THE ANALYST
www.rsc.org/analyst

The resurgence of Coulter counting for analyzing nanoscale objects

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This review discusses recent advances in the science and technology of Coulter counting. The Coulter counting principle has been used to determine the size, concentration, and in favorable cases the surface charge, of nanometer-scale colloidal particles, viruses, DNA and other polymers, and metal ions. A resurgence of interest in the field of Coulter counting is occurring because of the advent of new technologies that permit fabrication of membranes containing single, robust, and chemically well-defined channels having smaller and more uniform sizes than could be prepared in the past. These channels are prepared from biological materials, such as self-assembling membrane proteins, and from synthetic materials such as polymers, carbon nanotubes, and silicon-based inorganic materials. In addition to particle characterization, there have been a few recent examples of using Coulter counters to study chemical processes, such as the dehybridization of DNA.

Introduction

This Highlight discusses new developments in the science and technology of Coulter counting, also known as resistive pulse sensing, that have extended the applicability of this simple and reliable method to the analysis of nanoscale objects, including polymeric beads, DNA and other polymers, viruses, and metal ions.

Coulter counters consist of two chambers divided by an insulating membrane that contains a single channel (Fig. 1a). Electrodes immersed in an electrolyte solution present in each chamber are used to drive an ionic current through the channel. If, in addition to the electrolyte, particles having a size on the order of the channel diameter are present in the solution, then they may enter the channel and thereby reduce the magnitude of the ionic current. The output of a Coulter counter is a plot of current *vs.* time (Fig. 1b) that contains a string of current pulses. Under favorable conditions these current pulses can be correlated to the size, mobility, and concentration of the particles.

The reason this fifty-year-old method is once again attracting attention is because of its simplicity, single-particle sensitivity, nanometer-scale particle-size resolution, and because the data it provides is rich in information.[1] The purpose of this article is to highlight the current state-of-the-art of the Coulter counting methodology, provide some illustrative examples of how it has been used to solve analytical problems, and then discuss how it might evolve in the future.

Background

The Coulter counter was patented in 1953 by W. H. Coulter, and throughout the last fifty years has been widely used in medical laboratories to determine biological cell concentrations.[1,2] For example, in the early years, Coulter counters were used mainly to provide accurate complete blood counts (CBCs). These first-generation devices relied on pressure-driven flow to move blood cells into the channels, which were made from glass and had channel diameters ranging from ~20 μm to 2 mm. Prior to development of the Coulter counter, CBCs were determined by counting blood cells immobilized on glass slides with the aid of a microscope. This process was time consuming and often inaccurate; the Coulter counter substantially improved throughput and accuracy.

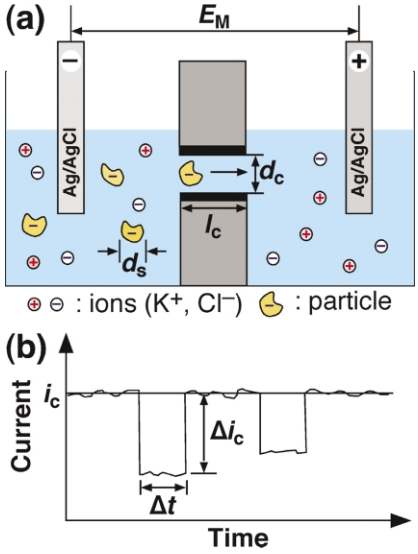

Fig. 1 (a) The general experimental approach for electric field-driven Coulter counting. A membrane containing a single channel divides two chambers containing an electrolyte solution. When an appropriate potential is applied across the membrane, an ionic current is driven through the channel. If particles of an appropriate size and charge are present, they will enter the channel and reduce the ion current. (b) Coulter counter data consist of a series of current pulses associated with the presence of particles within the channel. The height of the pulse, Δi_c, is related to particle size and the width, Δt, corresponds to the particle transit time. In favorable cases, data such as these can provide information about the size, charge, and concentration of the particles.

In the 1970s, DeBlois and Bean demonstrated that submicron-sized analytes could be detected when passing through the channel of a Coulter counter.[3] Specifically, they showed that polystyrene beads as small as ~90 nm in diameter could be detected and that their size could be accurately measured utilizing track-etched tapered pores in polycarbonate having diameters ranging from 0.4–0.5 μm. They also demonstrated that it was possible to detect and measure the sizes of viruses.[4] This was an important body of work, because it showed that the Coulter counting principle is valid even at the submicron scale and paved the way for the current generation of Coulter counters that rely on electric fields to drive analyte particles through the sensing channel.

The resurgence of interest in Coulter counting during the last few years has been a consequence of two factors. First, there is a pressing need for the development of new analytical methods for characterizing nanometer-scale analytes and, second, new methods for fabricating membranes containing single, well-defined nanometer-scale channels have been devised. These advances have resulted in Coulter counters that detect myriad types of nanoscale objects. Indeed, because Coulter counters are sensitive to size, any type of particle can, in principle, be detected without resorting to labeling. Other drivers of the technology include its simplicity, low power requirements, and inherent compactness (portability). At present, research into Coulter counting is focused in three areas. The first area is the development of new methods for

DOI: 10.1039/b404251b

fabricating small-diameter channels. Such channels fall into two categories: those based on synthetic materials and those based on natural pore-forming membrane proteins. The second area focuses on improving the durability of these channels. The third area is aimed at improving selectivity by either chemical modification of the channels or by labeling analytes to change their size or charge.

Operating principle

The Coulter counter response is based on the reduction in ionic current associated with the presence of a particle within a channel. The magnitude of the current decrease, or pulse height, can be used to determine particle size, the duration or the width of the current pulse can, under favorable conditions, be used to determine the charge carried by the particle, and the frequency of the current pulses is related to particle concentration.

The pulse height depends on the volume of electrolyte solution displaced by the particle, which represents a local increase in solution resistance. This effect is fully reversible: after the particle transverses the channel the current returns to its baseline value as shown in Fig. 1b. The effect of particle size on current is expressed mathematically by eqn. (1), which shows that the pulse height (Δi_c) is proportional to the third power of the diameter of a spherical (hence the subscript s) particle (d_s).[3]

$$\frac{\Delta i_c}{i_c} = S(d_c, d_s)\frac{d_s^3}{l_{c'}d_c^2} \qquad (1)$$

$S(d_c,d_s)$ is a correction factor that depends on the relative values of the channel diameter (d_c) and d_s. $l_{c'}$ is the channel length after correction for the so-called "end effect" ($l_{c'} = l_c + 0.8\ d_c$).[3] One very important prediction of this equation is that the use of smaller channels makes it possible to sense smaller particles.

In some cases the transport time is also important in a Coulter counting experiment. This is because if the geometry and chemical properties of the channel are sufficiently well defined, then the electrophoretic mobility of a particle, and hence its electrokinetic surface charge, can be determined. As shown in eqn. (2), in the absence of specific chemical interactions between analytes and the channel itself there are four possible transport mechanisms that can contribute to the velocity of a particle through the channel of a Coulter counter. With reference to eqn. (2), these are, respectively, the velocities arising from pressure-driven flow ($v_{s,PD}$), electrophoresis ($v_{s,EP}$), electroosmosis ($v_{s,EO}$) and diffusion ($v_{s,D}$).[5] In a Coulter counting experiment, diffusional transport is normally considered to be negligible, so only the other three terms need be considered further.

$$v_s = v_{s,\,PD} + v_{s,\,EP} + v_{s,\,EO} + v_{s,\,D} = \frac{d_c^{\,2}}{32\eta l_{c'}}\Delta P + \frac{\mu}{l_{c'}}E_M + \frac{\varepsilon\zeta_c}{4\pi\eta l_{c'}}E_M + \frac{D_s}{l_c C_s}\Delta C_s \qquad (2)$$

The width of the pulse signal (Δt) corresponds to the residence time of a particle within the sensing channel, and it provides the transport velocity of the particle (v_s) if the length of the channel is known. In eqn. (2), η is the solution viscosity, ΔP is the pressure across the channel, μ is the electrophoretic mobility of the particle, ε is the solution dielectric constant, ζ_c is the zeta-potential of the channel surface, D_s is the diffusion coefficient of the particle, C_s is the particle concentration in the chamber containing the source solution, and ΔC_s is the difference between the particle concentrations in the source and receiving solutions.

The frequency of signal appearance in Coulter counting data (J_s) is related to the average particle transport velocity ($v_{s,ave}$), C_s, and d_c (eqn. (3)):[5]

$$J_s = \frac{\pi v_{s,\,ave} d_c^{\,2}}{4} C_s \qquad (3)$$

This equation indicates that C_s can be determined from J_s by calibrating the device with a reference sample of known concentration.[5] If $v_{s,ave}$ and d_c are known, C_s can be determined from J_s without calibration.

As mentioned previously, early Coulter counters relied mainly on pressure-driven flow. Inspection of eqn. (2) indicates that the information available from devices based on this principle is limited to particle size and concentration. More recently, however, Coulter counting based on electrokinetic transport has been favored because this approach is easier to implement and can be more information-rich. In this case, diffusion and pressure-driven flow are negligible, and only electrophoresis and electroosmosis are considered. For channels having poorly defined geometries and/or chemical properties the effects of electroosmosis and electrophoresis cannot be distinguished, and therefore only information about particle size and concentration can be determined.

At present, there are two approaches being pursued for the development of smaller, better-defined channels for Coulter counters. One relies on self-assembled biological materials, and the other is based on channels mechanically or chemically fabricated within monolithic synthetic membranes. Each of these approaches has advantages that make it attractive for analyzing nanoscale objects. In the next section we will provide a brief overview of the fabrication of Coulter counters based on these two types of channels and then discuss their applications.

Coulter counters based on biological membrane proteins

A common approach for using biological channels for Coulter counting is illustrated in Fig. 2. Here, proteins, often α-hemolysin (α-HL), self-assemble within a fluid lipid bilayer to yield a channel ~2 nm in diameter.[6] The bilayer containing the channel-forming protein is attached symmetrically to a ~25 μm opening fabricated within a dielectric material such as Teflon. The permeability of ions through the Teflon and bilayer is very low, so when a voltage is applied the flow of ions is restricted to the protein channel. There are

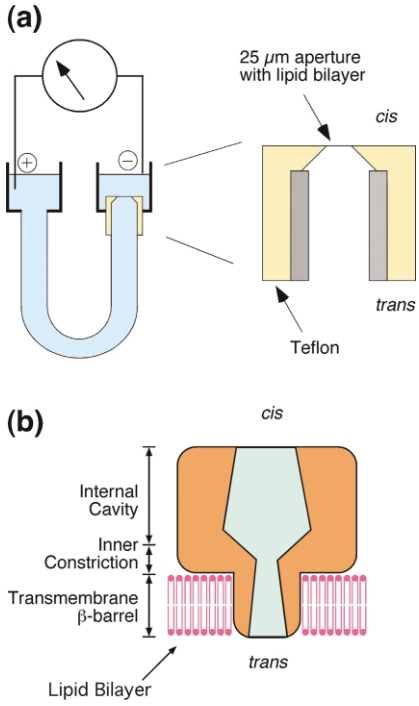

Fig. 2 (a) Schematic representation of the type of Coulter counting arrangement used for measuring transport through biological channels. Electrodes are immersed in an electrolyte solution contained within a U-tube. One side of the U-tube supports a Teflon membrane containing a micron-scale pore that is spanned by a fluid lipid bilayer. (b) Within the bilayer, a self-assembled protein forms a channel (~2 nm in diameter). An ion current flows through this channel when an appropriate bias is applied across the membrane. Current transients, such as those shown in Fig. 1b, appear as the object moves through the channel.

a few very good, in-depth reviews of this approach to Coulter counting that have appeared recently in the literature, and therefore we restrict ourselves here to a very brief overview.[7–11]

Deamer and Branton have shown that single-stranded DNA (ssDNA) and RNA pass through an α-HL channel in single-file motion; thus, their length can be directly correlated to the pulse width of the current *vs*. time data. They have also obtained information about the dehybridization kinetics of double-stranded DNA (dsDNA) as one strand passes through the channel and the other is stripped away.[6,9,12] In addition to their very small size, one of the most attractive aspects of α-HL channels is that the tools of genetic engineering, in conjunction with chemical modification, can be used to modify the channel and hence improve molecular specificity. For example, Bayley and coworkers have shown that β-cyclodextrin can be inserted into the α-HL channel to reduce its size, and that this provides a means for detecting small organic molecules.[13,14] This group has also shown that the α-HL channel can be modified with a probe strand of DNA, and that this makes it possible to distinguish between the residence time of complementary and mismatched target DNA.[15]

The advantages of this biological approach to Coulter counting are numerous. As mentioned in the previous paragraph, the geometrical and chemical properties of the channels can be reproducibly controlled through genetic engineering. Moreover, channels within membrane proteins are small, which means that the properties of important analytes, such as metal ions, nucleic acids, and other types of polymers, can be measured. Indeed, as mentioned earlier, many dynamic processes, such as dehybridization, can be studied.[12] For practical analytical applications, a significant drawback of biologically inspired Coulter counters relates to their stability. In the laboratory environment such channels have lifetimes, which are limited by bilayer stability, on the order of several hours. They have not been tested in the field.

Coulter counters based on synthetic membranes

In many ways, synthetic and biological channels have complementary properties. For example, channels prepared within synthetic membranes are usually (but not always) larger than protein-based channels, but synthetic channels are always more robust. In contrast to biological channels, the reproducibility with which synthetic channels can be prepared is usually rather poor (but in at least one case, highlighted later, this drawback is avoided). Likewise, the first examples of chemical functionalization of synthetic channels are just now being reported, whereas genetic engineering of biological channels has evolved to a sophisticated level.

Four basic approaches have been used to fabricate nanometer-scale channels within monolithic synthetic membranes. The most common of these is based on localized etching of the monolith using, for example, a focused ion beam (FIB). The second relies on various forms of lithography, the third involves the use of a template, and the fourth relies on the channels present within nanotubes. Each of these approaches is briefly introduced next.

Golovchenko and coworkers have reported a method for preparing very small channels by etching through a synthetic membrane with a FIB.[16,17] Specifically, they prepared a single channel within a Si_3N_4 membrane that was ~3–10 nm in diameter and ~5–10 nm in length. The size of channels prepared in this way approaches that of the biological channels discussed earlier, but they are chemically and structurally less well defined. It is very difficult, for example, to reproducibly control the size of the smallest channels. The obvious compensation is that these synthetic channels are structurally robust and perhaps chemically stable (although little is known in this regard). Like the biological channels, those fabricated by the FIB method have also been used to study DNA. Specifically, Golovchenko and his colleagues have measured the length and folding characteristics of dsDNA.[17] It was found that the smallest channels (3 nm) gave rise to resistive pulses attributable to unfolded dsDNA, while the largest channels (10 nm) detected various configurations of folded DNA. This study provides a compelling example of the use of Coulter counters for studying the conformation and mechanics of macromolecules at the nanoscale.

A chemical etching approach, which is closely related to the just discussed FIB fabrication method, was recently used by Siwy and coworkers to prepare channels in track-etched polyimide membranes.[18] Channels prepared in this way have a conical shape with a ~2–7 nm constriction at the tip. These channels were used to study transport of dsDNA, and the results were found to be similar to those reported for α-HL.[6]

Saleh and Sohn have demonstrated that very small channels can be integrated with microfluidic systems using relatively straightforward lithographic techniques.[19,20] This body of work is significant for a number of reasons, but from an analytical perspective the most important point is that it opens up the possibility of constructing low-cost, portable Coulter counters. As is almost always the case for advances in Coulter counting, the focus of these studies is on channel fabrication. In one case, this group used reactive-ion etching to fabricate open quartz channels having lateral dimensions ranging from 400 nm to 1 μm that spanned two reservoirs.[19] The quartz assembly was then sealed with a poly(dimethylsiloxane) (PDMS) plate and used to detect 87 nm diameter latex spheres that were electrophoretically driven through the channel using on-chip electrodes. In another case, micromolding[21] was used to prepare channels within PDMS monoliths that were also part of a microfluidic system.[20] This approach yielded channels having lateral dimensions as small as 200 nm that were used to sense the presence of λ-phage DNA. In a more recent report, the same sort of chip-based Coulter counter was used to measure the small difference in size of ~500 nm diameter, streptavidin-coated beads resulting from antibody binding to the surface.[22]

Our research group developed a template-based technique to fabricate channels as small as 1.5 μm in diameter. This was accomplished by suspending a glass-fiber template within a relatively large seed pore contained within a gold membrane, defining the channel by electrodeposition of additional gold around the template, and then removing the template.[23] In this approach, the size of the channel is determined by the diameter of the glass fiber, and thus a smaller channel can be reproducibly prepared by using a smaller diameter glass fiber.[23] These membranes were configured within a Coulter counter and used to measure the transport of 440 nm diameter polymer particles. Because the channel walls are gold, this method provides a simple means for modifying the channel interior with thiol-based, self-assembled monolayers to promote chemical selectivity. However, because gold is a conductive material this type of membrane has an inherently large capacitance. High capacitances are normally avoided in resistive pulse measurements, because they decrease the signal-to-noise ratio and reduce the time resolution of the experiment.

More recently, we showed that carbon nanotubes are probably the best choice for constructing synthetic Coulter counting channels.[5,24] There are a number of reasons for this assertion. First, carbon nanotubes have well-defined chemical and structural properties.[5,24] Second, nanotubes have interior diameters ranging in size from about 1 nm to greater than 150 nm. Third, the interior channel walls are uncharged, so that electrically driven transport arises only from electrophoresis.[5] This is significant, because eqn. (2)

indicates that under these conditions the transport problem becomes simple enough that the particle mobility can be determined without assumptions and or instrument calibration.[24] Few other channel materials, including all of those discussed here, possess this highly desirable characteristic. Therefore, biological channels, and those prepared from silicon-based materials or polymers, can only provide information about particle size and concentration but not particle mobility or charge.

Fig. 3 illustrates the method developed by our group for preparing single-channel membranes using multiwall carbon nanotubes (MWNTs).[5,24] First, a single MWNT is extracted from a mass of MWNTs using a sharp platinum tip. Second, one end of the nanotube is sealed by electrodeposition of a polymer. This prevents glue from entering the nanotube channel in the next step. Third, the nanotube is stretched and affixed to a TEM grid using epoxy. Fourth, the grid/MWNT assembly is encased within a liquid epoxy precursor and then polymerized at room temperature. Fifth, the resulting polymeric monolith is sectioned using a microtome. The resulting composite sections consist of a low-capacitance membrane surrounding a single MWNT channel. At present, the channels we have prepared range in diameter from 50 to 160 nm. Although there are no fundamental barriers to using much smaller diameter nanotubes, there are some practical problems that will have to eventually be addressed. For example, nanotubes having outer diameters smaller than about 100 nm are too small to be viewed and manipulated under an optical microscope. Accordingly, such small nanotubes would probably have to be synthesized *in situ* within the membrane. Finally, the membrane section is immobilized on a support structure and then clamped between two chambers for Coulter counting as shown in the final frame of Fig. 3.[24] Although fabrication of the MWNT-containing monolith (Fig. 3b) requires some skill, a significant advantage of this approach is that up to 300 membrane sections, each containing a channel having identical physical and chemical properties, can be microtomed from a single monolith containing a 400 μm long nanotube.

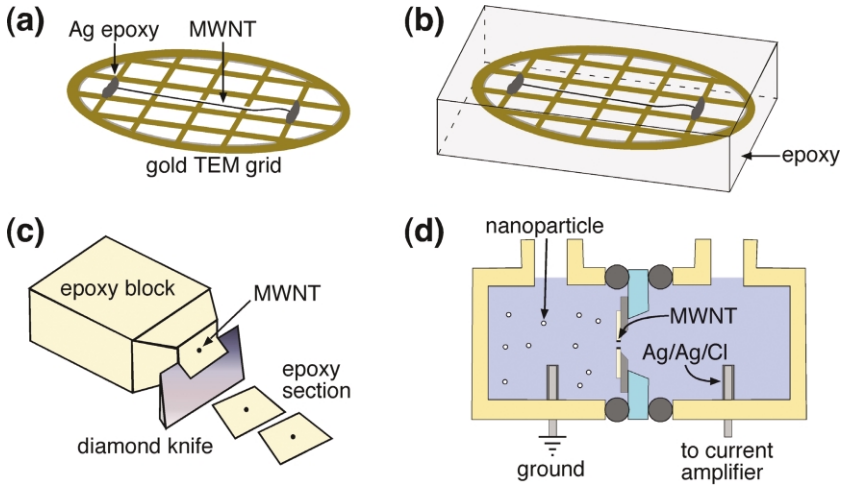

Fig. 3 Illustration of the procedure used to fabricate single-channel membranes containing multiwall carbon nanotubes (MWNTs). (a) The nanotube is stretched and affixed to a TEM grid using epoxy. (b) The grid/MWNT assembly is encased within a two-component epoxy. (c) The resulting polymeric monolith is sectioned using a microtome. (d) The membrane section is immobilized onto a support structure and clamped between two chambers for Coulter counting.

As mentioned earlier, carbon nanotube-based Coulter counters (CNCCs) are unique in that it is possible to determine the size and electrokinetic surface charge of individual nanoparticles, as well as the particle concentration. All of this information is available without calibration of the CNCC once the channel diameter, which is the same for every membrane cut from a particular monolith, and length, which is determined for each channel using a very simple electrochemical method, is known.[24,25]

We used CNCCs having an interior diameter of 132 nm and a length of 0.94–1.26 μm to simultaneously determine the size and surface charge of carboxylate-terminated polymeric nanoparticles ranging from 28 to 90 nm in diameter.[24] In contrast to TEM, CNCCs yield the size distribution of individual particles dispersed in solution. Dynamic light scattering (DLS) is a technique that can also provide size data for dispersed particles, but DLS normally only yields the average particle size; the size distribution can be obtained only through curve fitting and not by direct calculation as is the case for CNCCs.[25] In addition, CNCCs provide far more precise values for the electrophoretic mobility of nanometer-scale particles than other methods such as phase analysis light scattering (PALS).[25] We were able to take advantage of these unique properties of CNCCs to investigate the relationship between particle size and surface charge,[24] to differentiate individual polymeric nanoparticles having different surface charges but the same size,[24] and to determine the apparent surface pK_a of nanoparticles terminated with acidic functional groups.[26]

Summary, conclusions, and outlook for the future

The resurgence of interest in Coulter counting is a result of three recent developments. First, new technologies are available that make it possible to prepare smaller and more well-defined channels than has been possible in the past. Second, there is now unprecedented interest in characterizing nanoscopic objects. Third, there is a very strong commercial incentive to develop portable (small, lightweight, low power), sensitive, and selective biosensors that do not require labeling of the analyte. In this short Highlight, we have shown that Coulter counters have the right set of properties to address all of these issues. There is, of course, much more work remaining to be done.

As we look to a future when Coulter counters may become a routine laboratory instrument for studying and characterizing nanomaterials, there are two apparent frontiers. First, quite a bit of instrument development is required before Coulter counters will be routinely used for nanoparticle analysis. For example, smaller, more robust channels are required. It seems likely that hybrid channels, which combine the attractive properties of both synthetic and biological channels, will emerge to address this problem. It is important that such channels be relatively easy to fabricate in quantity and that their properties be reproducible. Synthetic approaches must be developed so that the chemical properties of the channel can be controlled to introduce selectivity and control over mass transport. Additionally, the hardware supporting the channel would benefit from a reduction in scale so that portable instruments can be developed. This probably means integration of channels into microfluidic system as has been described by Saleh and Sohn.[19,20,22]

Second, new applications for Coulter counting will evolve. At present these

instruments are primarily used to measure the size and concentration of nanoparticles (and in one case the electrokinetic charge).[24–26] However, just recently there have been several truly amazing examples describing the use of Coulter counters to better understand the structure and function of nanoparticles. This includes, for example, Branton's study of the dehybridization of DNA[12] and Bayley's report of detecting small organic molecules with modified α-HL.[13]

We hope this short overview of the Coulter counting principle, recently developed instrumentation, and the types of information that can be derived from this method will stimulate others to bring their particular talents to bear on the interesting problems associated with device development and chemical analysis.

Acknowledgement

We gratefully acknowledge financial support from the US Department of Energy, Office of Basic Energy Sciences (Contract No. DE-FG03-01ER15247).

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