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Review

Transverse diffusion of laminar flow profiles – a generic method for mixing reactants in capillary microreactor

The capillary is an attractive format for integrated microanalyses, which start with the injection of separate reactants into the capillary and their mixing inside the capillary. Due to the nonturbulent nature of flow inside the capillary, mixing reactants in a generic way is a challenging task. Three approaches have been suggested as a solution: mixing by electrophoresis, mixing by longitudinal diffusion, and, most recently, mixing by transverse diffusion of laminar flow profiles (TDLFP). This is the first review on TDLFP, describing: (i) the physical basis of the method, (ii) its theory, (iii) analytical and numerical solutions for the calculation of concentration profiles of mixed reactants, (iv) up-to-date applications, and (v) problems to be solved and future directions.

1 Introduction

The capillary is an attractive media for integrated microanalyses in multiple applications including kinetic measurements [1–5], selection of binding ligands from large combinatorial libraries [6–8], high-throughput screening [9], analyses of biomolecules [10, 11], and analyses of single cells [12–15]. The major advantage of the capillary in microanalyses is the integration of multiple functions in a single and simple capillary format. First, the capillary serves as a microreactor to conduct a chemical reaction under tightly controlled conditions in a nanoliter volume. Second, the capillary serves as a separation column for the separation of reaction products and remaining reactants by either capillary chromatography or CE. Third a capillary can be a cell for optical detection of products and remaining reactants after their separation. Moreover, the capillary can be interfaced with off-column electrochemical and mass spectrometric detectors. The diversity of suitable separation and detection methods offers ultimate analytical capabilities.

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Abbreviations: **EMMA,** elecrtophoretically mediated microanalysis; **FPP,** farnesyl pyrophosphate; **FTase,** farnesyl transferase; **FTI,** farnesyl transferase inhibitor; **GFP,** green fluorescent protein; **HTS,** high-throughput screening; **IMReSQ,** inject-mix-react-separate-and-quantitate; **TDLFP,** transverse diffusion of laminar flow profiles

To make use of the whole advantage of a nanoliter capillary microreactor, it is preferential not to pre-mix reactants outside the capillary. Such pre-mixing requires at least microliters of each reactant while only a nanoliter volume of the reaction mixture is required for capillary analysis. More than 99% of the reaction mixture not needed for analysis is, therefore, discarded when the mixture is prepared outside the capillary. If the reactants are injected into the capillary separately, only nanoliter volumes are consumed *per* analysis. Not only can the automated sequential injection of reactants into the capillary exclude unnecessary (and potentially error-prone) manipulations with solutions outside the capillary but it also allows highly economical analyses. Even in the worst scenario, when only ten injections are carried out from every aliquot of the reactant, the cost of material is reduced by a factor of 10. If the issues of evaporation and storage of materials between the analyses are solved, the consumption of material can be further decreased along with the associated cost reduction. Reducing material consumption is especially important for expensive reactants, such as proteins, and for multiple assays, such as those involved in high-throughput screening (HTS). Therefore, it is widely accepted that capillary microanalyses require sequential injection of individual reactants.

For integrated microanalyses to be generic, there should be a mixing method for sequentially injected reactants. A generic mixing method needs to be applicable to all kinds of soluble reactants with minimal necessary optimization. It has to be applicable to the mixing of more than two reactants injected separately (*e.g.,*

enzyme, substrate, and inhibitor). A generic mixing method also needs to facilitate fast mixing, so that reactions with fast kinetics could be studied.

Mixing is currently a bottleneck of integrated microanalyses. Three methods have been suggested for nonturbulent mixing reactants inside the capillary: mixing by electrophoresis, mixing by longitudinal diffusion, and mixing by transverse diffusion of laminar flow profiles (TDLFP). Here, we compare the three methods with respect to their ability to satisfy the requirements of being generic (see the previous paragraph). Finally, we focus on the comprehensive review of the TDLFP method which has never been previously reviewed.

2 Mixing by electrophoresis

Mixing by electrophoresis relies on differences in the electrophoretic mobilities of reactants. This method is the key to electrophoretically mediated microanalysis (EMMA), pioneered by Regnier and coworkers in 1992 [1, 2] and, since then, used by a number of groups mainly for studying enzymatic reactions. The fundamentals and applications of EMMA have been thoroughly reviewed elsewhere [16, 17].

For electrophoretic mixing, reactants can be injected either by the EOF or by pressure. The reactant with the lower electrophoretic mobility in the forward direction (from the inlet to the outlet of the capillary) is injected first, while the reactant with the greater electrophoretic mobility is injected second (Fig. 1A). When a high voltage is applied at time t_0, the reactants start moving to the right with apparent velocities v_1 and v_2, which are sums of the corresponding electrophoretic velocities and a common electroosmotic velocity. Electric field-driven translational movement is uniform across the capillary, which allows for very high-quality mixing by the "ideal" migration of the fast-reactant plug through that of the slow reactant. However, as the same translational movement moves both reactants to the right, mixing is achieved away from the inlet of the capillary. The distance from the capillary inlet to the rear front of the fully overlapping plugs depends on v_1, v_2, and the plug length. Assuming that the plug lengths are identical and equal to l, we can calculate this distance to be $lv_1/(v_1 - v_2)$.

As we mentioned above, electrophoresis can produce high-quality mixing, however, the method is not generic. For instance, electrophoresis cannot be used to mix reactants with identical electrophoretic mobilities. Furthermore, if the difference in apparent reactant velocities is less than the velocities, full mixing can only be achieved near the detection end of the capillary. Time t_1 required for complete mixing is equal to $l/(v_1 - v_2)$. If this time is longer than the characteristic time of reaction, the kinetics of such a reaction can hardly be studied. Accu-
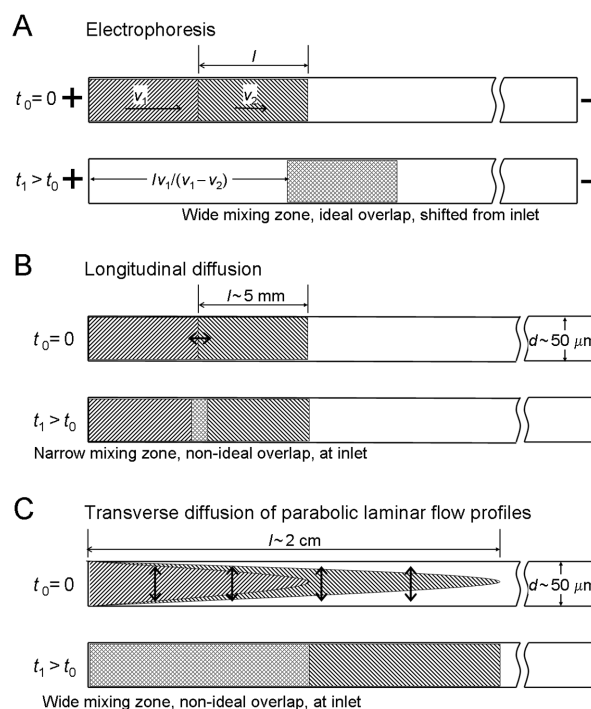
Figure 1. Schematic representation of mixing two reactants inside the capillary by electrophoresis (A), longitudinal diffusion (B), and TDLFP (C). The capillaries are shown out of scale and aspect ratio. See text for details.

rately predicting time of mixing is practically impossible if the reactants are dissolved in different buffers, which is the usual case for studies of molecules prepared from biological samples (*e.g.*, cell lysate). Finally, if more than two reactants need to be mixed, the optimization of mixing becomes very tedious and highly impractical. To the best of our knowledge, mixing more than two separately injected reactants by electrophoresis has never been reported.

3 Mixing by longitudinal diffusion

Mixing by longitudinal diffusion (Fig. 1B) was suggested in 1996 by Taga and Honda [3] to overcome some limitations associated with mixing by electrophoresis. This method is called an at-inlet technique to emphasize that no translational movement of the reactants toward the end of the capillary occurs in contrast to mixing by electrophoresis. It was used as a mixing method in EMMA, in particular, for studies of enzymatic reactions [18].

When being mixed by longitudinal diffusion, reactants are injected into the capillary either by pressure or by electroosmosis. If pressure is used for injection, it is typically assumed that the injection flow velocity is relatively low, so that the plugs of reactants are cylindrical (similar to electroosmotic injection). For our analysis, we assume that the length of the cylindrical plug is approximately

5 mm and the diameter is approximately 50 μm making the length-to-diameter ratio approximately 100. These values are typical for capillary-based microanalyses.

Mixing by longitudinal diffusion is applicable to all reactants, assuming diffusion is fast. However, the speed of diffusion is a limiting factor here. The time t_{diff} required for molecules with diffusion coefficient D to diffuse through a plug of length l is $t_{diff} = l^2/2D$. Even for a small molecule such as fluorescein, with $D = 3 \times 10^{-6}$ cm²/s, the time required to diffuse (t_{diff}) through a plug with l = 5 mm is greater than 11 h. Diffusion of such a molecule through even a relatively short 1 mm-long plug takes approximately 30 min.

Longitudinal diffusion mixing is typically used in a sandwich format, in which one reactant is surrounded by two identical plugs of another reactant [3]. Two mixing zones are formed in such a case, and if the mixing time is long enough, the mixing zones can overlap. The sandwich method decreases the diffusion distance for the outside reactant diffusing inwards by a factor of 2, and, thus, reduces mixing time by a factor of 4. Nevertheless, this does not dramatically improve its mixing speed.

Mixing by longitudinal diffusion overcomes two limitations of mixing by electrophoresis. First, it can mix reactants with identical electrophoretic mobilities (*e.g.*, neutral reactants) since diffusion is a fundamental property of all molecules. In addition, mixing by longitudinal diffusion does not result in translational movement of reactants, thus, the mixing zone is always at the inlet of the capillary. The rest of the capillary is, therefore, left for analytical separation. However, mixing by longitudinal diffusion is also not generic. It is limited to very short plugs of small molecule reactants, and also limited to no greater than two separately injected reactants.

4 Mixing by TDLFP

4.1 Physical basis of TDLFP

TDLFP was proposed as a first generic method for mixing reactants inside the capillary capable of rapidly mixing all kinds of reactants and applicable to more than two separately injected reactants [19]. Conceptually, for mixing by TDLFP, solutions of reactants are injected into the capillary by pressure as a series of consecutive plugs (Fig. 1C). The pressure used for injection is high enough and the injection time is short enough to make the front of the laminar injection flow parabolic. Thus, each next plug deeply penetrates into the previously injected plugs, creating long longitudinal interfaces between them. The reactants can, then, be mixed by transverse diffusion through the longitudinal interfaces. Note that this scenario contrasts with mixing by longitudinal diffusion through transverse interfaces (see Fig. 1B). To completely diffuse in the transverse direction, the reactant

needs to diffuse a distance no greater than the capillary inner diameter. In our example, the capillary inner diameter is equal to 50 μm (typical value). A fluorescein molecule, given as an example above, can diffuse through this distance in only 4 s. Even for a large molecule, such as a protein, with a diffusion coefficient of 10^{-7} cm²/s, transverse diffusion takes only 2 min. Longitudinal diffusion for a molecule with D = 10^{-7} cm²/s through a 5 mm long plug would take an astonishingly long time of 14.5 days. This example emphasizes the dramatic gain in the speed of mixing when conditions are met for mixing by TDLFP.

Multiple sequentially injected plugs will have longitudinal interfaces with neighboring plugs at a distance shorter than the capillary radius. Diffusion time required to mix multiple reactants is no longer than the time required for mixing two reactants, this time is defined by the capillary inner radius. In mixing by longitudinal diffusion, the diffusion distance increases with the number of reactant plugs and the diffusion time required grows as a square of the length of plugs. Thus, in contrast to longitudinal diffusion, TDLFP is suitable for mixing more than two separately injected reactants.

TDLFP satisfies the requirements setup for a generic capillary microreactor mixing method; it can facilitate fast mixing of multiple separately injected reactants of all kinds.

For capillary microanalysis with TDLFP-based mixing to be applicable to quantitative studies of reaction kinetics, it is necessary to know concentration profiles of the mixed reactants. The following sections explain the theory of TDLFP and different approaches to the determination and optimization of concentration profiles of the mixed reactants.
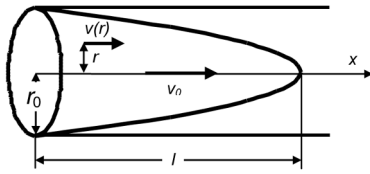
4.2 Theory of TDLFP

4.2.1 General analysis

A relatively comprehensive theory of TDLFP has been developed over the last 3 years [19, 20]. If the reactants are injected into the capillary by differential pressure, the following processes contribute to mass transfer: translational movement along the capillary, diffusion along the capillary, and diffusion across the capillary. The mass transfer is mathematically described by the following equation:

$$\frac{\partial n}{\partial t} = -v(r,t)\frac{\partial n}{\partial x} + D\left(\frac{\partial^2 n}{\partial x^2} + \frac{1}{r}\frac{\partial}{\partial r}r\frac{\partial n}{\partial r}\right)$$

$$v(r,t) = v_0(t)\left[1 - \left(\frac{r}{r_0}\right)^2\right]$$

$$D\frac{\partial n}{\partial r}\bigg|_{r=r_0} = 0 \tag{1}$$

Figure 2. Major parameters used in the theoretical consideration of TDLFP.

where n is the concentration of the reactant, D its diffusion coefficient, r the distance from the axis of the capillary in the transverse direction, x the distance from the capillary inlet in the longitudinal direction, t the time from the beginning of the injection, r_0 the radius of the capillary, $v(r,t)$ the injection velocity of the solution as a function of r, and $v_0(t)$ is the injection velocity on the axis of the capillary ($r = 0$). Figure 2 illustrates the major parameters used in the theoretical consideration of TDLFP.

In all previous reports on TDLFP, it was assumed that the pressure pulse was rectangular. However, due to the inertia, an experimental pressure pulse can considerably deviate from the rectangular one. Therefore, in this review, we generalize our consideration to a pressure pulse of an arbitrary shape by introducing $v_0(t)$ as a function of t (v_0 is proportional to the pressure). We assume that $v_0(t)$ has a maximum value, v_{max}, which will be used below to define dimensionless velocity. We also assume that the characteristic length of the injected plug, l, is related to the injection time, t_{inj}, as $l = t_{inj} v_{max}$, and l is much greater than the diameter of the capillary, $l/r_0 \gg 1$. In this case, the time required for transverse diffusion, t_r, is much shorter than the time required for longitudinal diffusion, t_x:

$$t_r = r_0^2/D, t_x = l^2/D \quad t_x/t_r = l^2/r_0^2 \gg 1 \quad (2)$$

The assumption in System 2 allows one to neglect mixing by longitudinal diffusion, which is equivalent to the assumption that $\partial^2 n/\partial x^2$ in the top equation of System 1 equals zero. Accordingly, the differential equation in System 1 can be simplified to obtain the following system proposed by Taylor in 1953 [21]:

$$\frac{\partial n}{\partial t} = -v(r,t)\frac{\partial n}{\partial x} + D\frac{1}{r}\frac{\partial}{\partial r}r\frac{\partial n}{\partial r}$$
$$v(r,t) = v_0(t)\left[1 - \left(\frac{r}{r_0}\right)^2\right] \quad (3)$$
$$D\frac{\partial n}{\partial r}\bigg|_{r=r_0} = 0$$

For simpler analysis, System 3 can be transformed into a dimensionless form if the following dimensionless variables and parameters are introduced:

$v = n/n_{max}$ dimensionless concentration

$\tau = tv_{max}/l$ dimensionless time

$\rho = r/r_0$ dimensionless capillary radius

$\chi = x/l$ dimensionless distance from capillary inlet

$\omega(\tau) = v_0(t)/v_{max}$ dimensionless velocity for $\rho = 0$ (4)

Here, n_{max} is the concentration of the reactant in the vial prior to injection, this concentration is maximal as the reactant can only be diluted upon injection and mixing. Mixing may involve injecting plugs of different lengths (typically by varying the injection time). If the plug lengths are different, l is defined to be the length of the shortest plug. If the new variables from System 4 are applied to System 3, it can be transformed into the dimensionless equations:

$$\frac{\partial v}{\partial \tau} = -w(\tau)(1 - \rho^2)\frac{\partial v}{\partial \chi} + Yo\frac{1}{\rho}\frac{\partial}{\partial \rho}\rho\frac{\partial v}{\partial \rho}$$
$$\frac{\partial v}{\partial \rho}\bigg|_{\rho=1} = 0 \quad (5)$$

where

$$Yo = Dl/(v_{max}r_0^2) = Dt_{inj}/r_0^2 \quad (6)$$

The parameter Yo was given the name of York number after York University in Toronto, Canada. The York number is the single parameter in the first dimensionless Eq. (5). Therefore, if two mixing procedures are characterized by (i) similar initial and boundary conditions, (ii) identical time dependencies of the pressure (or identical $\omega(t)$), and (iii) similar York numbers, the mixing will be similar and will generate similar final distributions of reactants along the capillary axis. The York number may be presented *via* the dimensionless Schmidt number ($Sc = \eta/D$), dimensionless Reynolds's number ($Re = r_0v_{max}/\eta$, where η is the kinematical viscosity of the fluid), and dimensionless ratio of l/r_0:

$$Yo = L/(r_0ScRe) \quad (7)$$

It is also possible to present the York number through the Péclet number ($Pe = r_0v_{max}D$) and the dimensionless ratio of l/r_0:

$$Yo = l/(r_0Pe) \quad (8)$$

It is instructive to compare Yo values that correspond to a typical range of diffusion coefficients of the reactant and capillary radii (Table 1).

The York number is a dimensionless value that characterizes the extent of transverse diffusion during plug injection. Transverse diffusion during the injection decreases the quality of mixing. The York number can serve as a parameter to quickly and qualitatively compare the quality of mixing for different sets of experimental parameters. As a rule, the lower the York num-

Table 1. York numbers for different diffusion coefficients of reactants and capillary radii and for an identical injection time of 5 s

D (cm^2/s) \ r_0 (µm)	25	37.5	50
10^{-4}	80	40	20
10^{-5}	8	4	2
10^{-6}	0.8	0.4	0.2
10^{-7}	0.08	0.04	0.02
10^{-8}	8×10^{-3}	4×10^{-3}	2×10^{-3}
10^{-9}	8×10^{-4}	4×10^{-4}	2×10^{-4}

ber, the higher the quality of mixing. However, the York number should be used in such comparisons with caution as it is only a characteristic of a single plug injection. It would be more instructive to analyze a quantitative parameter of the quality of mixing that is defined by the post-mixing distributions of mixed reactants. As a first step toward such an analysis, analytical and numerical approaches have been developed for the determination of post-mixing reactant distributions along the capillary (concentration profiles, for short).

4.2.2 Analytical solution

Although System 5 provides a general basis for the detailed modeling of plug formation and the calculation of concentration profiles of reactants, its analytical solution is difficult unless simplifying assumptions are made. Solving this system analytically becomes feasible if either of the two terms on the right side of the top equation is negligible with respect to the other one. In other words, the analytical solution of System 5 can be found if either (i) the York number is assumed to be zero (this assumption is equivalent to assuming that either the transverse diffusion is negligible during the injection or the injection time is much shorter than the characteristic time of transverse diffusion) or (ii) the velocity is assumed to be zero (this assumption is satisfied during the vial change between the injection of different reactants when the differential pressure applied to the capillary ends is zero). If the assumptions are used separately, they are experimentally "meaningful". The assumption of negligible diffusion during injection simplifies System 5 to:

$$\frac{\partial v}{\partial \tau} = -\omega(\tau)(1-\rho^2)\frac{\partial v}{\partial \chi} \tag{9}$$

Eq. (9) describes the plug formation during the injection and has the following general solution:

$$\lambda = \int_{\tau_0}^{\tau} \omega(\tau')d\tau'$$

$$v = F(\chi - (1-\rho^2)\lambda) \tag{10}$$

Here, λ is the dimensionless length of the injected plug, which is positive for a positive injection velocity ($\omega > 0$).

If the reactant plug is injected from a vial, in which the concentration of the substance is n_{max}, the distribution of the concentration inside the capillary is described by the following equation:

$$v(\tau, \chi, \rho) = \theta(\chi - (1-\rho^2)\lambda) \tag{11}$$

Here, $\theta(\chi)$ is a function that equals one if $\chi > 0$ and zero otherwise. This function allows one to describe the distribution of the substance not only after but also prior to the injection ($\tau < \tau_0$). According to Eq. (11), if $\tau < 0$, the concentration of the substance inside the capillary ($\chi > 0$) is zero. At the interface between the capillary and the solution in the vial ($\chi = 0$), the concentration of the substance is equal to that in the vial. According to Eq. (11), the profile of the injected plug is parabolic.

Inside the capillary, the dimensionless concentration equals one within the plug and zero outside the plug. Knowing the shape of the plug allows for the calculation of the average concentration for each section of the capillary:

$$N(\tau, \chi) = 2\int_0^1 v(\tau, \chi, \rho)\rho d\rho \tag{12}$$

In particular, for the parabolic distribution described by Eq. (11), the average "*per*-section" concentration (note that the average concentration will be reached throughout the cross-section if the reactant is allowed to diffuse in the transverse direction for a time longer than r_0/D) is:

$$N(\chi) = \theta(\lambda - \chi)(1 - \chi/\lambda) \tag{13}$$

The χ-profile of this distribution is linear.

Generally, when multiple plugs of different reactants are injected, some time is needed to change the vials. This time cannot be less than a few seconds, meaning that the assumption of no transverse diffusion during the vial switch may be unreasonable for small molecules. If the assumption of no transverse diffusion cannot be made, System 5 needs to be solved for diffusion with the single assumption of no translational movement between injections. If we assume that $\omega = 0$, System 5 can be simplified to the following equation:

$$\frac{\partial v}{\partial \tau} = Yo \frac{1}{\rho}\frac{\partial}{\partial \rho}\rho\frac{\partial v}{\partial \rho}$$

$$\frac{\partial v}{\partial \rho}\Big|_{\rho=1} = 0 \tag{14}$$

As it was mentioned above, if the reactant(s) is allowed to diffuse in the transverse direction for a time no shorter than r_0^2/D, then the average concentration will be reached throughout the cross-section according to Eq. (12). The assumption of "complete" transverse diffusion can be easily achieved experimentally by allowing long enough time (longer than r_0^2/D and shorter than l^2/D) for all reactants to diffuse in the transverse direction between plug

injections. As a result, gradients in the direction perpendicular to the capillary axis will be eliminated for all injected plugs.

4.2.3 Simulation of TDLFP using the analytical solution

The analytical solution of System 5 developed in Section 4.2.2 can facilitate the simulation of TDLFP-based mixing and the calculation of reactant concentration profiles using different sets of simplifying assumptions. The analytical solution was used to simulate TDLFP-based mixing of three reactants while assuming that: (i) longitudinal diffusion is negligible at all times and (ii) transverse diffusion is negligible during plug injections and starts only after the injection of the last plug [19]. The second assumption is very stringent but can be experimentally fulfilled when mixing large molecules such as proteins. Figure 3A schematically illustrates the steps of mixing, using the above two assumptions and the resulting concentration profiles of the three mixed reactants calculated using the analytical model [20]. The five steps of simulated mixing are as follows. In the first three steps, identical plugs of the three reactants are injected. In Step 4, a blank buffer is injected to increase the length of longitudinal interfaces and, thus, increase the level of their spatial overlap. Finally, the reactants are allowed to diffuse in the transverse direction. The result of the simulation presented in Fig. 3A demonstrates that TDLFP-based mixing leads to a significant spatial overlap of the three reactants at the inlet of the capillary.

The analytical solution of System 5 was also used to simulate the mixing of two reactants in a sandwich mode with three simplifying assumptions (Fig. 3B). The three simplifying assumptions used to analytically obtain Solution 13 are: (i) negligible longitudinal diffusion during the entire mixing procedure, (ii) negligible transverse diffusion during single plug injection, and (iii) a long waiting period between plug injections to allow for transverse diffusion to eliminate any gradients of reactant concentrations in the transverse direction. The procedure is performed in four steps. First, a 1 mm-long plug of the first reactant is injected and allowed to diffuse in the transverse direction. Second, the second reactant is injected penetrating into the plug of the first reactant and the two reactants are allowed to diffuse in the transverse direction. Third, to improve the quality of mixing, an additional plug of the first reactant is injected and the reactants are allowed to diffuse in the transverse direction. Finally, to further improve the overlap of the mixed solutions, a plug of a bare buffer is injected and the reactants are allowed to diffuse in the transverse direction to reach the final concentration profiles depicted in the lower graph in Fig. 3B. It is important to emphasize that the resulting quality of mixing is much better than one can obtain from longitudinal dif-
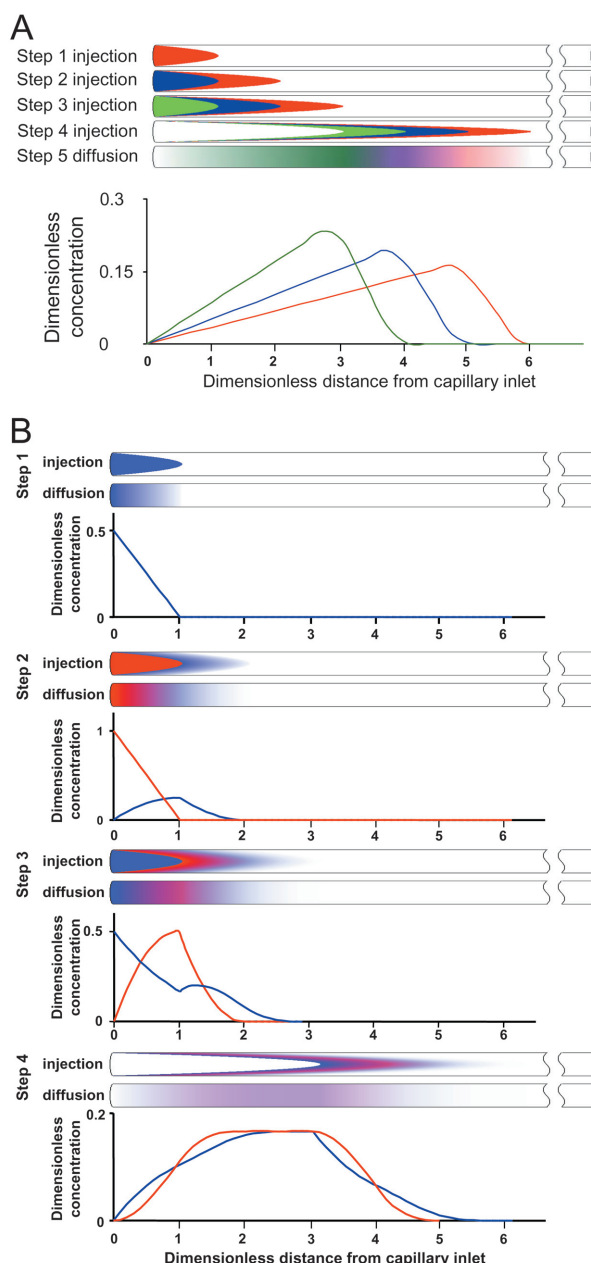
Figure 3. Simulated TDLFP-based capillary mixing of: (A) three reactants (red, blue, and green) assuming no diffusion until the last plug is injected and only transverse diffusion after the last plug is ingested, and (B) two reactants (blue and red) in a sandwich format assuming no longitudinal diffusion at any time, no transverse diffusion during the injection of every plug, and complete transverse diffusion between plug injections. The white color inside the capillary corresponds to pure solvent. The graphs show the simulated concentration profiles of the reactants along the capillary after corresponding steps of mixing. The colored plugs have identical volumes; the volume of the last (white) plug is equal to the sum of the volumes of all colored plugs.

fusion through transverse interfaces. Furthermore, the sandwich configuration provides a greater level of spa-

tial overlap than the standard sequence configuration depicted in Fig. 3A.

4.2.4 Numerical solution

Although the analytical solution of System 5 is very instructive for the qualitative study of TDLFP-based mixing, it depends on the assumption of no diffusion during the injection of individual plugs, the assumption used to obtain simplified System 9. This assumption is too stringent for the analytical model to be generic. For small molecules, transverse diffusion during the injection may be significant, making the model inaccurate. The inaccurate model cannot be used for quantitative applications, which require knowledge of the concentration profiles of the mixed reactants. Therefore, if the mixing process involves small molecules, the analytical solution of System 5 cannot be found and a numerical approach for solving the system has to be used.

The Monte Carlo method can be used for modeling the diffusion processes without "explicitly" solving System 5 [22]. This method, however, requires longer calculation times to achieve the same accuracy as the explicit numerical solution of System 5. We describe here a special case of System 5, which corresponds to injection by a constant pressure. In this case, $w = 1$ during the injection. To facilitate the solving of System 5 numerically, the equation is presented in the following form:

$$\frac{\partial v}{\partial \tau} = -(1 - u)\frac{\partial v}{\partial \chi} + 4\mathrm{Yo}\frac{\partial}{\partial u}u\frac{\partial v}{\partial u}$$

$$\left.\frac{\partial v}{\partial u}\right|_{u=0} = 0, \left.\frac{\partial v}{\partial u}\right|_{u=1} = 0 \tag{15}$$

A new variable, $u = r^2$, is introduced here. When numerically modeling System 15, it is preferable to use an algorithm that gives no distortion of concentration profiles in the absence of diffusion. Every iteration step in such a computation procedure is comprised of two sub-iterations. In the first sub-iteration, the process of longitudinal plug shift is modeled assuming no transverse diffusion. In the second sub-iteration, transverse diffusion is modeled without a longitudinal shift of the plug. The first sub-iteration is designed to average out the error in the modeling of longitudinal shifts after a large number of iterations. In the second sub-iteration, a numerically stable scheme of computation is used. Computational modeling is performed for a uniform three-dimensional grid with axes T, Y, and U, incremental steps Δt, Δy, and Δu, and lengths from zero to T, Y, and U, respectively. The resulting computational scheme is presented below:

$$n_{Y+Sy_{T,U},U}^{T+\frac{1}{2}} = n_{Y,U}^T$$

$$\frac{n_{Y,0}^{T+1} - n_{Y,0}^{T+\frac{1}{2}}}{\Delta t} = \left(\frac{2\mathrm{Yo}}{\Delta u^2}\right)\left(n_{Y,1}^{T+1} - n_{Y,0}^{T+1}\right)$$

$$\frac{n_{Y,U}^{T+1} - n_{Y,U}^{T+\frac{1}{2}}}{\Delta t} = \left(\frac{4\mathrm{Yo}}{\Delta u^2}\right) \times$$

$$\times \left[\left(U + \frac{1}{2}\right)n_{Y,U+1}^{T+1} + \left(U - \frac{1}{2}\right)n_{Y,U-1}^{T+1} - (2U+1)n_{Y,U}^{T+1}\right], 0 < U < \tilde{U}$$

$$\frac{(n_{Y,\tilde{U}}^{T+1} - n_{Y,\tilde{U}}^{T+\frac{1}{2}})}{\Delta t} = \left(\frac{4\mathrm{Yo}}{\Delta u^2}\right)\left(\tilde{U} - \frac{1}{2}\right)\left(n_{Y,\tilde{U}-1}^{T+1} - n_{Y,\tilde{U}}^{T+1}\right) \tag{16}$$

where shifts along the capillary axis, $Sy_{T',U}$, are calculated using the following algorithm:

$$Sy_{T',U} = \mathrm{round}\left\{\left[(1 - U\Delta u)T\Delta t - \sum_{T'=0}^{T'=T-1} Sy_{T,U}\right]/\Delta y\right\} \tag{17}$$

In Eq. (17), a function of rounding to the nearest natural number, *round*, is used. Variables in System 15 are related to the discrete variables in System 16 and Eq. (17) in the following way:

$$\tau = T\Delta t, \ \chi = Y\Delta y, \ u = U\Delta u \tag{18}$$

In addition, the following equality is fulfilled:

$$\tilde{U}\Delta u = 1 \tag{19}$$

This described computational procedure can be adopted by any researcher with computational experience using, for example, Excel solver.

4.2.5 Simulation of TDLFP using the numerical solution

Using the above computational approach, TDLFP-facilitated mixing of three reactants was simulated and studied under different conditions [20]. In particular, the influence of the York number, the length of the terminating solvent plug, "shaking", and double injection on the quality of TDLFP-facilitated mixing of three reactants was studied. Figure 4 shows the final distributions of the three reactants over the capillary length for the different scenarios of mixing. The order of reactant injection is: red, blue, then green. For each diagram, the York numbers of the three reactants are assumed identical and the injected volumes of the reactants are also identical. A terminal solvent plug of a volume 4 or 7 times the cumulative volume of the three reactant solutions is injected after the green reactant. This final injection plays an important role in improving the uniformity of the mixed reactants, leading to better mixing. The time between injections of individual plugs is assumed to be long
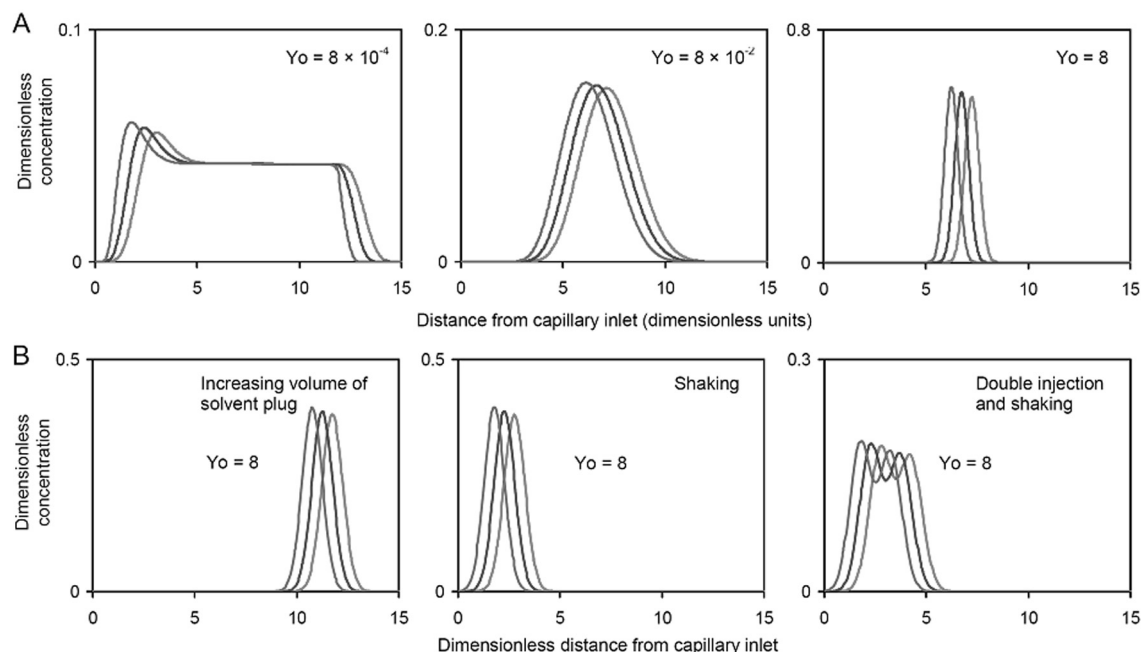
Figure 4. Numerical simulation of TDLFP-based mixing of three reactants with different York numbers (A) and different scenarios of mixing for the same York number (B). The graphs show the concentration profiles of red, blue, and green reactants after simulated mixing. In each diagram, the York numbers of the three components are identical. In Panel A, the simulation was performed for identical volumes of the red, blue, and green components followed by a terminating plug of solvent with a volume four times the cumulative volume of the three injected reactants. In Panel B, the improvement of the quality of mixing is demonstrated by: increasing the volume of the terminating solvent plug by a factor of 7/4 (left), using a "shaking" approach achieved by applying a reverse pressure pulse with duration and amplitude similar to that used for the injection of the terminating buffer plug (middle), and using double injection combined with the "shaking" approach, in which the injection of the three reactants is repeated with half concentrations, followed by injection of a solvent plug and "shaking" (right).

enough for the transverse diffusion to create complete uniformity of all reactants throughout the capillary cross-section. The quality of mixing is judged visually by the spatial overlap of the concentration profiles.

Results show that the mixing can be efficient only with a sufficiently low York number (Fig. 4A). The quality of mixing decreases with an increasing York number. This decrease becomes very significant when the value of the York number exceeds 1 (Fig. 4A, right). Low quality of mixing is attributed to fast transverse diffusion during the injection, preventing the formation of the needle-like structure of the injected plug and decreasing the efficiency of the interpenetration of the mixed solutions. One way to improve the quality of mixing is to decrease the York number by decreasing the injection time (see Eq. 6). Decreasing the injection time, while keeping the injected volume constant, can be difficult due to the hydrodynamic inertia. Commercially available instrumentation, however, makes it possible to generate short pulses (less than 1 s) at a suitably high pressure (more than 3 psi) to inject sufficiently long (more than 1 mm) plugs of solutions very fast. York numbers can be significantly decreased even for reactants with large diffusion coefficients.

Another way to improve the quality of mixing is to increase the volume of the solvent plug in the final step (Fig. 4B, left). Although increasing the length of the solvent plug decreases reactant concentration due to dilution, which may cause lower kinetic rates in concentration-dependent reactions, the dilution factor can be calculated by the mathematical models described above. This advantage allows for a-priori finding of the initial pre-injection concentration at which the desirable kinetics is achieved. One of the drawbacks of increasing the volume of the pure solvent plug is that the solution plugs travel further from the inlet of the capillary, increasing the overall volume of the mixture. This decreases the separation efficiency in the following step of microanalysis. Moreover, if the injected pure solvent is different from the separation buffer, the separation conditions may be significantly altered.

To overcome these problems, a "shaking" method, in which a series of negative and positive pressure pulses are applied to the capillary inlet after the final solvent plug, was proposed [20]. The negative pressure reverses the parabolic profiles and improves the quality of mixing. The alternating backward and forward movements perform this improvement repeatedly and are somewhat

analogous to mechanical shaking. The simulation shows that the quality of mixing for the "shaking" approach is comparable to the one with an increased volume of terminating solvent plug (Fig. 4B, left and middle). In the left graph of Fig. 4B, the terminating plug was 7/4 longer than that in the other graphs. While the effects of the longer terminating plug and shaking are similar, shaking has advantages of decreased dilution of reactants and shorter plugs. Further improvement of mixing quality can be achieved by combining the shaking approach with double injection, in which each reactant is injected separately twice with half of the concentration in each injection. For instance, after consecutive injection of the three reactants into the capillary with half concentrations, each reactant is injected again in the same order and with same concentrations. Combining the shaking approach with double injection significantly improves the quality of mixing without injecting a relatively long solvent plug (Fig. 4B, right) even in the case of a large York number.

4.2.6 Software for simulation of TDLFP

Several versions of software were designed to simulate TDLFP in Excel (as a flexible interface) and Object Pascal (as a DLL library, containing calculations which were based on System 16). The most recent versions of software have been published in the Research section of the following web page: www.chem.yorku.ca/profs/krylov. There are two versions of software interface that can be downloaded from the site. One version of software calculates concentration profiles of TDLFP-mixed reactants as an intermediate step in the determination of K_d of aptamer–protein complex formation. Another version calculates concentration profiles of an arbitrary number of reactants mixed by TDLFP but doesn't have options for modeling the reaction kinetics. Both versions have a detailed Help option.

4.3 Experimental proof and use of TDLFP

4.3.1 Mixing of two reactants

The first experimental proof of TDLFP mixing was demonstrated with two reactants: enzyme β-galactosidase and substrate fluorescein-mono-β-D-galactopyranoside [19]. β-Galactosidase catalyzes the hydrolysis of the substrate with the release of fluorescein. The enzyme and substrate were mixed using a mixing scheme as shown in Fig. 3B. The order of the four plugs was: substrate, enzyme, same substrate, and the solvent (buffer solution). After mixing, the enzymatic reaction was allowed to proceed for varying periods of time. The reaction was stopped by electrophoretical separation of the enzyme from the substrate; the product (fluorescein) was also elecrtophoretically separated from the unreacted sub-
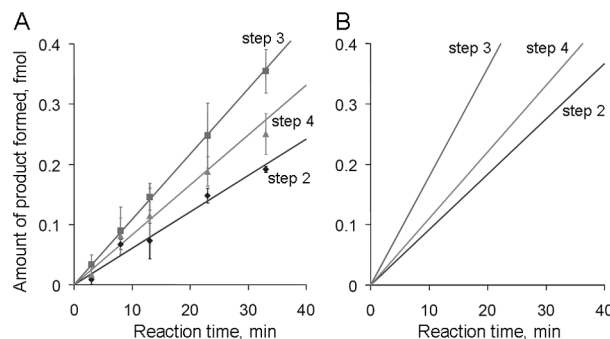

Figure 5. Experimental (A) and simulated (B) kinetics of enzymatic glycosylation of fluorescein-mono-β-D-galactopyranoside. The enzyme and the substrate were mixed by TDLFP using a procedure depicted in Fig. 3B. The simulated kinetics was calculated by integrating product formation along the capillary length using experimental values of K_m and k_{cat}.

strate. Due to the high efficiency of CE, this separation takes no longer than a few seconds. This time is much shorter than the time required for TDLFP-based mixing, thus, it can be ignored in the overall calculation. The quantity of the product was measured with a fluorescence detector at the distal end of the capillary. It was found that the enzymatic reaction proceeded in time- and concentration-dependent fashions, thus, confirming that the reactants were mixed.

Next, the experimental reaction kinetics was compared with the kinetics predicted by the analytical TDLFP model. The simulated kinetics was calculated by integrating product formation along the capillary length. This model used the values of the catalytic rate constant, k_{cat}, and the Michaelis constant, K_m, which were determined in a separate experiment by mixing the reaction components in a vial. Figure 5 compares experimental and simulated reaction kinetics for 2-, 3-, and 4-step mixing procedures described in Fig. 3B. The experimental initial reaction rates were not only in qualitative agreement but also in good quantitative agreement. The simulated reaction rates for steps 2, 3, and 4 were greater than the experimental ones by a factor of approximately 1.5. This difference between experimental and simulated rates is most likely due to the model's not taking into account transverse diffusion during plug injection. This result demonstrated that TDLFP can efficiently mix two reactants. The result also suggested that accurate quantitative prediction of the reaction rate requires more accurate modeling of TDLFP (modeling without the assumption of no diffusion during injection) which can only be achieved numerically.

Recently, TDLFP mixing of two reactants was used to facilitate the determination of the equilibrium dissociation constant of an affinity complex between a protein and a DNA aptamer [23]. A DNA aptamer is a ssDNA oligo-
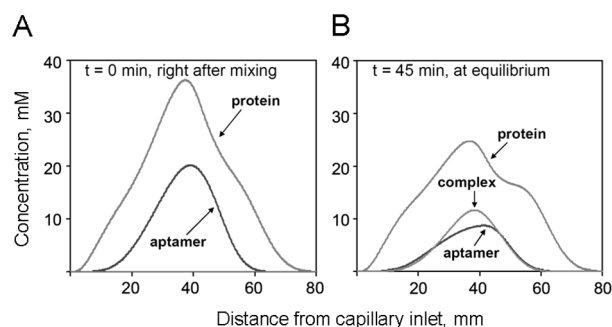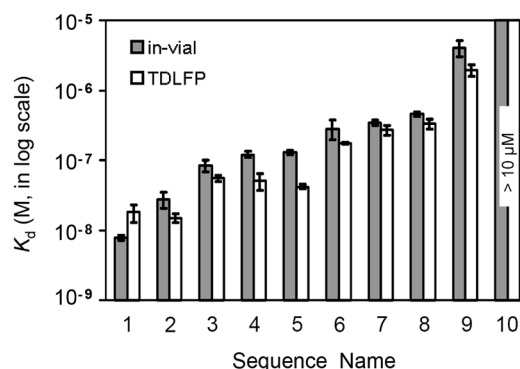
Figure 6. Numerically simulated concentration profiles of the protein, aptamer, and the protein–aptamer complex, right after mixing by TDLFP (A) and after equilibrium is reached (B). The mixing scenario was similar to that shown in Fig. 3B.

Figure 7. Ranking of DNA aptamers selected for MutS protein. K_d values for protein–aptamer interaction were measured with a KCE method known as nonequilibrium CE of equilibrium mixtures for mixing in a vial (gray bars) and TDLFP mixing in a capillary microreactor (white bars). The vertical axis is in a logarithmic scale.

nucleotide capable of binding its affinity target with high selectivity and affinity. Aptamers are selected from large libraries of random DNA sequences (10^{12}–10^{15} different sequences) by an affinity method. Kinetic capillary electrophoresis (KCE) is a conceptual platform for the development of affinity methods that can, in particular, be used for highly efficient selection of DNA aptamers. Selection of aptamers [6, 7] with KCE methods can be achieved with only a few rounds and, thus, leads to highly diverse pools of aptamers. Screening a large number of candidate aptamers for their affinity to the target protein is a challenging task. Screening an aptamer's affinity for the target protein in a capillary integrated microanalysis promises to facilitate high throughput and allows even minute consumption of the protein.

TDLFP was used as a mixing method for a proof-of-principle work on integrated microanalysis for measuring the affinity of aptamers [23]. Experimentally, TDLFP mixing of the aptamer and its target protein was achieved using a scenario similar to that described in the β-galactosidase example above and depicted in Fig. 3B. After mixing, the protein and aptamer were allowed to react and form a protein–aptamer complex. The incubation was long in order to approach the equilibrium. The complex was then separated from the unreacted aptamer and both the complex and unreacted aptamer were quantitated fluorescently using a fluorescein dye covalently linked to the aptamer.

Then, combined simulation of two processes, TDLFP mixing and complex formation, was used in combination with experimentally determined amounts of the complex and unbound aptamer to calculate the equilibrium dissociation constant, K_d, of the complex (Fig. 6). In contrast to the β-galactosidase example, TDLFP simulation was performed numerically without assuming the absence of transverse diffusion during plug injection. Numerical simulation provides greater confidence in the

accuracy of simulated concentration profiles. To facilitate the determination of K_d, the binding reaction was modeled with varying K_d values, and simulated amounts of the complex and unbound aptamer were compared with the experimental values. The value of K_d, at which the simulated and experimental amounts of the complex and unbound aptamer were the closest, was considered to be the correct one. The accuracy of K_d determination was examined by measuring K_d with a conventional method, in which protein–aptamer binding was performed in a vial and the reaction mixture was sampled for KCE-based determination of K_d [23].

Figure 7 compares K_d values measured in a TDLFP-facilitated capillary microanalysis (white bars) and the conventional CE analysis (gray) bars. There is a difference between the values obtained with the two methods. The origin of this difference most likely lies in a deviation of the real pressure dependence on time from a rectangular pressure pulse used in the simulations. The deviation is, however, not significant and allows the use of the TDLFP-based integrated microanalysis for preliminary ranking DNA aptamers and identifying suitable aptamers for more accurate measurements of K_d.

4.3.2 Mixing of four reactants

The advantage of mixing by TDLFP over mixing by electrophoresis or longitudinal diffusion is the ability of TDFP to mix more than two separately injected reactants. Mixing four reactants has been recently experimentally proven [24]. Enzymatic farnesylation of a pentapeptide, catalyzed by protein farnesyltransferase (FTase or E), was used in this work. The reaction involves two substrates, a fluorescently labeled CVGIA peptide (S1) as an acceptor of the farnesyl group and farnesyl pyrophosphate (FPP or S2) as a donor of the farnesyl group. The inhibition of this reaction was studied; therefore, the reaction mix-
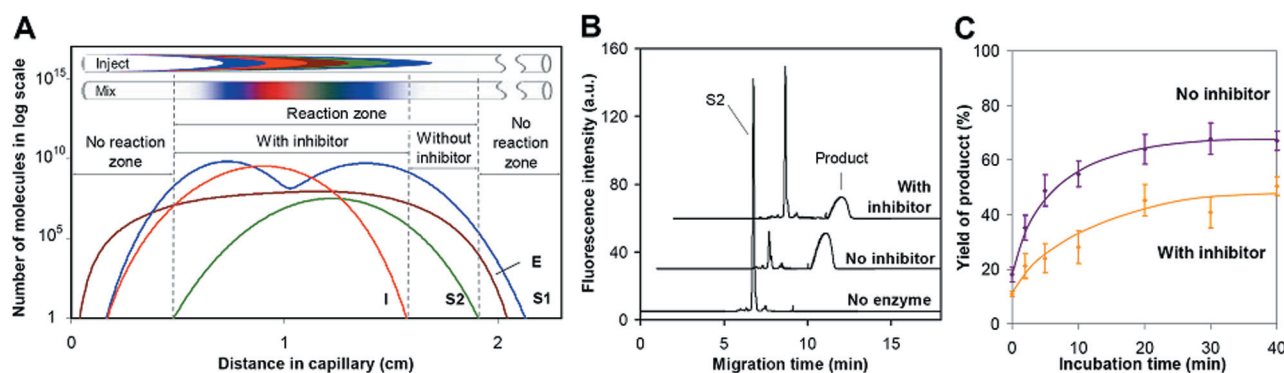
Figure 8. TDLFP-mediated capillary microanalysis (IMReSQ) for inhibition of enzymatic farnesylation of the fluorescently labeled CVGIA peptide. Panel A: Simulated distribution of reaction components in the capillary after injection (top) and after mixing (bottom) for the following plug order: enzymatic buffer (white), FPP (S1, blue), fluorescent pentapeptide substrate (S2, green), FTase enzyme (E, brown), inhibitor (I, red), FPP again, and enzymatic buffer again. Panel B: experimental results of IMReSQ analysis of FTase inhibition by a representative inhibitor (FTI-276) after 40 min incubation following injection and mixing of reaction components depicted in Panel A. The pre-injection concentrations were: S1 (75 μM), S2 (0.2 μM), E (3 μM), and I (50 μM). Panel C: Kinetics of product formation with and without inhibitor (50 μM FTI-276).

ture had to include four components: the enzyme, two substrates, and the FTase inhibitor (FTI or I).

A new term was introduced to describe TDLFP-facilitated integrated capillary microanalysis: "inject-mix-react-separate-and-quantitate" (IMReSQ) [24].

First, TDLFP mixing of the four reaction components using computer simulation with the numerical solution was studied. An algorithm for the optimization of the plug order in TDLFP mixing had not yet been developed, therefore, strictly speaking, the plug order could not be optimized. However, the concentration profiles of the mixed reaction components along the capillary for any given order of plugs could be simulated (Fig. 8A). Plug orders were tested using such a simulation. Two simple criteria were used to assess whether the tested order was reasonable or not: the number of plugs had to be small, while the spatial overlap of the reactants after mixing had to be significant. The plug order chosen for the experimental mixing was: S1, S2, E, I, and S1 again (Fig. 8A). Plugs of an enzymatic buffer were injected before and after injecting the reaction components to: (i) isolate the reaction mixture from an electrophoresis buffer containing a surfactant and (ii) improve the quality of mixing, respectively. The simulated post-mixing concentration profiles of the four components did not overlap perfectly, however, they revealed a significant reaction zone with all four components present (Fig. 8A). There was also a reaction zone with S1, S2, and E, but without I, suggesting that complete inhibition with this mixing scenario was not achievable.

In TDLFP, the required mixing time is defined by the time of transverse diffusion of the largest molecule, FTase. Computer simulation showed that, for the experimental conditions used, the sufficient mixing time was less than 1 min. The reaction time was longer than the

mixing time, which suggested that only a negligible amount of the product was formed during mixing.

Second, it was experimentally demonstrated that TDLFP actually mixed the four reactants and that the product formation could be observed along with the inhibition. The absence of either reactant meant that the concentration of reactants in the injected plug were zero (a pure enzymatic buffer was injected instead of the corresponding reactant). In this part of the study, the FTI-276 inhibitor, which had been previously proven to inhibit mammalian FTase, was used to test the inhibition of FTase from *Entamoeba histolytica*, a parasite. When the concentration of E was zero, no P was formed, and a single peak of S2 was detected (Fig. 8B, lower trace). In the presence of E but without I, S2 was converted into P and, accordingly, two peaks observed after P were separated from the remaining S2 (Fig. 8B, middle trace). Finally, in the presence of I, the reaction rate was lower and the amount of P formed during the same incubation time was smaller. The peak of P was smaller while the peak of the remaining S2 was higher (Fig. 8B, upper trace) than those in the absence of I. When the incubation time varied, the reaction kinetics could be studied. In the absence and presence of I, classical Michaelis–Menten kinetics was observed (Fig. 8C). Thus, all four components were mixed to the level at which the rate of P formation depended on I.

Third, the ability of the IMReSQ method to quantitatively rank the potencies of inhibitors was tested. Conventionally, IC_{50} values (inhibitor concentrations that cause 50% reduction of the reaction rate) are used to rank inhibitors. IC_{50} depends on enzyme and substrate concentrations and is, thus, inapplicable to IMReSQ, in which the solutions are not ideally mixed. To quantitatively rank inhibitors by IMReSQ, a new parameter, PIC_{50},

Table 2. IC$_{50}$ and PIC$_{50}$ for the inhibition of FTase-catalyzed farnesylation of CGVIA determined by traditional (in-vial reaction) and IMReSQ methods, respectively

Inhibitor	Traditional IC$_{50}$ (μM)	IMReSQ PIC$_{50}$ (μM)	PIC$_{50}$/IC$_{50}$
FTI-276	1.1 ± 0.3	11.2 ± 3.2	10.2 ± 2.9
FTI-277	2.1 ± 0.4	18.9 ± 3.8	9.0 ± 1.8
FTI-651	71 ± 17	800 ± 70	11.3 ± 2.7
FTI-656	61 ± 18	620 ± 90	10.2 ± 3.1

was suggested. PIC$_{50}$ is a pre-injection inhibitor concentration that causes a 50% reduction in the reaction rate. Ranking of several potential inhibitors of FTase obtained by a "traditional" method, using IC$_{50}$, and by the IMReSQ method, using PIC$_{50}$, was compared. In the traditional method, microliter volumes of S1, S2, I, and E were mixed in a vial and incubated to form P. A nanoliter volume of the reaction mixture was injected into the capillary and P was separated from the remaining S2 and their amounts were quantitated. The PIC$_{50}$/IC$_{50}$ ratio was identical within the error limits for all inhibitors (Table 2).

The constant PIC$_{50}$/IC$_{50}$ ratio suggests that IMReSQ can be used for quantitatively ranking the potencies of the inhibitors. Four inhibitors of mammalian FTase (FTI-276, FTI-277, FTI-651, and FTI-656) were found to be potent for FTase from *E. histolytica*. These compounds can, therefore, be used as templates for antiparasite drug development. To conclude, TDLFP can facilitate the mixing of very complex reaction mixtures in the capillary and, thus, facilitate IMReSQ-integrated microanalyses for all types of enzymes.

4.3.3 TDLFP-facilitated cell lysis for chemical cytometry

The generic capabilities of TDLFP contributed to further advances in chemical cytometry [25]. Chemical cytometry is a collective term applied to techniques that employ highly sensitive chemical analysis instrumentation and methods to quantitatively study the chemical contents of single cells [14]. In chemical cytometry, the cell to be analyzed is first lysed to release its chemical contents from the cellular compartments and dissolve them in the surrounding buffer solution. The molecules of interest are then separated by CE or capillary chromatography and detected by LIF, electrochemistry, or MS. CE–LIF is the most widely used combination of separation and detection in chemical cytometry as CE can separate all kinds of biomolecules, and LIF can be used for the detection of single molecules.

TDLFP has been proven to facilitate highly efficient cell lysis inside the capillary for chemical cytometry [25]. A mild surfactant, Triton X-100, was used as a lysing agent. It was either dissolved in the run buffer or its solution was introduced into the capillary by pressure as a short plug prior to cell injection. The cell was then injected by pressure within a plug of a physiological buffer. TDLFP was expected to mix the surfactant with the physiological buffer leading to surfactant's contact with the cell and subsequent cell lysis. Cell lysis by a short plug of the surfactant was investigated to exclude the surfactant from the run buffer (a surfactant in the run buffer can worsen the quality of CE separation).

To detect cell lysis, Madin–Darby canine kidney cells, expressing green fluorescent protein (GFP), were used in the experimental study. Figure 9 shows electropherograms of intact and lysed cells corresponding to different scenarios of mixing. A very sharp spike-like peak with a migration time of 2–3 min was observed for intact GFP-expressing MDCK cell (Fig. 9a). When a run buffer was supplemented with a lysing agent, a wide peak corresponding to the GFP molecules dissolved in the run buffer was detected (Fig. 9b). Hence, the spike-like peak was employed as a criterion of a cell not being lysed, while a wide peak of GFP was an indication of successful cell lysis inside the capillary. A wide peak of GFP combined with a sharp spike was interpreted as partial cell lysis, with a part of the cells remaining intact.

The TDLFP-based cell lysis was performed using two- and three-step injection, in which a plug of the lysing agent was introduced only before (two-step injection), only after (two-step injection), or both before and after (three-step injection) a plug of the physiological buffer with a cell. The observed results confirmed the hypothesis that TDLFP could facilitate the mixing of the surfactant with the physiological buffer and cause cell lysis.

The TDLFP-based cell lysis was validated by comparing pressure- and EOF-driven injections. In general, plugs injected by EOF could be mixed only by longitudinal diffusion if not mixed by electrophoresis (see Section 2). Longitudinal diffusion was expected to be much less efficient than TDLFP due to the high length to diameter ratios of the plugs (see Section 3). The experimental results were compared for two scenarios: the first, when a single cell was injected by pressure for the TDLFP experiment, and the second, when a single cell was injected by electroosmosis for the longitudinal diffusion experiment. When the surfactant and the cell were injected into the capillary by electroosmosis, the cells were left nonlysed in 80% of experiments, and the cells were partially lysed in 20% of experiments. In case of pressure-driven injection of the lysing agent and a cell, the cells were completely lysed in 80% of experiments and partially lysed in 20% of experiments. An even higher efficiency (100%) of TDLFP-driven cell lysis was achieved using the "sandwich" approach, in which plugs of the lysing agent were injected prior to and after the cell injection. These experiments confirmed that TDLFP-facilitated efficient diffusion of the lysing agent toward
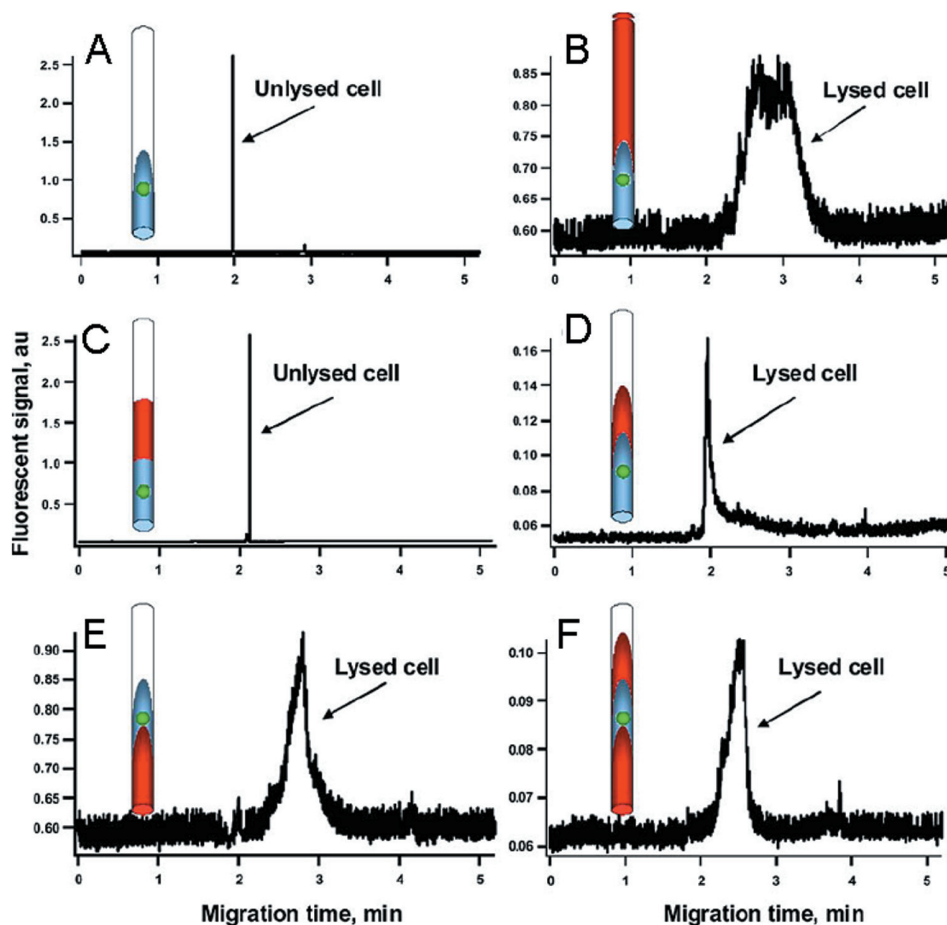
Figure 9. TDLFP-facilitated cell lysis in a capillary microreactor. Electropherograms of GFP-expressing cells under different scenarios of cell injections/lysis. Inserts show the corresponding scenarios of injection. The white color corresponds to the bare run buffer. The red color corresponds to the lysing agent (Triton X-100) dissolved in the run buffer. The blue color corresponds to the physiological buffer (PBS), in which the cells are suspended. The cell is shown as a green circle. Panel A: The inlet and outlet reservoirs and the capillary contain the bare run buffer. A cell is injected by suction. Panel B: The inlet and outlet reservoirs and the capillary contain the lysing agent. A cell is injected by suction. Panel C: The inlet and outlet reservoirs and the capillary contain the bare run buffer. First, a plug of the lysing agent is injected in the capillary by an electric field. Second, a cell is injected by an electric field. Panel D: The inlet and outlet reservoirs and the capillary contain the bare run buffer. First, a plug of the lysing agent is injected in the capillary by suction. Second, a cell is injected by suction. Panel E: The inlet and outlet reservoirs and the capillary contain the bare run buffer. First, a cell is injected by suction. Second, a plug of the lysing agent is injected by suction. Panel F: The inlet and outlet reservoirs and the capillary contain the bare run buffer. First, a plug of the lysing agent is injected by suction. Second, a cell is injected by suction. Third, another plug of the lysing agent is injected by suction.

the cell and promoted complete cell lysis. Lysis inside the capillary was methodologically and instrumentally simple. Also, it guaranteed no losses of cellular components. In addition to cell lysis, TDLFP can be potentially used in chemical cytometry to mix cellular components with labeling reactants, affinity probes, inhibitors *etc.*

5 Future directions

The three methods of mixing reactants in capillary microreactors (electrophoresis, longitudinal diffusion, and TDLFP) require different levels of attention in future research.

Mixing by electrophoresis has been relatively well studied. Its utility was proven for mixing two reactants with different electrophoretic mobilities. While the mixing of more than two separately injected reactants by electrophoresis does not seem to be practical (see Section 2), it would still be interesting to see if someone can experimentally demonstrate such mixing.

Mixing by longitudinal diffusion is a highly inefficient process (see Section 3). In our view, TDLFP rather than longitudinal diffusion could be the major reason of the observed mixing in the "at-inlet" experiments, which was always ascribed to longitudinal diffusion. We think that mixing by longitudinal diffusion does not require any further consideration or experimentation.

Mixing by TDLFP is the most generic way of in-capillary mixing of reactants. While much has been done to prove the principle of TDLFP and develop its theory, the method can still be considered in its infancy with regards to the number of its practical applications. To make the method practical, a number of issues have to be addressed.

An algorithm for the optimization of TDLFP mixing has to be developed. To develop such an algorithm, a quantitative parameter that characterizes the quality of mixing has to be introduced. This issue is not trivial. Mixing is arguably the oldest chemical procedure practiced by humankind; nevertheless, a quantitative parameter to characterize the quality of mixing has never been introduced. The historical reason for this deficiency is clear. Classical mixing is a stochastic process in which the solutions are broken into microvolumes by vortexes and reactants in microvolumes are finally mixed by diffusion. The stochastic nature of classical mixing does not allow the introduction of a nonstochastic function to describe the efficiency of mixing. Mixing by TDLFP leads to well-defined concentration profiles of the reactants. Knowing the concentration profiles makes it possible to build a function that can characterize the quality of mixing. It is obvious that such a function would be applicable to all three types of mixing considered in this review, as all of them are vortex-free and, thus, lead to well-defined concentration profiles of the reactants. Therefore, the development of a quantitative parameter to characterize the quality of mixing has to be the immediate task for researchers working on the design of capillary microreactors and their applications.

To make the output of TDLFP mixing accurate, two types of parameters have to be defined with good accuracy: the diffusion coefficients, D, of all the reactants and the time-dependence of the flow velocity $v_0(t)$ in the center of the capillary ($r = r_0$, see Eq. 1). Diffusion coefficients can be estimated based on the molecular weight of molecules [26–28]. Diffusion coefficients can also be experimentally determined by a number of methods including several CE-based methods [29–31]. As for $v_0(t)$, it can be accurately calculated using the Poiseuille equation:

$$v_0(t) = \frac{1}{4\eta}\frac{P(t)}{L}r_0^2 \qquad (20)$$

Here, η is a kinematic viscosity, r_0 a capillary radius, L a capillary total length, and $P(t)$ is the pressure differential as a function of time. The accurate determination of $v_0(t)$ requires the knowledge of accurate values for η, L, r_0, and $P(t)$. While the first three parameters can be accurately determined, the determination of the fourth one, $P(t)$, is somewhat a challenge. Commercial CE instruments utilize differential pressure for sample injections but do not return the exact pressure profile. It is assumed that pressure profiles are rectangular, and such an assumption

works well for injections in regular (not based on TDLFP) applications of CE. However, this assumption, if it is not accurate, can lead to significant errors in calculated concentration profiles of reactants mixed by TDLFP. Therefore, it is important that capillary instruments used for TDLFP mixing are equipped with accurate fast response in-line pressure gauges for the determination of $P(t)$.

Another instrumentation requirement for efficient TDLFP is fast robotics for rapidly switching reactant vials to reduce dead time between injections. The dead time leads to parabolic profile dissipation and a decrease in the quality of mixing. Although, it should be noted that dead time will not decrease the accuracy of concentration-profile determination if the transverse diffusion during dead time is taken into account. Capillary instrumentation with fast vial switching is not yet available. While designing such instrumentation is not an immediate task, it is important that it should be addressed prior to the TDLFP method hitting its first industrial applications.

An important aspect in the development of the TDLFP method is user-friendly software for the calculation of concentration profiles, and for further use of these concentration profiles in calculating the reaction rate or product yield integrated over the length of the capillary microreactor. So far, most computer programs working with TDLFP were designed (in Excel and Object Pascal) as experimental tools and, thus, do not have very user-friendly interfaces. However, these programs are available for researchers free of charge from the Research section on the following web page: www.chem.yorku.ca/profs/krylov. New type of software will be needed to optimize the quality of mixing when a quantitative parameter is developed to characterize mixing quality.

Calculation of concentration profiles of TDLFP-mixed reactants is the first step in the application of TDLFP. The next step depends on the specific application. If the goal is to evaluate the mechanism of reaction and find rate constants, then the sequence of operations that need to be processed by the software is the following:

(i) Calculate concentration profiles (along the capillary) axis of reactants R_1, R_2 *etc.* mixed by TDLFP: $R_1(x), R_2(x)$ *etc.*

(ii) Propose a hypothetical reaction mechanism with the formation of products P_1, P_2 *etc*:

$$R_1 + R_2 \underset{k_-}{\overset{k_+}{\rightleftharpoons}} P_1 + P_2$$

(iii) Calculate reaction rate (integrated along x) as a function of k_+ and k_-: $Rate(k_+, k_-)$.

(iv) Compare the experimentally measured reaction rate with the calculated one.

(v) The values of k_+ and k_-, for which the difference between the experimental and calculated rates are

minimal, are most probable for the proposed hypothetical mechanism. If the minimal difference between the rates is too large, another hypothetical mechanism needs to be proposed and considered.

TDLFP and IMReSQ-type microanalyses based on it can save a considerable amount of time and materials and help to automate routine experiments such as screening of large numbers of inhibitors, affinity ligands, substrates *etc*. The major industrial interest in TDLFP and IMReSQ is due to their suitability for HTS with nanoliter consumption of reactants. HTS applications require multi-capillary instrumentation with fast vial switching and an accurate pressure gauge, which is currently not available. In our view, development of such instrumentation must be an immediate goal of both academic and industrial researchers and engineers.

To conclude, we foresee that IMReSQ-type integrated capillary microanalyses will be widely used in HTS for drug development. TDLFP, as a generic method of mixing reactants in capillary microreactors, has a potential to greatly facilitate such analyses. TDLFP currently requires significant attention of the research community to address the outlined issues and develop the method to meet industrial standards.

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