(7) Observational findings will need to be followed up with in vitro or in vivo experiments, leading to the molecular mechanisms responsible for the observed interactions.

(8) Complex phenotype and genotype interactions require the analysis of their combined effects. The current statistical tools are limited in their ability to deal with this complexity. Therefore development of appropriate statistical tools will become indispensable for analysing and understanding the effects of variations in multiple genes, in combination with a large body of environmental and phenotypic information. Likewise, the information will need to be incorporated into predictive models that can then be used clinically to improve disease assessment and prevention.

(9) Finally, bioinformatics will allow a more comprehensive integration of the knowledge generated by different areas of research.

Summary

Nutrigenomics is evolving as a most promising area of research that will revolutionize nutrition research and public health. The increased ability to generate genotypic information, in combination with knowledge from the Human Genome Project and more comprehensive experimental designs, will dramatically improve our capacity to answer many of our current questions and to prove the validity of the goal behind this research; namely, to demonstrate that knowledge of an individual’s genetic background will facilitate more precise dietary counselling and intervention and more efficacious primary and secondary CHD prevention. The knowledge and models developed for cardiovascular disease will also be applicable to the other major diseases that plague modern society.

References


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Application of disposable plastic microfluidic device arrays with customized chemistries to multiplexed biochemical assays


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Abstract

Plastic microfluidic array platforms and synergistic multiplexed assay chemistries are under development for a variety of applications, including assays of gene expression, proteomics, genotyping, DNA sequencing and fragment analysis, sample preparation and high-throughput pharmaceutical discovery. The low production costs of plastic substrates makes possible economical single-use device arrays, eliminating cleaning and sample-to-sample carryover contamination. Hundreds of microchannels and reservoirs are readily included on a single microtitre-plate-size substrate, enabling the manufacture of highly parallel fluidic array systems to increase throughput and speed.

Introduction

The revolutionary miniaturization of analytical instrumentation and methodologies is nowhere...
more evident than in microfluidic systems. By accomplishing common laboratory procedures from filtration and mixing to separation and detection in planar ‘chip’ format, this technology can revolutionize a host of existing and emerging bioanalytical applications. Rapid progress in microfluidics relies upon advances in microfabrication technologies, customized assay chemistries, materials development and packaging concepts, marrying everything from biology to electrical engineering in a single monolithic microsystem.

Based upon interconnected networks of microchannels and reservoirs with tiny volumes, microfluidic devices are well matched to the demands for small volume, rapid response, parallel analyses and minimal cross-contamination that characterize applications in biotechnology. Accordingly, many promising applications of microfluidic systems are found in genetic analysis, pharmaceutical discovery and medical diagnosis. This review focuses on plastic microfluidic systems, which have sufficiently low manufacturing costs to be both disposable and adaptable – in the form of highly customized designs for each application – to a wide range of problems.

Progress towards microfabricated liquid-phase analytical systems made a revolutionary leap when electrokinetic phenomena were proposed as a means to motivate and separate dissolved species on planar chips. Electrophoresis provides high-resolution, low-dispersion separations, while electro-osmosis can pump dissolved charged and neutral species, as well as solvent, through capillaries and microchannels using electric fields. When filled with ion-containing aqueous solution, channels made from glass and similar materials have a pH-dependent fixed charge bound to their wetted surfaces. At neutral to basic pH values, this charge is negative. Some moderately polar polymers, such as poly(methylmethacrylate) and poly(carbonate), also have measurable fixed negative surface charges at appropriate pH values. Immobile surface charge attracts a layer of partially solvated charge-compensating (positive) ions from solution; application of a high electric field along the length of the channel causes the layer of counter-ions to move along the walls. For sufficiently small channel diameters (typically 10–100 μm), movement of a sheath of solvent at the walls entrains the liquid in the entire capillary. The resulting liquid motion is often described as ‘plug’ flow: the velocity is essentially uniform at all points across the diameter of the channel. Consequently, a bolus of material that starts at one end of the column is not distorted in shape as it moves down the channel under electro-osmotic motivation.

The capability to motivate and separate exceptionally small liquid volumes with precise electrical control is a powerful tool, which is complemented by an additional scaling-related consequence of using microchannels: two or more channels can intersect to form a tee, cross, or other such structure. By controlling the potential on each channel separately, fluid streams can be metered quantitatively to create mixtures of desired composition. In essence, this electronically controlled metering mimics valve functionality. The combination of electrokinetic pumping, valving and separation within interconnected networks of microchannels on planar substrates was described in the early to mid-1990s by Soane and Soane [1], and groups at Ciba-Geigy [2-4], the University of Alberta [5-7], Oak Ridge National Laboratory [8,9], the University of California at Berkeley [10,11], and PerSeptive Biosystems [12]. These workers demonstrated implementations of separations of solution species using microfabricated chip devices, and several groups explored ‘integrated’ chip capillary electrophoresis (CE), wherein various analytical protocols (mixing, reaction and separation) were included on a monolithic chip [13-15].

Materials and methods

Plastic substrate materials

Soane and Soane [1] and Ekström et al. [16] proposed the use of polymeric materials in chip CE. While the microfabrication of inorganic materials such as silicon or glass can be accomplished inexpensively when chip sizes are small ( 1 cm²), larger fluidic devices ( 100 cm²) are often a better match for applications such as the separation of DNA fragments or the screening of large numbers of pharmaceutical candidates. In addition, the reservoir drilling and sealing of inorganic substrates (by high-temperature fusion bonding of a glass cover layer, for example) are relatively expensive processes. In contrast, plastic parts made by techniques such as injection moulding, casting or embossing can be quite inexpensive (the manufacturing cost of an injection-moulded compact disc is presently less than U.S. $0.30). Thus plastic fluidic devices can be made so cheaply as to be disposable after a single use.

The chemical, optical, electrical and mechanical properties of both the base card material...
and the sealing layer must be compatible with the chemical reagents and procedures, the electric field-driven motivation of solvent and solute through the microchannels, and the method selected to detect the analytical targets. Fortunately, a number of organic polymer materials have demonstrated compatibility with these requirements, including various polysiloxanes, polyacrylates (‘acrylics’), polylefins and polycarbonates.

**Microfluidic device fabrication**

Among the methods for forming capillary-size channels in polymeric substrates, ‘direct’ techniques, such as mechanical machining or laser ablation, are not the most economical, and in some cases produce surfaces that are too rough for high-resolution electrophoresis. Replication methods, including injection moulding, compression moulding, embossing and casting, shift the burden of forming a high-quality surface to the creation of the template, master or mould tool from which the polymer part is to be formed. Such techniques are demonstrably economical when large numbers of identical plastic parts or devices are formed using extant manufacturing methods and machinery.

One approach, similar to that used in the commercial manufacture of compact discs, involves two primary steps: (1) formation of ‘open’ microstructures, such as channels and reservoirs, on one surface of a base layer of polymer, and (2) sealing of the features in the base layer by a plastic cover layer [17,18]. To form the base layer, fluidic features are first microfabricated on a glass or silicon wafer or plate using standard photolithographic patterning and etching techniques. Next, a metal mould tool is created by electroplating a few tenths to several millimetres of metal, creating a precise replicate ‘electroform’ with inverse topology. This tool can be used on an embossing machine or an injection or compression moulding system. Polymer substrates are then manufactured in volume from melted or softened polymer resin or sheet to create extremely smooth and precise channels in the finished fluidic device or card. The open microchannels and reservoirs of a moulded or embossed fluidic device are sealed to form closed capillaries by bonding to the surface a smooth plaque or thin film of polymer material; sealing is accomplished using an adhesive interlayer or by chemical or thermal techniques that fuse the two layers.

The microchannels are typically tens of micrometres in diameter and follow complex circuit-like paths that can be centimetres in length. Reagents are fed to these channels via reservoirs, typically 1–2 mm in diameter and penetrating the depth of the card (~1 mm), resulting in total reagent volumes of 1–10 μl per reservoir. Hundreds of reservoirs and metres of channels can be densely packed into multiplexed designs that form 96 or even 384 assay patterns on a single 8 cm × 12 cm disposable card.

**Detection**

Miniaturization of analytical instrumentation requires the detection of ever-decreasing sample amounts. Although techniques such as laser-induced fluorescence (LIF) are sensitive to decreases in the interrogated volume, they maintain impressive detection limits when coupled with microfabricated devices: single-molecule detection has been reported [19]. More routinely, LIF using confocal detection can detect molecules, labelled with appropriate fluorophores, at concentrations of approx. 10 pM; with nanolitre detection volumes, the number of molecules detected is hundreds to thousands. Confocal epifluorescence detection with a pinhole positioned in the image plane, coupled with high-numerical-aperture objectives, enhances the signal/noise ratio by rejecting background radiation and collecting light efficiently. An alternative strategy is to use near-IR-absorbing fluorophores, since background fluorescence is typically less in this wavelength region. Interestingly, under conditions of photobleaching of the polymer chip, the background fluorescence from appropriately selected plastic materials when excited by laser light at 488 nm is comparable with the background from a glass microfluidic device of similar thickness.

**Results and discussion**

**Screening of pharmaceutical candidates**

The search for new pharmaceutical candidates is a multibillion-dollar effort, with an annual growth rate approaching 20%. While molecular design has made giant strides, the interactions of complex biological molecules are not understood sufficiently for side-effect-free drugs to be designed on the first pass. More typically, hundreds of thousands of test compounds from the libraries of pharmaceutical companies are screened against targets such as enzymes, receptors and cells. Following this primary screen, a small subset of promising ‘hits’ is studied further with secondary screens and clinical trials to identify undesirable side effects and prove efficacy before a satisfactory choice can be made. Combinatorial chemistry has
increased exponentially the number of test compounds available, while advances in genomics have broadened the variety of targets, giving rise to the need to perform an exponentially increasing number of screening assays. However, even 1 μl of expensive or hard-to-obtain target or test compound can dominate the cost of an assay. Therefore it is critical to limit reagent volumes so that libraries of pharmaceutical compounds can be screened against many more targets.

A common method for screening test compounds involves mixing microlitre volumes of enzyme, fluorogenic substrate and a candidate inhibitor and, after a fixed incubation period, quantifying the amount of product formed via fluorescence detection. Such homogeneous assays are typically performed in the 200 μl-volume wells of a standard 96-well plate or 25 μl-volume wells of a 384-well plate. Utilizing modern robotic systems, 100000 test compounds can be screened in a single day.

ACLARA has developed a microtitre-plate-format device (the 'Arteas™' system) that enables sub-microlitre reaction assembly and incubation with standard robotic dispensers, incubators and readers common to high-throughput pharmaceutical screening labs. This microfluidic card employs moulded reservoirs and microstructures that fix in place a nanovolume meniscus and create a hydrostatic height differential, providing passive evaporation replenishment, as depicted in Figure 1. Precious reagents (enzymes, substrates) can be added in 10–100 nl volumes directly into the assay well, without special evaporation abatement procedures or equipment. At 500 nl assay volumes, the design of this card enables assays with 50 times less fluid volume than a standard 384-well plate, or 10 times less volume than a 1536-well plate.

For more challenging assays in which signals from multiple fluorescent species are not easily decoupled, or when library compounds are so scarce and expensive that multiplexing is warranted, separation techniques can be employed. Assay precision and reproducibility are enhanced further by spiking the mixture with known concentrations of internal standards with well characterized separation times. We have developed a microfluidic format that offers the advantage of integrated incubation and electrophoretic separation on a single plastic card. Assays of activity for kinase-, phosphatase- and protease-catalysed reactions have been demonstrated; separations are performed over a wide range of pH and assay conditions. Separations are rapid, occurring in seconds, because the action of the enzyme typically alters significantly the charge/mass ratio of the product relative to that of the substrate. An electropherogram obtained using a plastic fluidic system to assay the substrate and product of a kinase-catalysed reaction is shown in Figure 2.

**DNA sequencing**

Sequencing the human genome has been accomplished at a remarkable pace, due in large part to the utilization of capillary-array electrophoresis (CAE) instruments, which provide increased efficiency and reduced costs compared with slab-gel electrophoresis systems. Capillary bundles filled with high-molecular-mass sieving polymers,
such as linear polyacrylamide, allow rapid, high-resolution separations and automated capillary filling. Fundamentally, sequencing separations in microchannels are quite similar to those in CE: the speed and resolution are strongly influenced by the separation matrix, temperature, electric field strength and separation length. Significant differences between the two techniques include the method of sample introduction. In CE with gel-filled capillaries, samples are injected electrokinetically into one end of the separation capillary, with sample quantity being dependent on field strength, injection time and the electrophoretic mobility of the DNA fragments. By contrast, the microchannel format employs a cross-channel injection procedure that allows a controlled, reproducible injection plug size that is independent of injection time [20]; it also reduces electrophoretic injection bias (efficiency of injection) for DNA fragments of different lengths. Secondly, as the number of capillaries in an array increases, the array becomes increasingly difficult to manufacture, and optical alignment for detection is more challenging. In contrast, planar microfluidic channel arrays are manufactured in fewer steps and channel-to-channel alignment is fixed, making multiplexed detection strategies more robust.

DNA sequencing separations in plastic (acrylic) microchannels have been accomplished using devices produced via a hot embossing process. We have recorded four-colour DNA sequencing electropherograms of many samples run in 18-cm-long acrylic microchannels. The data correspond to the correct identification of 640 bases with 98% accuracy in under 30 min.

**Multiplexed assay of gene and protein expression**

Quantitative information provided by changes in the relative patterns of expression of genes and, ultimately, the relative quantification of the proteins that they encode, can speed progress in understanding a range of critical biochemical interactions, including those that must be deciphered in order to develop new, more effective pharmaceuticals. We have developed unique chemistry (the ‘eTag**™** assay system) that allows simultaneous multiplexed quantification of tens of genes and/or proteins in a single, homogeneous solution-phase assay. Molecular binding events, including the hybridization of a nucleic acid target sequence with a matching probe sequence or the specific interaction between a protein target and an antibody, lead to the release of fluorescently labelled ‘eTag reporters’ in direct proportion to the concentration of each target species. These reporters are then separated according to their unique and well characterized electrophoretic mobilities, with quantification via LIF. A library of some 200 reporters has been synthesized to date; Figure 3 details the electrophoretic separation of 41 of these molecules. Importantly, cell lysates containing cleaved eTag reporter mixtures – cleaved in proportion to the concentration of each protein or nucleic acid target present – can be analysed directly on conventional CAE systems or with integrated microfluidic-card-based systems. The capability to monitor both genes and proteins simultaneously in a single

**Electropherogram showing the separation of 41 different fluorescent eTag reporter molecules, each of which can be used as a quantitative indicator of a specific target nucleic acid sequence or protein in a multiplexed assay of cell lysate**

The capability to separate tens of reporters on a chip or using a CE instrument enables multiplexed gene expression, protein assay or single nucleotide polymorphism detection, a.u., arbitrary units.
reaction has been demonstrated by concurrent assay of the interleukin-6 mRNA and protein from Thp-1 cells, as a function of time, in a single experiment. The results showed a peak in the expression of the interleukin-6 gene after an induction period of approx. 2 h, and much later, after some 18 h of induction, a peak in the expression of interleukin-6 protein. Current limits of detection for this technology are in the low picomolar range, with further improvements anticipated.

**Conclusions**

The assay results presented in this paper represent a few examples of the integrated analytical processes that can be performed in microfluidic devices, with emphasis on the power of electrophoretic separations to increase accuracy, speed throughput and facilitate highly parallel analyses. Other chemical processes, such as reaction and purification steps, are being integrated with separations on microfluidic devices to realize the 'lab-on-a-chip', 'sample-to-answer' goal, a vision with the potential to revolutionize the process of biochemical analysis.

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**Exploring Nature's plasticity with a flexible probing tool, and finding new ways for its electronic distribution**

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**Abstract**

Concepts and results are described for the use of a single, but extremely flexible, probing tool to address a wide variety of genomic questions. This is achieved by transforming genomic questions into a software file that is used as the design scheme for potentially any genomic assay in a microarray format. Microarray fabrication takes place in three-dimensional microchannel reaction carriers by in situ synthesis based on spatial light modulation. This set-up allows for maximum flexibility in design and realization of genomic assays. Flexibility is achieved at the molecular, genomic and assay levels. We have applied this

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Key words: gene expression profiling, genotyping, in situ synthesis, microarray, microfluidics.

Abbreviation used: SNP, single nucleotide polymorphism.

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