

Optimization of capillary electrophoresis conditions for in-capillary enzyme-catalyzed microreactions

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Abstract

This paper describes the use of capillary electrophoresis (CE) to relate electrophoresis conditions of in-capillary enzyme-catalyzed microreactions to product distribution profiles. Migrating in a capillary under electrophoresis conditions, plugs of substrate and enzyme are injected separately, and allowed to react. A mathematical relation is obtained whereby the electrophoresis parameters, voltage (V), enzyme concentration [E], and mixing time of reaction (M) at the applied voltage, are correlated to product ratios. This concept is demonstrated using as a model system the conversion of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide, reduced form (NADH) in the oxidation of glucose-6-phosphate (glc-6-P) to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). The simulated results are shown to be in good quantitative agreement with experimental data. These procedures provide a direct relationship between electrophoretic conditions and product distribution of microscale reactions using CE. This technique offers a new and versatile approach to analyzing enzymatic reactions on a microscale. ©1999 Elsevier Science B.V. All rights reserved.

Keywords: Capillary electrophoresis; Enzyme-catalyzed microreactions; Electrophoresis parameters; Electrophoretically mediated microanalysis

1. Introduction

In recent years CE has emerged as a powerful and versatile separation tool for the study of minute quantities of samples [1–3]. This remarkable versatility coupled with a mechanism of analyte resolution that is complimentary to more traditional techniques, such as high-performance liquid chromatography (HPLC), has led to widespread application of this technology

in many laboratories [4]. CE differentiates charged species on the basis of mobility under the influence of an applied electric field gradient and provides for rapid analysis of small amounts of water soluble, charged samples with high resolution.

A powerful collection of analytical methods has been obtained by exploiting the selectivity and signal amplification possible with biomolecules for chemical analysis. Two of the most prevalent examples are immunoassays and enzyme assays [5,6]. A recent trend in the development of these assays has been incorporated by the use of CE and its respective principles and capabilities. CE enzyme assays can be divided into

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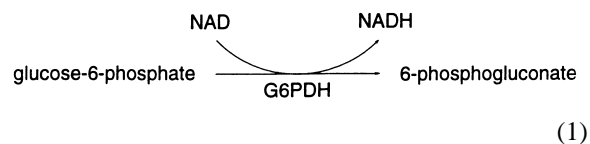
three distinct formats: (i) a ‘pre-column’ assay which is performed by sampling from an assay solution and separating substrate and product; (ii) an ‘on-column’ assay which is performed by electrophoretically mixing enzyme and substrate plugs within the capillary and detecting the product on-column, and (iii) a ‘post-column’ assay which is performed by mixing substrate (or enzyme) with enzymes (or substrates) that have already been separated by CE [5]. The second form of assay is of particular interest in that the enzyme assay (mixing, incubation, separation, and detection) occurs within the capillary, thus reducing the volume of the assay from microliters to nanoliters. This class of assays is referred to as ‘electrophoretically mediated microanalysis’ (EMMA) [7,8].

In EMMA, differential transport velocities are used as a useful mixing technique [7,8]. Spatially distinct zones of chemical reagents of different electrophoretic mobility are made to interpenetrate under the influence of an applied electric field (Fig. 1) [9]. EMMA techniques have been used to initiate chemical reactions by mixing analyte and analytical reagents, control reagent contact time, separate the reactants and detectable products, and transport the detectable products to a detector [2,6–28]. Several groups have utilized the EMMA concept to examine a number of biochemical systems [2,6–28]. For example, Regnier et al., have studied the control of product detection time in the EMMA of alkaline phosphatase and β -galactosidase [7]. Yeung et al., used a modification of Regnier’s technique in the very sensitive detection of lactate dehydrogenase using a combination of on-column reaction and CE [13]. Even more recently, Harrison et al., used a chip device to detect antiovine serum albumin antibody in mouse ascites fluid [28].

For CE to become a more widely used analytical technique, both a better understanding of the factors that influence separation and a more general and systematic approach in method development are required. Method development strategy must focus on determining appropriate conditions of electrophoresis and how to obtain these conditions in an efficient fashion.

This paper describes the use of CE to relate electrophoresis conditions of in-capillary enzyme-catalyzed microreactions to product distribution profiles. This concept is demonstrated using as a model system the conversion of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinu-

cleotide, reduced form (NADH) in the oxidation of glucose-6-phosphate (glc-6-P) to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) (Eq. (1)). This study provides the basis for future analysis of other enzyme systems.



1.1. Derivation of analysis

Using the experimental data, we derived an equation relating V , E , and M by the least-squares fitting method. An analytical function f can be represented with m different values of an independent variable x_i ($i = 1, 2, 3, \dots, m$) and the corresponding measured values of a dependent variable y_i ($i = 1, 2, 3, \dots, m$). The quantities a_j ($j = 1, \dots, n$) are independently adjustable parameters that at the beginning of the analysis are either known or unknown and only approximately. This form of fitting determines the best values of n adjustable parameters. Hence, we have determined the best, though not the exact solution, to a set of m simultaneous equations for n unknowns (Eq. (2)):

$$y_i = f(\alpha_1, \dots, \alpha_n; x_i) \quad (2)$$

For $m = n$, the corresponding set of simultaneous equations can be solved exactly for the parameters α_j . The least-squares fitting is defined by Eq. (3):

$$\delta_{\alpha_1} \chi^2 = \delta_{\alpha_2} \chi^2 = \dots = \delta_{\alpha_n} \chi^2 = 0 \quad (3)$$

Here, δ signifies variation with respect to infinitesimal and independent variation of j . Therefore, χ^2 is defined by Eq. (4):

$$\chi^2 = \sum_i [y_i - f(\alpha_1, \dots, \alpha_n; x_i)]^2 \quad (4)$$

At a minimum of the χ^2 hypersurface, Eq. (5) states that

$$\frac{\partial \chi^2}{\partial \alpha_1} = \frac{\partial \chi^2}{\partial \alpha_2} = \dots = \frac{\partial \chi^2}{\partial \alpha_n} = 0 \quad (5)$$

For the derivative with respect to α_1 we obtain Eq. (6)

$$2 \sum_i [y_i - f(\alpha_1, \dots, \alpha_n; x_i)] \frac{\partial f}{\partial \alpha_1} = 0 \quad (6)$$

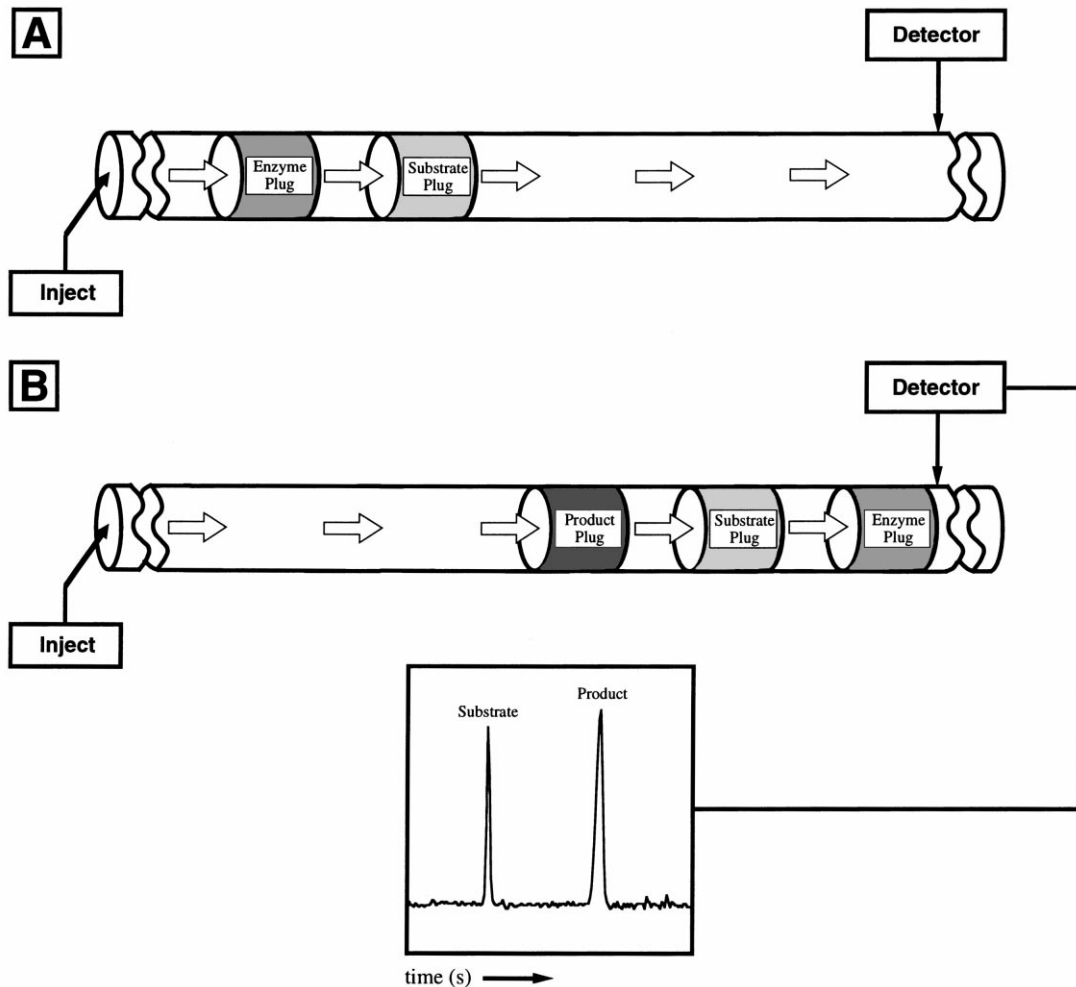


Fig. 1. Schematic representation of an in-capillary enzyme-catalyzed microreactor (A) before reaction and (B) after reaction.

For the general case when the function f is not a linear function of the α_j , it can be expanded as a Taylor series around the calculated trial values α_1^0 of the parameters (Eq. (7)).

$$y_i = f(\alpha_1^0, \dots, \alpha_n^0; x_i) \quad (7)$$

In the present work the expression for y_i is defined as in Eq. (8):

$$y_i = a + bV + cV^2 + dE + eE^2 + fM + gM^2 \quad (8)$$

Here V is voltage, E is enzyme concentration, M is the mixing time and a – g are the coefficients for the equation.

To simplify the equation, A_{jk} is defined as in Eq. (9):

$$A_{jk} = \sum_i \frac{\partial f}{\partial \alpha_j} \frac{\partial f}{\partial \alpha_k} \quad (9)$$

The quantities A_{jk} are the elements of an $n \times n$ square matrix. Equation 6 can be rearranged as follows:

$$A_{11}\Delta\alpha_1 + A_{12}\Delta\alpha_2 + A_{13}\Delta\alpha_3 + \dots + A_{1n}\Delta\alpha_n = h_1 \quad (10)$$

Similarly,

$$A_{21}\Delta\alpha_1 + A_{22}\Delta\alpha_2 + A_{23}\Delta\alpha_3 + \dots + A_{2n}\Delta\alpha_n = h_2 \quad (11)$$

$$A_{n1}\Delta\alpha_1 + A_{n2}\Delta\alpha_2 + A_{n3}\Delta\alpha_3 + \dots + A_{nn}\Delta\alpha_n = h_1 \quad (12)$$

Equations 10–12 constituting a set of n simultaneous linear equations in n unknowns, are known as the *normal equation*.

Since the experimental data yield a nonlinear plot, the second order of parameters are considered. Therefore, there are seven equations and seven unknowns yielding a matrix that can be solved with a graphing program like Mathematica. An equation can then be generated which relates V , E , and M to the product ratio N which is defined as the amount of product formed divided by the sum of product formed and remaining substrate.

2. Experimental

2.1. Chemicals and reagents

All chemicals were analytical grade. Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide, reduced form (NADH), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), and alcohol dehydrogenase (ADH, EC 1.1.1.1) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. Mesityl oxide was purchased from Calbiochem (San Diego, CA, USA). Stock solutions of G6PDH (1.0 mg/ml; 190 units/mg protein) and NAD (500 μ M) were each prepared by dissolving the lyophilized protein and cofactor in buffer (30 mM Tris-200 μ M glucose-6-phosphate; adjusted to pH 7.8 by addition of 1.0 M HCl).

2.2. Apparatus

The CE system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA.). The capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) was of uncoated fused silica with an internal diameter of 50 μ m, length from inlet to detector of 30.5 cm, and a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software. The conditions used in CE were as follows: voltage, 30 kV; current, 42.0 μ A; detection, 260 nm; temperature, 30 \pm 0.1 $^{\circ}$ C.

2.3. Procedures

A sample solution (1.2 nl) containing G6PDH in water was introduced by pressure injection onto the capillary equilibrated with buffer. A solution (1.2 nl) of NAD was next introduced by pressure injection and the electrophoresis run at a given contact voltage (1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0 kV) for the desired mixing time (0.2, 1.0, 1.5 min) followed by electrophoresis at 30 kV to complete elution of all species.

3. Results and discussion

We examined the conversion of NAD to NADH by G6PDH in the oxidation of glc-6-P to 6-phosphogluconate. It is a simple reaction that can be utilized to demonstrate the ease of in-capillary enzyme-catalyzed microreactions. G6PDH is an enzyme with molecular weight of 104 kD comprised of two subunits of approximately 55 kD. In this reaction G6PDH catalyzes the net transfer of a hydride ion to NAD from C1 of glc-6-P to form 6-phosphogluconate. Glc-6-P, a cyclic hemiacetal with C1 in the aldehyde oxidation state, is thereby oxidized to a cyclic ester.

Plugs of G6PDH and NAD were introduced sequentially into the capillary by pressure injection. The first analyte injected is the protein because it has a lower apparent mobility (higher electrophoretic mobility) compared to NAD. Glc-6-P was present as part of the buffer and the enzyme concentration was held constant. Upon application of a voltage (1.0 kV) (we define this initial voltage as the contact voltage), migration of G6PDH and NAD takes place towards the detector side and mixing of the two components is achieved electrophoretically. After 0.2 min (we refer to this as the mixing time at the respective contact voltage) the voltage is then increased to 30 kV and the sample is electrophoresed until completion. Upon reaction, the remaining NAD, generated NAOH, and G6PDH are electrophoretically driven towards the detector with different velocities. Both NAD and NADH are negatively charged species and are observed as two separate peaks. The mobility of NAD under these conditions is greater than the mobility of NADH and it is observed first in the electropherogram followed by

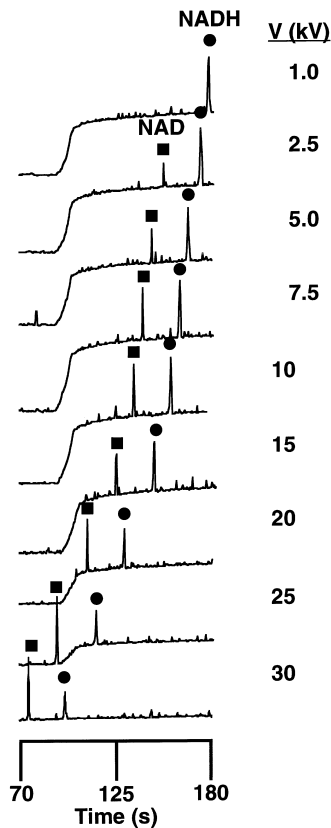


Fig. 2. A representative series of electropherograms showing the conversion of NAD to NADH by G6PDH on increasing voltage.

NADH. G6PDH is used in catalytic amounts at quantities too small to be detected. At pH 7.8 the conversion of NAD to NADH by G6PDH is fast and is easily observed in the series of electropherograms. For this type of in-capillary assay the amount of product formed is directly related to the applied voltage. The amount of NADH generated is determined by the time period between injection and when the enzyme passes the detector window. At higher voltages, G6PDH is transported to the detector within a shorter time period, allowing less product to be formed. Nine different contact voltages ranging from 1.0 to 30 kV were used. Fig. 2 shows a representative series of electropherograms obtained for the conversion of NAD to NADH by G6PDH on increasing the contact voltage. Repeated injections of G6PDH and NAD and subsequent in-capillary reactions yielded both repeatable migration times and amount of NADH generated.

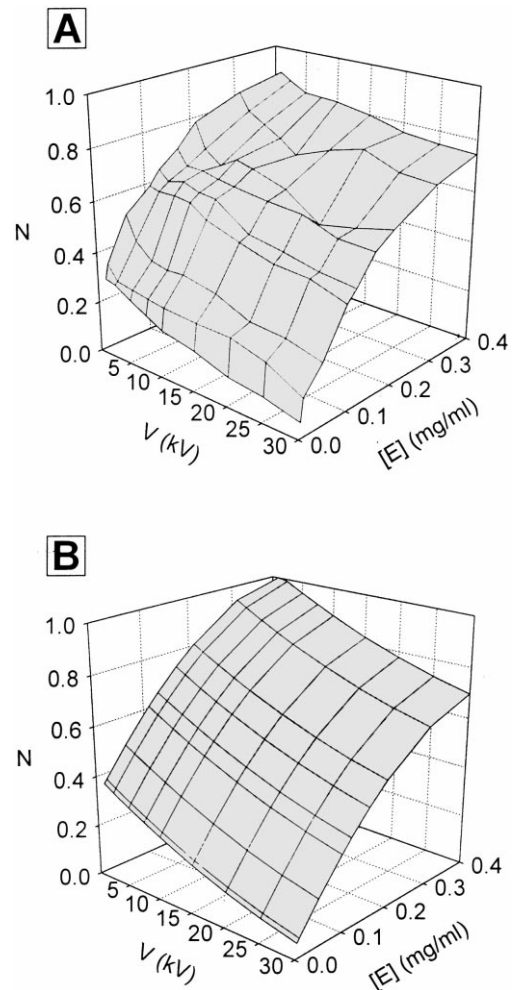


Fig. 3. Product distribution profiles for 0.2 min (A) mixing time (B) experimental calculated using Eq. (13).

Upon completing the series of contact voltages a higher concentration of enzyme was used and the experiment conducted as before. The concentration of G6PDH was then increased and the sample run again yielding an additional series of electropherograms. Nine different concentrations of G6PDH ranging from 0.01 to 0.4 mg/ml were injected yielding a CE product distribution profile.

Fig. 3A is a CE product distribution profile detailing the extent of the reaction. Here, N is defined as the ratio of NADH formed in the microreaction divided by the sum of NADH formed and remaining NAD. As can be seen, at low concentrations of enzyme and/or

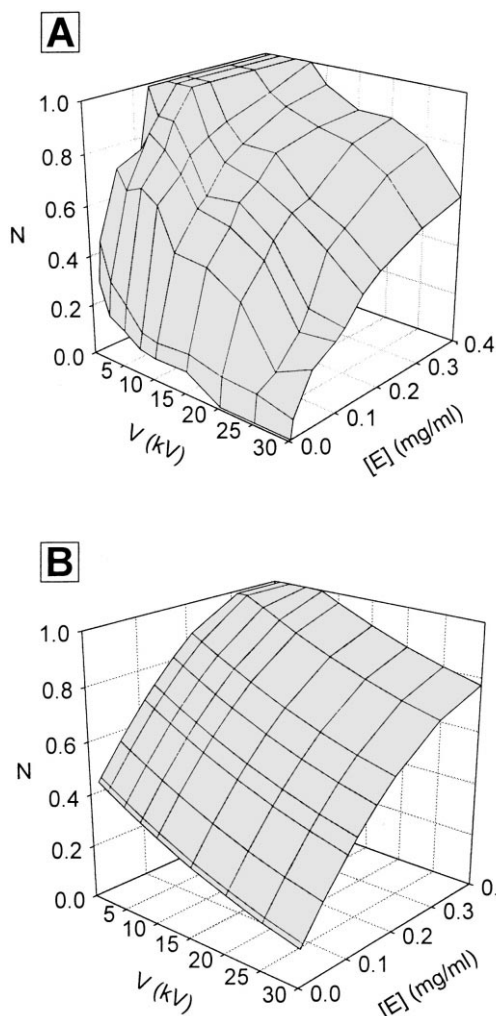


Fig. 4. Product distribution profiles for 1.0 min (A) mixing time (B) experimental calculated using Eq. (13).

at high contact voltages, the amount of NADH formed is small. Upon increasing the concentration of enzyme and/or decreasing the contact voltage, a greater conversion of NAD to NADH is observed. Two other CE product distribution profiles were obtained at mixing times of 1.0 and 1.5 min shown in Fig. 4A and Fig. 5A, respectively.

Solving the matrix from the three contact voltage experiments generated Eq. (13)

$$N = 0.37952 - 0.016095V + 0.00016466V^2 + 2.9450E - 3.2775E^2 - 0.031144M + 0.10626M^2 \quad (13)$$

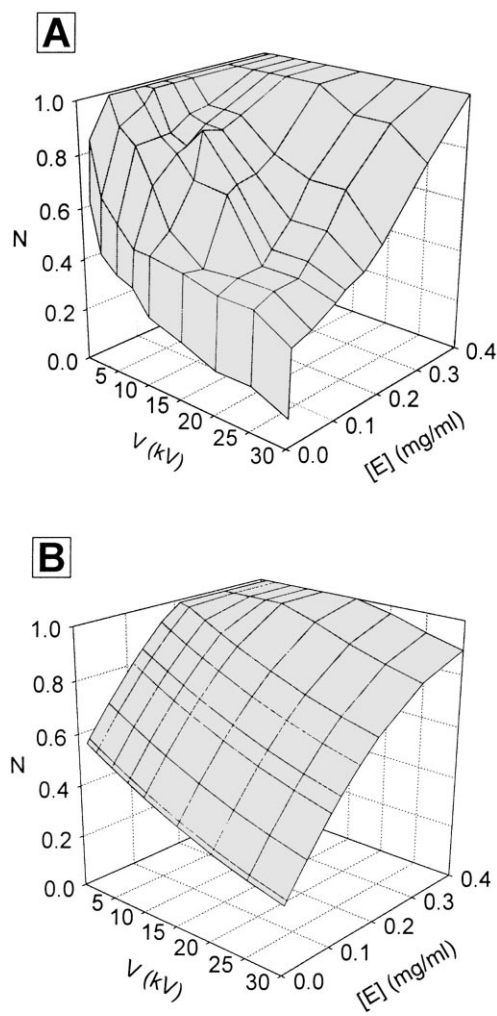


Fig. 5. Product distribution profiles for 1.5 min mixing time (A) experimental (B) calculated using Eq. (13).

that best fit the three sets of data. Equation 13 correlates V , E , and M to the product ratio N . In this equation V is the voltage (V), E is the enzyme concentration (mg/ml), and M is the mixing time (min). Fig. 3B, Fig. 4B, and Fig. 5B are the product distribution profiles calculated using Eq. 12 for mixing times 0.2, 1.0, and 1.5 min, respectively. Table 1 lists the values for N obtained experimentally (N_{exp}) and theoretically (N_{theor}), as well as percent error, using Eq. 13 for a random series of experiments at varying enzyme concentration, contact voltage, and mixing time. As can be seen, Eq. 13 provides an

Table 1
Comparison of the values of N as obtained by experiment and by Eq. (13)^a

Contact voltage (kV)	Mixing time (min)	[E] (mg/ml)	N_{exp}	N_{theor}	Error (%)
10.0	1.5	0.10	0.682	0.686	0.56
2.5	0.60	0.20	0.825	0.818	0.92
7.5	1.2	0.15	0.772	0.752	2.67
30.0	0.30	0.40	0.703	0.699	0.66
25.0	1.00	0.14	0.492	0.503	2.28
15.0	0.80	0.15	0.567	0.586	3.30
15.0	0.30	0.35	0.812	0.805	0.99
30.0	0.5	0.25	0.564	0.587	3.99
20.0	0.20	0.35	0.757	0.751	0.81
2.5	0.6	0.10	0.602	0.622	3.22

^aThe conversion of NAD to NADH is fast (on the time scale of CE).

accurate means of calculating product ratios obtained from in-capillary enzyme-catalyzed microreactions using CE.

We used G6PDH as a model system for several reasons: (i) it does not absorb to the walls of uncoated capillaries; (ii) we have data describing its electrophoretic behavior in other circumstances; (iii) it is commercially available and inexpensive; (iv) cofactor NAD and NADH are both negatively charged (minus one and two, respectively) and have different electrophoretic mobilities, and (v) the conversion of NAD to NADH is fast (on the time scale of CE).

4. Conclusions

This report demonstrates that it is practical to use CE to relate electrophoresis conditions of in-capillary enzyme-catalyzed microreactions to product distribution profiles. A mathematical relationship is obtained whereby arbitrary values for contact voltage, enzyme concentration, and mixing time of the reaction can be used to obtain simulated values for the extent of the reaction. Enzyme-catalyzed reactions are based on the fact that electrophoretic mobilities of reagents and products are different under an applied voltage and may be utilized to mix the reactants, separate the product from enzyme and substrate, and yield sharp product peaks for subsequent analysis and quantitation.

This study points the way to further applications of the microreactor concept in the area of immunoassays and enzyme assays.

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