Microfluidic arrays for high-throughput submicroliter assays using capillary electrophoresis

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Microfluidic arrays enable the required quantities of precious reagents to be greatly reduced in the measurement of enzymes of interest to the pharmaceutical industry. Reductions of the assay volume to the submicroliter range does not cause a reduction in sensitivity relative to other methods, because of the use of confocal fluorescence optics. High-resolution separations enable the addition of internal standards as well as the use of impure reagents.

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▼ In lead discovery programs, pharmaceutical companies are increasingly screening large compound libraries against increasing numbers of targets. This expansion is driven by the availability of combinatorial libraries and the successful identification of new targets from large-scale genomics projects. Today, the pharmaceutical industry conducts hundreds of millions of primary screening tests every year. The annual total could increase to several billion tests by 2005.

Growth in the number of screening tests conducted means that faster screening systems are required, together with lower costs per test. Reduction of the assay volume is one way to reduce reagent costs, which currently average approximately US$0.50 per test and could total US$1.00 per test. Multiwell plates with smaller well volumes than standard 96-well plates (assay volume ≤100 μl) provide a simple method to reduce assay volumes. Plates with 384 wells (assay volume ≤50 μl) and 1536 wells (assay volume ≤10 μl) are now widely available[1,2]. However reducing assay volumes while using traditional detection methods (scintillation proximity, luminescence, fluorescence polarization)[3] often compromises sensitivity as well as the signal-to-noise ratio.

Optical instrument systems can read 384- and 1536-well plates in the same time required for conventional 96-well plates[4]. Multiwell plates with even higher densities have been produced but these generally require specialized, proprietary dispensing and handling technologies such as the PlateTrak (Packard Instrument Company, Downers Grove, IL, USA). However, the speed, complexity and number of unit processes (i.e. dispensing, mixing, incubation and physical separations) usually limits throughput.

Reducing assay volumes to a few microliters or less presents major technical problems. These challenges include evaporation control, fluid dispensing and mixing. One microliter of water will evaporate from an open well in only a few minutes, and this effect reduces assay precision. At submicroliter volumes, electrostatic effects and trapped bubbles compromise the performance of fluid dispensers.

Mixing in small-volume wells can be unreliable as the inertial energy of liquids can be insufficient following dispensing and diffusional mixing is too slow. New microdispensing technologies could alleviate some of these problems[5]. For standard high volume (≈100 μl) assays, current technology will still be adequate as test throughput increases. Other assays, however, become impractical because of the limitations of high-throughput/low-volume dispensing.

Microfluidic systems for submicroliter-scale HTS

Microfluidics is a term applied broadly to technologies that move fluids through microscopic

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Box 1. Mechanism of capillary electrophoresis

Capillary electrophoresis (CE) is an analytical technique that can achieve rapid high-resolution separation of water-soluble components present in small sample volumes. Separations are based on the electrically driven movement of ions. In HTS, analytes are separated according to differences in electrophoretic mobility, dependent on the charge-to-size ratio. Separation is optimized by variation of the buffer pH and, in some applications, of a polymer additive that provides sieving based on molecular size. In conventional CE in glass or silica capillaries, analytes migrate toward the same electrode because of a bulk-flow of solvent known as electro-osmosis.

Electro-osmosis results from an excess of counterions in the solution double layer close to the capillary or channel wall that balance the charge of the ionized surface groups. In the commonly used electrophoresis buffer pH range (4.0–9.0), electro-osmotic flow is greater in silica capillaries or channels etched in glass than in channels molded from plastic. Electro-osmotic flow can be reduced or eliminated by chemically modifying the internal walls of the separation channel, or by using plastic materials such as acrylics, which have a low surface-charge density. Separation performance in CE is influenced by the pH of the separation medium, electric field strength, separation distance and temperature; application of a high voltage and a short separation length translate into rapid, efficient separation. Separated analytes are usually detected in the capillary/channel as they pass a fixed detection point. Laser-induced fluorescence detection is typically used to provide the high detection sensitivity.

channels to perform experiments. Microfluidic devices employ non-mechanical, electrokinetic processes (electrophoresis, electro-osmosis) to move solutions or suspensions of buffers, analytes and reagents (including high-MW biologicals, such as enzymes, and cells; Box 1). Microfabrication technologies can produce arrays of microfluidic devices to conduct a large number of analyses on a single device. Integrating sample handling, mixing, incubation, separation and detection into a single microfluidic device creates miniaturized total chemical analysis systems (microTAS)[6–9]. Microfluidic-based systems require submicroliter volumes of reagents and samples. Aside from HTS applications, nanoscale arrays offer advantages for many industrial, medical and diagnostic applications[10].

Several companies are developing capillary electrophoresis (CE)-based microfluidics for HTS. Typically, such technologies are funded in part by pharmaceutical industry partners in exchange for exclusive or partly exclusive early access to the technology. ACLARA BioSciences has developed single-use, plastic arrays enabling up to 12 microfluidic processes and CE analyses to be performed in parallel. This technology will be described in more detail later. Caliper Technologies (Mountain View, CA, USA) has introduced single-channel glass chips in partnership with Agilent Technologies (Palo Alto, CA, USA). These devices use electrokinetic injection of library compounds into a chip where the compounds merge with a stream of reagents to form a 'train', and this is then analyzed by CE separation in series[11]. Cetek (Marlborough, MA, USA) uses a 'mobility shift' method to identify target-binding ligands in libraries of synthetic compounds and natural extract libraries, enabling the ranking of binding affinities. This method uses electrophoresis in one or more conventional silica capillaries as the analysis method[12–14].

CE as a microfluidic method

Aside from the reduction in sample/reagent use, CE methods have other advantages for use in HTS. Firstly, CE provides the added dimension of separation by separating the assay signal from the chemical background in the sample and/or reagent(s). For example, CE-based separation enables protein kinase assays to be performed with impure (≈50%) peptide substrates. This enables assay development without the requirement for expensive and lengthy purification procedures. CE methods could offer advantages over techniques such as homogeneous time-resolved fluorescence (HTRF), where reagent preparation can be problematic[15,16].

Secondly, microfluidic array-based CE separations are extremely rapid and efficient, requiring only a few seconds (Fig. 1). The inherently rapid transport of mass [such as solutes (ions) and, in some circumstances, water] in microchannels translates directly to faster separations and a higher resolution[17]. For example, high-resolution DNA separation in microfluidic devices was recently demonstrated[18]. Performing assays in parallel using multiple-head optical systems enables throughputs of up to 100,000 assays per 16-hour working day. Reagent mixing on the arrays is rapid using mechanical means. A key feature of microfluidic CE systems is the injection scheme. Reaction mixtures are generally delivered from one capillary, and then separated in a second capillary that intersects the first capillary. This process confines the sample for analysis to a very low volume (typically <1 nl).

Thirdly, for high sensitivity, most microfluidics-based CE methods use laser-induced fluorescence (LIF) detection. However, other detector designs such as UV, electrochemistry[19] and chemiluminescence[20] are also used. Recently, single-molecule detection using LIF detection was demonstrated[21]. The multiplexed confocal optics developed for the LabCard (ACLARA BioSciences) device provides two exitation wavelengths (488 and 633 nm) (Fig. 2). The detection limit achieved using these optics is ≈50 pM for both fluorescein and Cy-5 fluorescent dyes.

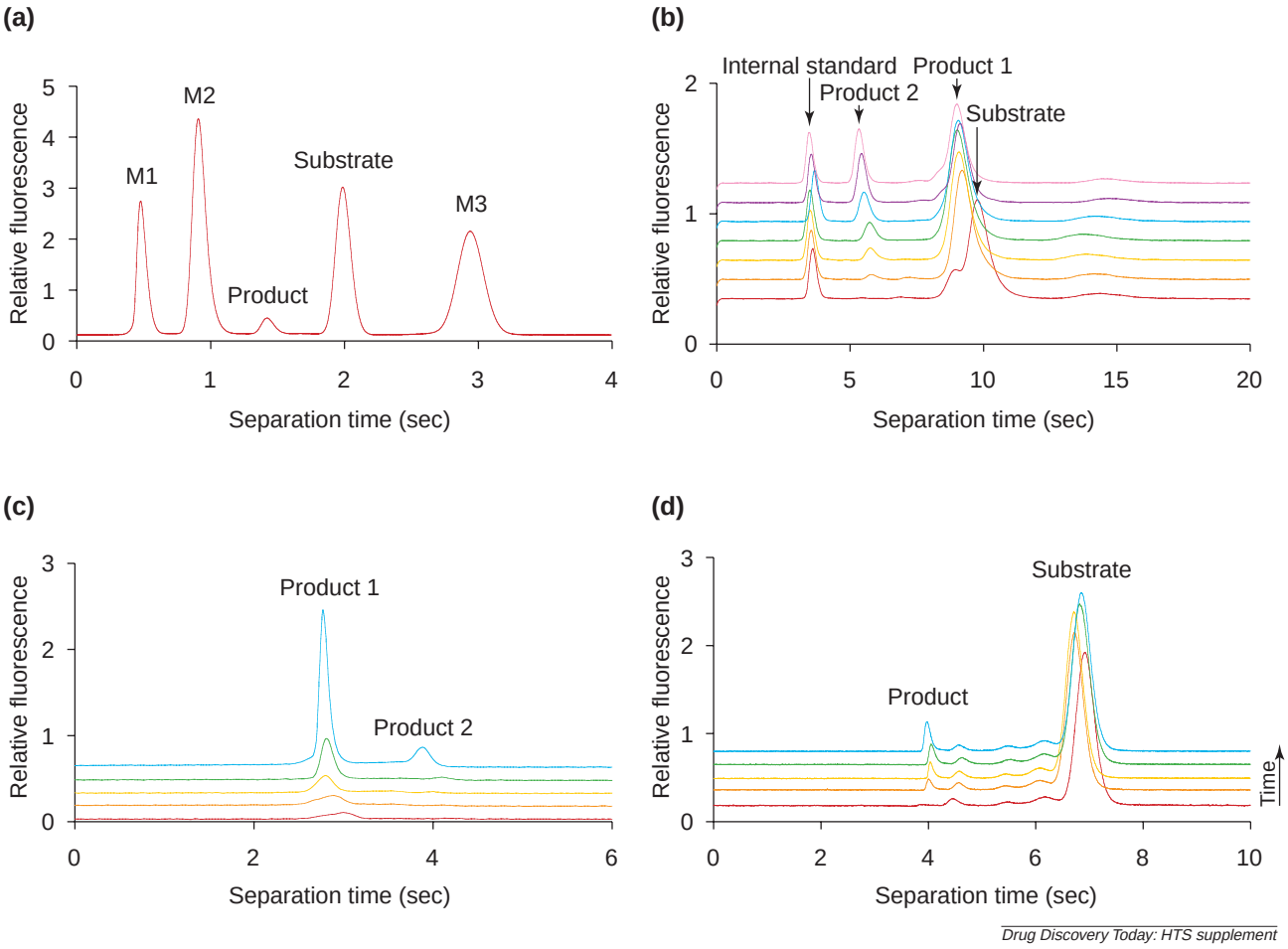

Figure 1. Assay results using a LabCard (ACLARA BioSciences) for a variety of enzymes. Data were collected using a 'breadboard' instrument equipped with an argon-ion laser focused in the separation capillary. Detection was by a photomultiplier tube. (a) An assay of Src-kinase using a separation voltage of 800 V cm^{-1}. A fluorescein-labeled peptide substrate and kinase product were separated. The markers (M1, M2 and M3, added for internal control) were in the micromolar range, and the product concentration was ≈500 nM. In (b), (c) and (d), several electropherograms corresponding to the assay time course are shown offset on the vertical axis for clarity. In reality, the baselines of each electropherogram would be at the same relative fluorescence level. (b) An assay of a protease (Cathepsin-L), using a fluorescein-labeled peptide substrate. The time-course of product formation over a few minutes is shown. Two products were formed and can both be used to measure enzyme activity. (c) An assay of alkaline phosphatase, where fluorescein bisphosphate was incubated with the enzyme for several minutes. Two products were formed, fluorescein monophosphate (product 1) and fluorescein (product 2). (d) An assay of phospholipase-C, using a fluorescein-labeled substrate. Fluorescent impurities in the substrate did not interfere with the assay.

Flexibility in chip design: serial versus parallel format, single versus multiple usage

Microfluidic CE devices can be designed for single or multiple use. In a sequential format, the device processes multiple assays in the same separation element (i.e. structure in a chip in which the separation is performed). In a parallel format, the device processes each assay in a separate separation element and the device contains multiple elements, thus permitting multiple assays in parallel. Silicon and glass 'master chips' combined with plastic injection molding or embossing is used to make inexpensive, single-use (disposable), microfluidic arrays.

Single-use arrays combined with parallel processing and reading offer the following advantages over reusable devices with sequential processing:

- They preclude carryover of compounds between assays that can result in contamination.
- There is no buildup of reagents because of adsorption in capillaries.
- Screeners can increase the sensitivity of enzyme assays by increasing incubations time (e.g. 10–100 min), while sequential devices must employ short (seconds) incubation times to have a useful throughput.

(a)

(b)

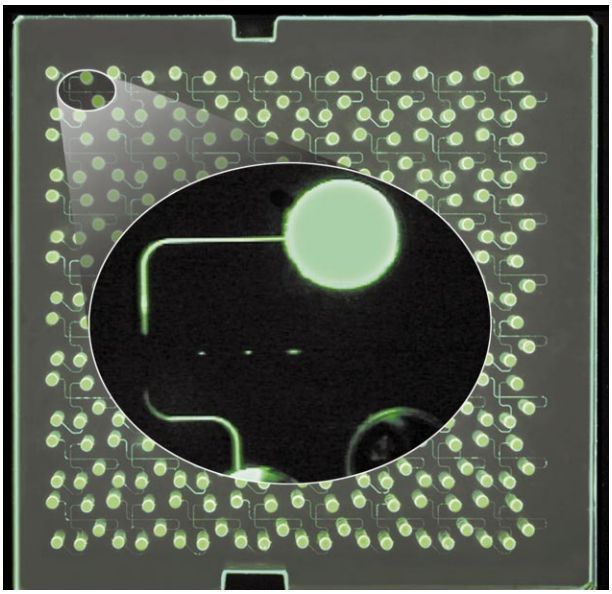

Figure 2. (a) Plan view of a LabCard (ACLARA BioSciences). The card has the same exterior dimensions as a 96-well plate but it is only 2 mm thick. Separation elements (96) are arrayed on a 9 mm spacing. The device is made by bonding a structure formed by injection-molding acrylic resin that has channel and well features to a thin film of plastic. Each separation element has four small wells that serve as contacts for electrodes during capillary electrophoretic separation. Wells are connected by two capillary channels. One well serves as a site for assay incubation. After completion of an assay incubation, reaction products are injected electrophoretically. Well dimensions are ≈1.5 mm in diameter, capillaries are ≈5 mm in length and 70 × 30 μm in cross section. (b) Photomicrograph (insert) of a separation of the SRC kinase assay product in a 64-channel LabCard device.

- Serial devices can be prone to blockage by debris from reagents and library compounds.

Figure 2 shows an example of a CE-microfluidic array. Incubations can be performed in volumes as low as 300 nl within the array or can be carried out in a standard plate at larger volumes (1–5 μl) and then transferred to the array for measurement. ACLARA is developing dispensing technology to transfer reagents and library compounds into LabCard devices. The dispensers can precisely deliver 50–200 nl of liquid into wells on the card and evaporation control is achieved by mechanical means such as adhesive lids.

Microarray CE enzyme assays

Data with several different types of enzyme assays validate the utility and value of microfluidic CE arrays for HTS (Refs 11,22,23) (Gibbons, I. *et al.* Screening of enzyme inhibition by capillary electrophoresis in single-use LabCard microfluidic devices. *5th Annual Meeting of the Society for Biomolecular Screening*, 13–16 September 1999, Edinburgh, UK). The assay format uses a fluorescent or fluorogenic substrate (and/or a means to couple the enzyme reaction to a fluorescent product) and produces a fluorescent product that is separated by CE from the fluorescent substrate and other fluorescent background. Fluorescent reagents (or materials to produce them by simple chemistries) are commonly available as commercial products. For example, kinase assays can be carried out using fluorescein-labeled peptides that are readily produced from commercial reagents.

The separator design shown in Fig. 2 yields highly sensitive and rapid assay measurements for protein kinases, phosphatases, proteases, cytochrome P450s and phospholipases (Fig. 1; Gibbons, I. *et al.* Screening of enzyme inhibition by capillary electrophoresis in single-use LabCard microfluidic devices. *5th Annual Meeting of the Society for Biomolecular Screening*, 13–16 September 1999, Edinburgh, UK). In these assays, the CE separation takes only a few seconds and coefficients of variance values are routinely less than 10%. Using confocal fluorescent optics, optical sensitivity of significantly less than 1 nM for the measured species (substrate and product in the case of enzyme measurements) has been demonstrated for fluorescein- and Cy-5-labeled substrates (Gibbons, I. *et al.* Screening of enzyme inhibition by capillary electrophoresis in single-use LabCard microfluidic devices. *5th Annual Meeting of the Society for Biomolecular Screening*, 13–16 September 1999, Edinburgh, UK). However, in enzyme assays, sensitivity in terms of the enzyme concentration is limited by fluorescent chemical impurities in the reagents rather than by optics, and is often higher or at the least comparable with 'conventional' assay methods. For example, the src kinase[24] assay enables the measurement of 50 pM enzyme with only a few minutes incubation. With this level of sensitivity, usage of the most expensive reagent (typically the enzyme) is at least 100-times lower than that for conventional assays run at 100 μl volumes.

As can be seen in Fig. 1, internal standards are usually added to reagents (for example, to the enzyme reagent). By measuring the ratio of the enzyme product to the internal standard, the precision of the assay can be significantly improved.