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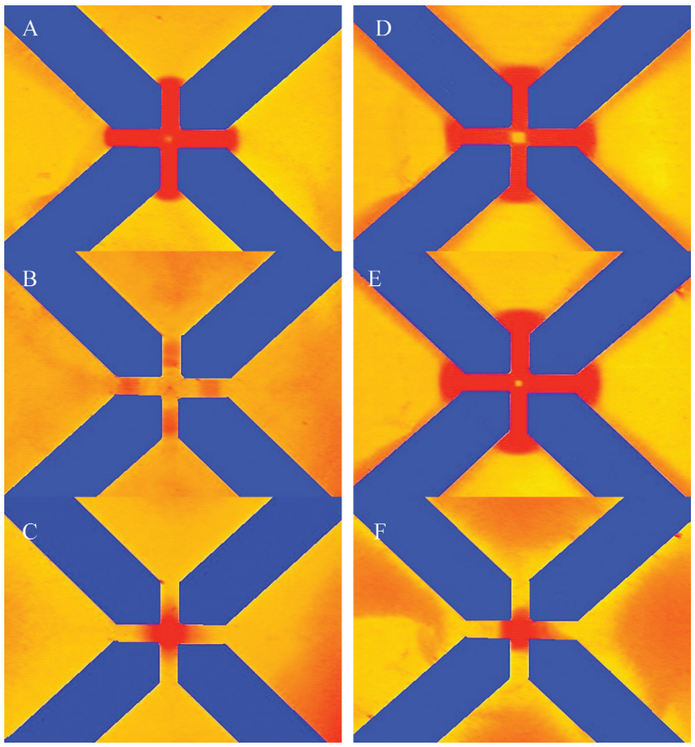

perspective

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Perspective

Nanobead Electrokinetics: The Enabling Microfluidic Platform for Rapid Multi-Target Pathogen Detection

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Introduction

Dielectrophoresis (DEP) is an electrokinetic nanobead-manipulation technique that can provide the final piece of a robust continuous-flow microfluidic platform for multitarget high-throughput biomarker/DNA screening and miniature diagnostic kits. Both technologies promise to create enormous research and employment opportunities for Chemical Engineers. The DEP platform offers sensitivity, rapid response (<10 min), field portability, reliability, unmatched economy, and provides a platform from which multitarget analysis can be performed. An integrated single-chip electrokinetic module, with embedded DEP microelectrodes and other flow-control components, is especially suitable for a portable device or as a unit in a parallelized network. This article reviews the advantages of an electrokinetic platform, particularly the DEP components, and the remaining technical and scientific challenges facing the realization of such a platform.

Diagnostic assays are biochemical techniques for detecting and identifying pathogens (harmful bacteria, viruses, organisms, etc.,) and diseased cells, or the molecular biomarkers they release. In the last two decades, there has been an explosion of research breakthroughs in diagnostic assay science. As a result of these advances, pathogen diagnostics has become dramatically more rapid, specific, sensitive and field-applicable. Arguably, the holy grail associated with this field is its capacity to provide for early and rapid cancer detection. Despite decades of cancer drug development, the mortality rate for most cancer patients remains unacceptably high if the diagnosis is made late in the disease progression. In contrast, early and type-specific diagnosis of cancer would immediately and dramatically lower this unacceptable mortality rate. Similarly, swift and pathogen-specific diagnosis of acute infections like sepsis, a deadly and rapid bacteria infection of the blood that is often lethal in a matter of days, would also significantly increase the patient survival rate. In addition to speed and specificity, device portability allowing for field use is also highly desirable. Portability would be particularly useful for field applications, such as epidemic control (identifying severe acute respiratory syndrome (SARS), or avian-flu viruses at airports, for example), detecting *E. Coli* in food products and water sources, and identifying antibody-resistant tuberculosis (TB), or malaria bacteria in third-world countries. For consumer oriented diagnostic kits, the sample-contacting components of the portable kit must be disposable, and, hence, the economy of fabricating the disposables is important. Unlike cancer biomarkers, medical pathogens typically appear in relatively large concentrations. In contrast, bioterrorism and environmental applications often involve smaller number of targets, and sensitivity is a main issue. In short, specificity, speed and sensitivity are the key performance measures for diagnostic assays, with portability and economy also important for field-intended kits. As gauged by these measures, recent scientific breakthroughs in diagnostic science have been transformative, and have had enormous implications on health care, environmental monitoring, and the biotechnology industry. For example, the century-old culturing technique of detecting bacteria with antibiotic screening requires days, whereas modern immunoassays for the same task, developed in the last decade, often take only hours.

Such dramatic advances and frenzied research activity has been catalyzed by the invention and subsequent development of the polymerase chain reaction (PCR), which not only revolutionized molecular genetics by allowing rapid DNA cloning, but also contributed extensively to rapid and specific genetic identification of pathogens. PCR is capable of rapidly amplify-

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ing a DNA sequence initially present in minute concentrations, ultimately producing millions of identical DNA molecules, thus, essentially increasing the detection sensitivity toward the respective DNA sequence exponentially. Successful isolation of antigen or protein-specific antibodies, and synthesis of sequence-specific DNA probes, has also contributed to the excitement. Amplified DNA sequences, biomarker molecules present in low concentrations, and pathogens can be selectively captured and removed from a large sample. The docked targets can then be detected with the latest detection techniques, particularly optical sensor technologies that are based on fluorescent tagging or emission, and Raman, IR, or UV spectroscopy. Traditional DNA microarrays epitomize the new fluorescent genetic identification techniques that integrated these new advances in both the assay and sensing technologies. Genetic diagnostic techniques are now routinely used in any medical diagnostic laboratory. Your neighborhood supermarket uses enzyme-linked immunosorbent assay (ELISA), or related assays to detect *E. Coli* in its produce department. While culturing remains the gold standard in accuracy for bacteria detection, its long response time would soon render it obsolete against the new assay techniques. (For example, TB bacteria require one week to culture.) A major effort is consequently underway to compile a library of cancer biomarkers, and the holy grail of high-throughput and rapid screening of these biomarkers by the new assay techniques could be within reach in the near future.

Unfortunately, these modern assay and detection techniques have reached a bottleneck that has prevented them from reaching the next plateau and spawning commercial diagnostic devices and turnkey instrumentation. The new assays are typically encumbered by expensive and heavy laboratory equipment, and often require extensive manual supervision and handling. Some of the instrumentation needs have been met. For instance, electrical engineers can now fabricate, with variable cost, on-chip optical sensors for fluorescence, absorbance, Raman, or electrochemiluminescence detection. Fluorescent readout from a DNA microarray, for example, no-longer requires a lab-bound fluorescent confocal facility. Portable PCR kits or chips are now commercially available. However, the hour-long response time for most assays remains too high for portable field-use devices or for high-throughput biomarker screening. The DNA microarray notwithstanding, most integrated assay kits still perform single-target detection because of the lack of a turnkey multiplex platform. Almost all use the batch format with low-throughput for the same reason. Because of the low-throughput, the target count is low with the batch format, and its sensitivity remains suspect and inferior to the standard culturing approach, particularly for debris-filled samples.

An Enabling Microfluidic Platform

It is commonly believed that a robust, enabling microfluidic platform can remove the aforementioned remaining obstacles, and lead to a new generation of rapid multitarget diagnostic devices. The lab-on-a-chip microfluidic platform would allow a large-throughput continuous-flow format by moving discrete samples or continuous streams from one station to the next within a single chip, and without human intervention. The platform should be reliable—just as in a large chemical plant, replacement of one unit can shut down the entire process. Disposable chips for field-use applications stipulate a platform that is cheap to fabricate. Reliability and economics rule out micromechanical platforms, as 3-D etching remains prohibitively expensive and moving mechanical parts are prone to frictional wear at the microscales. Not surprisingly, despite the emergence of over 30 microfluidic startups in the last five years, not a single commercially successful microfluidic platform has emerged for lab-bound high-throughput screening and portable diagnostic kits. As a result, significant advancements within major (multibillion dollar) environmental and health-science monitoring industries is at stake.

There are more specific scientific reasons why a robust microfluidic platform can lead to commercially viable diagnostic kits. The bottleneck for diagnostic response time is due to mass-transfer limitations. Aforementioned advances in the molecular biology of diagnostics have reduced the associated kinetic time scales to seconds and minutes.[1,2] As such, the key to reducing the hybridization time of a DNA microarray from hours to minutes is not in developing better probes (biochemistry), but rather better microfluidics that can remove the inherent mass-transfer limitation. Micromixing, which uses convection to enhance the DNA-DNA docking rate, is one solution. However, the Peclet number for most microfluidic devices is small, less than 100.[3,4] The classical $Pe^{1/3}$ convective mass-transfer enhancement due to convection near a surface then predicts only a unit-order enhancement in the mass-transfer rate. Sensitivity and portability limits can also be relaxed through improvements in microfluidics. The large surface area to volume ratio within a microchannel allows more surface probes to be functionalized to the walls of the channel, which significantly increases the probability of capturing targets present in minute concentrations, and, thus, improves the sensitivity of the respective diagnostic assay. The issue of portability obviously requires the fabrication of robust, smaller kits, which will be accomplished through employment of appropriate microfluidics. It may even be possible to further enhance device specificity by concentrating desired targets (that is, specific DNA segments) near the probes by microfluidic means.

I see a parallel of this final enabling technology within semiconductor chip fabrication, which brought us laptops, mobile phones, and the internet revolution. Just as microcircuit fabrication provided the enabling technology for semiconductor physics, microfluidics will enable diagnostic assay techniques to become a major biotechnology sector. The first few decades of the 21st century may see growths within the diagnostic kit industry similar to those seen for the semiconductor industry in the seventh and eighth decades of the 20th century.

Regarding on-chip fluid manipulation, a new AC electrokinetic platform has emerged that meets many of the needs of this enabling microfluidic platform (see an earlier review by this author[5]). A variety of electrokinetic components, such as micropumps, microvalves, and micromixers, have been developed in the last five years.[6–9] These components contain no moving parts, and, hence, are not prone to wear like mechanical components, and are cheap to fabricate as they involve primitive and well-known microcircuit fabrica-

tion techniques. Additionally, these AC electrokinetic platforms are extremely portable as they can be driven by handheld power supplies like those within a cell phone. The high-frequency (>100 kHz) AC field typically utilized has a period shorter than the Faradaic reaction time of the respective voltage, and, consequently, bubbles and net generation of ionic products do not occur at the electrodes. As such, electrodes can be embedded within the chip to allow for more precise fluid management.

Nanobeads for Sensitivity and Speed

There are, however, still significant microfluidic obstacles involving speed and sensitivity of target capture that must be overcome. The eventual microfluidic platform will most likely utilize surface functionalized probes similar to those found on the pixels of DNA microarrays. Surface-based platforms have been developed for substrate-enzyme reaction, DNA-DNA hybridization, and protein-DNA or protein-protein docking. One of the major advantages of the surface-based technique is multitarget diagnostics, with one pixel probe designed for a single target. Even if only one pathogen is to be detected, multiple DNA targets from its genome are often required to make an accurate identification. The large local density of surface-based probes within a traditional microarray offers a higher capacity for fluorescent sensitivity than unanchored probes in the bulk solution. However, the trade-offs for the traditional pixel platform include capture efficiency and long diffusion time—which accounts for the previously described slow response time and sensitivity bottlenecks. At low-concentrations, the diffusion length of the molecule to the probe approaches that of the longest vessel dimension, and its capture probability scales as the pixel area divided by the vessel area, which is a rather miniscule number. The long response time of the array can also be estimated by the DNA diffusion time. With the low-diffusivity of the target DNA at 10^{-7} cm^2/s for a 20 kB single-strand DNA, a diffusion time of hours is required for the target to reach the surface functionalized probes within a mm-high sample assuming realistic PCR amplified concentrations. This mass-transfer limitation becomes even more acute for peptide and protein biomarkers. Only several such molecules exist in 1 mL of a typical sample, translating into a diffusion length of 1 cm in the large sample. The diffusion time is then days as it scales as the +2 power of the diffusion length.

The most promising solution to this mass-transfer bottleneck lies in the employment of a nanobead platform, where probes are functionalized onto the surface of submicron nanocolloids.[10] The small dimension, large number, and large surface area-to-volume ratio of these beads offer several attractive features. A 100 microliter sample of 1% micronsized colloid suspension contains a billion colloids with a total surface area of 1 cm^2. Compared to a pixel area of 1 μm^2 for traditional DNA microarrays, these beads offer a capturing area that is eight-orders of magnitude larger. If the colloid geometry does not affect the docking dynamics, this could translate into a comparable increase in sensitivity. In the same sample, the average separation between beads is three-orders of magnitude smaller than the linear dimension of the sample. For the case of a small number of target molecules present, this translates into a maximum of six-orders of magnitude reduction in diffusion time, which is much higher than any convection-enhanced mass-transfer rate. There are other potential benefits for this bead platform. If these beads can be assembled and dispersed within the microchannels, they can form micro-CSTRs, microchromatographs, and microplug-flow reactors, and, hence, invoke advantages of these reactor designs: a yield better than the thermodynamic yield for an open-flow CSTR, separation to enhance selectivity of parallel docking reactions, and low dispersion to enhance the yield of irreversible reactions.

The chemistry for functionalizing nanobeads, nanowires, liposomes and carbon nanotubes (CNT) with oligomers, probes, fluorophores, and carboxyl groups in order to render them hydrophilic has also been actively developing in anticipation of this bead platform. The most commonly used beads are silica, latex, quantum dots, and gold colloids. Although latex particles with uniform size can be most easily synthesized, silica chemistry is best understood, and the functionalizaton of silica nanobeads with different chemical and molecular probes is now routinely carried out. Probe and fluorophore attachment to CNT is also relatively simple. The same art for CdSe and other nanowires is also nearly developed. Nanowires allow easier coding,[10] and CNTs offer better specificity as molecules do not adsorb indiscriminately on them due to electrostatic interaction. Colloid bar-code tagging for identification is now a mature technology. Different fluorescent dyes can be attached sequentially on a colloid, a liposome, or a nanowire to provide a fluorescent bar code.[10–12] All combined, a large library of bead probes for massively parallel multitarget, multiplex detection is offered. Identification and sorting of different beads is obviously simpler than carrying out the same tasks on beads with different barcodes.

However, such a bead platform can only be realized if a complementary bead-manipulation platform is developed to concentrate, identify, and sort these beads. When used in conjunction with a flow cytometer, the bead platform can identify and sort particles at the rate of 10^4 particles per second. Magnetic beads offer another complementary platform that is most effective for concentrating molecules and cells. The beads are often encapsulated with a shell that can be functionalized with antibodies or DNA probes. If the bar codes of individual beads can be individually probed, as in a cytometer, they can be sorted into different bins. A downstream assay of the sorted beads is still necessary although to determine if they have captured any target molecule, cell, or bacteria. For DNAs, the simplest technique is to label all of the targets fluorescently during PCR amplification, and then to simply measure the fluorescent intensity of the sorted beads. However, magnetic beads and cytometers still require expensive and lab-bound equipment. The small magnetic moment of magnetic beads requires large quadrupole magnets, which are not portable, for sorting tasks in the requisite continuous flow format. Similarly, portable cytometers that employ on-chip optical sensors and microfluidic platforms are still not possible because of the high-resolution required. Even for high-throughput screening in a laboratory, the size of such periphery equipment does not readily lend itself to the massively parallel format. Cytometry and magnetic beads will not be the enabling microfluidic technology for integrated diagnostic kits, at least not for portable ones.

An Integrated, Continous-Flow Dielectrophoretic Platform

It is this author's opinion that a bead platform based on dielectrophoresis (DEP), which is the use of an AC electric field to impart a particle force,[13,14] can deliver the final bead-manipulation technology for the enabling microfluidic platform. DEP refers to the migration of a particle (which need not be charged) under the influence of an electric field gradient. The electric field induces a particle dipole on each individual bead, and when exposed to a nonuniform field, the beads then experience a net force causing controlled migration, described as either positive DEP (p-DEP) or negative DEP (n-DEP) depending on whether the migration is toward or away from a high-field region. As the applied frequency is increased, most particles will switch from p-DEP to n-DEP, and, thus, exploiting differences in this "cross-over" frequency between particles provides a very effective means of rapidly imparting different particle forces, in fact particle forces in different directions, on distinct beads.

Recent studies summarized in the last section have shown that, with proper design, the DEP direction of nanocolloids can be reversed by molecular docking. This is dramatically demonstrated in the cover figure by patterns formed by nanocolloid suspensions in the vicinity of a quadrupole electrode. Nanobead suspensions with and without DNA-oligomer hybridization are seen to exhibit distinctly different patterns at different AC frequencies. The patterns shown can, in fact, be used to identify hybridization rapidly. The sensitivity of DEP mobility then suggests a DEP-nanobead sensing strategy for surface functionalized assays of molecular biomarkers and DNAs. The difference in force direction is obviously a much more effective means of separating and sorting beads than other separation techniques, such as chromatography, electrophoresis, and magnetic sorting, which rely only on differences in the force magnitude (bead mobility). Equally important is the fact that DEP sorting, unlike cytometry, does not require identification of the beads prior to sorting.

The DEP platform, like the electrokinetic flow-control components, is extremely portable because only microbatteries and microtransformers are required. Precision offered by embeddable electrodes still applies within this platform, and the same economic and wear advantages are also extended. As the on-chip optical sensors will be controlled electronically, a fully integrated electronic supervising structure for the entire chip can then be implemented with a minimum of actuators and sensors. In fact, other than possibly a few simple ball valves, there will be no mechanical moving parts on the chip. This would significantly reduce the fabrication cost of the chip. An electokinetic microfluidic platform can hence be easily integrated with on-chip detection with little fabrication effort. Feedback control and automation, both important to high-throughput screening, could then be easily implemented with a supervising microcircuit structure.

In the past five years, the first generation of DEP devices for label-free bacteria[4,15–20] concentration and detection have surfaced. In many cases, the DEP trapping is enhanced by a stagnation flow that arises from AC electro-osmosis on the electrode, by an external momentum source, or by nanocrystals or CNT.[20] Optical[4] and impedance spectroscopy,[17] functionalized probes on the collecting electrodes, or enzyme-based assays have been used to identify the trapped pathogens. Integration of this DEP platform with on-chip optical detection has also been reported.[21] However, these prototypes typically involve single-target detection or binary separation in a batch format. They, hence, do not fully exploit the massively parallel multitarget capability of the bead platform in an integrated, multiplex continuous flow DEP chip. More recently, three-dimensional (3-D) DEP trapping electrode gates and microrod arrays that allow through flow and some degree of separation have been reported, but not with the precise multiplex sorting capabilities required for the bead platform.[22–25] In fact, none of the reported DEP devices employ nanobeads for DNA or molecular detection, and, hence, are not expected to meet the desired specificity and sensitivity measures for high-throughput screening or portable diagnostics.

This year, we reported the first integrated multiplex continuous-flow DEP sorting chip (Figure 1) with three different sequential DEP components that allows sensor-free nanobead sorting and identification.[26] This chip can sort three different beads into three different channels at a speed of 100 beads per second. While this speed is two-orders of magnitude slower than that achievable using cytometry, the chip and its periphery equipment can be hand held, disposable, and fabricated at a cost of less than $1. Nearly all of the sorted beads can be trapped by a DEP trap within each channel without the use of a microfilter. The concentrated beads can then be further probed with on-chip or off-chip sensors and detectors. They can also be "counted" by simply measuring the impedance of trapping electrodes. The chips can be connected in series or in parallel, and then be used in a modular fashion to achieve massively parallel screening. This modular form facilitates scalingup to accommodate sample probing with massively large numbers of different targets, whenever the biomarker libraries are complete. It could even allow side streams and recycle streams as in classical unit-operation designs. F1

The different components of our integrated DEP chip module exploit the fact that different beads experience particle forces in different directions near the microelectrode components producing a high-electric field. The chip consists of three stages downstream of a coarse DEP debris filter: the first stage is a focusing unit that operates at the n-DEP region of all particles. It contains two side arrays of electrodes with a decreasing gap width and at a frequency higher than the ω_{CO} of most beads. The decreasing aperture of the gap focuses all beads in the continuous stream into a region less than 10 microns wide at the middle of the channel. The focused beads form a linear, single-file queue, and can then be interrogated individually downstream. The second unit contains three DEP sorters, each one consisting of an oblique electrode at the top substrate and a mirror image electrode at the bottom. The gap between the electrode pairs sustains a high-field that would repel n-DEP beads and allow p-DEP beads to pass, thus, effecting separation of these beads. The n-DEP beads would move along the oblique electrode pair, and then be released to the next sorter at a different streamline from the p-DEP beads. The beads can, hence, occupy four possible streamlines after the sorting unit: the original focused streamline plus the ones that pass through the tips of the three sorters. These streamlines can then be fed into four different channels. Given the resolution of the focusing unit

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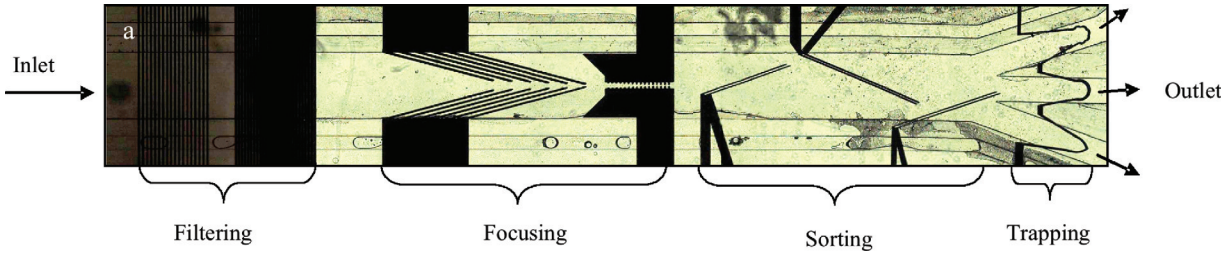

Figure 1. An integrated continuous flow DEP tip that allows multiplex detection of multiple targets.

shown in Figure 1, typically only three sorting channels are used at high-throughput operating conditions. By using different frequencies at different gates, three different beads can be sorted into three separate channels. In the final stage, a 3-D trap is fabricated to capture all of the beads in one channel, while the solution flows through the gap without extra hydrodynamic resistance.

When massively parallelized or serialized, this continuous-flow chip allows high-throughput, label-free sorting without using molecular-sieves or microfilters that introduce significant hydrodynamic resistance. By using frequencies specific to of certain nanobeads, the integrated sorters and traps offer much higher specificity than molecular nanosieves. Impedance measurement at the trap electrode can estimate the number of beads trapped. Trapping of a queue of nanocolloids is seen in Figure 2. Nearly 80% separation efficiency can be achieved for binary separation at about 100 particles per second. Hence, two or three modules in series can achieve 99% purity.

F2

Scientific Issues and Remaining Challenges

If beads with docked antigens (or hybridized genetic beads) can be sorted from the undocked ones, the above unit then offers a simple means of continuous-flow, multitarget detection with different beads and without (or in addition to) optical sensing or fluorescent labeling. However, this presupposes that the docked beads have a distinctly different DEP mobility from the undocked ones. Recent studies of DEP mobility shows that it is very size-sensitive (see Figure 3). As a consequence, a nanobead with the same dimension of the docked molecule should dramatically change its DEP mobility. Also, DNAs are conducting molecules and their docking can significantly increase the particle conductivity of a small nanocolloid relative to that of the buffer. For example, Figure 3 shows that two nanocolloids with a size ratio of 6 exhibit a cross-over frequency that is only a factor of five different for conductivities lower than 1 mS/m. At higher conductivities, the cross-over

F3

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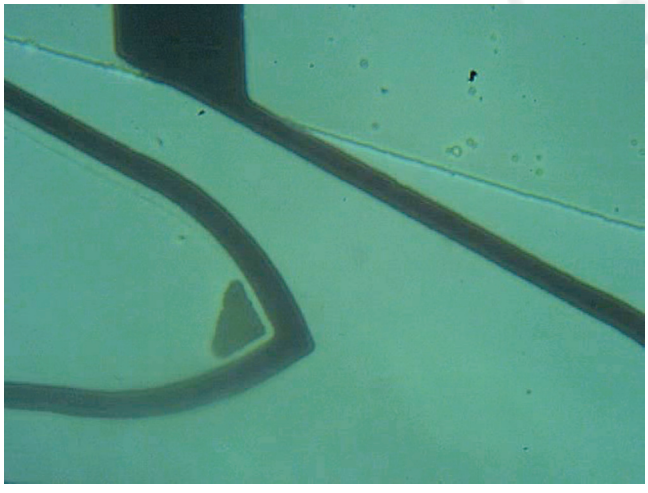

Figure 2. A focused and sorted single-file of 500 nm colloids are trapped at one of the sorting channels of the continuous flow sorting chip in Figure 1.

The queue feeds into the bottom vertex of the triangle of assembled beads at the trap. The pointed trap consists of identical and aligned electrodes on the top and bottom surfaces of the closed microchannel. The buffer solution flows through the gap between the electrodes, while the nDEP beads are filtered by the high-electric field at the gap, particularly at the sharp tip.

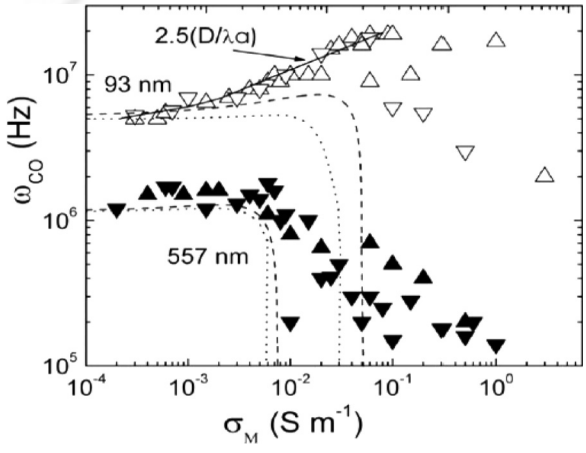

Figure 3. Cross-over frequency of 93 nm and 557 nm as measured by Green and Morgan.[27]

The dotted line is the classical MW theory with a conducting Stern layer which underpredicts by an order of magnitude for high-conductivity buffers. The shaded line is the extended theory of Emonilia and Morgan that includes double layer tangential conduction. The simple scaling theory is shown as a full curve for the 93 nm nanobead. Double-layer polarization is extremely important for nanocolloids when $\lambda \approx \alpha$ or $\lambda \ll \alpha$.

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frequency is different by two-orders of magnitude! Careful buffer preparation and colloid design must, hence, be carried out.

Unfortunately, there is little theoretical guidance. In the last five years, a flurry of experimental and theoretical articles on DEP have shown that electrolyte double-layer effects can defy the classical DEP theory, particularly for the desirable nanocolloid size for DEP sorting (see review by Green and Morgan[27]). For example, the cross-over frequency predicted by this classical MW theory is size-independent

$$\omega_{CO} = \frac{1}{2\pi}\sqrt{\frac{(\sigma_P - \sigma_M)(\sigma_P + 2\sigma_M)}{(\varepsilon_M - \varepsilon_P)(\varepsilon_P + 2\varepsilon_M)}} \quad (1)$$

where ε is the permittivity and the conductivity for the particle (P), and buffer medium (M), but we have already seen in in Figure 3 that nanosized latex particles in water clearly shows a strong particle-size dependence. Some (see review by Ermolina and Morgan[28]) have suggested that the particle size effect stems from Stern layer surface conductance, which scales inversely with the particle size. The resulting cross-over frequency is then $\omega_{CO} = 1/2\ \pi(K_s/\sqrt{2}a\varepsilon_M)$, where K_s is the surface conductance. However, we find by fitting literature cross-over data with this model that K_s would need to be particle-size-dependent.[29] It is also unappealing that adsorbed ions can fundamentally change the particle conductivity. We have found ω_{CO} to scale as $(D/\lambda a)$, and to increase with medium conductivity[29] for realistic buffer ionic strengths, as seen in Figure 3. This peculiar behavior and the various double-layer relaxation times still need further investigation.

How the conformation of the docked molecule affects the cross-over is also largely unknown but the length of the probe seems to play an important role.[30] CNT and slender nanowires are found to have far higher DEP mobilities (due to field focusing by the slender geometry[20,31]), more selective molecular capture, negligible dye adsorption, and may, hence, be the optimum nanobeads for the DEP platform—they are certainly easier to barcode.[10] In fact, we have found that CNTs dock with bacteria much more readily than nanospheres, and the docked CNTs can actually enhance the DEP of the aggregate—CNTs become DEP transporters of the pathogen.[20] Buffer tuning is relatively straightforward as zwitterions, ionic liquids, and other additives can easily change the medium permittivity and conductivity.[19] Even with some remaining issues, it is this writer's expectation that the nanobead-DEP enabling microfluidic technology for a large multitarget diagnostic market will be ready in the upcoming years.

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