence intensity increased from the complete quenched

signal to a peak fluorescence of 50% of the original signal, which indicates that the protons diffused levelling the pH through the cell in a time lapse of ~ 10 s.

H. Calculation of the minimum pH

Figure S11 Current vs voltage corresponding to the rise of the 5th cycle of the CV shown in fig. 2 in the article. Inset: Gaussian fit used for the area calculation, in green the current vs voltage after the subtraction of the capacitive currents, the red is the Gaussian fit. Data from the Gaussian fit are reported in table SI 1. CV was made at 0.05 V/s.

For the calculation of the redox charges involved in the proton exchange reactions we used the 5th cycle indicated in green in fig. 2 (c) in the article. Figure SI 11 reproduces the part of the cycle involved in the oxidation and the selected area hatched in grey. The inset shows the peak after the background subtraction and the fit using a Gaussian function (in red). The peak properties are summarised in SI table 1:

SI Table 1 Peak properties corresponding to the inset of SI Figure 8.

Peak type	Peak area (C)	Max Height (A)	FWHM (s)
Gaussian	1.7 e-3	2.3 e-4	7.1

Taking into account that the sweep rate was 50mV/s, the FWHM of the peak can also be expressed with the potential: FWHM = 355 mV.

The area of the cell of the WE can be approximated from the design in fig. 1 (b) as half of the area of the circle of 1 cm diameter that the electrodes occupy:

$$A \sim \frac{1}{2} \pi (0.05 \text{ dm})^2 = 3.9 \times 10^{-3} \text{ dm}^2$$

We used the initial height of the cell before platinisation (3.5 μm) without consider the volume reduced by the porous platinum as an upper limit of the total volume. Thus the total volume of the cell is ~ 140 nL. Using eq. 2 we get the minimum pH:

$$pH = -\log \left[\frac{Q}{F \cdot V_{cell}} \right] = -\log \left[\frac{1.7 \times 10^{-3} C}{9.65 \times 10^4 C \text{ mol}^{-1} \cdot 140 \times 10^{-9} L} \right] = 0.9$$

I. Quenching of fluorescence with two electrode configuration

Figure S12 (a) Current bias (in black) and voltage response (in red) corresponding to square pulses of 0.8 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.

Quenching of the pH fluorescence indicating pH values below 5 could be achieved increasing the current pulse amplitude as shown in figure 8 (a).

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5

Multiplexed control of acidity

Multiplexed control allows the possibility to drive different reactions at the same time thereby increasing the overall yield and the combinatorial throughput of the reactions. Miniaturisation is the route to increase the number of parallel processes. Here in this chapter we present a new miniaturised design capable of operating multiplexed reactions in few hundreds of μm spots similar to the ones of a microarray. We show by demonstration that the working model of pH actuation is compatible with further miniaturization and providing large control over the pH range, in which the contrast can be maintained for several minutes. Quantitative control of the acidity is also possible. The multiplexed control of acidity in the microreactors is presented and the conditions of reversibility for long lasting cycles have also been investigated.

Miniaturised control of acidity in multiplexed microreactors

Abstract

The control of acidity influences the structural assembly of biopolymers that are essential for a wide range of applications. Its miniaturization can increase the speed and the possibilities of combinatorial throughput for their manipulation, similarly to the way that the miniaturization of transistors allows the high throughput of logical operations in microelectronics. Here we present a device containing multiplexed micro-reactors, each one enabling independent electrochemical control of the acidity in ~ 2.5 nL volumes, with a large acidity range in aqueous solutions from pH 3 to 7 and an accuracy of at least 0.4 pH units. The attained pH within each microreactor (with footprints of ~ 100 μm for each spot) was kept constant for long retention times (~ 10 minutes) and over repeated cycles >100 . The acidity is driven by redox proton exchange reactions, which can be driven at different rates that influence the efficiency of the device in order to achieve more charge exchange (larger acidity range) or better reversibility. By the performance in the acidity control the miniaturisation and the possibility to multiplex paves the way for the control of combinatorial chemistry through pH and acidity controlled reactions.

*This chapter is based on the publication D. Balakrishnan, W. Olthuis, C. Pascual Garcia, "Miniaturized control of acidity in multiplexed microreactors.", arXiv:1908.02465 [physics.app-ph].

5.1 | Introduction

Acidity is a parameter that can drive chemical reactions, its control is one of the most important strategies in processes as important as the assembly of DNA [1- 4], the control of polymer structures [5,6] or the solid phase synthesis of biopolymers like peptides [7], nucleotides [8] and saccharides [9], which are the basis of combinatorial chemistry. The miniaturized control of these reactions has the potential to provide several advantages: the increase in the kinetics by reducing the distance between the molecules, the possibility to increase the number of simultaneous reactions providing a multiplexed control to achieve high combinatorial throughput with high-density arrays and the reduction of consumption of reagents, minimizing the costs of production. One approach to the miniaturization of chemical reactions controlled by acidity is the classical approach of solid phase synthesis to deliver reagents and immobilize them into a substrate, while applying the same acid treatment into different spots [7,10]. On the other hand, producing the acid locally has the advantage that would allow to introduce reagents in parallel and multiplex the chemical reactions. This strategy decreases greatly the time of high throughput assays. A successful approach to this was driven by Price et al. [11], who used a spin casted photo acid generators introduced in a photosensitive resist. With this method they produced arrays with spots of area $50 \times 50 \mu\text{m}^2$. A similar method was reported with an array of reactors that used photo acid generators immobilized with other reagents in the reactor volumes for the synthesis of DNA [12]. Here the generated acid was confined in the space of the reactor volume and the photo generating molecules were flushed into the reactors for every chemical reaction. Photoactive molecules were also used to release OH^- ions to alter the pH to basic conditions, while controlling enzymatic reactions on DNA [13]. Though photo-activated processes have shown that they can drive reactions in acid and basic conditions in miniaturized size, they require complex equipment and processes.

An alternative approach to generate the acid locally is to use electrochemical redox processes involving proton exchange reactions. Southern and Egeland [14] reported a device consisting of reaction chamber containing an array of microelectrodes on a glass substrate with an electrolyte containing redox active molecules that could exchange protons with the electrolyte. When a current was applied to the microelectrodes acid was generated in close proximity to them. As the redox reactions can be reused several times, this method has also the advantage that it can be used for several cycles without changing the assembly. However, the fast diffusion of protons limited the range of acidity and the contrast between spots to achieve high reaction yields [15]. To avoid the diffusion of protons on the local control of acidity was improved by implementing a porous substrate on the elec-

trodes and scavenging molecules in the electrolyte [16]. The reactions were confined inside the porous substrate in proximity to the electrodes, and protons would be annihilated outside. While this method improved the local control of acidity to limit the reaction sites into few tenths of micron, the acidity range was still limited. Recently we reported a device to control acidity in nL volumes using a platform that consisted of an electrochemical cell with working, counter and reference electrodes separated by long channels that acted as diffusion barriers [17]. The barriers were designed to avoid the reduction of protons at the counter electrode and to confine the proton concentration in the working electrode cell. Using polymerized 4-Aminothiophenol (ATP) as redox active molecules functionalized on the electrode surfaces an applied current generated the acid through the oxidation reaction. We reported a binary actuation of the pH in a range from 7 to 1, the stability of the attained pH for longer than 15 minutes and the reversibility of the oxidation and reduction reactions.

Here we present a device able to produce large acidity contrasts in confined microreactors and maintain it for times much longer than what the diffusion of the protons in free space would allow. The new device contains four electrochemical cells with each containing a working, reference, counter electrode cells separated by diffusion barriers. The new reactors use less than 2.5 nL volumes in an area of ~ 0.4 mm². In addition to the binary mode of pH actuation from 7 to 2.6, we also show the quantitative control of pH with an accuracy of ~ 0.4 pH units. Furthermore the multiplexed control of acidity in two of the reactors showing contrast pH changes are presented. The performance of the device in terms of the reversibility of the acidity control and the stability of attained pH are also reported.

5.2 | Results and Discussion

5.2.1. | Design of the chip and description of the setup

Figure 1 (a) shows the microscope image of the center part of one of the fabricated chips that includes four microreactors. The enlarged view of one of them is also shown in Figure 1 (b) and consists of working, counter and reference electrodes which are confined in each of the different cells. The working principle of each cell is similar to the one in our previous publication [17]. The working electrode cell is separated from the counter and reference electrode ones through two separate long channels that act as diffusion barriers. The electrodes are functionalized with 4 Aminothiophenol (4ATP). After a polymerization process of these molecules, they dimerize to a redox molecular state [18], which at low applied voltages reversibly exchange protons with the solution thereby changing the pH locally on each of the electrode cell. The long channels were designed to

Figure1: (a) Microscope image of the multiplexed device chip containing four fully functional electrochemical microreactors. (b) Enlarged view of one the microreactor with WE, RE, CE cells separated by diffusion barriers. (c) Photographic image of the mounted platform. (d) Schematic representation of the microfluidic platform in (i) closed and (ii) open position with movable optical setup on top.

avoid the reduction of protons at the counter electrode. The diffusion of protons along the channel was considered to be one dimensional and the barriers were designed so that the volume of the channel was much smaller than the volume of the electrode cells to avoid the change of acidity in each micro vessel during the diffusion time of protons. The diffusion time of the protons τ depends on the length of the barrier L and diffusion constant of protons ($D=9 \cdot 10^{-5} \text{ cm}^2/\text{s}$) and can be calculated as, $\tau=L^2/\pi^2 D$ [19]. The channel length of $\sim 0.8 \text{ cm}$ was designed to provide a diffusion time of 11 minutes for the protons to reach the counter electrode. The width and the height of the barrier were $\sim 25 \text{ }\mu\text{m}$ and $\sim 7.2 \text{ }\mu\text{m}$ respectively. The volume of the electrode cell was $\sim 2.5 \text{ nL}$. Different identical chips were fabricated first with the deposition of electrodes by optical lithography and e-beam evaporation of gold and then the channel barriers for the cells were patterned with optical lithography on a spin coated SU8 epoxy resist. The detailed steps of fabrication were explained in the supporting information figure SI-1. An electrochemical platinisation was performed on the electrode surfaces to increase their surface area. Finally, the electrodes were functionalized with 4ATP to form a self-assembled monolayer of redox active molecules on top of the porous electrodes.

The chip was mounted on a custom-made microfluidic platform schematically represented in Figure 1 (c), the closed position that includes the optical set-up in Figure 1 c (i) and the open position (ii) is shown. The top part of the platform consists of an optical window, microfluidic inlet and outlet and

electrical contacts that connect the chip to an external switch box which connects also to a potentiostat. The bottom part also includes a movable piston that allows to move the chip up and down to close and open the cell. The chip was sealed with an O-ring that helps to isolate the liquid from the contact pads during the open position. The platform was mounted under an optical microscope that allowed to monitor the pH using the fluorescence marker carboxy semi-naphthorhodafluors (cSNARFs). We used 1 M concentration of Potassium chloride solution with 0.5 μ M cSNARF as electrolyte. For pH control measurements, the microscope was moved to the respective electrode cells of the different microreactors to follow the changes in the spectra of the fluorescence marker that arises due to the redox active reactions at the electrode surface. Figure 1 (d) shows the image of the mounted platform. More details of the experimental setup were described in our previous works with chips where the reactors were not miniaturized [17,20]. When the chip was closed by the glass window from the top, the epoxy resist ensures the isolation of the electrode cells and channels. On the current chip designed we used a circular arrangement of electrodes and channels at the center so that they occupy most of the flat available surface of the spin coated resist. Additionally the concentric arrangement of channels could relax the tensions producing less cracks on the processed samples.

5.2.2. | Control of acidity in one microreactor

The acidity in the cells was controlled by the reversible redox reactions of the 4ATP that exchange protons with the electrolyte. The redox active states of 4ATP were created by polymerizing electrochemically the self-assembled monolayer [21] with the chip in open position to reduce the external resistivity existing when the electrodes are connected only through the diffusion barriers (closed cell configuration). Following the electropolymerization, the platform was flushed with fresh electrolyte and placed in closed position to constraint the volume for pH control measurements. Each cell on the chip was connected to a potentiostat channel with three-electrode configuration. Cyclic voltammetry (CV) was used to drive the redox reactions on the polymerized 4ATP layer. The microscope was focused on the WE to monitor the acidity changes through the fluorescence spectrum during CV measurements. The pH inside the electrode cell was monitored by the SNARF signal which has two peaks, the one at 590 nm is dominant in acidic pH and the other at 655 nm is dominant in basic pH. The relative intensity of the two peaks can be used to calibrate the pH. Due to the miniaturized volume and low concentration of SNARF we used an excitation wavelength at 565 close to the absorbance of SNARF molecule and to avoid the stray light we used a filter around 600 nm. Then we followed the pH variations with the intensity of the peak of SNARF at 655 nm. The correspondence between

the 655 nm fluorescence peak intensity normalized at the initial pH 7.4 and the real pH is shown in the calibration plot SI figure 4. To follow the fluorescence each

Figure 2: The graph shows (a) the voltage, (b) the current and (c) the normalized intensity of the fluorescence marker plotted against time. (d) Current is plotted against voltage for the cycle 2 (data corresponds to the shaded region in (b)). The inset shows the Gaussian fitting of the peak in red dotted plot and the subtracted data of current vs voltage is shown in black. (e) SNARF fluorescence spectrum showing the maximum and minimum intensity peaks corresponding to the pH value 7 and 2,6 respectively.

spectrum was integrated for 1 second during the CV.

Figure 2 (a), (b) and (c) show the bias voltage, the corresponding current and the 655nm peak fluorescence normalized to the peak intensity at pH 7.4, respectively, plotted against time during 5 cycles. The voltage range used was -0.75 V to +0.75 V at a rate of 50 mV/s. The positive voltage sweep from 0 V to 0.5 V gives rise to an oxidation peak like the one shown in figure 2 (d), which corresponds to the grey shadowed region shown in the second cycle of figure 2 (b). At the end of the oxidation peak a decrease in the fluorescence intensity is observed in figure 2 (c), which shows the decrease of the pH in the cell. The negative voltage sweep from 0 to -0.5 V produces a reduction peak shown in the region shadowed in blue in figure 2 (b). At the end of this peak, an increase in the

fluorescence intensity was observed in (c) that shows that the neutral pH was recovered. This cycle repeated successively showing reversible changes on the fluorescence intensity driven by the reversible redox states of the 4ATP molecule that releases protons during oxidation reaction and recovers them from the electrolyte during reduction reaction thereby controlling the acidity in the working electrode cell. In figure 2 (b), we also observed a small peak before the main oxidation, which was detected in the successive cycles. The origin of this different oxidation could be attributed to the underlying Au in the electrode due to incomplete platinisation in the electrodes which could produce an oxidation of the 4ATP with slightly different potentials and that we observed only in some samples where the electrodes were not completely platinized. The pH attained in the working electrode cell could be followed until the detection limit of the SNARF maker at pH 5 (see calibration plot shown in figure SI-2). Below this limit the 655 nm peak completely quenches and no more changes in the intensity of the 655 nm peak with respect to the pH was observed. In our CV experiment we started with the electrolyte that was prepared with KCl at pH 7. The minimum pH beyond the range of the SNARF marker (figure 2 (e)) could be estimated calculating the total charge exchanged by the electrons in the cell (Q) and attributing that charge exchange to redox reactions producing only protons. As the pH change is more than two units, as monitored by the SNARF, the final concentration exceeds largely the initial one at neutral pH, and as the measuring time is much smaller than the diffusion time of protons through the diffusion barriers The pH could be calculated converting Q into number of protons and retrieving the concentration using the volume in the cell:

$$\text{pH} = -\log[Q/(F \cdot V_{\text{cell}})] \quad \text{Eq. (1)}$$

where F is the Faraday constant and V_{cell} is the volume of the working electrode cell (~ 2.5 nL). The charge Q was obtained from the integrated area under the oxidation peak and the scan rate. In figure 2 (d) the oxidation peak for cycle 2 indicating the area under the peak (shaded region in figure 2 (b)) is shown along with the inset presenting the Gaussian fitting and the results from the fitting are tabulated in the table 1. The minimum pH attained in the cell resulting from the equation (1) was calculated as 2.6.

5.2.3. | Retention time and Quantitative control of the acidity range

An accurate control of the acidity in the cell would allow the possibility to orthogonally control chemical reactions specific to different pH values. In our device we tested quantitative control of the acidity by using pulse voltammetry as shown in Figure 3 (a) and (b). Different voltages with pulses of 20 s were applied while monitoring the pH through the fluorescence intensity Figure 3 (a). After the

first pulse of 0.8 V was applied we decreased the amplitude in steps of 0.05 V until reaching 0.55 V. Figure 3 (b) shows the normalized fluorescence intensity with respect to the different voltage pulses plotted against the time. After the first

Figure 3: The graph shows (a) the voltage pulse and (b) the corresponding fluorescence spectra with different pH values plotted against time. (c) The graph shows the voltage pulse and the corresponding fluorescence spectra plotted against time.

voltage pulse at 0.8 V the fluorescence intensity decreased to 0.1 while at 0 V the initial fluorescence intensity was recovered. A similar response was also observed during the second voltage pulse at 0.75 V, probably because the pH marker was out of range. For the next voltage pulse at 0.7 V the fluorescence intensity was slightly higher than that of the previous pulse showing a control of the pH. At 0.65 V the intensity was higher than the previous pulse at 0.7 V and this process was repeated for the next consecutive pulses too. The corresponding minimum pH values were denoted in figure 3 (b), showing that in addition to the binary mode of pH actuation (7 to 2.6), we were also able to demonstrate quantitative control over the acidity concentration with ΔpH of ~ 0.4 units.

We tested the stability in terms of retention time of the attained pH in the electrode cell by applying a voltage pulse from -0.15 V to 1.2 V for 3 cycles to

observe a reversible pH actuation and then we opened the circuit at 1.2 V. The change in the fluorescence intensity was monitored for 10 minutes, which is similar to the calculated diffusion time of the protons through the barrier (11 minutes). Figure 3 (a) shows the applied voltage pulse and (b) the fluorescence spectra plotted against time. In the third voltage pulse at 1.2 V the fluorescence intensity was completely quenched. Until 300 seconds (5 minutes) we observed no noticeable change in the fluorescence intensity. After five minutes a slight increase in the intensity was observed. At 600 seconds (10 minutes) the pH maintained in the cell was < 5 as it was obtained from the calibration plot. These measurements show that the design of the device along with the model of diffusion barrier is compatible with miniaturization and the ability to sustain the acidic conditions for 5 minutes already opens applications to the occurrence of different chemical processes.

5.2.4. | Reversibility tested over 100 cycles

Figure 4 (a) SNARF fluorescence spectra plotted for the CV cycles 2, 25, 50, 75, 100 in the voltage range from -0,75 V to +0,75V with an offset in Y axis. (b) shows the plot of the CV cycles against the normalized fluorescence intensity of the snarf spectra indicating the maximum and minimum intensity. (c) The graph shows the scan rate plotted against the voltage at the oxidation peak.

We studied the reversibility of acid control monitoring the CV during 100 cycles with the fluorescence marker. The voltage was swept from -0.6 V to 0.7 V at a scan rate of 100 mV/s and the fluorescence spectra were recorded with integration times of 1 second each. Figure 4 (a) shows the spectra corresponding to cycles 2 (black), 25 (red), 50 (green), 75 (blue) and 100 (purple) at different voltages plotted with a vertical offset as indicated in the graph. All the spectra reflect the behavior of pH already explained depending on the oxidation/reduction of the 4ATP. Initially, in cycle 2 (in black) during the voltage sweep at -0.6 V (top spectrum) the fluorescence peak at 655 nm corresponds to the neutral pH in the electrode cell. Towards positive voltages around 0.7 V (fourth spectrum) the 655 nm peak was quenched due to the increase of acidity made by the 4ATP oxidation and at -0.3 V (bottom spectrum) the increase in peak intensity witnesses the recovering of more basic conditions. A similar behavior in each of the cycles 2, 25, 50, 75, 100 were observed where the peak at 655 nm appeared and quenched indicating neutral and acidic pH conditions in the cell respectively. Figure 4 (b) shows the cycle dependence of the maximum and minimum peak intensities of the 655 nm plotted in red and black, respectively. After 25 cycles (40 minutes) we observed an increase of both intensities, which reflects a rise in the overall acidity of the system. After flushing the cell with fresh electrolyte, we were able to recover the same intensities repeating the experiment with the same chip (data not shown). This effect of progressive increase of basic conditions obtained with the closed cell could be attributed to the leakages of the protons, and/or to defects in the 4ATP functionalization at the electrode surface which would results in holes in the electrode interface where direct exchange of electrons with protons to reduce them into Hydrogen gas (H_2) could occur during the negative voltage bias. Both effects can decrease the overall proton concentration in the cells resulting in an increase in the basicity in the microreactor. Nevertheless, the minimum pH attained in the cell after 100 cycles was still under 5 from our calibration.

The kinetics of the redox reactions was also monitored with CV cycles at different scan rates from 10 mV/s to 70 mV/s. We observed a shift in oxidation peak potential. In Figure 4 (c) the scan rate is plotted against the voltage of the oxidation peak. The oxidation peak voltage was around -0,2V for upto 40mV/s. Then with increasing scan rates, the oxidation peak voltage shifted to higher voltages, which may be because those rates did not allow enough time for the redox reaction to occur. This indicates that an increase acidity control with lower bias could be used until 30 mV/s, although that would increase largely the control of multiple cycles, and the reversibility shown in figure 4 (a) provides already a pH contrast of at least two units for more than 100 cycles.

5.2.5. | Multiplexed control of acidity in two cells with different phase

Figure 5- shows multiplexed control of acidity in cell 1 and cell 2. (a) The voltage applied in cell 1 and cell 2 is shown in black and red respectively, and (b) the corresponding fluorescence spectra is plotted against time. The dotted plots of cell 1 and cell 2 represent the projection of the spectra (c) Microscope image of the chip highlighting the WE of cell 1 and cell 2. (d) and (e) shows the enlarged view of the dotted area in (a, b).

The chip with multiple cells was designed to allow simultaneous control of acidity in different cells. To control this feature two channels of the potentiostat were connected and we adjusted the CV cycles to apply the voltage out of phase introducing delay time at the start of the CV of channel 2. We used a voltage range of -0.3 V to 0.7 V at a scan rate of 20 mV/s in both channels. To measure the fluorescence signal in each cell using our single channel spectrometer we alternated the position of the cells two times in each cell every 5 cycles to complete twenty cycles. Figure 5 shows (a) the applied voltage and (b) the corresponding fluorescence intensity plotted against time in cells 1 and 2 in black and red, respectively. The WE cells 1 and 2 are shadowed in black and red respectively in Figure 5 (c). To visualize the behavior of acidity, the data from the previous 5 cycles of the two cells was projected in dotted lines for the cycles where the microscope objective was on the other cell, since simultaneous measurement of

fluorescence intensity at the two cells was not possible. Both cells 1 and 2 (plotted in black and red, respectively) show the behavior already described with oxidation and reduction at ~ 0.7 V and 0.1 V respectively, and the fluorescence following the acidic behavior of the redox reactions.

The fluorescence is shown to be out of phase on both cells, following the respective bias actuation. It is also remarkable that the projected fluorescence plotted in dotted lines for both cells shows very good continuity with the real fluorescence, which indicates that during the time the fluorescence is not monitored in each cell, the acidity follows the expected behavior. The enlarged views of the CV cycles in the shaded areas in figures 2 a and b are shown in (d) and (e), respectively. The changes in fluorescence intensity with respect to the applied voltage bias could be seen along with the contrast fluorescence changes in the other cell which is denoted as a projection. For 20 cycles alternating pH conditions acidic – neutral was demonstrated in cell 1 and cell 2 maintaining the contrast conditions. No potential leakage was observed. As conclusion we drove the multiplexed control of the acidity in these two miniaturized cells in an independent way.

5.3 | Conclusions

We fabricated a miniaturized device that holds four microreactors to control the acidity in miniaturized cells. The control of pH was demonstrated using the electrochemical reactions of redox active molecules functionalized on the surface of the electrodes. The microreactor was designed to confine the protons in electrode cells while the reduction during negative biases was avoided using diffusion barriers separating working and counter electrodes. A fluorescence marker in the electrolyte was used to track the pH. We were able to control the acidity in the individual microreactor from pH 7 to complete quenching of the marker at 5. The minimum pH attained in the cell was 2.6 as was calculated from the charge transfer of the polymerized 4ATP molecules. We demonstrated that regardless the miniaturization, the pH was controlled similarly to our previous article, which demonstrates that the acidity control is independent of the footprint of the microreactors. Further miniaturization of the device would be possible thanks to our design. We showed a quantitative control of pH with an accuracy 0.4 pH units varying the amplitude of the applied potential pulses. The microreactor retains a stable pH in the electrode cell for 10 minutes by the confinement of protons, thanks to the tailored diffusion barriers. This time control would allow to drive chemical reactions such as synthesis of bio-polymers that we expect to occur faster than in macroscopic vessels because the diffusion of reagents within this cell is limited with the miniaturized volumes. The reversibility of the acidity control was tested over 100 cycles. The minimum pH corresponding to the 100th cycle was still under

<5. This shows that the system has good control over the reversibility providing a dynamic acidity control and well suited for combinatorial chemistry applications that allows the assembly of molecules on the same spot. For example one of the current challenges in the peptide synthesis is the control of proton concentration. Current methods use 50 % concentration of trifluoroacetic acid to remove the protecting groups. The pH corresponding to this concentration is ~ 5.9 (more information is provided in the supporting information). The generation of acid through the redox reactions of 4Aminothiophenol occur also in organic solvents (data not shown). The multiplexed control of acidity was demonstrated in two cells on the same chip using simultaneous CV measurement that are out of phase to maintain different pHs without any crosstalk. This shows that our device could be used for combinatorial chemistry applications where the regulation of the large acidity range would enhance the throughput and the yield of chemical reactions.

In summary, we have demonstrated to the best to our knowledge, the largest control of the acidity in terms of acidity range and retention time, driven by electrochemical means in cells miniaturized in the range of hundred microns. We also showed that the pH could be independently activated in different cells. We believe that the key features of our device large pH range, quantitative control, reversibility and multiplexing could be used to control chemical reactions with increased throughput assuring stability of acid contrast between nearby spots.

5.4 | Experimental Section

Fabrication of the chip

The chip was fabricated using optical lithography. A maskless aligner – MLA 150 from Heidelberg Instruments was used to pattern the designs. The series of experimental steps involved in the fabrication process are detailed in the supporting information. The substrate was spincoated with optical resist and design of electrodes with contact pads are exposed and developed. Later 5 nm of Titanium and 50 nm of Gold was evaporated using a e-beam evaporator. The chip was placed in acetone for lift-off. Then the chip was spin coated with SU8 3010 epoxy resist to make the second layer. The design of the diffusion barriers and the electrodes are exposed on the chip using the maskless aligner. The chip was then developed and hard baked for 10 minutes. The electrodes are electrochemically platinized [17]. The chip was later cleaned in a UV ozone cleaner for 30 minutes and functionalized with 0.5 mM concentration of 4 Aminothiophenol in absolute ethanol for 24 hours.

Electropolymerization and acidity control on the platform

The cell was placed in open position and through the switch box the electrodes are connected to the potentiostat (Pstat 1) by three electrode configuration. A CV program was applied in the range of -0.25 V to + 0.7 V at a scan rate of 50 mV/s for 3 cycles.

Multiplexing experiments were performed in cell closed position with the first potentiostat (Pstat 1) connected to cell 1 of the chip and the second potentiostat (Pstat 2) connected to cell 2 of the chip through the switch box. A CV program was applied between -0.3 V to -0.65 V at a scan rate of 20 mV/s for 20 cycles. The microscope objective was focused at cell 2 working electrode for the cycles 1 to 5 and 11 to 15. The objective was focused at cell 1 working electrode during the cycles 6 to 10 and 16 to 20.

Chemicals and Instrumentation

4 Aminothiophenol, Potassium chloride and absolute ethanol were purchased from Sigma Aldrich. Carboxy semi-naphthorhodafluors was purchased from Molecular Probes Inc. For electrolyte preparation and cleaning purposes Millipore filtered water was used. For the electropolymerization and pH control experiments Solatron Modulab XM Pstat 1mS/s potentiostat (Pstat 1) was used. For multiplexing experiments, Gamry Potentiostat REF600-05104 (Pstat 2) was used.

5.5 | References

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5.6 | Supporting Information

A. Fabrication of the chip

Figure SI-1: Fabrication steps of the chip

The chips were fabricated using optical lithography. Figure SI-1 shows the sequence of experimental steps that were followed to fabricate the chip: 3 x 3 cm SiO₂/Si substrates with 50 nm oxide layer were used. 1- First an optical resist was spin coated on the substrate, 2 – using a maskless aligner the design of the electrodes were patterned on the substrate with a UV source of wavelength 375 nm, 3 – The substrate was then loaded into the E-beam evaporator to deposit 5 nm Ti and 50 nm Au layers, 4 – After evaporation the substrate was placed in Acetone for lift off process, 5 – For the second layer SU8 3010 resist was spin coated on the substrate, 6 – The design of the diffusion barriers were patterned using the maskless aligner, 7 – The electrodes were electrochemically platinized, 8 – The electrodes were functionalized with 4-ATP. More details regarding the platinisation of electrodes were described in our previous publication [10].

B. pH calibration of the fluorescence marker

In our experiments the fluorescence marker carboxy semi-naphthorhodafluors (carboxy SNARFs) was used to detect the pH changes in the electrode cells. Before performing the pH control experiments on the platform, first the changes in the intensity of the fluorescence marker is calibrated for different pH solutions. 5 μM concentration of carboxy SNARFs were mixed with buffer solutions of different pH (3 to 7). The marker was excited with 530 nm diode source and the measured intensity showed two peaks at 590 and 650 nm. The pH can be detected by following the relative intensities of the two peaks which is shown in the black data points in figure SI-2. In our platform the area of illumination on the electrode cells is ~ 60 μm. In order to maximize the fluorescence efficiency, the marker was excited using a 580 nm wavelength LED source which is close to the maximum of

absorption and we used a dichroic mirror with a band pass filter at 590 nm to cut the stray light. With this setup another calibration plot for the excitation wavelength 580 nm and the corresponding SNARFs spectra at the different pH buffer solutions were measured (black open triangle). Due to the band pass filter only the peak at 650 nm could be clearly seen so to follow the pH changes with this peak, we normalized the 650 nm peak intensity to the intensity at pH 7.2. This is shown in the calibration plot in red. In our experiments, we followed the pH using the intensity of the 650 nm peak due to the short integration time to monitor the changes of pH with applied potential and the band pass filter. More details regarding the calibration plot is reported in our previous publication [10].

C. Details of Electropolymerisation

Figure SI-2: pH calibration plot of the fluorescence marker [10]

Surface functionalization of 4Aminothiophenol forms a self-assembled monolayer on the platinum electrode surface. In order to make them redox active, the 4ATP layers were electrochemically polymerized. The substrate was mounted on the platform and the electrolyte was filled in the microreactors. Using the contact pads the WE, RE and CE were connected to the potentiostat. The platform was placed in open position and cyclic voltammetry experiment was programmed on the potentiostat. CV cycle in the voltage range -0.25 V to +0.7 V at a scan rate of 50mV/s for 3 cycles was applied. Figure SI-3 shows the plot of applied potentials and the corresponding current for 1 (red), 2 (blue) and 3 (green) cycles. The slope of the current at 0.6V decreases with consecutive cycles indicating the polymerization of the 4ATP.

Figure SI-3: CV plot corresponding to the electropolymerisation of the 4ATP layers on the electrode surface

D. Calculation of concentration of acidity in organic solvents

For solid phase synthesis of peptide, 40% concentration of Trifluoroacetic acid (TFA) [SI 1] is used to remove the protecting groups from the monomers allowing the assembly of the molecules. 99% of TFA has a molar concentration of 12.95 M [SI 2], below the concentration of protons was calculated for 6 M concentration of TFA (~ 50%) in the organic solvent acetonitrile. The pKa of Trifluoroacetic acid in acetonitrile is 12.65. $K_a = 2.238\text{E-}13$.

For acid base reactions, acid dissociation constant is expressed as,

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

Where HA is the acid that dissociates into A⁻ the conjugate base of the acid and H⁺ hydrogen. Since TFA is a very strong acid the conjugate base is also H⁺ ion.

$$K_a = \frac{[H^+]^2}{[HA]}$$

For 6M concentration of the acid [HA], the concentration of [H⁺] is 1.16E10-12. The pH corresponding to this proton concentration is 5.93.

References:

[SI 1] Ronald Eri. Reid, Solid phase peptide synthesis. Effect of trifluoroacetic acid concentration on the removal of the tert-butyloxycarbonyl protecting group, J. Org. Chem., 41,6, 1027-1031 (1976).

[SI 2] https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Product_Information_Sheet/t6508pis.pdf

6

Conclusions and Future Perspectives

A brief summary and conclusions of the thesis along with several future applications for this work are presented.

6.1 | Conclusions

The regulation of acidity has the potential to drive chemical reactions that manipulate and alter the structural assembly of biopolymers. In this thesis I was able to successfully demonstrate the miniaturized control of acidity in different designs of microreactors on a microfluidic platform in aqueous electrolyte.

The first step towards the goal of this thesis has been the study of proton exchange electrochemical reactions for the generation of acid. We chose to use the redox reactions of the molecule 4ATP to control the acidity. This molecule has the advantage that undergoes redox reactions at low voltages, it can produce reversible exchange of protons and it is easy to functionalise on metallic electrodes as it can form well-ordered self-assembled monolayers on Au or Pt substrates. The monolayers of 4ATP could be polymerised using different methods to dimerize and become redox active molecules that can exchange protons reversibly. In **chapter two**, we studied the potential to improve the control of acidity maximising the charge exchange linked to the proton generation investigating the 4ATP and their corresponding dimerized forms along with their electrochemical properties. We used electrical, optical and plasma polymerisation to dimerize the original electrodes of 4ATP monolayers, and we observed a different majority of population regarding the structure of the dimers for each polymerisation method. The electrochemical behaviour of the polymerised structures for the different methods was measured and compared with respect to their charge transfer and reversibility of the redox reactions over 50 cycles. The results show that the electropolymerised substrates exhibit higher charge transfer capacitance than the optical and plasma polymerised ones for the initial cycles but after some cycles (50 in our case) all the polymerised forms showed a similar charge transfer capacitance. This shows that the electropolymerisation methods could provide more acidity at the beginning, also with the advantage of a very simple implementation since the same electrochemical setup for the control can be used in the polymerisation. However optical and plasma polymerised substrates showed higher reversibility due to their head-to-head polymerised structure and could be well suited in the future for wafer scale fabrication processes.

The next step was the design of a system to demonstrate the control of acidity. **Chapter three** explains the design and the working of the microfluidic platform and the fabrication of the chips. Each chip that we designed contains a miniaturised electrochemical cell with three electrode configuration working, reference and counter electrode separated by diffusion barriers. The electrodes were functionalized with 4ATP and were electrochemically polymerised to exchange protons through the redox active reactions. The barriers were designed to avoid the reduction of protons at the counter electrode and to confine the protons

in the working electrode cell. The working model of pH actuation shows that the acidity concentration depends on the surface coverage of the 4ATP molecules that exchanges the protons and the height of the cell. So the surface area of the electrodes was increased through platinisation to increase the number of protons to obtain more acidic conditions. The chip was replaceable, and different designs of electrochemical cells could be implemented. The platform was designed to be opened for the exchange of reagents and closed to contain the protons and the reagents in the cell using a pneumatic actuator combined with an O-ring designed to actuate in parallel in all the cells of the chip. This design differs from the traditional serial process implemented in microfluidics where the actions and cells are implemented in a channels one after another. The platform also has an optical window that allows real time monitoring of acidity changes in the cell through a fluorescence.

In **chapter four**, we demonstrate the acidity control in nanolitre volumes. To this goal, first we used the electropolymerisation of 4ATP on the microfluidic platform in open position using cyclic voltammetry for few cycles and following the changes in acidity with a fluorescence marker. Then the platform was closed to confine the volume of the cell (~ 120 nL) and with cyclic voltammetry were controlled the acidity during several cycles. The oxidation and reduction reactions of the 4ATP were able to reversibly release and uptake the protons into the electrolyte for each CV cycle producing pH actuation. The acidity range achieved was from pH 7 to beyond the detection range of the marker at pH 5. The minimum pH reached was calculated from the Faradaic currents and it was pH ~ 1 . To the date of this thesis, this is one of the largest ranges of control of electrochemically generated acid shown in literature. The attained pH changes were able to remain constant in the working electrode cell for more than 15 minutes due to the tailored diffusion barrier without any leakage. This could enable the possible chemical reactions to take place for biopolymer applications. Two electrode configuration of acidity control were also shown eliminating the use of reference electrode, which would allowing the possibility to reduce the space or add more reactors, and facilitate the integration with electronics for parallelisation. In summary, we were able to demonstrate the acidity changes in one miniaturised microreactor cell that provides large acidity range with reversible control and able to confine the protons for several minutes.

The next task was to address the objective to improve the manipulation of biopolymers through further miniaturisation and multiplexed processes. **Chapter five** shows a chip with more microreactors enabling multiplexed acidity control in volumes down to ~ 3 nL. The new design of the chip contained four electrochemical cells with each containing working, reference and counter electrodes separated by

diffusion barriers similar to our previous design. The acidity actuation was produced by applying a voltage bias controlling the redox reactions of 4ATP and monitored through the fluorescence marker as in the previous chapter. With the new chips we were able to control the acidity reversibly from pH 7 until ~ 3 for few cycles due to the fabrication conditions and less platinisation achieved, which is not a limitation for the future. However, the working principle actuated in similar way, which shows that the working model of pH actuation is compatible with miniaturisation. In addition to the binary mode of pH actuation (from 7 to 3) the device were able to provide a quantitative control with an accuracy of at least 0.4 Δ pH units. The quantitative control could allow in the future the use of orthogonal chemical reactions that use specific ranges of acidity. The reversible control of the pH changes was tested up to 100 cycles using voltage bias. The results show that from first to 25th cycle the acidity was switched from pH 7 to 3 during each cycle. Beyond the 25th cycle there was a slight increase in the acidity levels until 100 cycles. The pH level maintained in the reactor at the end of 100 cycles was <5 , which could still be able to control the different chemical reactions. Reversible control of acidity in future could increase the combinatorial diversity in biopolymer synthesis reactions. The device was able to maintain the attained acidity in the electrode cells for longer than ten minutes allowing the necessary time for the chemical reactions to occur, which at the time of this thesis is reported, is the best results for stability of electrochemically generated acid in a miniaturised cell. The multiplexed control of acidity was shown in two of the electrode cells that were driven to attain contrast pH changes without any leakage or cross-communications. As the platform offers also a parallel exchange of reagents the system would allow the parallel synthesis of several biopolymers.

In summary we have realized a miniaturized device that addresses one of the most important challenges of combinatorial chemistry by providing the best control of acidity for the manipulation of chemical reactions. The key features of the system offer a large acidity range, the capacity to maintain acidity for several minutes, the ability to provide multiplexed control and the reversible and quantitative control of actuation.

6.2 | Future Perspectives

I have presented a device that could produce acidity changes in one or more microreactors to control chemical reactions. Acidity is a key parameter to control the structural stability of bio-polymers as they influence the intermolecular forces causing temporal or permanent changes to their structure. The temporal changes include the folding and unfolding of polymer structures. For example, in a particular case of DNA¹ (i-Motif) made by a four stranded helix structure, the specific folding depends strongly on the acid. Some studies show the relevance of

these structures in the gene expression which could be better understood with a systematic control of the acid. Acidity can also be used to control the intermolecular forces between DNA causing them to be apart ($\text{pH} > 8.5$) or close together ($\text{pH} < 4.5$). In literature it has been reported that the control of the pH is used to increase or decrease the reaction rate in DNA hybridization². Acidity could also induce permanent changes such as the activation of enzymes, which are triggered at different pHs, for example to control the hybridizing DNA to perform pH programmable DNA logic operations³. Another application with DNA is the control of the DNA replication⁴.

In a different domain, an example related to permanent changes is the solid phase synthesis⁵ of peptides or nucleotides that uses acidity to de-protect the monomers that allow the assembly of the next molecule. The reversible and multiplexed control of our system provides the possibility to drive different reactions in different reactors at the same time that could increase the combinatorial power and overall yield of the reactions. The device and the proton actuation reaction of 4ATP are compatible with organic solvents that allows the system to be used for organic synthesis reactions (peptide synthesis). Some challenges still remain, like the separation of the amino groups of the 4ATP from the synthesizing groups, and the size of these cells is in the order of few hundred microns, but the design allows further miniaturisation, which combined with the compatibility of the design with CMOS circuits provides to these results a potential big impact for the generation of biopolymer microarrays.

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6. D. Balakrishnan, W. Olthuis, C. Pascual Garcia, Miniaturisation of acidity control bursting combinatorial chemistry, 2019, Lab on a chip World Congress, United States of America (*oral presentation*).

Samenvatting

De geminiaturiseerde regeling van zuurgraad biedt de mogelijkheid om de snelheid te verhogen van chemische reacties die worden gebruikt voor de synthese van verschillende polymeren, zoals DNA en eiwitten door sturing van hun structuur en door gemultiplext gebruik voor te stellen in verschillend aanstuurbare gebieden om zodoende de opbrengst in combinatorische scheikunde te verhogen. De elektrochemische mogelijkheid om de zuurgraad te regelen zou een compacte, goedkope technologie bieden, die samen met de integratie van microfluidica en elektronica kan dienen als micro-Total-Analysis System voor vraaggestuurde vervaardiging of manipulatie van biopolymeren. De uitdaging ligt hier in de geminiaturiseerde productie en opsluiting van de protonen met een ontwerp dat gemultiplexte processen toelaat. In dit proefschrift tonen wij de voortgang in het onderzoek ten aanzien van bovenstaand doel. Eerst bestuderen we het molecuul 4 Amino-thiolphenol (4ATP) dat na polymerisatie omkeerbaar protonen kan genereren via een elektrochemische redoxreactie. Verschillende wijzen om 4ATP te polymeriseren zijn onderzocht. Het elektrochemische gedrag van de verschillende vervaardigingswijzen ten aanzien van hun mogelijkheid tot ladingsuitwisseling en de omkeerbaarheid van de redoxreacties is gemeten en vergeleken. Vervolgens tonen we het ontwerp van het microfluïde platform dat de microfluïde en elektrische verbindingen integreert. Het platform kan een uitwisselbare chip inklemmen dat de elektrochemische reactor bevat. Het werkende model en het aantonen van geminiaturiseerde regeling van zuurgraad in één microreactor chip (~100 nL volume), gemonteerd op het platform, wordt getoond, waarbij het zuur omkeerbaar wordt gegenereerd door het maken van protonen uit de gepolymeriseerde 4ATP moleculen. De stabiliteit van het gemaakte zuur door opsluiting van de protonen in de cel is ook onderzocht. De gemultiplexte regeling van de zuurgraad is onderzocht in een tweede ontwerp dat verder geminiaturiseerde elektrochemische reactoren op chip bevat met een afzonderlijk celvolume van ~3 nL. De gemultiplexte regeling wordt aangetoond op het platform met twee elektrochemische reactoren die worden aangestuurd om een verschillende zuurgraad vast te behouden.

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The results described in this thesis were obtained through collaboration with many people. The collaborators and their contributions are listed below. Dr. Cesar Pascual Garcia from Luxembourg Institute of Science and Technology and Dr. Wouter Olthuis from MESA+ group, University of Twente provided all the inputs for the execution of the thesis work through fruitful discussions. Dr. Damien Lenoble and Dr. Siva Shankar Krishnamoorthy from Luxembourg Institute of Science and Technology provided the guidance and support for the successful completion of the project.

Chapter 2

Divya Balakrishnan contributed on the performance and design of experiments, writing of the manuscript and preparation of the figures. Dr. Guillaume Lamblin and Dr. Jean Sebastien Thomann contributed with the interpretation of the data and preparation of the manuscript. Dr. Jerome Guillot performed and interpreted the XPS experiments and contributed to the writing of the manuscript. Dr. David Duday design and performed the Plasma polymerisation experiments and contributed to the writing of the manuscript. Prof. Dr. Albert van den Berg and Dr. Wouter Olthuis contributed to the design and interpretation of the experiments and to the writing of the manuscript. Dr. César Pascual-García provided the original idea of the study, contributed in all the steps of the design and performance of the experiments and to the full preparation of the article.

Chapter 3

Divya Balakrishnan contributed on design of experiments, analysis of data, writing of the manuscript and preparation of the figures. Mathieu Gerard contributed to the design and assembly of the microfluidic platform. Dr. Doriane Del Frari assisted in electrochemical measurements and data analysis. Dr. Stephanie Girod contributed to the fabrication of electrochemical chips through optical lithography. Dr. Wouter Olthuis contributed to the design

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Chapter 4

Divya Balakrishnan contributed on the performance and design of experiments. Dr. Guillaume Lamblin and Dr. Jean Sebastien Thomann contributed with the interpretation of the data and preparation of the manuscript. Prof. Dr. Albert van den Berg and Dr. Wouter Olthuis contributed to the design and interpretation of the experiments and to the writing of the manuscript. Dr. César Pascual-García provided the original idea of the study, contributed in all the steps of the design and performance of the experiments and to the full preparation of the article.

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Divya Balakrishnan contributed on the design of experiments, analysis of data, writing of the manuscript and preparation of the figures. Dr. Wouter Olthuis and Dr. César Pascual-García contributed to the design and interpretation of the experimental data and to the writing of the manuscript.

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