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Calibrant-Free Analyte Quantitation Via a Variable Velocity Flow Cell

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Abstract

In this paper, we describe a novel method for analyte quantitation that does not rely on calibrants, internal standards, or calibration curves but, rather, leverages the relationship between disparate and predictable surface-directed analyte flux to an array of sensing addresses and a measured resultant signal. To reduce this concept to practice, we fabricated two flow cells such that the mean linear fluid velocity, U , was varied systematically over an array of electrodes positioned along the flow axis. This resulted in a predictable variation of the address-directed flux of a redox analyte, ferrocenedimethanol (FDM). The resultant limiting currents measured at a series of these electrodes, and accurately described by a convective-diffusive transport model, provided a means to calculate an “unknown” concentration without the use of calibrants, internal standards, or a calibration curve. Furthermore, the experiment and concentration calculation only takes minutes to perform. Deviation in calculated FDM concentrations from true values was minimized to less than 0.5% when empirically derived values of U were employed.

TOC graphic

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Introduction

The need for quantitative chemical analysis extends far beyond the chemistry laboratory to fuel and alternative energy industries,^{1–2} catalysis,³ forensics,⁴ environmental analysis and remediation,^{5–6} clinical analyses,⁷ *in vitro* diagnostics,⁸ and many other areas. In some cases, the amount of analyte can be accurately determined using simple measurements and known physical constants (*e.g.*, gravimetric and coulometric methods).⁹ It is much more likely, however, that quantitation will rely on the use of calibrants and calibration plots.¹⁰

The use of standards or calibrants to aid in determining unknown analyte concentrations is a long-established and highly effective analytical procedure. The standard curve method,¹¹ in which the response of an unknown is compared to those of a series of standard solutions, is useful when the matrix of the sample does not suppress or enhance the response with respect to the matrix of the standards.¹² Addition of an internal standard to a sample gives rise to a method in which analyte concentration can be determined relative to that of the internal standard; this approach minimizes possible matrix effects. An alternate calibration scheme to that of the internal standard method is the standard addition method (SAM). The SAM, which results in an absolute concentration determination rather than one that is based on relative responses, is attributed to mining chemist Hans Hohn¹³ and is detailed in his 1937 book on polarography, *Chemische Analysen mit dem Polarographen*.¹⁴ The SAM is generally preferable to the use of calibration curves, especially in cases where the analyte matrix is unknown or difficult to replicate, but is not always practical.¹⁵

These three calibration methods address the historical needs of accurate analyte quantitation, but as the benefits of simultaneously monitoring ever increasing numbers of analytes – analyte panels – are contemplated,^{16–23} implementation of historical calibration methods becomes more awkward¹⁹ and, in some cases, intractable. For example, when attempting to analyze hundreds of markers in a limited-volume sample in which matrix effects cannot be easily overcome, it is not possible to employ a traditional calibration scheme. It was our intent to develop and reduce to practice a method of quantitative analyte analysis that does not rely on calibrants and could address the quantitative conundrum posed by large analyte panels. Our approach leverages the fact that, in some simple convective systems (*e.g.*, rotational or variable-width channel laminar flow cells), the amount of analyte impinging a discrete location – the analyte flux at an address – can be made to vary in a predictable manner and in a manner that can be described analytically. In such a system, the different analyte-induced responses from a set of these addresses can be fit to the analytical expression using only analyte concentration as the fitting parameter. In this manner, the concentration of a single analyte or a panel of analytes can be determined without the need of calibrants, standard curves, or internal standards.

There are a number of benefits that could potentially be realized if the need for calibration curves was removed, including reductions in labor, time, and materials. These benefits would prove especially valuable in light of the growing focus on large-scale multiplexed tests with respect to cancer diagnostics^{21–22} and personalized medicine.^{20, 23} The concept examined herein may also have utility in instances where the availability of consistent high

quality standards is problematic, a situation often encountered in infectious disease diagnoses (e.g., standards of potential antigenic markers for tuberculosis).^{24–25}

In this paper, we explore the reduction of the envisioned calibrant-free quantitation scheme to practice through the use of an electrochemical redox couple and a simple flow cell. Though only the first step in the process, we anticipate this platform will ultimately prove valuable in the assessment of *in vitro* diagnostic marker panels. Our test platform is comprised of a fluid cell, Figure 1, in which the mean linear velocity (U , cm/s) of an analyte-containing fluid changes as the fluid moves across a series of gold electrodes, or addresses, aligned along the central flow axis of the cell. The addresses are positioned within a polydimethylsiloxane (PDMS) flow channel whose sidewalls converge to give rise to different U at each electrode. At a fixed volumetric flow rate (V , mL/min), this controlled change in U results in a predictable change of the analyte flux to each electrode. It is this phenomenon that serves as the basis for our proposed quantitation strategy.

The theoretical underpinnings for this concept draw upon the classic treatment by Levich of the convective-diffusive transport of a dissolved redox couple in conjunction with a rotating disc electrode.²⁶ In subsequent work,²⁷ Levich solved the transport process for the channel electrode analog (laminar flow over a planar electrode; see Figure 2). With this flow architecture, the steady-state limiting current, i_{lim} , is proportional to the cube root of the mean linear velocity over the electrode, $U^{1/3}$, and is expressed as

$$i_{lim} = \pm 1.165nFC_0w \left(\frac{l^2 D^2}{h} \right)^{1/3} U^{1/3} + k \quad (\text{Eqn. 1})$$

The sign of Equation 1 indicates the direction of electron transfer (anodic current is positively signed), n (equivalents/mol) is the stoichiometric electron-transfer coefficient, F is the Faraday constant (96,485 C/equivalent), C_0 is the bulk concentration of the soluble electroactive species (mol/cm³), D is the diffusion coefficient of the redox couple (cm²/s), w is the electrode width (cm), l is the electrode length (cm), $2h$ is the channel height (cm), U is the mean linear velocity of the fluid over the electrode (cm/s) in a rectangular channel, and the y -intercept, k , is the current realized without flow.²⁸ The quantitative accuracy of Equation 1 for a variety of applications has been demonstrated,^{29–32} and has served as the basis for electrochemical velocimetry measurements.^{33–35}

When a parameter m is defined as the combined constants in Equation 1, we can write

$$i_{lim} = \pm mC_0U^{1/3} + k \quad (\text{Eqn. 2})$$

in which the product of m and C_0 is the slope. In this form, it is readily apparent that if all of the constants are accurately known, C_0 can be determined from the slope of a plot of $U^{1/3}$ vs. i_{lim} .

If a fluid containing an electroactive species, *Red*, of unknown concentration flows at a fixed V through the cell depicted in Figure 1, U will systematically increase from the inlet to the outlet as the fluid flows along the central axis of the cell. As a result, the flux of reactant to each electrode will increase as the fluid moves from address 7 to address 1. If a sufficiently positive potential is applied to the electrode, *Red* will be converted to *Ox* (Equation 3) and the transport-limited current, i_{lim} , will be proportional to C_o , n , and $U^{1/3}$.

In conjunction with Equation 1, the measured i_{lim} for any electrode could then be used to calculate C_o in a solution of unknown concentration if all of the physical constants are accurately known. Of special importance is having accurate knowledge of the analyte's diffusion coefficient, particularly in instances where the viscosity of the samples differ from that given in the literature. For example, 1,1'-ferrocenedimethanol's (FMD) diffusion coefficient of 6.4×10^{-6} cm/s would decrease (according to the Stokes-Einstein relation) to 3.5×10^{-6} cm/s if the medium was changed from water (0.89 cP) to human serum (1.64 cP).³⁷ Given that $i_{lim} \propto D^{2/3}$, the i_{lim} of an FMD measurement in serum would be expected to be ~ 67% of the current measured in water, if all other conditions were equivalent. However, as a series of i_{lim} could be measured for a given V (*i.e.*, one i_{lim} at each of the electrodes of Figure 1), C_o can be more accurately determined from a plot of i_{lim} vs. $U^{1/3}$. When these data are fit to Equation 2, the sole fitting parameter is C_o , providing a straightforward means to determine analyte concentration. Additional sets of i_{lim} for a series of V could be added to the data set, resulting in a higher degree of statistical certainty due to an increased number of sampled points.

For Equation 1 to be valid, the following conditions must be met: (1) fluid flow must be laminar (*i.e.*, Reynolds numbers, $Re < \sim 2000$);³⁸ (2) the measured current must be mass transport-limited, and not diminished by heterogeneous kinetics;³⁹ (3) the concentration of the electroactive species within the layer depleted by the neighboring upstream electrode is replenished to the bulk analyte concentration (*i.e.*, C_o) before encountering the next downstream electrode; and (4) the active electrode area ($A = w \times l$) must not change over the course of the measurements. These conditions can be satisfied by taking advantage of the laminar flow conditions which typify microfluidic constructs and by using a nonadsorbing, kinetically fast electroactive species, which conceptually would enable the repeated use of the platform over a range of flow rates bounded by the pump apparatus (low V values) and by the heterogeneous electron-transfer reaction rate (high V values). Stipulation 2 implies that kinetic information can be gleaned by employing the type of flow cells described here, and indeed, this has been recently explored in related work.⁴⁰

In this paper, we present results of the proposed calibrant-free quantitation concept using a model redox analyte, 1,1'-ferrocenedimethanol (FDM). These preliminary data demonstrate that C_o can be determined from the linear relationship between i_{lim} and $U^{1/3}$ within a range of V , absent any experimental cell characterization. However, as will be shown, the quantitative accuracy of the method is increased by implementing an improved cell design and replacing theoretical values of U with empirically determined values.

Experimental Section

Flow Cell Fabrication

The channel electrode array was fabricated in a multistep process. Purified 18.2 M Ω -cm water, used throughout this work, was drawn from a Barnstead NanoPure water purification system. Glass substrates (25×75 mm microscope slides, Fisher Scientific) were sonicated in an aqueous 10% Contrad 70 (Decon Labs) solution, followed by rinsing with absolute ethanol (Decon Labs) and high purity water, and drying under a nitrogen stream. The size and shape of the electrodes were defined by resistively evaporating Cr/Au through milled aluminum shadow masks onto the cleaned substrates. The deposition was conducted at low pressure (10^{-8} to 10^{-7} millibar) at 0.4 Å/s in an Edwards Auto306 evaporator. The substrates were slowly rotated during deposition. A 10-nm adhesion layer of chromium (99%, Alpha Aesar) was first deposited, followed by deposition of 200–300 nm of gold (99.995%; Alpha Aesar). These substrates were stored in ambient laboratory conditions until use.

Aluminum shadow masks – thinned to ~0.5 mm to limit shadowing during the deposition process – and acrylic PDMS molds (PN 8560K168, McMaster Carr), fabricated using an in-house CNC-milling machine (CNC Masters, Irwindale, CA), were used to construct two versions of the flow cell: Ver. 1 and Ver. 2. Both versions contained 0.079 cm-wide (1/32 in.) electrode leads and electrical contact pads 0.318 cm (1/8 in.)-wide. The Ver. 1 design used circular electrodes that were 0.238 cm (3/32 in.) in diameter, duplicated on a 1.065 cm pitch; the PDMS flow channel had a 14° divergence. The electrodes used for Ver. 2 were rectangular in shape (0.190 cm×0.355 cm [$w\times l$], with a 0.040 cm [1/64 in.] corner radius patterned on a 1.065 cm pitch. The Ver. 1 and Ver. 2 flow cells were each milled to have channel widths (d) of 0.590, 0.842, 1.094, 1.346, and 1.598 cm over the center of electrodes 2–6, respectively.

Flow Cell Assembly

The patterned microscope slides were cleaned immediately before attachment of the PDMS flow channels by rinsing first with ethanol and then water, followed by a brief (a few seconds) immersion in piranha solution (3:1 conc. sulfuric acid: 30% hydrogen peroxide). Sulfuric acid and peroxide were obtained from Fisher Scientific. *Great care should be taken, and the proper safety equipment utilized, when working with piranha solutions, which are extremely caustic, can reach high temperatures, and may be explosive under certain conditions.* After rinsing with water and drying with a stream of nitrogen, the electrode leads exposed within the flow channel were passivated by carefully applying a thin layer of UV-curable photopolymer (Norland Optical Adhesive #61) with a fine-tipped paintbrush under stereoscope examination. The photopolymer was cured by exposure to UV light in a homemade light box for ~1 h.

PDMS channels were formed by pouring uncured PDMS (Sylgard 184 elastomer kit, Dow Corning) over the milled acrylic molds (cleaned with ethanol immediately prior to use) and curing at 60–70 °C for a minimum of 1 h. The cured channels were then removed from the mold, rinsed, and stored in absolute ethanol until use. Before assembly, the PDMS channels

were dried with a stream of nitrogen, and tubing ports were formed directly above addresses 1 and 7 using 2 and 3 mm diameter biopsy punches (Miltex), respectively.

The cured PDMS flow channels were adhered to the glass substrates with a thin layer of uncured PDMS. Application was performed by spin-coating the PDMS at 2000 rpm onto a 4 in. silicon wafer (University Wafer), and the PDMS channels were placed onto the coated wafer to transfer a thin, even coat of PDMS. The coated PDMS channels were then aligned onto the glass substrates such that the active electrode areas were positioned along the center of the flow channel. Proper alignment also resulted in a 1 mm wide electrical contact pad for each electrode. Thin-walled Teflon tubing was inserted into the ports and again, uncured PDMS was used as a sealant to prevent leakage at the tube-channel junctions. The assembled cell was allowed to dry for ~12 h at ambient and cured 60–70 °C for at least 1 h, a procedure that gave a leak-tight seal.

Flow Cell Operation

During operation, the flow cells were inverted and the inlet tube (1/8 in. OD), located at the wide end of the channel above address 7, was placed in a reservoir containing the analyte solution and a Ag/AgCl reference electrode (saturated KCl; BAS Inc.). The exit tube, located at the narrow end of the channel above address 1, was connected to a 60 mL Luer-lock plastic syringe that was mounted in a Harvard Apparatus PHD 2000 syringe pump. The syringe pump was operated in the refill mode and was calibrated gravimetrically. Either electrode 1 or 7 were used as on-chip auxiliary electrodes.

All electrochemical data were collected using a CH Instruments 600A potentiostat one electrode at a time. A homemade wiring harness and smooth-jawed copper clips were used to make electrical connections to the electrode assembly.

Amperometry of 1,1'-ferrocenedimethanol

FDM (Sigma Aldrich, 98%, diffusion coefficient⁴¹ of $6.4 \times 10^{-6} \pm 3.9 \times 10^{-7}$ cm²/s) solutions were prepared in 1.00 M NaCl (Fisher Scientific). Immediately prior to analysis, the working electrodes (2–6) were activated by cycling the potential between +0.4 and +1.5 V vs. Ag/AgCl (satd. KCl) in 0.1 M H₂SO₄ at a sweep rate of 50 mV/s for a minimum of 15 cycles.⁴² The observed voltammetric waves showed the characteristic signatures for the formation and subsequent reduction of gold oxide. After cleaning, the cells were rinsed with electrolyte solution and the working solution was introduced. Each electrode was interrogated over the entire range of flow rates before moving to the next electrode. Fluid was allowed to flow for several seconds prior to stepping the applied potential from 0.000 V to +0.600 V vs. Ag/AgCl such that steady-state fluid dynamics were established prior to amperometric data collection. Measurements in the supporting electrolyte were used to account for background responses.

Some pulsation was observed in all of the current-time traces that were acquired during flow. The magnitude of these pulses, when normalized to the average limiting current, i_{lim} , decreased with increasing flow rate and ranged from 6.3 to 2.3%. The frequency of these pulses was independent of the type of syringe used; their existence was attributed to the operation of the syringe pump. For purposes of data presentation, the pulsatility was

removed from the current-time traces (Figure 4 inset) using a running-average smoothing algorithm (Sigma Plot ver. 11.0; sampling proportion of 0.2).

Modeling

In order to characterize the fluid dynamics present during cell operation and optimize cell design, simulations were run using COMSOL Multiphysics. Cell geometries were imported from the three-dimensional drafting figures used for cell fabrication (AutoCAD). No-slip boundary conditions were imposed on the channel walls, and the system of equations for the fluid ports, boundaries, and fluid were solved using the COMSOL finite difference solver. Constant flow rate and constant pressure conditions were specified for the inlet and outlet, respectively. This modeling yielded fluidic stream lines and velocity profiles as a function of position for both versions of the flow cell. Re values were calculated from the obtained velocity values, and the largest of these ($Re = 107$ at $V = 10$ mL/min) was still well within the laminar flow regime.

Results and Discussion

The key to success of our calibrant-free quantitation concept is the ability to vary analyte flux over a series of addresses in a predictable manner at a given volumetric flow rate, V . To realize optimal flow cell geometries, two were selected from several modeled designs. The first, Ver. 1, is depicted in Figures 1 and 3. The cell consists of an array of electrodes and a PDMS fluid channel with an inlet and outlet port. The seven gold electrodes are aligned along the center of the axisymmetric flow channel. The sidewalls of the flow channel undergo a steady 14° decrease in wall separation from fluid inlet to outlet, which results in a continuous increase in the mean linear velocity, U . This channel angle was chosen to result in the largest change in U , given the footprint of the underlying glass support. At $V = 10$ mL/min, this architecture yields a range of $0.90 < U < 4.93$ cm s⁻¹. These values were calculated by:

$$U = \frac{V}{2hd} \quad (\text{Eqn. 4})$$

where $2h$ is the channel height (0.10 cm) and d is the width of the channel directly over the center of each electrode. The ratio of the smallest and largest U – maximum relative U or U_r – capable of being realized at one V is an important design metric; in this case $U_r = 5.48$. A flow cell with an electrode array spanning a large range of U increases the ability to resolve between different analyte concentrations. Additionally, a large range of U helps to ensure that measurable currents are observed, while also minimizing the possibility that heterogeneous redox kinetics affect the measured signal.

Equation 4 was used to obtain the mean linear fluid velocity values employed in all of the calculations presented in this work. However, in an effort to understand the fluidic conditions present locally in the cells during operation, finite elemental analysis (COMSOL) was employed. This modeling provided the fluid velocity profiles present in the Ver. 1 flow cell during flow; a simulation result displaying fluid velocity stream lines in the cell at $V = 5$

mL/min is shown in Figure 3. As expected, the stream lines show that the requisite laminar and unidirectional fluidic conditions are not met for electrodes 1 and 7, both of which reside directly under the fluid ports. Those electrodes were therefore excluded from these analyses and functioned instead as on-board auxiliary electrodes, which reduces U_r to 2.71.

Shown in Figure 4 are limiting current densities, j_{lim} (i.e., i_{lim} normalized to electrode area), measured at electrode 2 over series of V ranging from 1 to 10 mL/min. These data were acquired by flowing aqueous FDM (0.809 mM in 1.00 M NaCl) through the cell and stepping the applied potential from 0.000 V to +0.600 V vs. Ag/AgCl (satd. KCl) 5 s after flow initiation. The quiescent period was used to establish a steady-state flow profile prior to the anodic potential step. The electrolysis potential was chosen to be sufficiently positive of the formal reduction potential of the FDM/FDM⁺ redox couple ($E^{\circ'} = +0.23$ V vs. Ag/AgCl) to ensure that the measured current was transport-limited and not diminished by the rate of the heterogeneous electron transfer reaction.

The resultant current was recorded for 45 s, and the average for the last 30 s recorded at electrode was plotted as a function of $U^{1/3}$. These data display excellent linearity over the entire range of $U^{1/3}$ ($R^2 = 0.999$), which indicates that the measured responses were not affected by reaction kinetics. The error bars represent the standard deviation of the current during the sampling period, and are omitted from subsequent presentation of amperometric data for increased clarity. When fit using Equation 2, a concentration of 0.703 mM is calculated from the slope ($124 \mu\text{A cm}^{-2}/(\text{cm s}^{-1})^{1/3}$) – a relative error of –13.1% – which confirms that analyte flux is directly proportional to analyte concentration and that the treatment synopsized by Equation 1 is a promising approach to calibrant-free quantitation. However, this result does not validate the approach.

The complete data set, i.e., electrodes 2 through 6 at each of the seven V , is shown in Figure 5. The amperometry data was collected from one address for one flow rate at a time.

When evaluated electrode-set by electrode-set at each flow rate, it is clear that j_{lim} vs. $U^{1/3}$ for each set at a given V is linear (sets of identically colored data points in Figure 5). The calculated C_o from the best-fit slope for all of the data ($120 \mu\text{A cm}^{-2}/(\text{cm s}^{-1})^{1/3}$), is 0.68 mM – a –16% relative error from the true FDM concentration. We believe that the discrepancies in true vs. calculated FDM values arise from a number of factors. First, a value of l was chosen to equal to the width of a square having the same area as the 0.238 cm circular electrodes in the flow cell. The derivations from Levich were aimed at rectangular rather than circular electrodes; circular electrodes require a different mathematical treatment. The lower overall efficiency (~84%) for the electrolysis of a liquid flowing over a disc electrode as compared to a rectangular electrode⁴³ points to the need of a modified theory to accurately describe our Ver. 1 flow cell. Second, the constant 14° convergence of the channel meant that any misalignment in the long axis of the PDMS channel with the underlying substrate resulted in a concomitant miscalculation in U .

One approach – akin to the assignment of a “cell constant” in classic bulk electrolysis⁴⁴ – to address these probable issues is to use a known concentration as a standard and calculate the unknown using a ratio equality of standard vs. unknown:

$$\frac{[\text{Standard}]}{[\text{Unknown}]} = \frac{\text{Slope}_{\text{standard}}}{\text{Slope}_{\text{unknown}}} \quad (\text{Eqn. 5})$$

In other words, if the flow cell is calibrated using a known standard, the concentration of an unknown solution can be determined with a high degree of accuracy. To test the validity of this method, we introduce a second FDM concentration. Shown in Figure 5 is the second j_{lim} vs. $U^{1/3}$ plot for 0.675 mM FDM; using Equation 2, C_0 calculated from the complete data set is 0.563 mM – a relative error of –17%. However, if the approach described by Equation 5 is used, an FDM concentration of 0.669 mM is calculated, which errs by less than –1% from the true value of 0.675 mM. This tactic, while valuable, does not truly align with our vision for quantitation methodology, and is not as impactful as the ability to directly quantitate an analyte concentration with only the knowledge of the device geometry and D of the analyte of interest.

Another method to improve the predictive value of our quantitation scheme is to address the shortcomings of the Ver. 1 cell. To that end, we designed a Ver. 2 flow cell. As shown in the Figure 6 schematic, the electrodes used with this cell were designed with rectangular geometries to increase the appropriateness of Equation 1 to describe measured j_{lim} . The Ver. 2 cell was designed to provide the same usable range of $U^{1/3}$ over electrodes 2 through 6 and the same channel wall spacing, d , as the Ver. 1 cell. The 14°-channel wall divergence was replaced by a stepped design in an attempt to provide a more constant U over each electrode. The electrodes were designed to be spaced (a distance d') sufficiently from the cell wall necking points (points where changes in d occur) so the fluid flow was fully developed (*i.e.*, $d' >$ the lead-in length) before reaching the electrodes.⁴⁵ Values for d' were optimized for each electrode, at each necking point, and over the range of Re numbers applicable to our experiments.

As the no-slip boundary condition at the sidewall surfaces results in a plateaued flow profile across the channel, the electrodes were positioned a distance d'' from the sidewall to minimize the variation in U over w . The modeling results that informed that decision are presented in Figure 7, where the x -component of the fluid velocity, u_x was computed over the width of the channel (d) along the centerline of each electrode and at a z -position equal to 0 – the center of the channel height (see Figure 2.). Analysis of these modeling results resulted in $w = 0.190$ cm. The electrodes were equally spaced from each other (s value) in a manner such that the closest point-to-point distance was $2l$, which resulted in $l = 0.355$ cm. This design choice was motivated by the need to satisfy stipulation 3. While not relevant to the data presented in this work (as electrodes were measured one at a time, rather than simultaneously), our simulations (not shown) suggested that stipulation 3 was not completely satisfied in the current electrode configuration; future iterations of cell design will attempt to address this issue.

To test whether the predictive abilities of the new cell were improved relative to Ver. 1, the experiments whose results are presented in Figure 5 were repeated with the Ver. 2 cell. The chronoamperometric data, collected using identical experimental procedures and the results of i_{lim} as a function of $U^{1/3}$ for every electrode and flow rate, are presented in Figure 8. The results of a linear fit to the 2–6 set of electrodes at a single V , which is the preferred analysis method, displayed errors for each V of $\sim -10\%$. The value of C_o computed from the best-fit slope ($7.71 \mu\text{A s}^{1/3} \text{cm}^{-1/3}$) in conjunction with Equation 2 was 0.77 mM , a -3.9% error relative to the true value of 0.801 mM . This result demonstrates improvement in the quantitative predictive accuracy of the Ver. 2 cell relative to Ver. 1.

We believe that the discrepancy between the calculated and true concentration values is largely a result of the fluid velocities being poorly described by the simple geometric expression of Equation 4. Potential sources of the error include the compliant nature of the PDMS channel and any fluidic disturbances caused by the photopolymer film used to passivate the electrode leads. Therefore, we sought to measure U at each electrode for each V used and employ those empirically-determined values rather than theoretical U . Ostensibly, this fluid velocity calculation would need only be performed once per flow cell version.

Empirical U at each electrode and each V were determined by measuring i_{lim} of a 0.818 mM FDM solution. The i_{lim} values and the true C_o were then used to solve for U at each electrode and V . The empirical U values obtained in this manner were found to vary by 7.29×10^{-4} to $4.67 \times 10^{-2} \text{ cm/s}$ from the theoretical values calculated using Equation 4. To evaluate performance improvements using these empirically determined U , values of i_{lim} measured for an “unknown” FDM concentration (0.591 mM) were plotted against the empirical $U^{1/3}$ (Figure 9). Upon inspection of those data, the improvement in agreement of the data from electrode to electrode and across all V is immediately clear. Linear fits of i_{lim} vs. $U^{1/3}$ were applied to all data points (all V at all electrodes). The slopes of these fits and resultant concentration calculations, along with comparison to the true value of 0.591 mM , for each of the subsets, appear in Table 1. The standard deviations listed were calculated from a propagation of the errors associated with the parameters in Equation 1. The correction applied by the use of empirical U greatly improved the calculated FDM concentrations in relation to the true value, as the magnitude of the errors were $< 1.5\%$ for all data sets. The error was found to decrease with increasing V , which gives insight into the selection of the optimal V to use in future analyses. Preliminary results suggested a lower limit of $V = 2 \text{ mL/min}$ due to the pulsatile nature of flow generated from a syringe pump, which was found to be more problematic at low values of V . In order to remove complication associated with pulsed flow, future experiments are planned to utilize an alternate pump and pulse dampers.

Conclusions

Though currently in a rudimentary state, we have reduced to practice a concept of calibrant-free analyte quantitation and believe that lessons learned through these studies can be applied to more complicated systems in which quantitation is currently difficult. The approach hinges on the ability to realize disparate and predictable analyte flux to a series of “sensing” addresses. Signals measured as a function of that flux are succinctly described by

an analytical expression and the inherent analyte concentration is calculated using only knowledge of the platform's geometry and descriptors of its convective nature. To demonstrate the validity of this approach, two flow cells were constructed such that the linear fluid velocity, U , was made to vary systematically over a series of electrodes. This resulted in a predictable variation of the address-directed flux of a redox analyte, ferrocenedimethanol (FDM). The resultant limiting currents measured at a series of these electrodes and accurately described by a convective-diffusive transport model provided a means to calculate an "unknown" concentration without the use of calibrants, internal standards, or a calibration curve. Furthermore, the experiment and concentration calculation takes only minutes to perform.

Using this approach, agreement between calculated FDM concentrations and the true values varied between 4% and 10%. Believing this discrepancy is due to inaccurate theoretical estimates of U , we employed empirical U values derived using a known FDM concentration. The improvement in calculated vs. true concentration agreement was immediately clear and better than 0.5% discrepancies were observed at volumetric flow rates larger than 2 mL/min. Though demonstrated in a laminar flow cell, this approach is equally valid for any system in which disparate analyte flux can be realized at an array of addresses. The approach is not limited to convective systems; cell designs employing diffusive, migration, capillary, absorption, evaporation, magnetophoretic, or precipitation mass transport phenomena could be used to determine analyte concentration in a calibrant-free manner.

Though in its current form the proposed concept is not well suited for quantitation in immunocomplexation systems, we suggest that a modified version of this method may be useful for calibrant-free quantitation of antibody-recognized analytes (*e.g.*, in multiplexed heterogeneous immunoassays such as serum-based cancer screening).¹⁶ Convective-driven immunosorbent assays function through analyte complexation at antibody-functionalized addresses,⁴⁶ typically under nonequilibrium conditions.⁴⁷ Depending on binding kinetics, this process may not necessarily be transport limited and results in constantly changing address areas and analyte affinity, and depleted protein concentrations (C_0) at downstream addresses. These phenomena result in situations not likely to satisfy stipulations 2, 3, and 4 (*i.e.*, mass transport-limited, constant C_0 , and invariant address area) and additional considerations must be made.

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Figure 1. Self-calibrating electrode array (Ver. 1). Values of U were calculated using Eqn. 4 at $V = 10$ mL/min.

Figure 2.

Coordinate definition and architecture of channel electrode design. The height and width of the channel, $2h$ and d respectively, are such that laminar flow is predicted over each address of length ℓ and width w . (Figure reprinted from ref 36 with permission from Elsevier.)

Figure 3.

Results of a COMSOL simulation displaying the fluidic stream lines present in Ver. 1 cell ($V = 5 \text{ mL/min}$). Unidirectional laminar flow is observed throughout the cell except the areas directly below the tubing connection ports, which is made more apparent in the magnification inset.

Figure 4.

Chronoamperometric analysis of FMD (0.809 mM in 1.00 M NaCl) for electrode 2 of cell Ver. 1 at multiple flow rates. Data points are the average limiting current density, j_{lim} , from 15–45 s after a potential step to +0.6 V vs Ag/AgCl was applied. The error bars are the standard deviation of the current during this time period. $U^{1/3}$ was calculated using Eqn. 4 with a channel width (d) at electrode 2 of 0.590 cm. Inset: smoothed chronoamperograms for each flow rate.

Figure 5.

Experimental limiting current densities, j_{lim} , obtained using Ver. 1 cell and two FDM solutions. (A) The complete data set of Figure 4. (B) A second complete data set for 0.675 mM FDM. Line equations for the linear least-squares best fit are shown.

Figure 6.

Design of Ver. 2 flow cell. The stepped shape of the channel walls was designed to maintain a uniform U over the entire length, l , of each electrode. Flow is from right to left.

Figure 7. COMSOL simulation results displaying the variation of fluid velocity, u_x , as it varies across the width of the cell (y-position) for electrodes 2–6 at $V = 5$ mL/min in the Ver. 2 cell. Velocities were computed at a height in the center of the cell ($z = 0$). The dashed lines represent the electrode width (w).

Figure 8. Chronoamperometric results obtained for the analysis of 0.801 mM FDM using cell Ver. 2. The calculated concentration differed from the true value by -3.9% .

Figure 9.

Chronoamperometric analysis of FDM (0.591 mM) using a Ver. 2 cell, electrodes 2–6 at $V = 2.00, 3.00, 4.00, 5.00, 7.00,$ and 10.00 mL/min. Empirical values of $U^{1/3}$ used in this plot were obtained after analysis of an 0.818 mM FDM solution using the same cell and experimental procedure.

Table 1

FDM Concentration Calculations.

Data Set ^a	Slope ($\mu\text{A s}^{1/3} \text{ cm}^{-1/3}$)	Calc. Conc. (mM) ^b	% Error ^c
All points	5.96	0.59	-0.2
Add 2	6.00	0.60	1.5
Add 3	6.00	0.60	1.5
Add 4	5.95	0.59	-0.2
Add 5	5.96	0.59	-0.2
Add 6	5.99	0.60	1.5
2.00 mL	5.84	0.58	-1.9
3.00 mL	5.87	0.59	-0.2
4.00 mL	5.88	0.59	-0.2
5.00 mL	5.91	0.59	-0.2
7.00 mL	5.91	0.59	-0.2
10.00 mL	5.91	0.59	-0.2

Notes:

^aValues for the slopes of the linear fits applied to the chronoamperometric data in Figure 9 are broken down by individual address (Add) and V . The resulting FDM concentrations are calculated with Equation 2.

^bThe calculated result is limited to two significant figures due to the precision of the diffusion coefficient.

^cThe percent error represents the comparison of the calculated concentration values to the true FDM concentration – 0.591 mM. The standard deviation (found from the propagation of errors associated with the parameters in Equation 1) was 0.03 mM for all calculated concentrations.