Design and optimization of a double-enzyme glucose assay in microfluidic lab-on-a-chip

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An electrokinetic driven microfluidic lab-on-a-chip was developed for glucose quantification using double-enzyme assay. The enzymatic glucose assay involves the two-step oxidation of glucose, which was catalyzed by hexokinase and glucose-6-phosphate dehydrogenase, with the concomitant reduction of NADP^+ to NADPH. A fluorescence microscopy setup was used to monitor the different processes (fluid flow and enzymatic reaction) in the microfluidic chip. A two-dimensional finite element model was applied to understand the different aspects of design and to improve the performance of the device without extensive prototyping. To our knowledge this is the first work to exploit numerical simulation for understanding a multisubstrate double-enzyme on-chip assay. The assay is very complex to implement in electrokinetically driven continuous system due to the involvement of many species, which has different transport velocity. With the help of numerical simulation, the design parameters, flow rate, enzyme concentration, and reactor length, were optimized. The results from the simulation were in close agreement with the experimental results. A linear relation exists for glucose concentrations from 0.01 to 0.10 g l⁻¹. The reaction time and the amount of enzymes required were drastically reduced compared to off-chip microplate analysis. © 2009 American Institute of Physics. [doi:10.1063/1.3250304]

I. INTRODUCTION

Microfluidic chips or lab-on-a-chip (LOC) devices are used in a wide variety of chemical, (bio)medical, food, and environmental applications.^{1,2} Typical assays implemented on a LOC device include enzymatic assays,²⁻⁶ immunoassays^{2,7,8} and DNA (Ref. 9), and protein analysis.¹⁰ The scaling down of the assays in microfluidic chips results in (i) a reduction in reagents, (ii) short analysis time, (iii) increased sensitivity, and (iv) a higher degree of automation, portability, and disposability.¹ In addition, through miniaturization, multiple analytes can be simultaneously detected in a small volume of a sample.^{1,11}

With respect to enzymatic assays, microfluidic chips have already proven their strength to different aspects such as determination of enzyme kinetic parameters,^{12,13} quantification of targeted substrates,⁴ bioactive screening, and identification of possible inhibitors.^{14,15} However, the designing of LOC devices involved in enzymatic assays is difficult since the different operational parameters (such as applied voltages, microfluidic channel dimensions, enzyme concentrations, etc.) strongly influence the efficiency of the assay.

Quantification of glucose is vital for clinical diagnosis and food analysis. Quantification of glucose in submicroliter volumes of fluid in a microplate was previously attempted both in food sample¹⁶ and biological fluid¹⁷ using commercially available enzyme assay kits. One of the ad-

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vantages of enzyme based optical analysis of glucose is that it is free from interference of other molecules.¹⁸ This assay involves a two-reaction enzymatic assay with nicotinamide adenine dinucleotide phosphate (NADPH) as end product. Vermeir *et al.*¹⁶ performed this assay in a microplate with a volume of 200 μl ; the assay required 2 μl of enzyme solution and took about 30 min to complete. Here we aim to implement this sequential enzyme assay in a microfluidic chip to significantly reduce both the reagent consumption and the assay time. An inverted fluorescence microscopy setup is used to visualize the different flow processes and to monitor the end product. The transport of the different components (enzymes, samples, and buffer) of the enzymatic assay is achieved by electrokinetic flow. The involvement of many species [such as glucose, hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PDH), NADP^+ , and adenosine-5'-triphosphate (ATP)] and the intermediate product in electrokinetic driven system results in different species velocity, which complicates the mixing process.

Mathematical modeling has proven to be a useful tool to understand the different aspects of a sensor device or to improve the performance of the device without extensive prototyping.^{18–20} Numerical simulation tools are specially required for LOC development due to the ability to analyze tightly coupled physical domains, to help elucidate experimental results, to validate analytical models, to determine values of variables that cannot be measured experimentally, show design feasibility, obtain experimental results rapidly at relatively low cost, and to test and optimize device design.^{7,19,21} Even though modeling of electrokinetic transport in microfluidic LOC device is widely reported,²² there exists little work on modeling complex enzyme assay in such devices.

In this study we demonstrated how a complex double-enzyme glucose assay can be systematically performed in a standard cross-channel microfluidic chip. This was assisted by numerical simulations of a coupled multiphysics model solved in a two-dimensional (2D) geometry. The governing equations within the microfluidic chip are the general and well known fluid flow equations for electrokinetic flow, and mass transfer equations coupled with developed reaction kinetics, which characterize the transport and enzymatic reactions of all species involved. In this work, we couple the above equations, implement the reaction kinetics, design the geometry, provide physical and enzymatic model parameters, and select a suitable solution algorithm for this system. An interface program for model optimization and simulation automation is written. Using the numerical simulation, the flow pattern at different stages of the assay is optimized by changing the applied potentials in the reservoirs. Both simulation and experimental results of the transport study were reported to provide a complete picture of the assay. The effects of other operating parameters such as flow rates, amount of enzyme, and length of the microreactor on the assay signal were extensively studied.

II. MATERIALS AND METHODS

A. Experimental

1. Reagents

Fluorescein sodium salt, NaHCO_3 , and the different components (glucose, ATP, NADP^+ , and MgCl_2) of the enzymatic assay were all purchased from Sigma-Aldrich (St. Louis, Missouri, USA). To visualize the different flow processes in the microfluidic chip, fluorescein solution in a concentration of 0.1 mg l^{-1} was prepared using carbonate buffer (40 mmol l^{-1} , pH9.0). Glucose solutions were prepared freshly every day. All solutions introduced in the reservoirs of the chip were filtered through a 0.22 μm filter (millipore).

2. Microfluidic chip

The enzymatic assay was implemented on a standard cross channel microfluidic chip, fabricated by Micralyne (Edmonton, Canada). The chip was made from low fluorescence Schott borofloat glass and contains four reservoirs identified as A, B, C, and D, as shown in Fig. 1(a), connected by the microchannels that have 50 μm width at the top and 10 μm at the bottom and depth of 20 μm at the center. All reservoirs are 2 mm in diameter and 1.1 mm in depth. Each

FIG. 1. (a) Schematic representation of the three stages of the gating process applied for dispensing a controlled amount of sample from reservoir A to C. The values for the potentials applied at each stage in the reservoirs are shown in Table II. (b) Schematic representation of the experimental setup. Filtered light from the source is directed by the dichroic mirror to the objective and then reaches inside the microchannel. Fluorescence light emitted from fluorophore molecules inside the microchannel will be filtered and directed to CCD camera. Both the camera and the power supply to the LOC device are computer controlled.

reservoir was filled with $2.6 \mu\text{l}$ of solution. Microchannels were sequentially rinsed using 1 mol l^{-1} NaOH, de-ionized water, and freshly prepared buffer between each experiment.

Electrokinetic transport was achieved using a computer controlled high-voltage sequencer (Labsmith, Inc., Livermore, USA) and the electric field between the intersection and the respective reservoir is denoted as E_A , E_B , E_C , and E_D . In order to assure a good connection between the reservoir and the high-voltage sequencer, the microfluidic chip is placed in a homemade chip holder. Positive potentials were applied from the high voltage supplier to the electrode inserted in the liquid filled reservoir of A, B, and D, while electrode in C was grounded.

3. Enzymatic assay

The enzymatic assay involves the two-step oxidation of D-glucose to D-gluconate-6-phosphate with the concomitant reduction in NADP^+ to NADPH according to the following equations:

This assay is previously executed off-chip in a high concentration triethanolamine (TEA) buffer ($pH7.6$).¹⁶ The use of high concentration buffer in electrokinetically driven microfluidic chip not only reduced the electro-osmotic flow but also caused significant Joule heating effect. Hence, kinetic experiments were performed in a microplate (in an analysis volume of 200 μl) at 5, 20, 40, and 100 mM carbonate buffer ($pH9$). The analysis showed that the use of the carbonate buffer has no significant influence on the kinetic of the enzymatic reaction and does not favor higher buffer concentration compared with TEA buffer. Moreover, working at high pH condition minimizes fouling of enzymes on the surface of microchannel and also maximizes the electro-osmotic flow mobility. The carbonate buffer (40 mM) was therefore used in the chip.

Reservoir A contained the enzyme solution, about 14.5 and 7.3 U ml^{-1} HK and G6PDH, respectively. Reservoir B contains the substrate mixture composed of 5.92 g l^{-1} ATP, 2.37 g l^{-1} NADP^+ , 1.24 g l^{-1} of MgCl_2 , and glucose solution. Reservoirs C and D and the microchannels were initially filled with buffer solutions. The on-chip enzymatic assay is executed practically by introducing three stage flow process [shown in Fig. 1(a)] in the microfluidic device. First, both the substrate mixture and enzymes are pushed into the crossing, which is called gating. Second, after reaching steady state flow, enzyme is systematically injected from reservoir A to the reaction channel (long channel connecting reservoir C to the intersection) for a short time. Finally, switching back to the first configuration allows a plug of enzyme to be transported using the carrier fluid that contains the substrate mixture. Consequently, enzymes are then sandwiched by the mixture of glucose and coenzymes resulting in the formation of the fluorescence molecule, NADPH, which is stoichiometrically equivalent to the glucose consumed in the reaction. This method exploits the electrophoretic system to effectively mix reagents of different electrophoretic mobility and separate the product of the reaction using picoliter to nanoliter volumes of reactants injected to the system.²³ The uniqueness of this work is that such a complex assay can be performed in simple cross microchannel using electrokinetic gated injection. Moreover, with the use of the gated injection method many repetitions can be done within a single experimental setup.

4. Fluorescence microscope and imaging setup

The chip holder with the microfluidic chip is mounted on an inverted fluorescence microscope (IX-71, Olympus, Tokyo, Japan), which is illustrated schematically in Fig. 2(b). Images of the microfluidic channels were captured by a cooled (-65°C) electron multiplier charge coupled density (CCD) camera (C9100-13, Hamamatsu, Shizuoka, Japan) with an exposure time of 120 ms. The acquired images have a resolution of 512×512 pixels with a 16 bit dynamic range. This resolution corresponds to a field of view region of $215 \times 215 \mu\text{m}^2$ using a $56\times$ magnification factor. Fluorescein (λ_{ex} 490 nm, λ_{em} 514 nm) was visualized with the aid of filter set U-MWIBA 3 (Olympus), whereas NADPH (λ_{ex} 360 nm, λ_{em} 460 nm) was detected using filter set U-MWU2 UV (Olympus). The camera orientation was aligned carefully in order to capture the center of the cross in the middle of the image for flow analysis. Background images and bright-field images were taken to eliminate temporal fluorescence intensity and pixel noise. The image I was normalized using $I^* = (I - I_{bg}) / (I_{bf} - I_{bg})$, where I_{bg} and I_{bf} are background image and bright-field image, respectively.²⁴ Digital image processing was performed on the recorded image using a program written in MATLAB (R2006b, The Mathworks, Inc., Natick, USA). These data were used for further data analysis.

FIG. 2. Experimental and simulation results for gated injection of fluorescein from reservoir A to C. (a) is loading of fluorescein to the valve, (b) and (c) are the injection of fluorescein into the reaction channel (channel connected to reservoir C), (d)–(f) are the transport of the injected plug down into the reaction channel. Red is maximum concentration and blue refers to zero sample concentration.

B. Governing equations

1. Fluid flow

The following assumptions are made in the formulation of the mathematical models: The fluid is Newtonian and incompressible; flow is steady and the flow and transport processes are effectively 2D; the Debye-layer thickness is small compared with the characteristic channel dimensions; Joule heating is insignificant for electric fields below 800 V cm^{-1} ; and the solution has constant thermodynamic properties and there is no interaction with the channel wall.^{5,6,19,22,24} The governing equations for the electrokinetic fluid flow are the conservation equations for mass [continuity equation, Eq. (2)] and momentum [Navier–Stokes equation, Eq. (3)],

$$\nabla \cdot \mathbf{u} = 0, \quad (2)$$

$$\mathbf{u} \cdot \nabla \mathbf{u} = -\nabla p + \eta \nabla^2 \mathbf{u} + \mathbf{F}, \quad (3)$$

where $\mathbf{u}$ (m s^{-1}) is the velocity vector, p (N m^{-2}) is the pressure, η ($\text{kg m}^{-1} \text{s}^{-1}$) is the dynamic viscosity of fluid, and $\mathbf{F}$ is the externally applied force.

The electrokinetic flow phenomena were modeled by introducing the axial electrostatic force in the double layer as a source term in the Navier–Stokes equations,^{5,22}

$$\mathbf{F} = \rho_e \mathbf{E}. \quad (4)$$

The electric field $\mathbf{E}$ has two contributions, the first one from the external electric potential (ϕ) and the second one from the potential (ψ) due to the charge on the wall. When the double-layer thickness is very small and the charge at the wall is not very large, the Debye and Huckel approximation is valid, and the potential distribution near the wall is governed mainly by the ψ potential and is not affected by the external electrical field.²² In addition, at very low flow veloci-

TABLE I. Kinetic constants of the enzymatic reactions and diffusion coefficients of the different components involved in the enzymatic glucose assay at a temperature of 298 K (Refs. 26–30).

Enzyme	$D \times 10^{-10}$ ($\text{m}^2 \text{s}^{-1}$)	Substrate	K_M (μM)	K_i (μM)	$V_{\max}$ ($\text{mol m}^{-3} \text{s}^{-1}$)
HK	0.8	D-glucose	130		4.85
		ATP	100	310	
G6PDH	0.65	G6P	48		2.42
		NADP ⁺	19	21	

ties, the potential can be decomposed and solved independently.²⁴ The externally applied electric potential (ϕ) would be then governed by the Laplace equation,²²

$$\nabla^2 \phi = 0. \quad (5)$$

2. Model simplification

When the thickness of the double layer is much smaller than the size of the microchannel, the velocity gradients inside the electric double layer can be neglected.^{22,24} Hence, the slip velocity at the channel wall can be calculated from the local potential gradient by the Helmholtz–Smoluchowski equation^{6,21}

$$\mathbf{u}_{\text{eo}} = -\frac{\varepsilon_0 \varepsilon_r \zeta}{\eta} \nabla \phi = \mu_{\text{eo}} \nabla \phi, \quad (6)$$

with $\mathbf{u}_{\text{eo}}$ (m s^{-1}) as the electro-osmotic velocity, ζ (V) the zeta potential, and μ_{eo} ($\text{m}^2 \text{V s}^{-1}$) the electro-osmotic mobility. The working buffer has an electrical conductivity of 0.32 S m^{-1} and electro-osmotic mobility of $4.9 \times 10^{-8} \text{ m}^2 \text{V}^{-1} \text{s}^{-1}$. This value was obtained experimentally using current monitoring techniques and is in close agreement with literature values.^{22,25}

Hence, the source term from Navier–Stokes equation is replaced by this velocity, which is imposed as a slip velocity at the wall boundary. Using this approach, the computational memory and time are significantly reduced especially during the modeling of complex microfluidic systems.

3. Species transport

The general mass transport equation in microfluidic chips includes terms for convection, diffusion, migration, and reaction,^{5,6}

$$\frac{\partial C_i}{\partial t} - D_i \nabla^2 C_i - \frac{z_i F D_i}{RT} \nabla \cdot (C_i \nabla \phi) + \mathbf{u} \cdot \nabla C_i = r_i, \quad (7)$$

with C_i (mol m^{-3}) as the concentration of the i th species, D_i ($\text{m}^2 \text{s}^{-1}$) the diffusion coefficient of the i th species shown in Table I, z_i the electrical charge of the i th species, F (C mol^{-1}) the Faraday constant, R ($8.314 \text{ J K}^{-1} \text{mol}^{-1}$) the universal gas constant, T (K) the temperature, and r_i ($\text{mol m}^{-3} \text{s}^{-1}$) the rate of production or consumption of the i th species in the reaction [see Eq. (8)].

The second, third, and fourth terms on the left represent the diffusion, migration (electrophoresis), and advection (electro-osmosis) mode of mass transfer, respectively. Migration implies that positively charged species move from a positive potential to a negative potential and vice versa for negatively charged species.

4. Enzyme reaction model

Both HK and G6PDH perform two-substrate reactions using random sequence mechanism. HK binds D-glucose and the ATP-Mg^{2+} complex,^{26,27} G6PDH also acts in a similar mechanism

with respect to NADP⁺ and ATP.²⁸ For in-depth analysis on the kinetic mechanism of two substrate enzymatic process we refer to Marangoni.³¹ The initial rate equation for such mechanism is given by^{6,27}

$$r_1 = -\frac{dC_{\text{Gl}}}{dt} = \frac{dC_{\text{G6P}}}{dt} = \frac{k_{\text{cat,HK}_g} C_{\text{HK}} C_{\text{Gl}} C_{\text{ATP}}}{K_{i,\text{ATP}} K_{M,\text{HK}_g} + K_{M,\text{HK}_g} C_{\text{ATP}} + K_{M,\text{HK}_{\text{ATP}}} C_{\text{Gl}} + C_{\text{Gl}} C_{\text{ATP}}}, \quad (8)$$

$$r_2 = -\frac{dC_{\text{G6P}}}{dt} = \frac{dC_{\text{NADPH}}}{dt} = \frac{k_{\text{cat,G6P}} C_{\text{G6PDH}} C_{\text{NADP}^+} C_{\text{G6P}}}{K_{i,\text{NADP}} K_{M,\text{G6P}} + K_{M,\text{G6P}} C_{\text{NADP}^+} + K_{M,\text{NADP}^+} C_{\text{G6P}} + C_{\text{NADP}^+} C_{\text{G6P}}},$$

where K_M is the Michaelis–Menten constant (mol m⁻³), k_{cat} is the rate constant (s⁻¹), and K_i is the inhibition constant (mol m⁻³). Kinetic data and diffusion coefficients of enzymes are presented in Table I. The diffusion coefficients of ATP, NADP⁺, glucose, G6P, and NADPH at a temperature of 298 K are estimated to be 3.7, 3.3, 6.7, 5.8, and 3.3 × 10⁻¹⁰ m² s⁻¹, respectively.³⁰

C. Boundary conditions and numerical procedures

The numerical simulation requires boundary conditions to close the system of Eqs. (2)–(8) described above. The voltages were applied at the inlets and outlets while the potential gradient was set to zero at the microchannel walls. Flow is assumed purely electrokinetic driven; the absence of hydrodynamic pressure gradient was then modeled by setting an atmospheric pressure at all the inlets and outlets. When the thickness of the electric double layer is much less than the dimensions of the microchannel (approximately 1.52 nm in our case), the fluid flow model is greatly simplified by imposing the Helmholtz–Smoluchowski slip velocity [Eq. (6)] at the channel walls. For the species transport equation, all the walls were set to no mass flux (i.e., impermeable walls) and the concentration of the different species was determined by the concentrations in the reservoirs. The concentration gradients of all species at the outlets were set to zero.

All simulations were done with the commercially available finite element method software COMSOL (version 3.5, Comsol, Inc., Burlington, MA) on high speed computer (AMD Opteron linux cluster node with 4 Gbytes of random access memory). The differential equations were solved in a spatially discretized 2D geometry of the microfluidic chip, neglecting the channel depth that is equal everywhere.²² To save the computational resource, the reaction channel beyond 20 mm is neglected during the computation and the corresponding potential in this artificial boundary is recalculated.^{5,6} Using mesh sensitivity analysis, mesh size that governs a mesh-independent solution was chosen. Accordingly the mesh that consists of 44 308 triangular elements has been computed for an absolute and relative tolerance of 1 × 10⁻⁶. The assay was a three step process that obliges change in boundary conditions according to the flow patterns. COMSOL has also a user-friendly solver manager to first solve the steady state flow equations with nonlinear solver and use the results for mass transport transient simulation. An interface program written in MATLAB is used for model optimization and simulation automation.

III. RESULTS AND DISCUSSIONS

A. Validation of fluid flow and reagent transport

To validate the numerical models of fluid flow, electrical field distribution, and molecular transport in the microfluidic channels, flow experiments were first executed with fluorescein solution. Thereto, reservoir A was filled with this solution and a gating flow pattern was envisaged in the microfluidic channels. In the first stage, buffer flows from reservoir B to the reaction channel (intersection and channel connecting reservoir C) while fluorescein solution transports from reservoir A to the intersection and then toward reservoir D. In the second stage, the potentials were changed to deliver a plug of sample into the reaction channel. Figure 2 presents the simulated and the experimental results of the concentration field in the intersection of the microchannel during the three stage electrokinetic injection procedure using the potential values denoted in

TABLE II. Applied potentials in the three step gated valve injection process.

Stages	Time (s)	A (V)	B (V)	C (V)	D (V)
Loading	18	1000	950	0	800
Dispensing	2	1000	940	0	940
Transporting	30	1000	950	0	800
Enzyme assay					
Loading	30	1000	1000	0	800
Dispensing	2	1000	940	0	940
Transporting	120	760	900	0	760

Table II. Minimal mixing was observed at the interface between the buffer and the fluorescein solution in the first stage, Fig. 2(a). After 18 s from start of the experiments, the concentration at the intersection reached a steady state profile. Then the potentials in reservoirs A, B, and D were changed (stage 2) and the fluorescein begins to flow into the reaction channel toward reservoir C [Figs. 2(b) and 2(c)]. After 2 s, the potentials were switched back to their initial values. The third stage is aimed at producing a plug of fluorescein solution that would be pushed into reservoir C by the new buffer stream [Figs. 2(d)–2(f)].

The numerical solution should be independent of the mesh size. This can be checked by monitoring the solution as a function of mesh refinement. The computation domain is discretized with a triangular mesh. The region in the crossing, particularly the area around the corners, needs to be well refined. Solutions from three different mesh sizes were compared and the dependence of the mesh on the solution was evaluated. Computational results from coarse mesh (the maximum mesh size of 30 μm), finer (the maximum mesh size of 10 μm), and finest (the maximum mesh size set to 5 μm) are presented in the inset of Fig. 3(a). The solution is independent of meshing for maximum mesh size below 10 μm and all computations were performed at this mesh size.

A close agreement was obtained between the simulations and the experimental concentration contour plots the results in the three different stages of the injection process. The fluorescence intensity was measured at 77 μm away from the intersection into the reaction channel and Fig. 3(a) presents the intensity profiles from the simulated and the experimental results. The repeatability of the experiments was particularly good with a coefficient of variation (CV) of $\pm 3.6\%$ on the average peak height of the signals. Here, also an excellent agreement between the simulated and the experimental results was observed. The discrepancies between the simulation and the experimental result could be due to simplification of the 2D geometry at the cross section. This is because the cross section of the actual channel has a D-shape, which results in a more complex geometry at the intersection. As the electric field distribution highly depends on geometrical shape, the real flow profile is likely to be more complex than predicted in the simulation. This could result in slightly different concentration profiles in the region very close to the intersection. We also experienced that a better agreement between simulation and experiment was obtained at locations very far from the intersection. Some errors could also be inherited from the value of the parameters used in the model (e.g., electro-osmotic mobility, diffusion coefficient, and electrophoretic mobility).

Both figures showed the presence of leakage in the first and third stages of the injection process, which is illustrated by elevated fluorescence intensities (shoulder of the signal). Theoretically, leakage of sample to the reaction channel should not happen when the flow rate in the channel toward D is larger than the flow rate from A, which also means that the flow rate from B is larger than the flow rate from A. As the electro-osmotic flow rate is proportional to the applied voltages, electric field strengths between the intersection and reservoir D should be greater than or equal to electric field between the intersection and reservoir A. The simulation result (not shown) certainly reveals that there is no fluid flow toward channel C. However, as we observe in the experiments this does not guarantee that leakage is avoided during the loading and transport stages

FIG. 3. (a) Signal intensity of fluorescein measured at $77\ \mu\text{m}$ from the center of the intersection into the reaction channel. The simulation agrees well with the experiment and the shoulder in the signal is due to leakage as a result of molecular diffusion. (b) Signals for sequential injection of fluorescein solution within 10 s interval.

(first and third stages), which cause a shoulder in the signal. The observed leakage is actually due to molecular migration and this is significantly influenced by the charge of the species involved. In the applied flow pattern, the negatively charged molecules have more susceptibility for leakage toward the reaction channel than positive or neutral molecules.³² Hence leakage can be eliminated by increasing the potential applied in reservoir B to allow some amount of buffer to flow toward reservoir D. In such a way fluorescein molecules are kept away from the inlets of the reaction channel. For example, by raising the potential in B from 950 to 1000 V (depicted in Table II) during the first and third stages, leakage is significantly minimized. Experiments executed using these new potentials proved no leakage and the repeatability of the experiments was good with a CV of 3.3% on the average peak height. In addition, to avoid an excessive flow of species from reservoir A to D, the potentials in the third stage were modified further with the help of the developed numerical model (see Table II).

In this experiment the buffer flow rate from reservoir B is $0.56\ \text{nl s}^{-1}$ and for a running time

of 150 s, the amount of liquid pumped out of the reservoir is 84.1 nl. This accounts for 3.2% of the buffer solution originally filled into the same reservoir and, hence, does not change the liquid level significantly, and the assumption for pluglike velocity profile holds throughout the experiment time. As shown in Fig. 3(b), ten successive plugs of fluorescein solution were generated with a 10 s interval in a single experimental run. This supports the previous assumption and illustrates the robustness of the injection procedure. Overall, the mathematical model predicted the experimental results with a very good level of accuracy, which makes the use of this model appropriate for optimization of other working parameters.

B. Enzymatic assay for glucose

The on-chip enzymatic assay is executed as follows. First, both the reaction mixture (a solution of ATP, NADP⁺, and glucose) and enzyme solution are transported into the intersection using a similar injection procedure, as shown in Sec. III A. As a result the microfluidic channel from reservoir A to reservoir D is filled with the enzyme solution while the microfluidic channel from reservoir B to reservoir C is filled with the reaction mixture. In the second stage a plug of enzyme is injected to the reaction channel for a specified time. In the third stage this plug is transported by the carrier fluid that contains the mixture. These fluid flow patterns are obtained by applying the set of potentials depicted in Table II in the reservoirs. As a result of these fluid flow patterns, enzymes are sandwiched between mixtures which initiate the enzymatic reaction resulting in the formation of NADPH. The concentration of this component depends on the amount of glucose in the sample and a linear relation ($R^2 > 0.99$) is established for glucose solution in the range from 0.01 to 0.10 g l⁻¹.

Model equations used to characterize the aforementioned flow pattern were coupled with enzyme kinetic model for simulating the double-enzyme glucose assay in the electrokinetically driven microfluidic system. Figure 4 depicts the simulation results of concentration profiles of components (such as glucose, glucose-6-phosphate, G6PDH, and NADPH) close to the intersection. Figure 4(a) shows that enzymes are in contact with the reaction mixture both in the diffusion region at the intersection and in the channel connected to reservoir D. Due to large diffusion coefficient of glucose, significant amount of NADPH was produced in these regions. In the second stage of the assay, the potentials are carefully chosen to avoid leakage of preformed product into the reaction channel. In this way, a controlled amount of enzyme solution was injected to the reaction channel. Using the third set of potentials, the plug of enzyme solution injected is transported in the reaction channel and a significant amount of NADPH is produced. Figure 4(b) illustrates the concentration profiles of the different components 1 s after the beginning of the third stage of the injection process.

The intensity of the assay signal depends on the amount of enzyme injected, flow rate, and length of the reactor where detection was done. Figure 5(a) illustrates the peak heights of the signal at 2, 5, and 8 mm away from the intersection for five different flow rates. The applied electric fields in the reaction channel corresponding to these flow rates are 50, 99.5, 150, 200, and 250 V cm⁻¹ and are referring to the third stages of the assay. The maximum flow rate of most biological assays performed in microfluidic systems is limited in the applied potential less than 250 V cm⁻¹.¹⁵ Working at lower flow rate means prolonging the residence time of substrates, which results in more product formation. Although, choosing longer reactor length resulted in more NADPH, this does not contribute to raise the height of the peak, rather the signal base widens and in some case this may even result in a decrease in the peak height. This is due to the electrophoretic effects of the assay product, NADPH. In such cases, use of short reaction channel (as short as 2 mm) might be preferable as it provides sufficient signal intensity in a short time. On the other hand, for higher flow rate conditions one should use sufficiently long reactors (i.e., putting the detector far from the inlet of the reaction channel). However, in this particular assay the height of the signals did not significantly increase for detection made beyond 5 mm.

The amount of enzyme solution is controlled by the duration of the second stage of the injection procedure. Figure 5(b) shows the normalized peak heights of the signal at eight different durations (0.5, 1, 2, 5, 8, and 12 s) in the simulation. Working at a flow rate of 38.5 nl min⁻¹, the

FIG. 4. Contour plots of glucose, G6PDH, G6P, and NADPH at the (a) end of the loading stage and 1 s after the (b) end of enzyme injection process. Red is maximum concentration and blue refers to zero concentration; color shown is not to scale.

corresponding amount of enzyme injected to the reaction channel would be 0.24, 0.48, 0.95, 2.39, 3.82, and 5.65 nl, respectively. This figure shows that the response is linear up to an injection time of 2 s and an increase in injection time of enzyme beyond 8 s does not significantly increase the peak height of the signal. Higher amounts of enzyme solution should be avoided to minimize fouling of them on the surface of the microchannel and ultimately reduce the analysis cost.³³

Experiments were made based on optimized potentials in the three stage assay. The repeatability of the experiments was good (CV less than 5%) and they compare well with the simulation results, as shown in Fig. 6(a). This figure shows normalized product profile both from experiments and simulation at two locations in the reactor, 2 and 5 mm from the crossing. The discrepancy between profiles is due to the complexity of the assay to model with simplified reaction models and due to the estimation of some parameters such as diffusion coefficients, enzyme kinetic parameters, and other parameters that depend on the properties of the buffer and temperature. The smaller second shoulder in the signal at 2 mm is due to the difference in net flow rates of all species involved in the enzymatic reactions. Both simulation and experimental analysis showed that the signals will merge and give a wide base signal when detected at distances further down the channel. The microfluidic LOC system uses nanoliter reaction volumes and the amount of reagents used reduced significantly. 0.95 nl of enzyme solution is involved in a single assay; however, the amount of enzyme filled in the reservoir is much larger than this value. Compared with off-chip

FIG. 5. (a) Peak height of the signal at different flow rate (corresponding to electric field of 50, 99.5, 150, 200, and 250 V cm^{-1} in the reaction channel during the third stage of the assay) and detection of the signal at three positions in the reaction channel (2, 5, and 8 mm away from the crossing). (b) Response of the assay signal to the change in the amount of enzyme injected into the reactor. Enzymes are injected for 0.5, 1, 2, 5, 8, and 12 s (corresponds to 0.24, 0.48, 0.95, 2.39, 3.82, and 5.65 nl of enzyme at a flow rate of 39.5 nl min^{-1}). The response is linear up to an injection time of 2 s.

microplate assay (using 96 microwell plate in 200 μl volume), the amount of enzyme used is still 15-fold lower and the assay time is significantly reduced (i.e., 30 times shorter).

A series of *in silico* experiments were also made to produce a calibration curve based on the intensity of the peak height of the signal. The result is presented together with its experimental counterpart in Fig. 6(b). There is an excellent linear relationship between the amount of glucose in the sample and the fluorescence signal with $R^2 > 0.99$ for glucose concentration from 0.01 to 0.1 g l^{-1} . Again a good agreement between simulation and experimental results was obtained.

FIG. 6. (a) Concentration profile of NADPH at 2 and 5 mm downstream in the reaction channel. The flow rate in the third stage of the assay is 33.7 nl min^{-1} and the amount of enzyme injected to the reactor is 0.95 nl. Experiments are indicated by solid lines whereas simulations are represented by dashed line with marker. (b) Concentration of glucose vs normalized signal from simulation and experiment. The results are linear in both cases (with $R^2 > 0.99$) for glucose solution ranging from 0.01 to 0.1 g/l.

IV. CONCLUSION

A double-enzyme reaction glucose assay was implemented in a microfluidic LOC device for the first time. The assay is very complex to implement in electrokinetically driven continuous system due to the involvement of many species, which has different transport velocity. The LOC uses nanoliter reaction volumes, using only 0.95 nl of enzyme solution to be injected to the reactor. The amount of enzyme filled in the reservoir is much larger than this value, but it is still 15-fold smaller than that required using 96 MicroWell plate in volume of $200 \text{ } \mu\text{l}$. The assay time

is also 30 times shorter. Mathematical models were used for the optimization of LOC systems, aimed to execute enzymatic assays by means of electrokinetic pumping. Flow patterns of injection, flow rate, amount of enzymes, and length of the microreactor were optimized using numerical simulation. Simulation results agreed well with the experimental results. A linear relation existed for glucose solutions ranging from 0.01 to 0.10 g l⁻¹ based on the peak height of fluorescence intensity signal. Numerical simulations are important both to facilitate new designs and better understand the complex microfluidic phenomena.

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