The effect of voltage on a conductivity gradient in a nanochannel and its application to protein trapping

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Abstract

Studies in the last two decades have demonstrated the use of protein biomarkers for early disease detection. Increasingly, these biomarkers have been found at very low concentrations with a high background of abundant proteins. Recent work in nanofluidics has allowed for the possibility of high concentration enhancement and separation – two processes that are essential for the detection and discovery of trace biomarkers. With recent demonstrations of the trapping and detection of these biomolecules using nanochannels, there is a need to better model and appreciate the physical mechanisms that are involved in the trapping of these biomolecules. This paper investigates the effect that voltage has on a conductivity gradient established within a nanochannel. This conductivity gradient is used to focus proteins by balancing the opposing electrophoretic and viscous drag forces – and it is established in a nanochannel that connects two microchannels containing solutions of different salt concentrations and hence conductivity. A conductivity gradient is directly observed using a fluorescent salt, then the trapping behaviour of a fluorescent protein, in a phosphate buffered solution containing salt, is investigated, while varying the applied voltage. This study concludes that voltage appears to have a significant effect on the conductivity gradient inside a nanochannel, and that this effect may have important implications for protein trapping inside a nanochannel.

Keywords: Electrostatics, nanofluidics, analytical chemistry.

Introduction

Understanding the behaviour of molecules in a nanochannel is of great interest for the detection and analysis of biomarkers in new nanofluidic devices. These devices include nanopore-nanochannel systems described in Ling's work (2010), as well as types of polymers and membranes discussed in Eijkel and van den Berg's review (2005) of developments in nanofluidics. Various studies have been conducted, and reviewed, on the benefits of exploring this relatively new field of nanofluidics (Bocquet et al., 2010; Eijkel et al., 2005). This area of study is unique because – unlike microfluidics – the dimensions (1-100nm) of nanofluidic devices are approximately equal to an important ion screening distance called the Debye length, λ_D:

$$\lambda_D = \sqrt{zz_0k_BT/2n_{bulk}z^2e^2}$$

Where ε is the dielectric constant of water, ε_0 is the permittivity of vacuum, k_B is the Boltzmann constant, T is the absolute temperature, n_{bulk} is the bulk ion concentration, z is the valency of the ions, and e is the charge of an electron (Daiguji, 2010).

Intermolecular interactions with solid surfaces affects the behaviour of ions in solution. Considering the length scales of the channels of fluid transport involved in this study (smallest length 80nm), electrostatic forces are considered, and not steric or van

der Waals interactions which occur at much smaller scales (Daiguji, 2010). Electrostatic interactions occur near the charged surfaces of the nanochannel walls. At these surfaces, counter-ions become attracted to the charge of the surfaces, while co-ions are repelled. This layer of counter-ions and co-ions is called the electrical double layer (EDL)—whose thickness is approximately equal to the Debye screening length. As this length is typically 1-100nm in ionic conditions, the EDL shields or screens the surface charge from the bulk solution—so the bulk becomes electrically neutral. In *microchannels*, the width of the channel is much larger than the Debye length—so the bulk of the fluid is neutral. On the other hand, the width of a *nanochannel* is approximately equal to the Debye length—so all of the solution may be inside the non-neutral EDL.

Previous studies have also identified the simultaneous concentration and separation of proteins in a nanochannel (Inglis et al., 2011). These and many other studies are based on the principle that trapping of charged molecules occurs where there is a stable zero position in the net velocity caused by opposing forces of viscous drag and electrophoresis (O'Farrell, 1985).

The relevant forces that act on a molecule in solution are: first, the viscous drag force F_D which can be approximated as the drag on a sphere in laminar flow, Stoke's drag (Berg, 1993):

$$F_D = 6\pi\eta r v.$$

Where η is the solution viscosity, r is the molecules radius and v is the difference between the solution velocity and the molecule's velocity; the second force is the electric force F_E which is just the molecule's charge, Q, times the local electric field, E:

$$F_E = QE.$$

A stable trap is formed when the sum of these two forces is zero and the derivative of the sum is negative. In any ordinary liquid-filled channel, an electric field will cause electro-osmotic flow, so there will be a roughly constant fluid velocity and a roughly constant electric field. For a negatively charged surface like glass, the electro-osmotic flow, and the drag force, is toward the lower voltage electrode, right to left in all figures here.

The electric field is right to left, but the protein used here is negatively charged, so the electric force acts left to right. The nanochannel used in this study sustains a gradient in conductivity because the microchannels at either end have different conductivities and are continually refreshed by flow along their length. This forms a unique micro-nano-microchannel system comprising three fluid-filled channels whose vertical dimensions are in the micrometer, the nanometer, and the micrometer scale.

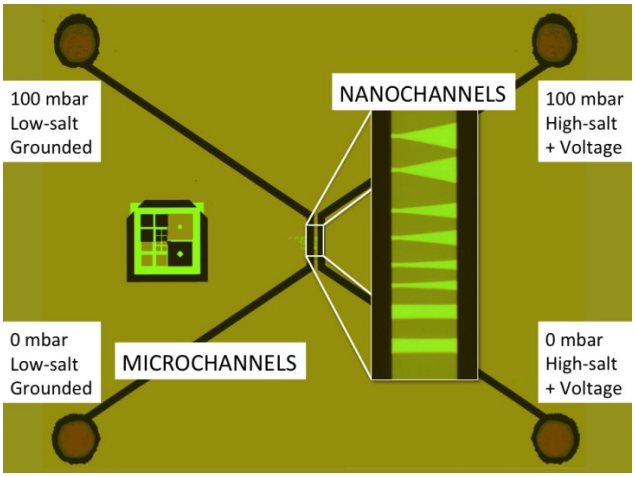
In such a channel, the electric field is not constant. The local electric field is inversely related to local conductivity, which is roughly proportional to ion concentration. Consequently, at the left side where the ion concentration is lowest, the electric field is highest. This high electric field at the low-conductivity end of the nanochannel is able to oppose the viscous drag force and trap a negatively charged protein. This paper shows how this gradient is affected by an applied voltage. This is achieved through direct observation with a fluorescent salt solution, and analysis of this effect on protein trapping.

Previous work by Inglis et al. (2011) showed that the nanochannel used here could be used to simultaneously concentrate and separate proteins. Translating this research to practical devices for biomarker detection will require a deeper understanding of the parameters involved. Here the following is demonstrated: how one critical parameter, applied voltage, affects the underlying conductivity gradient and the trapping of proteins. This understanding – coupled with further studies using mathematical modeling – may improve direct measurement, prediction and manipulation of this concentration gradient (inside the nanochannel). Since this device requires very small quantities of sample, this study appears to entail significant possible applications in single molecule detection and biomarker discovery – as well as improvements in nanofabricated devices.

Part I: Direct Visualization of the Gradient

Using methodologies described in previous studies (Inglis et al., 2011), a concentration gradient is created by connecting a high salt microchannel to a low salt one, using a series of nanochannels, as seen in Figure 1. A circuit (to establish an electric field) is created using platinum electrodes that are placed in the 4 circular holes at the ends of the microchannels. Only rectangular channels are analysed. The microchannels are 10 microns deep and the nanochannels are 80 nm deep. Each microchannel is 7.5 mm long. All microchannels are 200 um wide. Each nanochannel is 100 microns long. The rectangular nanochannels are 20 microns wide. The top ports are pressurized with 100 mbar and the right ports are electrically biased.

Figure 1. *Micrograph of the silicon-glass nanochannel device.*



The top and bottom microchannels are seen as dark lines. They are connected in the middle by a series of nanochannels (light green triangles and rectangles in the blow-up). The device shown in Figure 1 was fabricated at Macquarie University by first etching the microchannels to a depth of 10

um into silicon wafers by potassium hydroxide etching. The wafer was then coated in 110 nm of new thermal oxide into which 80 nm deep nanochannels were etched by CF_4 plasma.

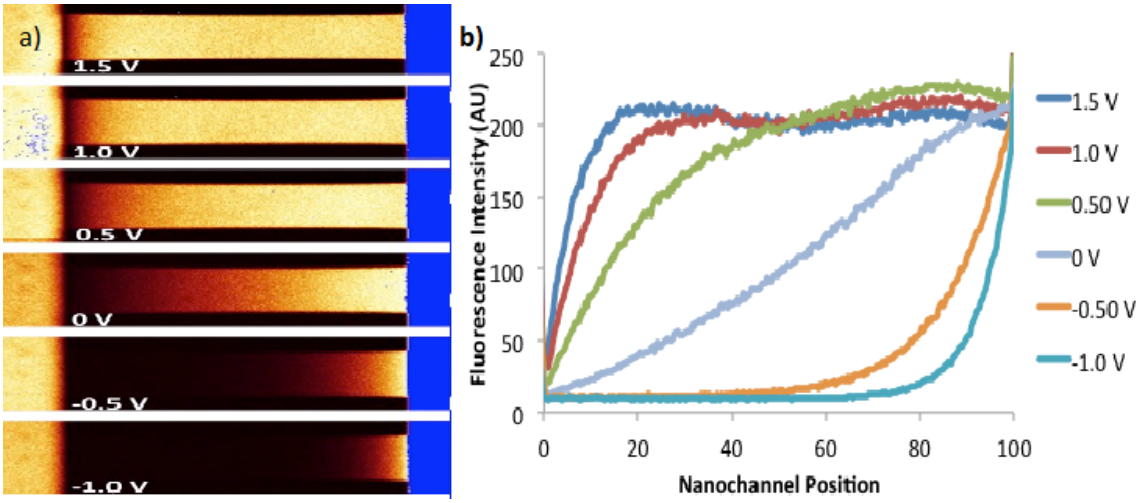
Previous work (Inglis et al., 2011) made assumptions about the shape of the concentration gradient. In this work, there are no such assumptions and direct visualization of this concentration gradient, using a buffered fluorescent salt, is demonstrated. To this aim, two molar parts TRIS are mixed with 1 molar part fluorescein acid (both from Sigma Aldrich) in de-ionized water. Full ionization of the fluorescein acid and partial ionization of the buffering TRIS base leaving a fluorescent solution with a pH near the pKa of TRIS, 8.06, is expected. The solution is diluted to 1.5 mM (combined concentration) to achieve complete solubility. This bright fluorescent salt solution can then be used to directly observe the behavior of ions in the nanochannel.

The concentration gradient is set up with 3 µM Tris Fluorescein (pH 8.0) in the left microchannel (see Figure 2a, low salt side), and 1.5 mM in the right (high salt side). A circuit is created, where the high salt, right microchannel is connected to the positive terminal of the voltage source, and the low salt, left microchannel is earthed.

Figure 2a illustrates the differences in fluorescence intensity along the nanochannel at positive and negative voltages—while Figure 2b allows us to analyse these results graphically. A fluorescence microscope was used and the images were analysed with a software program called ImageJ (Rasband, 1997-2011). At 0V (see Figure 2b), there is a roughly linear gradient between the high and low salt reservoirs, meaning very little solution flow. At positive voltage, electro-osmosis carries the high salt solution into the nanochannel. At negative voltage electro-osmosis carries the low salt

solution into the nanochannel. Furthermore, with increasing voltage, the gradient becomes steeper. The sharp gradient in ion concentration will cause a sharp gradient in the electric field. The following section shows how the shifting and sharpening of this gradient affects the molecular trap.

Figure 2. *Analysing the conductivity gradient of TRIS-Fluorescein.*



a) *Imaging the salt gradient of TRIS-Fluorescein. The right side is biased positively. The microchannels, being much deeper, are brighter than the nanochannels. The blue colour indicates intensity-saturation in the right side microchannel.* ***b)*** *Fluorescence intensity versus nanochannel position for the images in Figure 2a. Both the position and width are measured first in pixels—then converted to micrometers. The entire length of the rectangular nanochannels is 100um with 0 at the low salt side and 100 at the high salt side.*

Part II: Protein Trapping and Concentration

So far this paper has only discussed how the conductivity gradient behaves in the channel. Now it moves on to discuss how a fluorescent protein, R-phycoerythrin, diluted

into a standard buffer solution behaves. Here there is no fluorescent salt, only standard phosphate buffered solution, with varying NaCl levels and a fluorescent protein.

A concentration gradient is again established between a high salt microchannel (right), which also contains the protein (Phosphate buffer (PB) containing 241mM NaCl, pH 7.8, 25 nM R-PE), and a low salt side (left) (PB containing 4.8mM NaCl, pH 7.8). The protein can be added to either solution, but has a longer shelf life in the higher salt buffer. This time, the high salt side is again connected to the positive terminal of the voltage source and the low salt side to the ground.

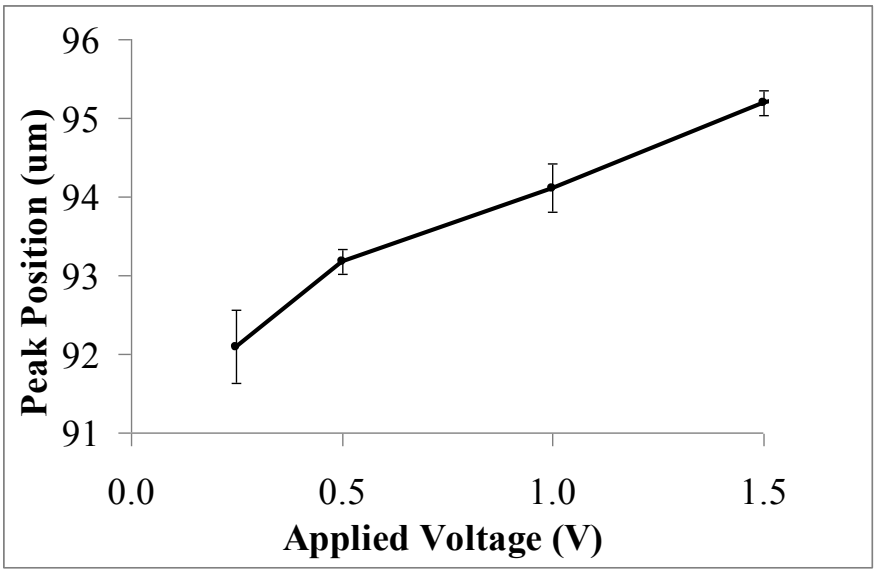
The circuit is connected up in the same way as was done with the TRIS-Fluorescein. To maintain consistent flow of protein and buffer in the system throughout the experiment, the microchannel terminals are connected up to a pressure-flow meter (approx. 50 mbar both microchannels; 100nL/min). Without flow in the microchannels the high salt buffer that is transported through the nanochannel would quickly increase the conductivity at the low salt reservoir, which would alter the conductivity gradient. It is expected that the salt gradient will behave as it did above – that is, that the positive voltage leads to a sharp drop in salt concentration at the left side, and consequently a sharp spike in electric field. Here, however, this gradient cannot be seen because it is formed in NaCl.

A negatively charged protein dissolved in solution will become trapped by the stable electric field spike at the low salt end which opposes the stable electro-osmotic flow. A more negatively charged protein will stack further to the right and a positively charged protein will not stack at all. The trapped protein forms a bright band or peak in

the nanochannel when viewed with a fluorescence microscope. The width of this band is determined by the diffusion constant of the protein and the strength of the trapping forces. The trapping force is proportional to the gradient of the force equation at the trapping point. As the fluid velocity is constant, this force is proportional to the gradient in electric field, or the gradient in ion concentration. This behavior is characterized by measuring the position, brightness and width of this bright peak.

Figure 3 below shows the peak position versus applied voltage. As the voltage is increased, the peak position of concentration (indicated by fluorescence intensity) moves forward in the nanochannel, towards the low salt microchannel reservoir—just like the concentration gradient moved forward with TRIS-Fluorescein. Relatively negligible standard errors are observed throughout Figures 3, 4 and 5. Note that at 0V and negative voltage, no peaks are observed.

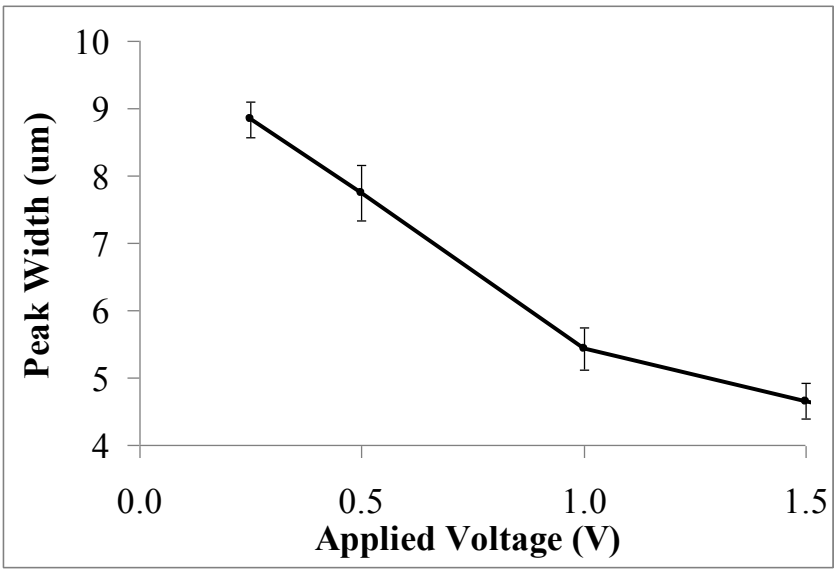
Figure 3. *Peak position versus applied voltage.*



Both the position and width are measured first in pixels—then converted to micrometers. The entire length of the rectangular nanochannels is 100um with 0 at the high salt side. The data points are averages of values taken over 3 experiments from the same device. The errors bars in these figures indicate standard error of the mean.

Figure 4 on the following page shows that peak width decreases as a function of applied voltage as expected from the sharpening gradient observed above (see Figure 2b). Peak width is defined as the full width at half the maximum height of the peak intensity. In the TRIS-Fluorescein experiments it was observed that the gradient moved toward the low-voltage, low-salt end and that the gradient of the salt solution increased. This higher gradient causes greater confinement forces which focus the peak and narrows its width.

Figure 4. *Peak width versus applied voltage.*

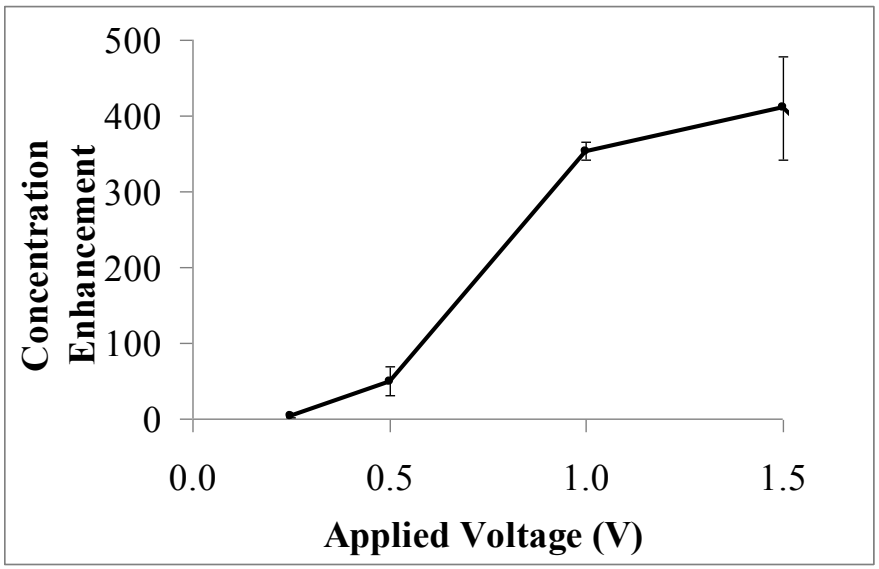


The data points are averages of values taken over 3 experiments from the same device. The errors bars in these figures indicate standard error of the mean.

The brightness of the peak is measured and compared with the brightness of the microchannel. Knowing the relative depths of the channels allow us to estimate the protein concentration in the nanochannel trap. If the concentration in the nanochannel is the same as it is in the microchannel it is expected that the brightness in the nanochannel will be lower by a factor of 80 nm divided by 10 micrometers. If the brightness of the peak is equal to the brightness of the microchannel then it can be deduced that the concentration in the peak is (10/0.08 which equals) 125 times higher than it is in the microchannel. This is named concentration enhancement.

Figure 5 below shows how concentration enhancement of R-phycoerythrin is affected by applied voltage. As the voltage is increased, the electric field is increased, which in turn increases the electro-osmotic flow and brings more molecules through the nanochannel in a given time, resulting in greater amounts of analyte being trapped. The peak intensity grows with time, but for simplicity the concentration enhancements are compared 1 minute after the voltage is turned on.

Figure 5. *Concentration enhancement at 1 minute versus applied voltage.*



The data points are averages of values taken over 3 experiments from the same device. The errors bars in these figures indicate standard error of the mean.

Discussion

This paper has illustrated that there is a significant observable effect of voltage on (1) the shape of the salt gradient and (2) the trapping of molecules inside the nanochannel. Positive voltage pushes the high salt solution into the nanochannel while negative voltage pushes the low salt solution into the nanochannel. Higher positive voltages move the gradient toward the low salt end and sharpen the gradient. This agrees with results obtained by trapping a protein on the gradient, which was shown in this project with the protein R-phycoerythrin. In these results, it can be seen that higher voltages push the trap toward the low salt end and this sharpens the focused band, indicating a higher gradient in the force.

The two experiments are quite different with regards to the conductivity in the nanochannels and consequently the electric double layer thickness. The TRIS-Fluorescein is in the micromolar range, giving Debye layer thicknesses that are 10s of nanometers—which is significant with respect to the channel depth. The protein work, on the other hand, uses millimolar salts, giving Debye layer thicknesses that are nanometres thick. While certain nanofluidic phenomena are more prevalent when the Debye layer thickness is comparable to the channel thickness, the protein work must be done in near physiological salt levels to keep the protein in its natural configuration. At very low salt levels the protein does not fluoresce. Despite the thousand-fold difference in conductivity between the two experiments strong agreement in behavior can be seen.

There are some concerns that may need to be addressed in future studies. The first is that it is difficult to mathematically model these observed effects—and this leads to some weakness in being able to define the relationship between parameters (applied voltage, conductivity gradient) exactly. Clearly there are some effects that can be observed, which can also be predicted—but these predictions have yet to be quantified—and as a result, future studies need to more precisely understand the fundamental physical principles at play (electric field, force). Current work is focused on large scale numerical modeling of the ions, proteins, surface charges, electric field, chemistry and solution flow in the channel.

Another concern is that it is difficult to determine the voltage inside the nanochannels. This is because it is difficult to connect electrodes directly to a

nanochannel—therefore, platinum electrodes are connected to microchannels as seen in Figure 1. Knowledge of electrical circuits (including Ohm's law) could be used to build an approximate electrical circuit model of the system. However, it has been observed that there appears to be significant leakage of current through the silicon wafer of the chips—so such a circuit model is invalid.

Conclusion

This work clearly illustrates the significant effect of the voltage on molecules of interest in nanochannels. There is now a need to quantify and accurately model these observed effects. This paper suggests that should these effects be modeled accurately, there may be scope for manipulation of these effects. An ability to control and use nanofluidic channels for molecular detection will be an attractive alternative to the myriad existing biomarker trapping methods—that require more sample and buffer, and more money, to use effectively.

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