Spatiotemporal Electrochemical Detection in Nanofluidic Devices

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in
nanofluidic devices**

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SPATIOTEMPORAL ELECTROCHEMICAL DETECTION IN NANOFLUIDIC DEVICES

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Chapter 1

General introduction

This chapter introduce briefly introduce the electrokinetic effects in nanofluidic devices. Electroosmotic effect and ionic current manipulation is emphasized since they become more important in the nanoscale than in the microscale. Electrochemical reactions will add another complexity in this system.

Electrochemistry counts among the most important classes of analytical methods due to its sensitivity, relatively low cost and versatility. It can be used either as a detection tool that directly translates information about the composition of a sample into electrical signals^{1–5} or as a preparation method to manipulate a sample prior to other forms of analysis.^{6–8} Because of the relative ease with which electrodes can be incorporated into more complex systems, electrochemistry is often used in conjunction with other methods. For example, the integration of electrodes into capillaries provides an excellent means of detection in many capillary-based analytical methods such as isotachopheresis (ITP)⁹ and chromatography.⁸

An area of great current interest is the realization of analytical techniques capable of dealing with minute sample volumes. This allows the realization of heavily parallelized assays but also analyzing intrinsically small samples such as the contents of single living cells. Modern micro-fabrication techniques make it relatively straightforward to bring the size of devices down to the nanometer scale. However, scaling down dimensions can lead to effects that are not easily perceived in conventional macroscopic systems to become dominant. In particular, due to the high surface-to-volume ratios of nanoscale devices, the properties of surfaces and the frequent interactions of analyte molecules with these surfaces can become disproportionately significant.

Figure 1. (a) Scheme of electroosmotic flow resulting from an applied electric field with overlapping electrical double layers (EDLs).¹⁰ (b) Schematic of the origin of

the streaming current from a pressure-driven flow in a charged channel. (Van Der Heyden, F. H. J., Stein, D. & Dekker, C. Streaming currents in a single nanofluidic channel. *Phys. Rev. Lett.* **95**, 116104 (2005).)

The best-known examples of this trend are electrokinetic phenomena, which play a considerable role in microfluidic systems. The best-known of these, electro-osmotic flow,¹¹ is illustrated in Figure 1(a). Here the ionic charge located in the electrical double layer (EDL) to compensate for the charge of a nearby surface is driven by an external electric field. Electrophoretic motion of the ions transfers force to the solvent, which allows generating fluid flow in a channel via the application of an electric field.¹² The converse is also true: an externally imposed flow (via the application of pressure, for example) causes the ions in the EDL to be carried along. This results in a net flow of charge, the so-called streaming current,¹³ as illustrated in Figure 1(b). In situations where no mechanism exists to dispose of this charge at the ends of the channel, the charge builds up and generates a potential difference between the ends of the channel, the so-called streaming potential. This shows that interplay between fluid and charge transport is intimately linked in microscale fluidic systems via the charge that is typically present at interfaces. Further, the type of response of a system does not depend only on local properties at a given location, but also on boundary conditions established elsewhere.

This trend is accentuated upon further downscaling of critical device dimensions. In microfluidic channels, the EDLs usually extend only a small fraction of the width of a channel and the EDLs from different surfaces do not overlap. Electrical conduction in the channel then remains dominated by the bulk properties of the electrolyte that occupies the central region of the channel. However, in nanofluidics devices, the channel height can become comparable to the Debye length, which characterizes the extent to which the EDL extends away from surface. This means that the electrical current resulting from an electric field along the channel axis becomes dominated by either cations (for a negatively charged surface) or anions. For a uniformly charged channel this has no direct consequence on

the observed transport properties. If charge inhomogeneity is introduced along the length of the channel, however, a situation is created in which different species carry the current in different spatially separated regions (in electrochemical parlance, the transference numbers for cations and anions vary as a function of position along the channel). Under steady-state conditions, in which the total ionic current has a constant value along the length of the channel, this means that various species of ions can be either accumulated or depleted in some regions. This behavior is analogous to the transport of electrons and holes in semiconductors and allows so-called nanofluidic diodes (Figure 2) and nanofluidic field transistors (Figure 3) to be constructed.^{14,15}

Figure 2. Schematic of forward- (a) and reverse-biased (b) nanofluidic diodes. In the forward-biased case, the asymmetry between cations and anions results in an enrichment of ions at the interface between the cation-rich region (left) and the anion-rich region (right). This state has a high conductivity. In the reverse-biased case, ions are instead depleted from the interfacial region, leading to a low steady-state conductance. (Daiguji, H., Oka, Y. & Shirono, K. Nanofluidic diode and bipolar transistor. *Nano Lett.* **5**, 2274–80 (2005).) Corresponding, the current-potential (I - ϕ) curve (c) is very asymmetric. (Daiguji, H. Ion transport in nanofluidic channels. *Chem. Soc. Rev.* **39**, 901–911 (2010).)

Figure 3. (a) Schematic of a nanofluidic field-effect transistor when the gate is positively charged. The formation of a depletion region at the left interface hinders current flow in this case. ((Daiguji, H., Oka, Y. & Shirono, K. Nanofluidic diode and bipolar transistor. *Nano Lett.* **5**, 2274–80 (2005).) (b) The ionic current I can be controlled via the charge on the gate electrode which controls the ion distribution below it. (Daiguji, H. Ion transport in nanofluidic channels. *Chem. Soc. Rev.* **39**, 901–911 (2010).)

These effects have been extensively studied in the last two decades. Apart from fundamental interest, this has been driven by the hope that they can be exploited to create new analytical methods that rely explicitly on nanoscale dimensions.¹⁶ Another important motivation is that new behavior is usually unwelcome when attempting to scale conventional methods down to nanoscale dimensions: they should then be avoided or, when this is impossible, understood and compensated for.⁹

The electrokinetic phenomena discussed above represent complex behavior that results from a relatively simple form of coupling between fluid and charge transport. Electrochemical reactions introduce a new dimension to this coupling by allowing the local injection of charge and the interconversion of species between different charge states. Spatial inhomogeneity is also automatically introduced in nanoelectrochemistry when electrodes with different voltage biases are collocated inside a channel. It is thus reasonable to expect that a rich array of effects involving the coupling between convection, migration and charge transfer will emerge in

electrochemical nanofluidics devices. This has so far remained largely unexplored.

Outline of the thesis

This thesis explores spatiotemporal electrochemical phenomena in nanofluidic devices and how these may create new possibilities for electrochemical analysis, in particular for the analysis of complex samples, single-molecule macromolecular assays and the monitoring of enzyme kinetics. This research relies on lithographically fabricated nanofluidics devices that were designed and realized specifically for these experiments.

Chapter 2 introduces the concept for a new electrochemical chromatographic technique. This makes use of the reversible adsorption of redox active species to solid surfaces to electrically create concentration perturbations and separate the signals from different species in a mixture.

Chapter 3 introduces several refinements to the electrochemical chromatography method, including the characterization of different operation modes and geometrical designs as well as, most importantly, the implementation of gate electrodes inside the nanochannels. The latter allows tuning the separation properties of the devices using an external potential applied to the gate.

Chapter 4 explores the apparent non-local coupling between different electrodes embedded in the same nanochannel, through which modifying the potential of an electrode affects the electrochemical signals detected downstream or even upstream under a pressure-driven, steady-state flow in concentrated supporting electrolyte solution. This behavior is explained by taking into consideration the local inhomogeneity that is created in the concentration of redox-active species due to the variations in diffusion coefficients for different redox states of the molecules.

Chapter 5 introduces a conceptually new assay based on the detection of single water-soluble conducting polymers. Polymer chains bridging two electrodes spaced by less than 100 nm can be detected via the electrical

current that they allow to flow between the electrodes. Telegraph-like fluctuations in the current are interpreted as resulting from intermittent contacts being made and broken via thermally induced conformational fluctuations of single polymers.

Chapter 6 compares the results of kinetic studies for an enzyme between conventional photo-spectroscopy and amperometry. As a test system we employ the enzyme tyrosinase, which converts monophenols into the corresponding quinones. The latter can be monitored electrochemically via amperometry at a microelectrode.

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Chapter 2

Potential-controlled adsorption and separation of redox species in nanofluidic devices I

Nanoscale channels and electrodes for electrochemical measurements are characterized by extreme surface-to-volume ratios and a correspondingly high sensitivity to even weak degrees of surface interactions. Here we exploit the potential-dependent adsorption of redox species to modulate in space and time their concentration in a nanochannel under steady-state flow conditions. Concentration variations created in this manner propagate downstream at a species-dependent velocity. We show that this effect can be exploited to amperometrically distinguish between species based on their time of flight through nanochannels.

An important challenge for the electrochemical analysis of complex samples such as bodily fluids or *in vivo* measurements is the limited selectivity of these methods to species that are active in the same electrical potential window.¹ Several approaches to mitigate the impact of interferents on target chemicals have been extensively explored, including electrode modification (with polymers, self-assembled monolayers, etc) that allow partitioning one or more analyte,^{2–4} surface modifications that tune the reactivity of the electrode surface,⁵ redox cycling for selectively amplifying chemically reversible reactions,^{6,7} and, very importantly, preferential adsorption of target species to the electrode surface.^{8,9} One particularly successful example of the latter is fast-scan cyclic voltammetry (FSCV) with carbon-fiber electrodes for *in vivo* neurological measurements, which permits monitoring rapid concentration dynamics of, e.g., dopamine in the complex environment of the brain.^{10–12} More recent examples include the selective detection of adenosine against a background of two major interferents, H₂O₂ and ATP,¹³ and a quantitative study of ferrocene derivatives on graphite.¹⁴

Despite these noteworthy successes, however, the analysis of complex mixtures generally remains a challenge. Part of the difficulty in applying, for example, FSCV more widely is that dynamic adsorption is often relatively weak. Interestingly, however, its impact is greatly amplified in miniaturized micro- and nanofluidic systems due to extremely high surface-to-volume ratios.^{15–17} While adsorption is normally perceived as placing unwanted limits on the detection performance of miniaturized electrochemical devices,^{17–19} the question of whether this interaction can alternately be exploited to enhance selectivity has so far remained unexplored.

Here we introduce a new approach based on amperometric measurements at microfabricated electrodes imbedded in nanochannels under pressure-driven flow control. We show that, for three reversible outer-sphere redox couples, potential- or redox-state-dependent adsorption to an electrode or a channel wall, respectively, can be employed to deplete or enrich the solution of redox species. The resulting perturbation in concentration

can be transported downstream by an advective flow, where it can be amperometrically detected via additional electrode(s). The transit time of the perturbation to the detector is also found to be modulated by adsorption,²⁰ and we exploit this effect to distinguish between two redox species in the nanofluidic device.

Our approach is illustrated in detail in Figure 1. The fluidic device, shown in Figure 1(a), consists of silicon oxide nanochannel (height 330 nm, width 5 μm) in parallel with a polydimethylsiloxane (PDMS) microchannel (height 3 μm , width 5 μm). This parallel-flow configuration allows creating a convective flow along the nanochannel while retaining the ability to exchange solutions within several seconds using moderate (<1 bar) applied pressure.^{21,22} The average linear flow speed in the nanochannel is proportional to the flow rate at the inlet of the microchannel, which is controlled by a syringe pump. The proportionality factor is estimated as 24 $\mu\text{m/s}$ flow speed per $\mu\text{l/h}$ pump rate based on the Hagen-Poiseuille law.²³ Two nanogap transducers are located 500 μm apart along the channel, each consisting of a pair of electrodes embedded in the floor and ceiling of the nanochannel (lengths of 102 μm and 108 μm for the top and bottom electrodes, respectively). Details of the device fabrication are given in Appendix A.

In a typical experiment, aqueous solutions of 50 μM 1,1'-Ferrocenedimethanol ($\text{Fc}(\text{MeOH})_2$), (Ferrocenylmethyl)trimethyl-ammonium (FcTMA^+) or ferrocyanide ($[\text{Fe}(\text{CN})_6]^{4-}$) in 1 M KCl flowed at a constant rate through the nanochannel. The potentials of the downstream top and bottom electrodes were held at 0.5 V and 0 V, respectively, versus an external Ag/AgCl reference electrode located downstream of the device. This corresponds to large oxidizing and reducing overpotentials, respectively, for all three species investigated. Thus, while these particular species have different formal potentials, we do not exploit this fact to discriminate between them in these proof-of-concept experiments. Instead, redox cycling takes place between the two electrodes for all three species with a diffusion-limited redox cycling current given by $I_{\text{rc}}(t) = nFADc(t)/z$, where n is

Figure 1. (a) Optical images of a device before etching of the nanochannel (top) and an etched device interfaced to a PDMS microchannel (bottom; the image appears grainy as it is captured through ~ 4 mm thick PDMS). The complete device consists of two nanogap transducers (1 & 2), a nanochannel used for separation (3), a microfluidic inlet (4, flow direction shown by the blue arrow) and a microchannel (5) in parallel with the nanochannel. (b) Working principle of the device, as described in the main text. Note that the upstream top electrode extends further than the corresponding bottom electrode, insuring that the redox state of molecules traveling downstream is set by the potential of the top electrode.

the number of electrons transferred per cycle, F is the Faraday constant, $A = 300 \text{ } \mu\text{m}^2$ is the overlap area between the two electrodes, z ($= 30 \text{ nm}$) is the electrode spacing, D is the diffusion coefficient of the redox species and $c(t)$ is the time-dependent average concentration of the redox species in the detection volume between the two electrodes. This expression also holds in the presence of advective flow because the transverse diffusion time ($\sim 80 \text{ } \mu\text{s}$) is much shorter than the transit time through the detection volume ($\sim 0.14 \text{ s}$ at the fastest flow rates considered here, corresponding to a Graetz number²¹ $Gz \sim 1.2 \times 10^{-4}$). This separation of time scales also insures that practically all molecules transported through the upstream nanogap equilibrate with the potential of its top electrode since the latter extends $2 \text{ } \mu\text{m}$ further downstream, as sketched in Figure 1(b).

The degree of adsorption to an electrode depends on the latter's potential.^{17,24} Here we exploit this effect to locally modulate the concentration of redox-active analytes in the channel. As illustrated in Figure 1(b), the potential of the upstream top electrode is initialized at 0 V (time t_1 in Figure 1(b)), then switched to 0.5 V at t_2 . This causes more analyte to get adsorbed to the electrodes, creating a plug with depleted concentration in the channel. This plug is advected along the nanochannel by the flow (t_3), resulting in a temporary decrease of the redox-cycling current I_{rc} when it reaches the downstream nanogap (t_4). As the flow brings fresh analyte into the nanochannel, both upstream and downstream transducers return to a new steady state (t_5). The opposite process occurs when the upstream potential is switched back to 0 V ($t_6 - t_8$). The redox-cycling currents through both top electrodes (and thus the local values of $c(t)$) are monitored continuously throughout this process.

Figure 2 shows typical results for all three redox species investigated. Figure 2(a) and 2(b) show the signals at the upstream and downstream top electrodes, respectively, for $\text{Fc}(\text{MeOH})_2$. Upon applying a potential step to the upstream electrode at t_2 , sharp capacitive spikes are observed in the currents at both electrodes, after which redox cycling is initiated at the upstream transducer. The upstream current does not directly jump to its

Figure 2. Redox cycling currents in the (a) upstream and (b) downstream transducers for $\text{Fc}(\text{MeOH})_2$ upon the application of a potential square wave to one of the upstream electrodes. The labels t_1 to t_8 refer to the corresponding sketches in Figure 1(b). (c), (d) Downstream responses for FcTMA^+ and $[\text{Fe}(\text{CN})_6]^{4-}$, respectively. (e) Upstream response observed simultaneously with (f) indicating that desorption instead of adsorption takes place upon stepping the potential at t_2 . The currents in panels (a)-(e) were measured at a pump rate of $30 \mu\text{l/h}$. (f)-(h) inverse of the time at which the peak currents is observed (proportional to the speed of propagation) versus the pump rate.

steady-state level, however, instead increasing gradually while the adsorption-depleted plug below this electrode is replaced by fresh solution.¹⁷ The 0.6 nA magnitude of the initial dip in the current corresponds to only 1.0 attomole of molecules that have adsorbed to the electrode upon stepping the voltage, illustrating the absolute sensitivity of the nanogap transducer. Several seconds later, a corresponding decrease in redox-cycling current is observed at the downstream transducer as the leading edge of the depleted region reaches its position. Surprisingly, however, the magnitude of this decrease, 1.06 nA, corresponds to a depletion of 1.8 attomole, which is larger than observed at the upstream electrode. This suggests that additional molecules, now in their positively charged oxidized form, were lost in transit via preferential adsorption to the SiO₂ channel walls. This, together with broadening of the leading edge of the plug due to diffusion, causes the downstream signal to be more spread out in time than the upstream signal.²⁰ With continued flow the surfaces eventually come to equilibrium with the solution again and the detected redox-cycling current returns to its steady state value. Similarly, at time t_6 a number of previously adsorbed molecules are released from the surface into the nanogap when the electrode potentials is stepped downward; the mirror process occurs and a peak appears in the downstream signal several seconds later.

Qualitatively similar behavior is observed with FcTMA⁺ (Figure 2(c)). For [Fe(CN)₆]⁴⁻, on the other hand, the opposite behavior is observed (Figure 2(d)): here stepping the top electrode to 0.5 V results in an increase of concentration at the top transducer which then slowly decreases back to a steady state (Figure 2(e)), followed by a corresponding subsequent increase in current at the downstream detector. This can be understood as reflecting decreased adsorption of ferricyanide at 0.5 V compared to ferrocyanide at 0 V.

Figures 2(f)-(h) shows the inverse of the peak current time (approximately proportional to the speed of propagation) versus the pump flow rate (proportional to the linear flow speed as discussed above). The two measured quantities are linearly related, further supporting the interpretation of

a front of different concentration being carried downstream at a constant velocity set by the flow rate.

Interestingly, Figure 2 shows that the transit times are different for each of the three species. They are also systematically influenced by the potential of the upstream top electrode, which sets the redox state of the molecules being transported downstream. We interpret this observation as further evidence that the redox molecules also undergo some degree of reversible adsorption to the SiO₂ channel walls, which slows down their transport to the detection region.^{20,23} The observed transit times indicate that, of the three species investigated, [Fe(CN)₆]^{3-/4-} interacts least and FcTMA^{+ /2+} most with the silicon oxide walls. We further conclude that the oxidized forms [Fc(MeOH)₂]⁺ and FcTMA²⁺ have stronger adsorption to the channel walls than the corresponding reduced forms, the opposite being true for [Fe(CN)₆]^{3-/4-}. While adsorption free energies are notoriously difficult to rationalize based solely on electrostatic arguments, we note that most of these trends are qualitatively consistent with the negative charge of the SiO₂ channel walls, the preferential adsorption of [Fe(CN)₆]⁴⁻ compared to [Fe(CN)₆]³⁻ being a notable exception.²⁵

Species-dependent transit times suggest the feasibility of separating species in nanochannels based on differences in adsorption. As a proof of concept, Figure 3(a) shows the response of the device to a 1:1 mixture of Fc(MeOH)₂ and FcTMA⁺ at different flow rates. As expected from Figure 2, the amperometric response in this case exhibits two peaks, each presumably corresponding to a single species. Measurements with different ratios of the two analytes (Appendix A) confirm the assignment of the first and second peaks to Fc(MeOH)₂ and FcTMA⁺, respectively, consistent with the data of Figure 2(f),(g). Combinations of [Fe(CN)₆]⁴⁻ with the other two species did not yield a clear peak response, on the other hand, presumably because of the opposite adsorption properties of this species leading to partial cancellation of their responses.

In order to further quantify the separation process, Figure 3(b) plots the retention factors k' extracted from Figures 2 and 3(a) as a function of pump

Figure 3. (a) Normalized redox-cycling currents for a 1:1 mixture of $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ at different pump rates. The traces have been offset vertically for clarity. (b) Corresponding retention factors k' of the two species measured in a mixture (solid symbols) as well as for the individual species at the same total concentration (open symbols). At the lowest flow rates, the FcTMA^+ peaks were not well resolved.

rate. This quantity is defined, in analogy with liquid chromatography,²⁶ as $k' = (t_R - t_M)/t_M$, where t_R is the time at which the peak current is observed following switching of the upstream electrode (the retention time) and t_M is the mobile phase transportation time calculated from the Hagen-Poiseuille law. The retention factors observed for both the individual species and the mixture exhibit a slight decrease with increasing flow rate, suggesting that shear forces from the solvent may have a slight inhibitory effect on adsorption. Interestingly, the retention factors for the mixture also differ significantly from those for the single-species solutions, being smaller for $\text{Fc}(\text{MeOH})_2$ and larger for FcTMA^+ in the mixture. This behavior is characteristic of competition between two species for the occupation of discrete adsorption sites in which one species displaces another from the stronger binding sites, as described for example by the multicomponent Freundlich isotherm^{27,28} (see Appendix A for further details).

These experiments demonstrate that potential-dependent adsorption of redox species can transiently influence their concentration – and hence the associated electrochemical signals – in nanoscale channels. While the specific devices employed here consisted of two thin-layer cells embedded in a channel, similar behavior can be expected in other nanoscale geometries and in porous materials with comparably high surface-to-volume ratios. While this complicates the interpretation of some electrochemical nanofluidic measurements, it also creates new opportunities. In particular, we have shown that potential-dependent adsorption can be employed to create localized concentration perturbations, enabling a form of electrochemical chromatography based on continuous sample flow and “on-demand” sample plug generation. The device employed here, while well suited for demonstrating the effect, did not maximize discriminating power, which we propose can be significantly improved through geometry optimization (e.g., shorter control electrodes and longer separation channel) and tuning of the adsorption properties of the channel (which here plays the role of separation column). The latter might be achieved in a controlled and tunable manner by placing an additional ‘gate’ electrode along the channel whose potential could be tuned to optimize separation.^{29,30} Finally, we note

that the device described can be realized in a variety of geometries including, e.g., a needle-shaped microprobe suitable for *in vivo* studies.

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Chapter 3

Potential-controlled adsorption and separation of redox species in nanofluidic devices II

In this chapter, we investigate several possible refinements to the electrochemical chromatography approach introduced in the previous chapter. In particular, we investigate the influence of a variant approach for generating concentration perturbations, the use of a separately biased ‘gate’ electrode and the role of the device dimensions.

In Chapter 2 we demonstrated that it is possible to locally modulate the concentration of redox species in a nanochannel using embedded electrodes and to detect the resulting perturbation as it is advected at a species-dependent velocity by an externally imposed flow. In principle, this provides a new route for distinguishing between species whose contributions to amperometric or voltammetric signals cannot be separated by conventional methods. A significant limitation of this approach, however, is that the extent to which different species are slowed down by adsorption in the channel cannot at this time be easily predicted or controlled. In the worst case, different species exhibit approximately the same degree of interaction with the channel walls and they cannot be separated at all by this method. In this chapter, we explore additional refinements to the electrochemical chromatography approach based on adjustments to the measurement protocols and the detailed structure of the devices.

Device geometry

Figure 1 shows optical images of the devices used in the experiments presented in this chapter. The devices share the same basic nanochannel architecture as in Chapter 2 but differ from each other in their dimensions and in the number of electrodes. This information is summarized in Table 1.

So-called type 1 devices have two nanogap transducers located 300 μm apart along the channel, each consisting of a pair of electrodes embedded in the floor and ceiling of the nanochannel (lengths of 32 μm and 34 μm for the top and bottom electrodes, respectively). The top electrode extends 2 μm further toward the center of the device than does the bottom electrode, while the bottom electrode extends below the inlet and outlet access holes located in the roof of the channel. The overlap length available for redox-cycling reactions is 30 μm . The fluidic channels have a height of 100 nm and a width of 5 μm along their entire length, both in regions with electrodes and without electrodes.

Figure 1. Optical images of the four types of devices employed here. Type 1 consists of two nanogap transducers separated by a long silicon oxide nanochannel. Type 2 is similar to type 1, but has with longer dimensions. Type 3 is similar to type 2 except that two gate electrodes are embedded in the floor and roof of the nanochannel. Type 4 devices are conceptually equivalent to type 3 but the lengths of the various sub-components are shorter. The type 4 device is shown with a PDMS channel overlayed on top of the nanofluidics; this is also possible with types 1 – 3.

Type 2 devices have a layout similar to type 1 devices and are identical to the devices employed in Chapter 2. Compared to type 1 devices, they exhibit longer dimensions. Details beyond the dimensions in Table 1 can also be found in chapter 2.

Type 3 devices have the same dimensions as those of type 2 but also incorporate two gate electrodes of length $496\text{ }\mu\text{m}$ in the floor and ceiling of the nanochannel region. The distance between the gate and both the upstream generator electrodes and the downstream detector electrodes is $2\text{ }\mu\text{m}$.

Type 4 devices bears the same components as devices of type 3, but have different dimensions. The nanochannel height is reduced to 100 nm ,

the redox-cycling-active length is 10 μm and the gate electrode length is 96 μm .

In each case the device is in a parallel configuration with a polydimethylsiloxane (PDMS) microchannel (height 3 μm , width 5 μm), as shown for a device of type 4 in Figure 1. This allows creating a convective flow along the nanochannel, as discussed in detail earlier in Chapter 2.

Table 1. Summary of device dimensions. The detection length is the length of the overlap area between the two opposed nanogap electrodes in regions where redox-cycling takes place. When no gate electrodes are present, the separation is simply the length between the two top electrodes in the detection regions. When a gate is present, this distance is broken into the length of the gate electrodes (middle number) and the gap between the gate and the transducers on either side.

Device type	Detection length (μm)	Separation(μm)	Gates
1	30	300	No
2	100	500	No
3	100	2/496/2	Yes
4	10	2/96/2	Yes

Controlling the pulse shape

The electrochemical chromatography principle of operation is based on the creation of a local perturbation of the analyte concentration at an upstream electrode or transducer which then propagates due to convection and is detected downstream. The upstream transducer also controls the redox state of the molecules propagating downstream. In particular, because the top electrode extends further downstream in our designs, practically all molecules exiting the upstream transducer have their redox state determined by the potential applied to the upstream top electrode (Graetz number¹ $Gz \sim 0.055$ for the fastest pump rate and highest channel used here). This means that, for example, stepping the upstream top electrode from from a reducing to an oxidizing potential not only creates a temporary plug of different concentration in the vicinity of this electrode, but also causes the redox state of the molecules traveling downstream in the nanochannel

to switch from the reduced form to the oxidized form. This provides an additional degree of coupling between the electrode potential and the spatiotemporal response of the concentration of molecules in the device. In contrast, switching the potential of the bottom electrode also leads to a concentration perturbation, but all molecules traveling downstream remain in the redox state set by the top electrode. The impact that this subtle difference may have on the propagation of the perturbation is difficult to assess theoretically without detailed models of the adsorption process, which are currently lacking.

To explore this question experimentally, we performed a series of experiments in which both the top and bottom electrodes were switched under otherwise identical conditions. We refer to these two procedures as ‘T mode’ and ‘B mode’, respectively.

In a typical experiment, the top and bottom electrodes of the downstream transducer remained biased at large oxidizing and reducing overpotentials, respectively (0 V and 0.5 V in practice, respectively). This yielded a diffusion-limited redox-cycling current for all species at the downstream transducer with a magnitude $I_{rc}(t) = nFADc(t)/z$, where n is the number of electrons transferred per cycle, F is the Faraday constant, $A = 300 \mu\text{m}^2$ is the overlap area between the two electrodes, z is the electrode spacing, D is the diffusion coefficient of the redox species and $c(t)$ is the time-dependent average concentration of the redox species between the two electrodes. One of the upstream electrodes was also held at a fixed potential (bottom electrode at 0 V for T mode, top electrode at 0.5 V for B mode), while the other upstream electrode (top for T mode, bottom for B mode) was periodically switched between 0 V and 0.5 V.

Figure 2 shows representative amperometric traces for a device of type 1 for $\text{Fc}(\text{MeOH})_2$ as analyte, the upper and lower panels corresponding to T mode and B mode, respectively. Both curves are qualitatively similar, a step of the modulated electrode from a reducing to an oxidizing overpotential giving rise to a region of lower analyte concentration in the device. The magnitude of the response is smaller for B mode, as might be

intuitively expected since the only effect of the potential modulation in this case is the creation of the depleted region. In both cases, an offset in the steady-state current appears between the two modulation potentials; this effect, which is more pronounced here than in Chapter 2 as a result of the different electrode dimensions, will be discussed in detail in Chapter 4.

Figure 2. Amperometric response for a type 3 device for $\text{Fc}(\text{MeOH})_2$ in T mode (upstream top electrode potential switched) and B mode (top and bottom panel, respectively) for a pump rate of $30\mu\text{l/h}$. In each plot, the dark red line represents the potential of the electrode being modulated.

In order to compare the T- and B-mode responses quantitatively, we again introduce the retention time, t_R , which is defined as the time interval between the moment the perturbation is generated at the upstream transducer and the time at which the downstream detection signal exhibits a maximum or a minimum. The inverse retention time essentially corresponds to the propagation velocity of the analyte along the channel. This quantity is shown for both modulation potentials in Figure 3 for three distinct analytes, namely, (Ferrocenylmethyl)trimethylammonium bromide (FcTMA^+ , $50\mu\text{M}$), 1,1'-Ferrocenedimethanol ($\text{Fc}(\text{MeOH})_2$, $50\mu\text{M}$) and potassium ferrocyanide ($[\text{Fe}(\text{CN})_6]^{4-}$, $50\mu\text{M}$). The data for both T and B

mode exhibit longer retention times for oxidized molecules for $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ (presumably caused by stronger adsorption), the opposite being true for $[\text{Fe}(\text{CN})_6]^{4-}$. These trends are consistent with those reported in Chapter 2 using type 2 devices.

Figure 3. Inverse retention time for the desorption and adsorption peaks versus flow rate for (a), (b) FcTMA^+ , (c), (d) $\text{Fc}(\text{MeOH})_2$ and (e), (f) $[\text{Fe}(\text{CN})_6]^{4-}$ in both T mode (left panels) and B mode (right panels).

Closer inspection, however, reveals a more subtle difference between T and B mode. While $1/t_R$ always depends approximately linearly on the pump rate, in T mode the slope of this relation depends on the potential of the top electrode for both $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ . This is expected if molecules in the oxidized form experience a more pronounced retardation due to stronger adsorption. In contrast, modulating the bottom electrode

does not cause a change in overall slope and only leads to a relatively small offset in $1/t_R$ (the flow-rate dependence of which is too small to ascertain within the scatter in the data). This is reasonable since in B mode only adsorption in the (relatively small) region of the modulated electrode should be affected by the change in potential. For $[\text{Fe}(\text{CN})_6]^{4-}$, which was earlier found to exhibit only a weak adsorption to the SiO_2 channel, the T- and B-mode curves are essentially identical, further strengthening the observation that only the region with electrodes present significantly influences their downstream travel.

Based on these observations, we conclude that both modes exhibit a qualitatively similar ability to modulate t_R and therefore that they can be expected to lead to a comparable ability to separate between species. The more extensive interaction with the channel walls in T mode however leads to sharper peaks with a larger amplitude. For this reason, it is reasonable to concentrate on T mode in further experiments.

Role of device geometry

In order to further estimate the effect of different geometries on performance, we performed numerical simulations for the expected signals under a range of different geometries. Assuming constant diffusion coefficients, the non-local response of a nanogap transducer to a step-like local excitation in a step-like excitation domain is equivalent to the cross-correlation function for the single-molecule response between these two different regions, as described earlier.² Even though this description only takes steady-state mass transport process into account, and does not account for complex nonlinear adsorption behavior, the results can still shed light on basic design rules at a qualitative level. In this spirit, we performed simulations assuming a 100 nm channel height and 50 $\mu\text{l/h}$ pump rate throughout.

As a first trial, we varied the separation distance between upstream and downstream transducers for gate-less devices, as shown in Figure 4(a). Unsurprisingly, for a series of nanochannel with a 30 μm redox active length for both upstream and downstream transducers, the resolution increases as the separation time increases. This effect is expected as longer convection times dominate over diffusion as longer separation times are employed. A second approach is to optimize the length of generator and detector. As shown in Figure 4(b), for a nanochannel bearing a 300 μm separation distance, the separation power increases with a shortening of the generator and detector electrode lengths. These simulations suggest that it is possible to significantly increase the ability of the devices to discriminate between different analytes by tuning the relative lengths of the electrodes and of the separation region.

Figure 4. Simulated cross-correlation function to simulate the response of a downstream transducer to a rectangular concentration pulse being created upstream. The pump rate is 50 $\mu\text{l/h}$ and channel height is 100 nm. (a) A 30 μm redox active length is assumed for both upstream and downstream transducers. The cross-correlation function is computed as a function of the distance between these two transducers along a uniform channel. (b) The separation is fixed at 300 μm , while the redox-active length is treated as a variable.

Figure 5. Comparison of retention factor k' for adsorption peaks between old (type 2, shown by hollow symbols) and new (type 3, shown by solid symbols) devices that were operated in T mode. Two species were tested, and they are $\text{Fc}(\text{MeOH})_2$ (squares) and FcTMA^+ (circles).

Shrinking the height of nanochannels is also expected to increase the relative adsorption of analytes to the electrode surfaces since higher surface-to-volume ratios increase the average interaction of analytes to the channel walls, leading to longer retardation times. To test this, chromatographic experiments were conducted in a device of type 1 with a 100 nm channel height. The results are compared with those obtained in a device of type 2 with a 330 nm channel height in Figure 5. The retention factor of an analyte is again defined as $k' = (t_R - t_M)/t_M$, where t_M is the time taken for mobile phase to pass through the device. This dimensionless factor describes how much the targeted analyte lags behind the mobile solvent. For species of $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ , who are in their oxidized state, k' is larger in the new shallower device than that in the old device. More importantly, the absolute difference in k' is enlarged as expected in the

Figure 6. Normalized currents from downstream top electrode in T operation mode for different flow rates. The sample solution is a 50 μM mixture of $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ with a 1:1 ratio.

shallower device, which can improve the separation performance. To further show the more powerful separation ability for devices of type 1 over those of type 2, an equimolar mixture sample of $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ with 50 μM total concentration was tested in devices of type 1. It is qualitatively clear from the normalized redox-cycling current from the downstream top electrode (Figure 6) that the relative resolution increases as the pump rate increases.

While not a complete study, these results indicate that device optimization have significant potential to improve the discrimination ability of chromatography devices.

Introduction of a gate electrode

In the device concept introduced in Chapter 2, the mechanism used for generating a concentration perturbation was based on potential-dependent adsorption.⁴ Most intuitively, the degree of adsorption of a redox species to the electrodes depends on the redox state of the molecules at the surface, which can be tuned between essentially full oxidation and full reduction by varying the potential of the electrode. More subtly, even at high overpotentials – where the molecules are fully reduced or oxidized – the degree of adsorption continues to exhibit a significant dependence on the electrode potential. It is therefore natural to expect that the mass transport of redox-active species being advected through the nanochannel can also be tuned in this manner.⁵ A strategy to improve the tunability of electrochemical chromatography devices is therefore to embed additional electrodes between the upstream (generator) and downstream (detector) transducers to modulate the transport process. In analogy with field-effect transistors, in which electronic transport is modulated by an electrode positioned parallel to the conduction channel, we refer to this electrode as a gate.

Upon switching the upstream top electrode to an oxidizing potential, approximately half⁶ the redox molecules in the volume below this electrode are in their oxidized state. These have a stronger affinity for the electrode than the reduced form to a reducing electrode, temporarily leading to a decrease in the local concentration of freely moving molecules. This concentration perturbation is then transported by hydrodynamic flow to the sensing transducer located at the other end of nanochannel, where it gives rise to a transient dip in the redox-cycling current. As new molecules enter into the channel from upstream, the concentration again reaches a new steady state everywhere along the nanochannel.

Qualitatively, it can be observed in Figure 7 that increasing the gate electrode potential 0 V to 0.3 V causes the time at which a maximum is observed at the downstream sensor to shift to later values. Furthermore, the perturbation increases in duration and decreases in amplitude with increasing gate potential. These features are consistent with the degree of adsorption in the channel region increasing with increasing gate potential, which causes more molecules to exist in the oxidized form in the channel. They therefore spend more time immobilized on the surface of the electrodes and are convected more slowly on average, corresponding to a longer retention time.¹ The simultaneously occurring diffusive transport then has more time to smear out the perturbation in space, leading to a longer transient response at the detector.

Unfortunately, when the gate potentials were set to values greater than 0.3 V, there was no clearly identifiable downstream response for FcTMA^+ and FcCOOH due to excessive peak broadening (combined with a shift in the baseline current, as discussed further in Chapter 4) and these results are omitted in the figure. For $\text{Fc}(\text{MeOH})_2$ a transient could still be observed thanks to the different electrode lengths and channel height; the trend of increased delays continues for gate potentials of 0.4 V and 0.5 V.

The middle row of panels in Figure 7 shows the response at the downstream detector when the potential of the upstream top electrode was switched from 0.5 V back to 0 V. The observed response is approximately the mirror image compared to the top row, the downstream current exhibiting a transient dip rather than a peak. The trends with gate potential are similar in the two cases.

To discuss these observations more quantitatively we once again investigate the retention time, t_R . The bottom row in Figure 7 compares the reciprocal retention times for the three species. In all cases, the presence of the gate leads to a clear retardation of mass transport along the channel compared to the cases without gate electrodes (colored dots versus dashed lines). Furthermore, the retention time exhibits a different dependence on

Figure 7. Effect of gate electrode on reciprocal retention time ($1/t_R$). The three columns correspond to measurements on (a) FcTMA⁺, (b) FcCOOH, and (c) Fc(MeOH)₂. The top row shows the redox-cycling current measured at the downstream top electrode after the upstream top electrode potential was switched from 0 V to 0.5 V (pump rate 20 μ l/h). The traces with different colors correspond to different potentials being applied to the gate electrodes. The middle row shows the response of the transducer after the potential of the upstream top electrode was stepped back to 0 V under the same other conditions. The bottom row shows the retention times for adsorption and desorption in device of type 3 (colored dots) as well as in gate-less devices of type 2 (dashed lines) for columns (a) and (b). The data in column (c) are from devices of type 4 with different length and height scales and cannot be directly compared.

gate potential for the three species, indicating that the relative adsorption between species can be tuned through the use of the gate.

The data of Figure 7 indicate that the retention time exhibits the same trend for oxidized and reduced molecules, apart from a relatively small offset in the I/t_R versus gate voltage relation. This is consistent with the comparison between T and B modes in the previous section. Although the measurements in Figure 7 were performed in T mode, the redox state of advecting molecules in the gate region is set by the potentials of the gate electrodes themselves and is thus independent of the modulated electrode potential. The residual offset must therefore be caused by the difference in transit time in the modulated electrode region, consistent with earlier trends suggesting that oxidized forms of ferrocene tend to adsorb more strongly to electrodes.

In summary, the experiments presented here make it clear that the separation of redox species based on electrochemical signals, while a classic topic, is still liable to improvement through new experimental modalities. The combination of new device geometries, materials, potential control and measurement protocols may create new avenues beyond conventional measurement platforms. Spatiotemporal control of analyte concentrations in particular offers opportunities that still need to be explored.

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Chapter 4

Electrochemically generated concentration inhomogeneity in nanofluidic devices

We report the (apparently nonlocal) electrochemical generation of concentration variations along the length of a nanochannel under steady-state fluid flow conditions. Faradaic processes break the expected symmetric concentration distribution about the geometric center of such devices due to variations of the mass transport properties of molecules in different redox states. Unlike current rectification in so-called ionic diodes, this can occur equally well in concentrated supporting electrolytes. Most counterintuitively, this interplay can cause the concentration or redox species upstream in a nanofluidic channel to be influenced by electrochemical reactions occurring downstream under steady-state fluid convection.

Electrochemical reactions provide one of the most practical mechanisms for transducing information about the chemical contents of a fluid sample into an electrical signal. Applications of electrochemistry as a detection modality span a broad spectrum including, in particular, both the health and environmental monitoring fields. Like most analytical methods, there is currently a strong drive to miniaturize electrochemical sensors and integrate them into microfluidic circuitry, as this can yield higher sensitivity, smaller sample volumes, reduced power consumption, and large-scale parallelization of measurements. Interestingly, however, doing so raises a number of important issues about mass transport in miniaturized fluidic systems. Recent decades have unveiled a number of complex phenomena in ionic transport including electroosmotic flows^{1,2}, streaming potential^{3,4} and currents⁵, isotachophoresis⁶ and other depletion-enrichment effects which give rise to, for example, current rectification in so-called ionic diodes⁷⁻¹². In all these phenomena, however, the charge carriers – mobile cations and anions – have a constant charge and therefore behave as indestructible particles that obey mass conservation.

Electrochemical processes, which transfer electrons between molecules in solution and/or with an appropriately biased electrodes, introduce an additional dimension to this already complex interplay between charge and fluid transport. They allow ions to change their charge state and thus their identity: in a formal sense, electron transfer annihilates one charged species and replaces it with a second, closely related species which exhibits a different charge, mobility and diffusion coefficient. This process has no analogue in conventional electrokinetics and brings with it both challenges in understanding even nominally simple experiments and new potential opportunities for enhanced detection.

Here we address the influence of electrochemical reactions on mass transport in a prototypical nanofluidic circuit. Our system consists of a nanoscale channel in which several electrodes are embedded and through which a solution containing electrochemically active molecules is transported, as shown in Figure 1. The concentration of redox molecules at a

given position in the nanochannel can be modulated by electrochemical reactions taking place not only upstream but also downstream of the detection region. We demonstrate that this counterintuitive behavior can be attributed to concentration gradients of the different charge states of the redox molecules. We anticipate that the same behavior will impact a wide range of fluidic systems in which electrochemical reactions in confined spaces are employed.

Figure 1. (a) Schematic illustration of the cross-section of the nanofluidic device consisting of three pairs of electrodes (gray rectangles) separated by insulators (magenta). The labels 1 correspond to the regions with embedded electrodes that act as upstream, gate and downstream transducers. Label 2 shows the axial separation between the electrodes. The y-axis has been exaggerated for clarity. (b) Optical image (top view) of a device.

As summarized in Figure 1(a), our system consists in a 100 nm high nanochannel with outlets at both ends. A 100 μm long nanogap transducer is positioned centrally along the nanochannel with additional 10 μm long transducers located at both its ends. Each transducer is composed of two electrodes serving as floor and roof of the channel in those regions. We refer to the large central pair of electrodes as the gate electrodes, or gate, and the two smaller transducers as the detectors. The bottom electrodes of

the detectors extend below the outlet holes of the device, while the top electrodes of the detectors are located 1 μm away from these orifices. The top electrodes of the detectors extend 2 μm further toward the gate compared to the bottom detector electrodes. The gate transducer is symmetric in length and separated from the two detector top electrodes by 2 μm . A photograph of a complete device is shown in Figure 1(b).

The measurements employed an aqueous solution of 50 μM (Ferrocenylmethyl)trimethylammonium (FcTMA^+) with 0.1 M potassium chloride (KCl) as supporting electrolyte. This solution was transported through the nanofluidic channel at constant flow rate using an external syringe pump (Pump 11 Pico Plus Elite, Harvard Apparatus; 1 $\mu\text{l/h}$ can generate a cross-section-averaged speed of 12.6 $\mu\text{m/s}$ in the nanochannel) by coupling the device with microfluidic channels in a parallel-flow configuration.¹³ The bottom and top electrodes of both transducers were held at reducing and oxidizing potentials for FcTMA^+ (0.0 V and 0.5 V, respectively) while the potential of the gate electrodes was varied, as summarized in Figure 2(a). Because FcTMA^+ is a simple, reversible outer-sphere species, this allowed a diffusion-limited redox current to be carried between the top and bottom electrodes of each of the detectors. This current is proportional to the total local concentration of redox species in the detection volume located between the two top and bottom electrodes; its magnitude is given by

$$I_{rc} = \frac{nFADc(t)}{z} , \quad (1)$$

where $n = 1$ is the number of electrons transferred in each cycle, F is Faraday constant, A is the overlapped region of top and bottom electrode, D is the diffusion coefficient, z is channel height and $c(t)$ is the average concentration of redox species in the redox cycling region. Thus, continuously monitoring the redox cycling current in the upstream and downstream detectors yields the local concentration at those locations.

Figure 2(a) shows the redox cycling currents at the upstream (red) and downstream (blue) transducers when switching the potential of the gate

Figure 2. (a) The redox cycling currents through short top electrodes in upstream (red) and downstream (blue), while stepping the gate electrode potential between 0 V and 0.5 V. The pump rate is 30 $\mu\text{l/h}$. (b) The ratio ($I_{0.5}/I_0$) of steady state current when the gate is biased at 0 V and 0.5 V for each transducer as a function of pump rate. The upstream ratio is plotted in red and the downstream ratio in blue.

electrodes between 0 V (under which conditions the redox molecules between the gate electrodes are all in the reduced form) and 0.5 V (all molecules oxidized in the gate region). A transient lasting tens of seconds is observed before the redox cycling current (and therefore the local concentration) settles back to a steady-state value. This transient reflects changes in adsorption levels in the device, as discussed in Chapters 2 and 3, and is not addressed further here. Surprisingly, however, the new steady-state current to which the electrodes converges varies depending on the potential of the gate electrode. In particular, the downstream current decreases and the upstream current increases upon switching the gate potential from 0 V to 0.5 V.

To further characterize this response, Figure 2(b) shows the ratios of the steady-state redox-cycling currents measured for gate voltages 0.5 V and 0 V, $I_{0.5}/I_0$. The upstream (red) and downstream (blue) transducers exhibit opposite responses at all flow rates, with the downstream and upstream detectors exhibiting a decrease and an increase,^{*} respectively. The magnitude of this response does not vary monotonically with flow rate, however, first increasing and then decreasing again with increasing flow rate. Additional, qualitatively similar experiments on the flow-rate dependence of the redox-cycling current in a gate-less geometry are presented in Appendix C.

To elucidate the origin of this apparent non-local coupling between electrodes, we first consider the assumptions underlying Eq. (1). This expression assumes that the redox-cycling current is set by diffusion alone. Migration in the transverse direction (i.e., perpendicular to the electrode surfaces) can be ruled out since in such highly concentrated supporting electrolyte the electric double layers (EDLs) limit electric fields to the immediate surface of the electrode (Debye length ~ 1 nm). The high salt concentration also suppresses electric fields along the channel axis

^{*} An exception is the ratio $I_{0.5}/I_0$ at the lowest pump rate measured (2 $\mu\text{l/h}$, which corresponds to a 25 $\mu\text{m/s}$ average convection speed), where the current decreases for both electrodes but by different amounts.

arising from ohmic drops¹⁴ and streaming potentials¹⁵. Similarly, electron-transfer kinetics at the electrodes can be assumed to be unchanged since all the electrodes are controlled independently and only the potential of the gate electrodes has been changed. We therefore conclude that the current variation can only result from variations in the local concentration at the two detectors. Specifically, the data indicate that not only does the concentration of redox species downstream of the gate vary with the gate potential, but also the upstream concentration. This is a counterintuitive observation when considering that the rate at which new solution is introduced into the channel is independent of the potential at the gate electrode.

What could break the symmetry between upstream and downstream in a device arrangement that is for all practical purposes symmetric about its center? In our effectively one-dimensional system,¹⁶ the local concentration of redox species is expected to obey the steady-state drift-diffusion equation,

$$\frac{\partial j_{R,O}}{\partial x} = D_{R,O} \frac{\partial^2 c_{R,O}(x)}{\partial x^2} - v \frac{\partial c_{R,O}(x)}{\partial x} = 0 \quad (2)$$

Here v is the average advection velocity of the fluid, $j_{R,O}$ is the total flux across the channel, $c_{R,O}$ is the local concentration and $D_{R,O}$ is the diffusion coefficient. We explicitly distinguish between the reduced (R) and oxidized (O) forms of the molecules: while molecules cannot be created or annihilated, they can interconvert between these two forms when they cross between different domains of the device.

For the simple symmetric case $D_R = D_O$, Eq. (2) has the simple solution $c_R + c_O = c_B$ and $j_R + j_O = v c_B$, where c_B is the bulk concentration of redox species outside the device, as expected for a uniform concentration fluid being transported through a uniform channel at constant velocity. In practice, however, the diffusion coefficient of redox molecules depends on their redox state (for FcTMA^+ in bulk aqueous solution, for example, $D_R/D_O \approx 1.1$ ¹⁷), which is known to influence the concentration distribution in steady-state redox cycling reactions.^{18–20} In addition, surface diffusion of adsorbed species along the surface may introduce additional differences

between the reduced and oxidized forms. In the presence of regions with different ratios c_R/c_O , as imposed by the presence of electrodes at different biases, Eq. (2) has the general solution

$$c_{R,O}(x) = a + be^{vx/D_{R,O}}, \quad (3)$$

which breaks the symmetry between the upstream and downstream directions and therefore introduces a possible mechanism for the observed interactions between the different regions of the device. Furthermore, Eq. (3) indicates that perturbations in local concentration caused by, for example, the boundary of an electrode can extend a distance of order $v/D_{R,O}$ from this boundary. For a 2 μm distance between the gate and the upstream and downstream transducers and a typical diffusion coefficient of $6 \times 10^{-10} \text{ m}^2/\text{s}$, the crossover between having strong and weak coupling between the gate and the transducers is then expected to occur for flow rates of 25 $\mu\text{l/h}$. For a typical slowing down of diffusion due to reversible adsorption by a factor 2–4²¹ this value goes down to 6–12 $\mu\text{l/h}$. Suggestively, these values are consistent with the pump rate at which a crossover is observed in Figure 2(b). Equivalently, these flow rates correspond to a Péclet number of order unity; higher flow rates correspond to mass transport being dominated by convection.

To further investigate this hypothesis, we performed simulations using COMSOL Multiphysics to solve Eq. (2) numerically in a two-dimensional geometry representing the device and the electrodes. These simulations are fully consistent with analytical results such as Eq. (3) once appropriate boundary conditions are included, but also account for fringing effects at the edges of the electrodes which are neglected in a purely one-dimensional description. We therefore concentrate on these two-dimensional results here.

Figure 3(a) shows the simulated local concentration of reduced (blue) and oxidized (red) species versus longitudinal position along the central axis of the channel for the simplest case where $D_R = D_O$. The total concentration of redox molecules ($c_R + c_O$) is constant over the length of the

Figure 3. Results of two-dimensional numerical simulations of the Nernst-Planck equation using COMSOL Multiphysics for the symmetric case $D_R = D_O$. (a) The concentration distribution of molecules of reduced (blue) and oxidized (red) states along the axial positions for convection velocity $90 \mu\text{m/s}$ when the gate electrodes potential is 0 V (upper panel) and 0.5 V (lower panel). (b) The ratio of the detected current for oxidizing and reducing gates, which correspond to the experimental situation of Figure 2(b). This ratio is simply unity for both cases.

Figure 4. Results of two-dimensional numerical simulations of the Nernst-Planck equation using COMSOL Multiphysics for the asymmetric case. (a) The concentration distribution of molecules of reduced (blue) and oxidized (red) states along the axial positions for convection velocity $90 \mu\text{m/s}$ when the gate electrodes potential is 0 V (upper panel) and 0.5 V (lower panel). (b) The ratio of the detected current for oxidizing and reducing gates, which correspond to the experiment results of Figure 2(b).

device and equal to c_B , the concentration in bulk solution. There are however sharp gradients in both c_R and c_O as a result of the presence of electrodes: depending on the potential on these electrodes, redox molecules inside each nanogap are either fully reduced (all electrodes in a given region at a reduced potential), fully oxidized (oxidizing electrode(s)), or partly oxidized and partly reduced (redox cycling conditions). In the latter case, half the molecules are reduced. Furthermore, while changing the potential of the gate affects the local reduced/oxidized ratio in the gate region, it has no impact on the overall distribution of redox molecules elsewhere in the device. Given this perfect symmetry between the reduced and oxidized form of the molecules, and their consequent interchangeability, it was indeed to be expected that the concentration at both transducers is independent of the potential of the gate or of the flow rate; this is shown explicitly in Figure 3(b).

What are the consequences of introducing a different diffusion coefficient for the R and the O form or the analyte molecules ($D_O \neq D_R$)? First, and most simply, within each nanogap undergoing redox cycling, the ratio of c_R to c_O adjusts so as to keep the compensating oxidation and reduction rates equal in magnitude. Consequently, the concentration of R and O are unequal. This effect is also present in the absence of convective flow and is well understood.¹⁸ Second, and more directly relevant here, equal gradients of R and O give rise to unequal fluxes when the diffusion coefficients are unequal; satisfying the constant-flux condition in the steady state (Eq. (2)), therefore necessarily requires breaking the symmetry between R and O. Taken together, these effects lead to a much more complex, spatially inhomogeneous structure for $c_R(x)$ and $c_O(x)$ in the steady state. The advective flow, which introduces a finite length for concentration adjustments between different domains as per Eq. (3), further complicates this interdependency between the concentrations in various regions of the device.

Figure 4(a) shows simulated concentration profiles along the longitudinal axis of a device for both polarities (oxidizing and reducing) of the gate

electrode. In direct contrast with the case $D_R = D_O$, where the concentration profiles were essentially constant within each electrode domain, significant gradients of concentration can be observed within the domains of each electrode pair. These gradients take place on a length scale D/v introduced in Eq. (3) and develop so as to compensate for the local variations in the O/R composition in different regions of the device. The corresponding changes in local concentrations lead in turn to variations in the average concentrations in the device, which are in turn detected in the experiment as offsets in the measured current.

The corresponding simulated redox-cycling current response is shown in Figure 4(b). As in the experiment, this exhibits an oppositely signed response for the upstream and downstream detectors that first becomes more pronounced and then wanes as a function of flow speed. The crossover advection velocity is also of the same order as seen in the experiment. For these simulations, values of the effective diffusion coefficients (including the role of adsorption to the channel surfaces) were $3.8 \times 10^{-10} \text{ m}^2/\text{s}$ for the reduced species and $5 \times 10^{-10} \text{ m}^2/\text{s}$ for the oxidized species. Additional experiments are needed to verify to what extent these empirically chosen parameters are universally applicable.

In summary, we have shown that electrochemical reactions taking place at one or more electrode in an electrochemical fluidic device can influence the concentration in other regions of the device. We attribute this effect to differences in the transport coefficients for molecules in different redox states. The shifts in concentration become more pronounced when sharp gradients of counter-propagating molecules in different redox states are created – for example at closely spaced electrodes with different biases – even if the overall concentration of molecules exhibits much weaker variations. This interaction diminishes under convective transport when the Péclet number exceeds unity. While here we have focused on differences in effective diffusion coefficients, we anticipate that this behavior is more general and that similar effects will appear in more complex scenarios

where mass transport also involves migration or local chemical equilibria with additional surface-bound or solvated species.

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Chapter 5

Electrical detection of single water-soluble conducting polymers at open nanogap electrodes

In this Chapter, we introduce a method for measuring the conductance of water-soluble, electrically conducting polymers. In analogy with previous chapters, these experiments employ pairs of electrodes separated by less than 100 nm. A simpler geometry in which the electrodes are exposed to a bulk reservoir is however employed instead of the electrodes being embedded inside a nanochannel. Polymers diffusing in the reservoir adsorb to the electrodes and form bridges between them, allowing an electrical current to pass. The electrical properties of the polymers are found to depend sensitively on their redox state, which is controlled via a reference electrode in the reservoir. At very low polymer coverages, discrete telegraph-like signals are observed which we attribute to individual polymers intermittently closing the gap between the electrodes.

At its core, a biosensor can be reduced to two components: (1) a recognition element, which interacts very specifically with a target molecule or larger entity of interest, usually via the formation of chemical bonds, and (2) a signal transducer, which translates the recognition event into a signal that can be read out. A broad variety of transducers exist that are based on optical, mechanical or electrical mechanisms, and combinations thereof. All-electrical signal transduction is particularly appealing in the context of integrating sensors with modern microelectronics. In particular, a transduction mechanism that is fully compatible with integrated circuits technologies employed for consumer electronics (most commonly complementary metal–oxide–semiconductor, or CMOS, technology) opens the door to create highly miniaturized, massively parallel assays which can be mass produced at remarkably low cost.^{1,2}

Several mechanisms exist that satisfy this criterion to a greater or lesser degree. The most developed approach is ion-sensitive field-effect transistors (ISFETs),³ which detect the charge at the surface of a gate electrode exposed to solution. This approach is however often limited by its inability to probe beyond the electrical double-layer created by screening ions and by sensitivity to non-specific electrostatic interactions.^{4–8} Alternatives include Coulter counters and nanopores,⁹ electrochemical methods based on amperometry,¹⁰ potentiometry,¹¹ and impedance spectroscopy at (arrays of) nanoelectrodes².

Here we take the first steps in exploring a newly proposed mechanism¹² that relies on the existence of long, water-soluble, conducting polymers. Polymers spanned between two electrodes that are biased at different electrical potentials allow electrical current to flow directly between the electrodes, a much more efficient charge-transfer mechanism than, for example, diffusion-limited redox cycling as investigated in the earlier chapters. The polymers can either be weakly adsorbed to the surfaces of the transducer, as illustrated in Figure 1(a), or remain fully solvated as a random

Figure 1. Illustration of the proposed electrical transduction mechanism. (a) A surface-adsorbed polymer established an electrical contact between two closely spaced electrodes. (b) Even if the polymer remains fully solvated, intermittent approaches to within electron tunneling distance of the electrodes due to Brownian motion is sufficient to allow a current to pass. (c) Sketch of a DNA detection scheme based on the scheme of panel (b). Binding of the target DNA (purple) with the probe strand (light blue) releases a shorter, transducer strand (dark blue) linked to a long conducting polymer. This is detected via the absence of conduction through the polymer.

coil and allow transient currents to flow when they temporarily contact the electrodes via Brownian motion, as shown in Figure 1(b).

An example of a type of application for which this mechanism could prove ideally suited is the detection of trace levels of short, sequence-specific (order 100 base pairs) DNA or RNA oligomers at the sub-picomolar level, a key enabling technology for several evolving strategies for the early detection of cancer.¹³ Detection at such low concentrations faces the formidable challenge of identifying a small absolute number of molecules in a large sample volume, a notorious problem for conventional sensing approaches based on chemical equilibrium between the target and its receptor.¹⁴ Ideally, a successful approach would combine single-molecule sensitivity and the possibility of being scaled up to allow massively paral-

lel measurements, both of which can potentially be achieved simultaneously using the scheme of Figure 1(c). Here a probe single-stranded DNA oligomer is tethered to the surface and hybridized with a short complementary strand linked to the conducting polymer. Upon strand displacement by the target DNA, the polymer is released into solution and the current between the electrodes exhibits a sharp decrease.

The goal of the research presented in this chapter is to establish whether conduction through water-soluble can be detected near or at the single-molecule level as sketched in Figure 1(a) and/or Figure 1(b). Because this is an exploratory process that could require a significant amount of trial and error while testing different candidate polymers and measurement protocols, it was decided to develop a form of nanogap device that could be easily produced in large quantities, would alleviate the need for time-consuming sacrificial layer etching, and would allow rapid throughput of measurements.

The fabrication process flow for the devices is sketched in Figure 2(a). A four-inch Si wafer (p-type, <100>) was insulated with 500 nm thick thermally grown SiO₂. The bottom electrodes were first patterned using photolithography. The photoresist (Olin OiR 907-17) was spin-coated on the wafer with 4000 rpm and exposed. After development in Olin OPD 4262, 3 nm Ti was deposited on the substrate as an adhesion layer at a rate of 0.03 nm/s with a pressure of 2×10^{-7} Pa. Immediately following this step, 30 nm Au was deposited as top electrodes at a rate of 0.08 nm/s with a pressure of 3.8×10^{-7} Pa. After this metal deposition step, the photoresist was lifted off together with both redundant metals in acetone and under sonication for at least one hour to form the bottom electrode. The insulating layers and top electrodes were then defined simultaneously using the same process. For this purpose, a metal stack of Ti/SiO₂/Ti/Au with thickness of 3/50/3/30 nm was evaporated and patterned so as to overlap with the bottom electrodes; the deposition rate and working pressure were the same as during the first step. Finally, the wafer is diced into square chips of 6.475 mm $\times$ 6.475 mm, each chip containing 10 nanogap devices with

two separately addressable electrodes per device. An optical image of a finished device is shown in Figure 2(b). The width of top electrode is 5 μm ,

Figure 2. (a) Microfabrication process flow to generate the nanogap devices. Starting with an insulating substrate, the bottom electrode is defined in a first lithography step (middle sketch) while an insulating spacer and the top electrode are defined simultaneously in a second lithography step (bottom sketch). (b) Optical image (top view) of a single chip with 10 separately addressable nanogap devices. A zoom-in of the region of one device where the two electrodes overlap, thus forming the nanogap, is shown in the inset.

and the overlapped length is roughly 65 μm . The bottom electrode is 3 times wider and several times longer than the top one for convenience of alignment in lithography process.

The chemical potential of the majority of neutral conducting polymers in aqueous solution usually resides in their (relatively wide) band gap.¹⁵ Thus, doping is routinely necessary to modulate the conductivity of conducting polymers and allow electronic transport. Withdrawing electrons from the polymer, which is termed p-doping in analogy with conventional semiconductor materials, is equivalent to oxidizing the polymer. Similarly, the injection of electrons into a polymer is analogous to polymer reduction. In other words, doping of a conducting polymer can be achieved through electrochemical reactions. It has been shown that the conductivity of thin films of many conducting polymers can be flexibly varied over several orders of magnitude by controlling their redox state electrochemically.^{16–18} Perhaps even more intriguing, electrochemical methods can be employed to synthesize such polymers from their constituent monomers; this has enabled conductance measurements at the single polymer level when a polymer was grown between two electrodes.^{17,19–21} In all of these measurements, an accurate control of the electrostatic potential of the solution is of critical importance. A reference electrode is normally needed and plays the role of a gate electrode for the polymers.^{22,23}

Figure 3 illustrates how our experiments were carried out. As shown in Figure 3(a), a three-electrode configuration was employed. We refer to the electrodes as the top, bottom and reference (or gate) electrode. No potentiostat or auxiliary electrode was employed since the current through the reference electrode remained small in the measurements.²⁴ As gate, a commercial Ag/AgCl reference electrode (BASi) was immersed in the sample fluid containing the polymer solution to modulate the solution potential. Figure 3(b) sketches the measurement cell configuration. The nanogap device was positioned in a custom-made socket (Interconnect Devices, Inc.) fitted with spring-loaded pins that could simultaneously make connections to all the contact pads of both top and bottom electrodes on the chip. An

opening in the top of the socket allowed positioning a PDMS reservoir with an opening at its bottom with the surface of the chip. The reservoir was then filled with the test solution and the reference electrode was inserted via an opening at its top.

Figure 3. (a) Sketch of the experimental configuration. The potentials of the top, bottom and reference electrodes were separately controlled with respect to the circuit ground and the currents through all three electrodes were simultaneously measured. A Ag/AgCl reference electrode placed in the solution reservoir effectively served as a gate electrode for polymers in solution. (b) Connections to the outside world. A custom-built socket held the device and made electrical contact with all of the electrodes simultaneously. A printed circuit board (PCB) routed the signals from the socket to external connectors. A PDMS reservoir or plug was positioned so as to make contact with the surface of the device via a holder that could be accurately translated using an *xyz*-micromanipulator. The plug was filled from above with solution and a reference electrode (not shown) was connected to the fluid via the same access point.

The experiments presented here were performed using water-soluble conducting polymers based on polythiophene. While polythiophene is itself insoluble, solubility is made possible via the attachment of suitable side groups. Figure 4 gives the structures of four variants of water-solubilized polythiophenes that are commercially available from Rieke Metals (USA). While soluble as received, higher concentrations of dispersed polymers can be achieved by heating the solution above room temperature or through the addition of tetrabutylammonium hydroxide to the solution (final concentration of approximately 3 mM). 0.1 M potassium chloride (KCl) was used as supporting electrolyte. Little prior knowledge of these commercial polymers being available, the size of each polymer has to be estimated. The contour length of each polymer is calculated based on the given molecular weight (M_w) of each polymer and the corresponding monomer weight; the number of monomer units is calculated by dividing M_w of polymer by that of corresponding monomer weight. The contribution of each

Figure 4. Molecular structure of four commercially available water-soluble conducting polymers.

- (a) Poly[3-(potassium-4-butanoate)thiophene-2,5-diyl] (Rieke Metals #4021);
- (b) Poly[3-(potassium-5-pentanoate)thiophene-2,5-diyl] (#4022);
- (c) Poly[3-(potassium-6-hexanoate)thiophene-2,5-diyl] (#4024);
- (d) Poly[3-(potassium-7-heptanoate)thiophene-2,5-diyl] (#4026).

Table 1. The contour length of the polymers shown in Figure 4 calculated based on the given molecular weight (M_w) of each polymer and monomer. The number of monomer units is obtained by dividing M_w by the weight of each monomer. The length of each monomer units is estimated as 3.81 Å.

	M_w polymer [kDa]	M_w monomer [Da]	Number of monomer units	contour length [nm]
#4021	12 - 16	206.3	68 ± 10	25.9 ± 3.8
#4022	30 - 40	220.3	159 ± 23	60.6 ± 8.8
#4024	55 - 65	234.6	255 ± 22	97.2 ± 8.4
#4026	55 - 65	248.4	242 ± 20	92.2 ± 7.6

monomer unit to the total chain length is estimated as 3.81 Å.* The results are summarized in Table 1.

Before initiating measurements, the conductance between the top and the bottom electrode was measured in both the dry state and in aqueous solution with only supporting electrolyte by applying a small voltage difference (10 mV) between the two electrodes. A low conductance (<0.2 nS) in the dry state demonstrated the absence of pinholes in the 50 nm thick SiO₂ insulation, while the measurement in solution established the baseline (typically <1 nS) against which the measurements with polymers were carried out.

In a typical experiment, the top electrode was biased at 10 mV, the bottom electrode was biased at 0 mV, and the potential of the gate electrode was swept back and forth continuously between -0.2 V and 0.05 V from the moment that the polymer solution and reference electrode were introduced into the PDMS plug. During the first several cycles, shown in Figure 5, only relatively small currents flowed through the top and bottom electrodes. A reduction peak was observed near 0 V and the corresponding oxidation peak appeared near -0.09 V for each of these electrodes (with the top electrode being shifted by approximately 0.01 V due to the slightly different bias on this electrode). The sum of these two currents is equal and

* The length of a monomer is estimated as the length of a C-C bond (1.41 Å) plus the width of the thiophene ring. For the latter, the bond angle for C-S-C is taken as 93 degree and the bond length of C-S as 1.70 Å.²⁶

opposite to the current through the gate electrode. These features indicate that these currents are of an electrochemical current nature and corresponding to the doping and de-doping of polymers present on the surface of electrodes. In addition, the amplitude of the current from each electrode increased with the increasing number of cycles (as shown by the arrows in Figure 5), indicating that the adsorption of polymers on the electrodes' surface (and presumably in the nanogap between them) is a dynamic process.

Figure 5. The currents through the top (black), bottom (red) and gate (blue) electrodes were recorded while the gate potential was being swept between -0.2 V and 0.05 V with a rate of 5 mV/s for a 2 μ M solution of polymer

Following this initial slow buildup, and typically quite abruptly, high currents eventually appeared for gate voltages above ~ 0 V, as shown in Figure 6. Contrary to the behavior shown in Figure 5, these currents had opposite signs at the top and bottom electrodes, while the current through the gate electrode remained modest as before. We attribute this abrupt onset of a conduction to a bridge being first formed between the two elec-

trodes over the course of the present gate potential cycle, creating a conducting pathway between the two electrodes where none had existed earlier.^{20,21} The conductance in this state was of order 1 mS, which indicates that the current was limited by the resistance of the contacting wires rather than the resistance of the polymeric junction itself. Upon sweeping the gate potential back to lower values, the polymers remained in the conducting state past 0 V and until the gate potential was as low as -0.075 V, at which point it returned to its initial value.

Figure 6. Cyclic voltammetry and simultaneous polymer conductance measurement. The black curve shows the current flowing through the top electrode (held at 10 mV) and the red curve the corresponding current through the bottom electrode (held at 0 mV) as a function of potential of the gate potential, V_g . The arrows indicate the sweep direction. Conduction through the polymers turns on sharply near $V_g = 0$ V when V_g is being swept toward positive values and drops again sharply near -0.075 V when V_g is swept in the opposite direction. The simultaneously measured current through the gate electrode (blue curve) indicates that these two features correspond to reduction and oxidation of the polymers, respectively. Type #4021 polymer, concentration 2 μM , scan rate 5 mV/s.

Investigation of the current through the gate electrode, also shown in Figure 6, shows that the abrupt switching potentials and the associated hysteresis match the potential of the electrochemical reactions taking place in the system: reduction of polythiophene near $V_g = 0$ V and the associated oxidation near $V_g = -0.075$ V. This indicates that, once the gap was bridged by polymers, the conductance between the two electrodes became controlled by the gate potential. As the gate potential went to positive values, the polymers were reduced and doped with electrons, allowing current to flow between the top and bottom electrodes. Similarly, when the gate electrode was swept to negative values such that the bridge was at least partially oxidized, the polymer became insulating and the current returned to a vanishingly small value. This trend suggests that the polythiophene used in our measurements conduct through the lowest unoccupied molecular orbital (LUMO, n-type).¹⁶ Observations drawn from different systems based on polythiophene exhibit either n- and p-type conduction,^{17,25} and it is not yet clear what factors determine the type of doping in our system.

Our primary objective was to measure the conduction from very few or single polymers, as proposed in Figure 1. Chronoamperometry measurements (that is, current versus time at constant potential) are ideally suited to this purpose if the system exhibits fluctuations in time. Thus, extensive chronoamperometry measurements were performed with particular emphasis on the time interval when conduction first begins to appear. A small potential difference (10 mV) was applied across the top (-40 mV) and bottom (-50 mV) electrodes while the potential of the reference electrodes was held at 0 mV. This is equivalent to a gate potential of +50 mV in Figure 6 and corresponds to a value at which the polymers are fully reduced and conductive. Before the addition of sample solution, a blank measurement with 0.1 M KCl solution was measured for approximately 15 min. No significant changes in current flowing between the top and bottom electrodes were normally observed during this time. The polymer solution was then introduced into the device. Nothing happened initially, but, after a few minutes typically, some discrete changes in current began to be observed at the top and bottom electrodes, as illustrated in Figure 7. These

consisted in telegraph-like, anticorrelated current plateaus with typical switching times on the order of several seconds. The simultaneously recorded gate current exhibited no such feature. We ascribe these transient events to the formation of intermittent polymer bridges between the electrodes, which allow a sizable current to flow between the top and bottom electrodes. The lack of gate current further supports this interpretation since the transient reduction of a small number of polymers would lead a current too small to be measured.

Figure 7. Chronoamperometry measurements for polythiophene sample #4021 during the initial formation of a conducting bridge between the (a) top and (b) bottom electrodes. A small potential difference (10 mV) was applied across top (-40 mV) and bottom (-50 mV) electrodes while the potential of reference electrode (c) was fixed at 0 mV. The curves are offset vertically as the raw data were filtered with 2 mHz high pass filter to remove the slow baseline drift.

The steps in the current in Figure 7 correspond to conductance changes of about 0.4 nS. However, while measurements on different devices typically lead to telegraph-like transients such as those of Figure 7, the size of

the steps varies over several orders of magnitude between experiments. This suggests that the steps do not correspond to the intrinsic conductance of a single polymer, which would be expected to be relatively uniform, but rather to that of a complex network of interconnected polymers whose last link closes the contact between the electrodes. This is reasonable since the average contour length of the polymers employed in the experiments, let alone their radius of gyration, is insufficient to bridge between the electrodes. Quantitative interpretation of these step-like signals must therefore await experiments in which the behavior of a single polymer can be isolated.

In summary, the experiments presented here show that it is possible to establish a conducting link between two closely spaced electrodes via surface-adsorbed polythiophene-based polymers in aqueous solution. The conductance of the molecules can be switched by controlling their electrochemical potential. Fluctuations in the configuration of individual or small numbers of polymers lead to discrete, time-dependent fluctuations in the conductance of the system.

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Chapter 6

Substrate-dependent kinetics in tyrosinase-based biosensing: amperometry *vs.* spectrophotometry

Despite the broad use of enzymes in electroanalytical biosensors, the influence of enzyme kinetics on the function of prototype sensors is often overlooked or neglected. In the present study, we employ amperometry as an alternative or complementary method to study the kinetics of tyrosinase, whose catalytic activity results in o-quinone products. We further compare our results for four monophenolic substrates with those obtained from ultraviolet-visible spectrophotometry and show that the results from both assays are in good agreement. We also observe large variations in the enzyme kinetics for different monophenolic substrates depending on the R-group at the para position. To further study this effect, we investigate the stability of quinone products in the enzymatic assay. This information can in principle be utilized to discriminate between different phenolic species by monitoring the reaction rate.

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Introduction

Enzymes are common recognition elements in electrochemical biosensors due to their specificity and the response of such sensors is therefore largely dictated by enzyme kinetics. A variety of methods such as radiometry¹, ultraviolet-visible (UV-Vis) spectrophotometry², manometry³ and electrospray ionization with ion trap mass spectrometry⁴ has been developed to study enzyme kinetics, with UV-Vis spectrophotometry being the most commonly employed as it is non-invasive, sensitive, inexpensive and allows the enzymatic reactions to be monitored continuously.

Tyrosinase is a copper containing protein that is able to bind dioxygen and catalyze the *ortho* hydroxylation of monophenols to *o*-diphenols (monooxygenase activity) and the oxidation of *o*-diphenols to the corresponding *o*-quinones (catechol oxidase activity) according to the following reaction:

The binuclear copper center in the active site of tyrosinase exists in three different oxidation states depending on the copper ion valence and ability to bind with molecular oxygen: Ty_{met} ($\text{Cu}^{\text{II}}\text{-Cu}^{\text{II}}$), Ty_{oxy} ($\text{Cu}^{\text{II}}\text{-O}_2\text{-Cu}^{\text{II}}$), and Ty_{deoxy} ($\text{Cu}^{\text{I}}\text{-Cu}^{\text{I}}$)⁵. About 85% of the enzyme in a fresh preparation at atmospheric pressure, room temperature, neutral pH, and in the absence of substrate is in the *met* form with some variation depending on the source of the enzyme.⁶ In this state, the active site is in the bicupric form and unable to bind oxygen. Thus, only a small fraction of the enzyme is present in the *oxy* form⁶. The *oxy* form of tyrosinase can catalyze both the monooxygenase and catechol oxidase reactions, whereas the *met* form lacks the monooxygenase activity. Activation from the Ty_{met} form to Ty_{oxy}

form takes place through the reaction of the enzyme with diphenols. Thereafter, Ty_{oxy} can react with monophenols to produce the *o*-quinones through diphenol intermediates. It is known that the catecholase activity of the enzyme is much faster^{7,8}, thus the rate of conversion of phenol to quinone is limited by the rate of the monophenolase activity. The *o*-quinones regenerate the *o*-diphenol in the medium, which in turn reacts with and activates the remaining enzyme in the *met* form. The transformation of the enzyme from *met* form to *oxy* form introduces a short delay at the start of its reaction with monophenols called the lag phase.

Phenolic compounds present in pesticides, petroleum, textile, and paper based materials released to the environment from industrial processes pose health and environmental concern due to their high toxicity. Measurement of phenolic compounds is also important in medical diagnostics and food inspection. In the past, analysis was mostly based on the spectrophotometric² or chromatographic⁹ approaches. Electrochemical detection of phenols is based on their electro-oxidation which requires high positive potentials. This process is very complex, resulting in deactivation of the electrode due to the formation of a passivation of polymeric film from electrogenerated phenoxy radicals¹⁰. There is an interest in biosensors based on the reaction of phenolic compounds with tyrosinase which would potentially be simpler to make, easier to use, and less costly to manufacture. Tyrosinase has broad substrate specificity toward phenols relevant for a wide range of applications¹¹. The enzymatic reaction of phenols can be monitored by various electrochemical detection techniques, including detection of dioxygen consumption¹², direct reduction of generated *o*-quinone¹³, and coupled mediated reduction of *o*-quinone¹⁴. Among these methods, amperometric detection of the biocatalytically generated *o*-quinones is most common.

Tyrosinase biosensors have been proposed with enzyme immobilized on the electrode surface using variety of techniques including entrapment in conductive polymers such as polyaniline¹⁵ or in a sol-gel membrane¹⁶, covalent binding on gold nanoparticles¹⁷ or self-assembled monolayers¹⁸, adsorption to carbon nanotubes¹⁹ or to zeolite particles²⁰, and entrapment

with chitosan²¹. Although immobilized tyrosinase on the electrode surface has been reported to result in increased enzyme stability and allows reusing the sensor for a few times²², in several cases it introduces problems with mass transfer of the substrate to the enzyme²³. A much simpler approach is to add free enzyme to the sample solution as suggested in the recent publication of Adamski et al.²⁴. Their approach utilizes a 10 minute integration of the amperometric signal for reduction of quinones produced from different phenolic substrates. Here, we show that the signal is however highly dependent on the nature of the substrate, which makes it less straightforward to use the suggested protocol for measurement of phenolic compounds.

Although structural and mechanistic data of tyrosinase exist²⁵, they have, thus far, not been extensively utilized in the design of enzyme-based sensors. For example, the *R*-group of phenols influences the reaction rate of tyrosinase *via* two factors, namely, the electronegativity of the *R*-group and the steric hindrance caused by the size of this group. The reactivity of tyrosinase decreases upon a transition of the substituent in the *para* position of phenols from electron donating to electron withdrawing²⁶, and tyrosinase does not react with bulky phenols²⁷. There are few studies on kinetics of tyrosinase for different phenolic substrates with alkyl groups side chain such as methyl-²⁸, ethyl-²⁹, and *tert*-butylphenol³⁰. To complete the series with the type of *R*-group substitutions, we have measured the turnover rate of tyrosinase for methyl-, ethyl-, isopropyl- and *tert*-butyl phenol using both UV-Vis spectrophotometry and amperometry techniques and show that the two methods are in good agreement. We also find that the nature of the *R*-group at the *para* position of the substrate has a strong impact on the obtained response.

Experimental details

Chemical Reagents

Chemicals were obtained commercially in analytical grade and used without further purification. Mushroom (*Agaricus bisporus*) tyrosinase

(4,276 unit/mg) was purchased from Sigma-Aldrich in lyophilized powder form. The powder was dissolved into a stock concentration of 10 μ M and stored at -80 °C. Phosphoric acid 85 %, 4-methyl phenol, 4-ethyl phenol, 4-isopropylphenol, 4-*tert*-butylphenol, and sodium hydroxide were obtained from Sigma-Aldrich. Stock solutions of the phenolic substrates were freshly prepared in 0.2 M sodium phosphate-buffered solutions (pH=6.7) before each experiment. Deionized and filtered water of a resistivity not less than 18 M Ω cm was taken from a Milli-Q Advantage ultrapure water system.

Instrumentation

Electrochemical experiments were performed in a two-electrode cell system (reference and counter electrode were short circuited together which was appropriate due to the low current levels) using a CHI832 potentiostat. The working electrode was a carbon fiber ultramicroelectrode (radius of 6 ± 1 μ m) from BASi. A 3 M Ag/AgCl electrode (BASi, USA) was used as reference electrode. UV-Vis absorption spectra were recorded using a Carry 50 spectrophotometer (Agilent Technology). All the experiments were carried out at room temperature (20 ± 2 °C).

Methods and Procedures

Procedure for UV-Vis Spectrophotometry Measurements Enzymatic assays were conducted using UV-Vis spectrophotometry with a scanning speed of 600 nm min⁻¹ and an interval of 1 nm following the appearance of the products in the reaction medium. Varying substrate concentrations were used against a fixed concentration of 100 nM tyrosinase unless stated otherwise. Absorbance was recorded at a wavelength of 400 nm. Reference cuvettes contained all of the components except the substrate, with a final volume of 2 ml. The absorbance data were analyzed according to the Beer-Lambert equation.

Determination of Diffusion Coefficients The diffusion coefficients for different quinones were obtained for each substrate using cyclic voltam-

metry at a carbon fiber ultramicroelectrode (radius of $6 \pm 1 \mu\text{m}$). The phenolic solutions were first oxidized thoroughly using a high concentration of tyrosinase ($1 \mu\text{M}$). The product was then detected by repetitive potential cycling until the current reached its maximum value. Assuming that all the phenol was converted to quinone by tyrosinase, the diffusion coefficient was calculated using the equation $I_{\text{lim}} = 4nFDrc$, where I_{lim} is the limiting current, n is the number of electrons transferred per molecule diffusing to the electrode surface, F is the Faraday constant, D is the diffusion coefficient, r is the electrode radius, and c is the bulk concentration of redox active reagent³¹..

Procedure for Electrochemical Measurements The substrates used in electrochemical assays were 4-methylphenol, 4-ethylphenol, 4-isopropylphenol, and 4-*tert*-butylphenol. Formation of the reaction product was monitored using the amperometry technique at -0.2 V vs. 3 M Ag/AgCl .

Analyzing of Kinetic Data The time-dependent velocity of the reaction, V , was calculated by differentiating the concentration of quinone product vs. time. The maximum value was then selected to determine V . Typically, this maximum velocity was obtained in the first few minutes of each experiment. The maximum velocity, V_{max} , and the Michaelis constant, K_m , were subsequently obtained by nonlinear least-squares fitting of V against $[S]$ according to the Michaelis-Menten kinetics model, $V = \frac{V_m[S]}{[S] + K_m}$.³²

Results and discussion

Study of Enzyme Kinetics: UV-Vis Spectrophotometry vs. Amperometry

Although the kinetics of tyrosinase have been mostly studied using spectrophotometry techniques², the application of the enzyme in biosensing usually focuses on electrochemical approaches. However, the electrochemical study of tyrosinase kinetics and its comparison and validation against spectrophotometric techniques have not been the subject of any

specific study. Here, we compare these two methods and discuss the consequences of tyrosinase's complexity in designing electrochemical sensors for phenolic compounds.

The quinone product of tyrosinase reaction with phenolic compounds is both electrochemically and spectrophotocchemically active. It is therefore possible to monitor and compare its production in the enzymatic assay over the course of a reaction using both techniques. In order to have a broad range of comparisons for these two techniques, the activity of mushroom tyrosinase for four different substrates (4-methylphenol, 4-ethylphenol, 4-isopropylphenol, and 4-*tert*-butylphenol) is explored. For UV-Vis spectrophotometry measurements, the appearance of the product (quinone) and its evolution to a chromatophoric compound is measured at a wavelength of 400 nm, where the quinone absorption is maximum. For electrochemical measurements, the response is based on the amperometric detection of the biocatalytically generated *o*-quinones. Figure 1 shows the data for both methods using 4-ethylphenol as an example.

Figure 1A shows the absorbance vs. time for different concentrations of 4-ethylquinone. For low substrate concentrations, the absorbance increases monotonically with time before leveling off on a time scale of minutes. At this point, the substrate has completely been depleted in the reaction medium and transformed into *o*-quinone and thus the absorbance no longer changes with time. The time it takes to reach to this transition point varies for different concentrations of the substrate. For example, for a concentration of 25 μM , this is obtained after two minutes whereas for a concentration of 500 μM , four minutes are required. The figure also shows that at high substrate concentrations, true steady state conditions are not achieved. In these cases, the absorbance instead reaches a maximum before decreasing again. The monophenol oxidase reaction also depends on the presence of oxygen in the environment. Therefore, at high concentrations, the amount of phenol converted is limited by the amount of oxygen available in the assay^{33,34}. This limits the highest analyte concentration that a tyrosi-

nase biosensor can detect. Figure 1B shows the data for the maximum reaction rates of different concentrations of 4-ethylquinone produced in the enzymatic assay *vs.* the substrate concentration. The reaction rate is approximately linear for the concentration range of 10 to 100 μM , above which it saturates to a maximum rate of $1.7 \mu\text{M s}^{-1}$.

Tyrosinase has a complex kinetics associated with the lag phase at the beginning of its reaction and does not completely follow the common Michaelis-Menten model. Nevertheless for simplicity, we parameterize our data in terms of maximum velocity, V_{max} , and Michaelis-Menten constant, K_m . The red line in Figure 1B shows the fit to this model. A turnover number of 17 s^{-1} per enzyme with a Michaelis constant of $130 \mu\text{M}$ was obtained for 4-ethylphenol for an enzyme concentration of 100 nM .

Figure 1. (A) UV-Vis absorption ($\lambda=400 \text{ nm}$) *vs.* time for different concentrations of 4-ethylphenol in 0.2 M phosphate buffer solution ($\text{pH}=6.7$) in the presence of

100 nM tyrosinase, (B) Plot of the reaction rate obtained from UV-Vis adsorption vs. 4-ethylphenol concentration and fit to the Michaelis-Menten model. (C) Amperometry results (at potential of -0.2 V) obtained for different concentrations of 4-ethylphenol under the same conditions as in (A). (D) Plot of the reaction rate obtained from amperometry results vs. 4-ethylphenol concentration and fit to the Michaelis-Menten model.

The final product of tyrosinase reaction with a monophenol is an *o*-quinone, which is electrochemically active and its reduction involves a two-electron process. The quinones can be reduced at the electrode surface when the required negative potential is applied according to the following reaction:

Ultramicroelectrodes, unlike macroelectrodes, have the advantage that they do not perturb the bulk reaction on experimental time scales. In particular, due to the small size of the probe and spherical diffusion of material to the electrode, a time-dependent depletion zone of *o*-quinone does not establish itself at the electrode surface and the concentration of quinones in the bulk does not change significantly. The measured current is simply proportional to the concentration of quinones as they evolve in time, directly analogous to absorption in UV-Vis spectrophotometry. A carbon fiber ultramicroelectrode was used for this experiment. Experiments were carried out at a potential of -0.2 V vs. Ag/AgCl. Figure 1C shows the electrochemical responses for reduction of 4-ethylquinone for four different concentrations. A similar trend to those of spectrophotometry measurements was observed. For each concentration, the cathodic current for reduction of 4-ethyl quinone increased superlinearly, then reached a plateau where all the phenol was used up and converted to 4-ethylquinone. The time to reach the maximum current increased with the substrate concentration in a manner similar to what we observed using spectrophotometry.

At high concentrations, the faradaic currents for reduction of quinone did not reach a plateau, but declined after reaching a maximum point. In addition to oxygen depletion, as in the UV-Vis case, this may be influenced by the benzene derivative products which form oligomers among themselves, especially at high concentrations. The oligomers have much lower diffusion coefficients than the monomer and, thus, the transport to the electrode surface becomes more sluggish. The electron transfer rate may also be decreased as the electronic structure of oligomers differs from that of monomers.

Figure 1D shows the plot of the reaction rate for different concentrations of 4-ethylphenol and the fit to Michaelis-Menten kinetics model. From this fit, a maximum rate of $1.4 \mu\text{M s}^{-1}$ and a Michaelis constant of $130 \mu\text{M}$ were obtained for an enzyme concentration of 100 nM , in good agreement with those of UV-Vis spectrophotometry.

Similar behavior was observed for both 4-methylphenol and 4-isopropylphenol, although longer times were required for the enzyme to convert 4-isopropylphenol to 4-isopropylquinone due to much lower turnover rates. This may be explained by the steric hindrance introduced via the isopropyl group at *para* position. The results obtained for all four monophenolic substrates are summarized in Table 1 from which we can see that there is a good agreement between both techniques. The variation between the two methods are comparable to the uncertainty between measurements using the same technique. These data illustrate that the electronic structure of the substituent on the substrate affects both the turnover rate of the enzyme and its affinity toward the substrate.

The reaction between tyrosinase and 4-*tert*-butylphenol differs from other substrates and in particular exhibits different kinetic behavior. Figure 2A shows the UV-Vis spectra for different concentrations of 4-*tert*-butylphenol. Due to the sluggish kinetics of tyrosinase for this substrate and limited resolution of our spectrophotometer, a higher concentration of tyrosinase ($1 \mu\text{M}$) was used in this experiment. For low concentrations of 4-

tert-butylphenol, similar to the monophenols studied earlier, the absorption initially increases and then reaches a plateau; however, much longer periods of time are required to reach to this plateau. For example, for a concentration of 50 μM , this is achieved only after 13 minutes. For higher concentrations, this plateau is not established even after 30 minutes. Surprisingly, the rate at which the absorption changes decreased at higher substrate concentrations, as plotted in Figure 2B. This is a different behavior from that of the other monophenols studied and is not consistent with the Michaelis-Menten kinetics model. This has been reported before by Ros et al.³⁰ and Fennol et al.³⁵, who related this behavior to both the suppressed autoactivation of tyrosinase from *met* to *oxy* form caused by binding of monophenols to the *met* form of the enzyme together with the high stability of quinone products.

Table 1. A comparison of the results obtained from amperometry and UV-Vis spectrophotometry experiments and their fit to Michaelis-Menten kinetics model. The concentration of tyrosinase is 100 nM in all studies except for 4-*tert*-butylphenol which is 1 μM .

Substrate	UV-Vis		Amperometry	
	Turnover(s^{-1})	K_m (M)	Turnover(s^{-1})	K_m (M)
4-methylphenol	16	7×10^{-5}	24	2.1×10^{-4}
4-ethylphenol	17	1.4×10^{-4}	14	1.4×10^{-4}
4-isopropylphenol	1.5	1×10^{-4}	2.5	1.5×10^{-4}
4- <i>tert</i> -butylphenol	0.12	-	0.16	-

Figure 2. (A) UV-Vis absorption ($\lambda=400$ nm) vs. time for different concentrations of 4-*tert*-butylphenol in 0.2 M phosphate buffer solution (pH 6.7) in presence of 1 μ M tyrosinase. (B) Plot of the reaction rate obtained from UV-Vis adsorption vs. the 4-*tert*-butylphenol concentration. (C) Amperometry results (at potential of -0.2 V vs. Ag/AgCl) for different concentrations of 4-*tert*-butylphenol under the same conditions as in (A). (D) Plot of the reaction rate obtained from amperometry data vs. the 4-*tert*-butylphenol concentration.

The results from amperometry in this anomalous case are also in good agreement with those obtained from absorption spectra. Figure 2C shows the amperometry results obtained for different concentrations of 4-*tert*-butylphenol. The maximum reduction current for 4-*tert*-butylquinone increases with increasing of 4-*tert*-butylphenol concentration, while the reaction rate initially increases with 4-*tert*-butylphenol concentration and then declines, similar to the data obtained from spectrophotometry method

(Figure 2D). These studies complement earlier data and show the influence of the substituent side chain at the *para* position on the hydroxylating rate of monophenols. Phenols with a small substituent side chain, such as 4-ethylphenol and 4-methylphenol exhibit higher turnover rate. On the other hand, phenols with larger molecular size substituent side chain, 4-isopropylphenol and 4-*tert*-butylphenol exhibit much lower turnover rates.

For spectrophotometry measurements, the detection of quinone is limited by the resolution of the instrument. The main factor limiting the sensitivity of amperometry method is instead the background current obtained in the absence of the analyte. The background current is mainly due to the presence of other electroactive substances such as oxygen. In this context, carbon electrodes have both the advantage of broader potential window when compared with the noble metals and that their catalytic activity toward the reduction of oxygen is poor.

Stability of *o*-Quinones

Quinones are known to be unstable and undergo several non-enzymatic reactions^{29,35}. Here, we study the stability of the biocatalytic product of tyrosinase reaction with monophenolic substrates by following the evolution of their UV-Vis absorption spectra. The wavelength is scanned from 700 to 350 nm and the change in UV-Vis absorption spectra is recorded for 90 successive scans over 1.5 hours.

Figure 3 shows a selection of the absorption spectra for evolution of *o*-quinones generated by oxidation of our four different monophenolic substrates using 500 nM mushroom tyrosinase. For 4-methylphenol (Figure 3A), the absorption peak at 400 nm increases with the reaction time for the first 30 minutes with a maximum absorption of 0.29, then the absorption decreases for the next 10 minutes to 0.16. Finally, it gradually increases again to reach an absorption level similar to that of 30 min (0.29 at λ_{max} = 400 nm) after 90 min. However, absorption at other wavelengths also slowly increases with time, especially around 500 nm. The progressive absorbance changes at other wavelengths can be attributed to the products

from the nonenzymatic autopolymerization of *o*-quinones which yield several unstable intermediates, as reported previously^{36,37}.

For 4-ethylphenol (Figure 3B), the absorption increases gradually at 400 nm for the first 40 min with a maximum absorption at 0.26, then decays with time to 0.20. The absorption at other wavelengths (mainly at 500 nm) instead constantly increases with time. The absorption increase for 4-isopropylphenol (Figure 3C) occurs for the first 60 min ($A=0.37$), after which it levels off and stops changing with time, whereas the absorption at 500 nm increases with time. Finally, Figure 3D shows that the absorbance peaks (at 400 and 500 nm) from the product of oxidation of 4-*tert*-butylphenol increase with time and do not saturate even after 1.5 hour. The contribution from the absorbance at 500 nm is insignificant compared with the other substrates studied here.

Figure 3. UV-Vis absorption spectra for the evolution of *o*-quinones from the reaction of 500 μ M of different monophenolic substrates (A) 4-methylphenol, (B)

4-ethylphenol, (C) 4-isopropylphenol, and (D) 4-*tert*-butylphenol with 500 nM mushroom tyrosinase for 90 minutes.

In all cases, the resulting *o*-quinones may undergo non-enzymatic auto-polymerization to produce colored compounds which cause the red shift of the products and increase of absorbance at other wavelengths. The quinone produced in biocatalytic reaction can react with (1) water which acts as a weak nucleophile, (2) itself, or (3) the product resulted from adding water, and (4) the intermediate radicals formed in the enzymatic reactions. The products of the tyrosinase reaction with 4-methylphenol and 4-ethylphenol are unstable quinones that evolve very rapidly whereas they are more stable in the case of 4-isopropyl- and 4-*tert*-butylphenol. The following relative stability is suggested from Figure 3:

Importantly, different substrates have different time scales to reach the maximum absorbance where all phenols are expected to be converted to quinones. The trend here is exactly opposite to the one presented above for stability. Thus, the response time associated with the oxidation of the substrates by tyrosinase proves to be very sensitive to the nature of the substitutes present on the aromatic ring. The stability of the enzymatically generated *o*-quinones in the reaction medium is thus also an important parameter for designing a biosensor based on tyrosinase.

Conclusion

Amperometry was shown to be a rapid and sensitive continuous method for analysis of enzyme kinetics where the change in current for oxidation or reduction of substrate or product is followed vs. time at a suitably fixed biased potential. The kinetic mechanism of tyrosinase on different monophenolic substrates is very complex and dependent on the substituent group at *para* position. This influences the measurement time for different monophenolic substrates and therefore, reliable calibration methods are a prerequisite for its application in biosensors.

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Appendix A

Fabrication of the nanofluidic devices

Figure A1. The four main steps in the microfabrication process flow. Left: cross-section along the channel axis. Right: cross-section perpendicular to the channel axis.

The fabrication scheme is illustrated in Figure A1. A 10 cm Si wafer was isolated by thermally growing 500 nm SiO₂ layer. On this isolated side, $108\ \mu\text{m} \times 3\ \mu\text{m} \times 20\ \text{nm}$ (length $\times$ width $\times$ height) platinum (Pt) layers were deposited as bottom electrodes by means of photolithography, metal evaporation and lift-off processes in succession (Step 1 in Figure S1). Wires leading to contact pads elsewhere on the chip were also simultaneously patterned. Following the same process as for the bottom electrode, a $720\ \mu\text{m} \times 5\ \mu\text{m} \times 330\ \text{nm}$ chromium layer (Step 2) and a $102\ \mu\text{m} \times 7\ \mu\text{m} \times 370\ \text{nm}$ platinum layer (Step 3) were subsequently deposited and patterned. These formed the sacrificial layer (which later becomes the fluidic channel when etched) and the top electrode, respectively. The height of both the nanochannel and the nanogap transducers was defined by the

thickness of the Cr layer. Afterwards, a passivation layer consisting of 90 nm/325 nm/90 nm thick PECVD SiO₂/SiN/SiO₂ was deposited. Access holes (4 μ m by 2 μ m) were patterned by photolithography and created by CHF₃/O₂ reactive ion etching through the passivation layer (Step 4). The wafer was diced by a saw (NBC-Z 2050) into 60 mm by 100 mm rectangular chips.

Before each measurement, a molded PDMS block with a microchannel design on one face was punched with a needle to create holes in which tubing (FEP Capillary Tubing, IDEX Health & Science) could later be inserted. This PDMS block was then treated in oxygen plasma together with a chip. Afterwards, the PDMS plug and the chipset were aligned under an optical microscope and bonded together. Finally, the sacrificial layer was etched by introducing Cr etchant into the PDMS microchannel, creating the nanochannel.

Details of the nanogap microfabrication and microfluidic interface have been described in more detail elsewhere.^{1,2}

Separation of mixtures with different ratios

Figure A2. The normalized redox cycling currents from downstream transducers. The analysts are mixtures of FcTMA⁺ and Fc(MeOH)₂ of different concentration ratios. The total concentration of redox molecules is kept constant at 50 μ M.

Figure A2 provides additional evidence that the first peak corresponds to $\text{Fc}(\text{MeOH})_2$ and the second one to FcTMA^+ . The amplitude of the first peak increases compared to that of the second one when the fraction of $\text{Fc}(\text{MeOH})_2$ increases.

Figure A3. The peak width and upstream transient time are plotted together in a log-log scale with respect to pump rate.

Retention factor

Figure A4. The peak time (t_{peak}) divided by t_M .

In Figure 3B in the main text, the retention number is not constant for each species in different conditions and seemingly shows an inhibitory effects when the convection velocity is high. We plot the ratio of peak t_{peak} to t_M with respect to convection velocity at different adsorption conditions in Figure A4, by computing the cross-correlation function.³ From the curve in orange, in which no adsorption effect is taken into account, we can see that the peak time is only an estimation of time of flight for the concentration plug transport. The curve also shifts upwards as the degree of adsorption increases. Therefore, based on the calculation shown in Figure A4, if adsorption adapts a higher degree at smaller pump rate and lower degree at high rate, the main trend in Figure 3B can basically be recovered.

Multicomponent Freundlich Isotherm

The multicomponent Freundlich isotherm^{4,5} is one of several successful semi-empirical models to describe the simultaneous adsorption of two or more species on a surface in contact with a solution. These species compete to occupy discrete binding sites with a distribution of binding energies, such that one species may displace another on the surface when both are present in solution.

In short, the model assumes an exponential distribution of binding energies for the individual species, such that each of the monocomponent adsorption isotherms in a two-component mixture obeys the Freundlich form,

$$\Gamma_{1,2} = K_{1,2} c_{1,2}^{n_{1,2}}. \quad (\text{A1})$$

Here $\Gamma_{1,2}$ and $c_{1,2}$ are the surface and bulk concentrations of the two species, respectively, while $K_{1,2}$ and $n_{1,2}$ are species-dependent constants. We have previously reported that adsorption of $\text{Fc}(\text{MeOH})_2$ on Pt electrodes from aqueous and acetonitrile solutions is well described by the Freundlich isotherm in concentrations ranging from 1 μM to 1 mM.⁶ In a mixture, this result is modified to become

$$\Gamma_1 = K_1 c_1 (c_1 + a c_2)^{n_1 - 1}, \quad (\text{A2a})$$

$$\Gamma_2 = K_2 c_2 (c_2 + c_1/a)^{n_2-1}. \quad (\text{A2b})$$

Here a is an additional dimensionless parameter, the so-called competition coefficient ($a = 1$ corresponding to the two species having the same probability of occupying a given site when present in the same concentration).

We now focus on the case considered in the experiment of Figure 3 in the main text. Let c_0 be the total concentration of both redox species. For a monocomponent system, $c_1 = c_0$ and $c_2 = 0$, or vice-versa. For the 1:1 mixture, $c_1 = c_2 = c_0/2$. The relative degree of adsorption is given by the ratio $\Gamma_{1,2}/c_{1,2}$. Substituting into eq. A2 then yields

$$\frac{(\Gamma_1/c_1)_{\text{mixt}}}{(\Gamma_1/c_1)_{\text{mono}}} = \left(\frac{1+a}{2}\right)^{n_1-1}, \quad (\text{A3a})$$

$$\frac{(\Gamma_2/c_2)_{\text{mixt}}}{(\Gamma_2/c_2)_{\text{mono}}} = \left(\frac{1+1/a}{2}\right)^{n_2-1}. \quad (\text{A3b})$$

Furthermore, assuming that adsorbed species are bound to a specific site on the surface and therefore immobile while adsorbed, the average time for a redox molecule to travel through the channel, t_R , is related to the travel time of the solvent by

$$t_R = t_M \left(\frac{\Gamma A + cV}{cV} \right). \quad (\text{A4})$$

Here V is the volume of the channel and A is the area of the adsorbing surfaces; the term in parenthesis represents the (inverse) fraction of the time that a molecule is mobile. Combining equations A3 and A4 with the definition of the retention factor, $k' = (t_R - t_M)/t_M$, finally yields

$$\frac{k'_{1,\text{mixt}}}{k'_{1,\text{mono}}} = \left(\frac{1+a}{2}\right)^{n_1-1}, \quad (\text{S3a})$$

$$\frac{k'_{2,\text{mixt}}}{k'_{2,\text{mono}}} = \left(\frac{1+1/a}{2}\right)^{n_2-1}. \quad (\text{S3b})$$

That is, as long as $\alpha \neq 1$, the relative degree of adsorption of one species increases while that of the other species decreases in the 1:1 mixture, leading to shifts in the retention factors consistent with the trends observed in Figure 3B in the main text.

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Appendix B

An important issue that was encountered during chromatography measurements was a slow evolution of the system properties with time. We attribute this time dependence to the slow modification of the electrode and channel surfaces by contaminants from solution, as is commonly observed in electrochemical measurements stretching over long periods of time without electrode regeneration.

Figure B1. 0.5 M H_2SO_4 cyclic voltammetry with 10 mV/s scan rate for the device used to generate results for Figure 2 and Figure 6. Panel (a) and (b) show the results from the two top electrodes. Panel (c) and (d) show the results from the corresponding bottom electrodes. The red curves give the characteristic curves for a freshly etched surface. The blue and black curves correspond to the surfaces subsequent to a series of single-species measurements; the black and blue curves represent the first cycle and the final (stable) cycle, respectively.

In electrochemical measurements with metal electrodes, cyclic voltammetry in 0.5 M H_2SO_4 is a standard method to activate and/or clean the electrode surfaces. Non-standard responses usually reflect features of the electrode surfaces.¹ Figure B1 shows results for the four electrodes of a device both before and after measuring three individual redox species (measurement time of about 22 hours). The red curves show the results for freshly exposed surfaces immediately following device etching. After a series of single species' measurement (and before subsequent measurements using mixtures), the surfaces were characterized again, as shown by the black and blue curves in Figure B1. In the first cycles (black curves), the current being especially high at oxidizing potentials (0.7 ~ 1.2 V). Based on these features, we infer that the electrode surfaces become contaminated throughout the long run time of the measurements.² After several cycles of cyclic voltammetry, all curves (blue) stabilized to the nearly standard expected shape for a platinum electrode.¹ Since these curves are still quite different from the initial red curves, we can reasonably expect that the property of electrode surfaces can permanently change after long time measurements.

Figure B2. Normalized currents from downstream top electrode in T mode. The sample solution is a 50 μM mixture of $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ with 1:1 ratio.

Given this fact that the surface property evolves over the course of the measurements, it is revealing to repeat the chromatography experiments using the same device on different days. Following measurements on $[\text{Fe}(\text{CN})_6]^{4-}$ and cleaning with H_2SO_4 , the same mixture of $\text{Fc}(\text{MeOH})_2$ and FcTMA^+ was tested a second time in the same device. Figure B2 gives the results for the normalized current in T mode both before and after the other measurements. In comparison with Figure 6 in Chapter 3, the shape of the adsorption peaks has substantially changed. Even though discrimination improved with pump rates up to $50 \mu\text{l/h}$, the baseline resolution was not reached for the same pump rate as earlier. Interestingly, however, even though, the resolution is quite poor, a series of separated desorption peaks was observed for the first time for the reduced state in this follow-up measurement.

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Appendix C

Here we present measurements performed with a very different geometry than in Chapter 4 where nonlocal coupling also becomes apparent. The geometry is identical to that of the devices in Chapter 2, with two transducers separated by a SiO₂ nanochannel. Stepping the potential of the upstream transducer leads to a change in the steady-state current at the downstream transducer. Interestingly, and as expected from Eq. (3) in Chapter 4, increasing the flow rate leads to a decrease in the magnitude of this influence at high flow rates.

Figure C 1. Redox-cycling current versus time at the downstream transducer for an oxidizing potential (0.5 V) at the upstream transducer. This current is normalized to the steady state current when the upstream top electrode was biased at a reducing potential (0 V).

Summary

The main focus of this thesis is to explore mass-transport processes for redox-active analytes in concentrated supporting electrolytes when they are driven by external pressure through nanofluidic channels with embedded electrodes. The principal devices employed in these experiments are so-called nanogap electrodes, which consist of two electrodes located in the floor and roof of the nanochannel. Electrochemical reactions taking place at the electrodes can profoundly disturb the concentration distribution of analytes along the axial direction of the nanochannel in several manners. First, changes in the redox state of molecules at different electrodes can cause significant variations in the distribution of analyte concentrations under steady-state conditions. Second, time-dependent changes in electrode potential can cause local, transient fluctuations in the analyte concentration. These represent a new class of phenomena in nanofluidics next to the more established electrokinetic and depletion-enrichment effects.

The large surface-to-volume ratio that exists in nanofluidic devices means that any interactions between analyte molecules and surfaces manifest themselves more strongly than in conventional systems. After a general introduction in Chapter 1, Chapters 2 and 3 of this thesis investigate how transient concentration disturbances can be created by the near-instantaneous switching on and off of an electrochemical reaction. A pressure-driven convective flow can then transport these transient concentration profiles downstream. Throughout this process, further interaction with the channel walls can cause different species to become separated, resulting in a new form of electrochemical chromatography. After demonstrat-

ing the basic effect, we explore how it could be further optimized by varying the geometry and controlling the potential of the channel walls via additional ‘gate’ electrodes spanning the entire length of the channel.

An originally unforeseen consequence of introducing multiple nanogap transducers and/or gate electrodes in a single channel is that it can lead to significant coupling between different regions of the system. Local changes in the redox state imposed by electrodes at different biases means that large gradients can develop between different regions. Chapter 4 addresses how, in some cases, this can result in a rearrangement of the analyte concentration distribution along the entire channel, leading to an apparent non-local coupling between electrodes.

In addition to these three core chapters, we explored further potential applications of electrochemistry in bioanalytical research. Chapter 5 describes exploratory attempts to detect single conducting polymer molecules as they form a conducting pathway between two closely spaced electrodes. This approach can be further developed to become a transduction mechanism in single-molecule detection assays suitable for detection of short DNA oligomers. Chapter 6 instead shows how electrochemical detection at microelectrodes can yield the same enzyme kinetic parameters as conventional photo-spectroscopy techniques, a preparatory work prior to studying the kinetics of single enzyme in nanogap devices.

Samenvatting

Dit proefschrift richt zich voornamelijk op het onderzoeken van massa-transport van redox actieve analyten in geconcentreerde elektrolyten in nano-fluïde kanalen met ingebouwde elektroden waarbij de analyten onderhevig zijn aan druk van buitenaf. Het belangrijkste device in deze experimenten zijn nanogaps welke bestaan uit een nanokanaal met twee elektrodes aan de boven en onderkant van dit kanaal. Elektrochemische reacties bij de elektrodes kunnen de concentratieverdelingen in de axiale richting van het nanokanaal op verschillende manieren grondig verstoren. Als eerste kunnen veranderingen in de redoxtoestand van moleculen bij de verschillende elektrodes een aanzienlijke verandering in de verdeling van analyt concentraties onder steady state condities teweeg brengen. Als tweede kunnen tijdafhankelijke veranderingen in het elektrodepotentiaal lokaal kortstondige fluctuaties in de concentratie van het analyt veroorzaken. Ze vertegenwoordigen een nieuwe groep van fenomenen in nano-fluïdica naast de meer bevestigde elektrokinetische en verarming-verrijking effecten.

De hoge oppervlakte tot volume verhouding in nano- fluïdica devices maakt dat interacties tussen analyt moleculen en de oppervlakten veel sterker zijn dan in conventionele systemen. Na een algemene introductie wordt in de hoofdstukken 1, 2 en 3 van dit proefschrift onderzocht hoe kortdurende concentratieverstoringen geïnitieerd kunnen worden door de elektrochemische reactie haast instantaan aan of uit te schakelen. Een door druk aangedreven convectiestroom zal dit kortdurende concentratieprofiel stroomafwaarts verplaatsen. Gedurende dit proces door interacties met de kanaalwanden kunnen verschillende soorten van elkaar gescheiden worden resulterend in een nieuwe vorm van elektrochemische chromatografie. Na het demonstreren van de primaire effecten proberen

we deze te optimaliseren door het aanpassen van de geometrie en door het potentiaal van de kanaalwanden te controleren via extra ‘gate’ elektrodes over de gehele lengte van het kanaal.

Een niet voorziene consequentie door introductie van meerdere nanogap transducers en/of gate elektrodes in een enkel kanaal is de sterke koppeling tussen de verschillende gebieden in het systeem. Lokale veranderingen in de redoxtoestand veroorzaakt door verschillen in bias op de elektrodes kunnen hoge gradiënten tussen verschillende gebieden teweeg brengen. In hoofdstuk 4 wordt uitgelegd hoe in sommige gevallen dit kan resulteren tot een herordening van de analyt concentratieverdelingen in het gehele kanaal wat weer leidt tot niet-lokale koppelingen tussen elektrodes.

In volgende hoofdstukken verkennen we potentiële applicaties van elektrochemie in Bio-analytisch onderzoek. Hoofdstuk 5 beschrijft exploratief onderzoek naar het detecteren van enkele polymeer moleculen wanneer ze een geleidend pad vormen tussen twee elektroden met een kleine tussenruimte. Deze benadering kan verder ontwikkeld worden tot een transductie mechanisme voor enkele molecuul detectie analyse systemen welke geschikt zijn voor het detecteren van korte DNA oligomeren. Hoofdstuk 6 laat zien dat elektrochemische detectie bij micro elektrodes dezelfde kinetische enzym parameters als bij conventionele foto spectroscopie technieken oplevert. Het is een voorbereidend werk tot het bestuderen van de kinetiek van enkele enzymen in nanogap devices.

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