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Essential Data and Techniques for Conducting Microbial Fuel Cell and other Types of Bioelectrochemical System Experiments

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Microbial fuel cells (MFCs) and other bioelectrochemical systems are new technologies that require expertise in a variety of technical areas, ranging from electrochemistry to biological wastewater treatment. There are certain data and critical information that should be included in every MFC study, such as specific surface area of the electrodes, solution conductivity, and power densities normalized to electrode surface area and volumes. Electrochemical techniques such as linear sweep voltammetry can be used to understand the performance of the MFC, but extremely slow scans are required for these biological systems compared to more traditional fuel cells. In this Minireview, the critical information needed for MFC studies is provided with examples of how results can be better conveyed through a full description of materials, the use of proper controls, and inclusion of a more complete electrochemical analysis.

Introduction

Studies of electrochemical activities of microorganisms have increased both in number and complexity over the past 100 years. The first studies were conducted by Potter[1,2] over one hundred years ago at a university in the UK that is currently known as Newcastle University. Potter used a very simple system (Figure 1) to show that an electrochemical potential was generated by different microbes when they were given certain organic substrates. He found potentials were generated by the yeast *Saccharomyces cerevisiae* and the bacterium *Bacillus coli* (later renamed *Escherichia coli*[3]), both of which fermented substrates in the medium. He failed to measure any electromotive force generated with three other microorganisms, but these other microbes did not grow in the medium. Fermentative growth of microorganisms can set up thermodynamic potentials in terms of substrates, pH and other gradients,[4,5] which can often lead to the generation of an electromotive force, but not appreciable current generation. Only microorganisms that generate substantial current densities without the use of exogenous electron shuttles or mediators, called exoelectrogens, are of primary interest today.

Since this first study by Potter,[1] over 3000 papers have been written in the general area of bioelectrochemical systems (based on a Thomson Reuters Web of Science search), mostly in the field of microbial fuel cells (MFCs), and the equipment needed to conduct these experiments has been substantially advanced. In contrast to simple circuits and materials used by Potter, a modern bioelectrochemical laboratory will use many different instruments for obtaining data, such as multimeters for low impedance voltage measurements and potentiostats for electrochemical analysis (Figure 1). As we learn more about factors that affect the generation of electrical current using microorganisms, it has become apparent that better controls, measurements, and care are needed when taking measurements and reporting results. The experiments by Potter are a good example in this regard. Both *S. cerevisiae* and *E. coli* are not considered to be exoelectrogenic microorganisms, as exogenous mediators need to be added with these strains to produce appreciable currents.[6,7] *E. coli* have been used in recent studies as a negative control,[8] although one group reported that current could be produced by one *E. coli* strain after successive transfers.[9,10] However, purity of the culture was not confirmed after these transfers, raising the possibility that current generation was due to contamination. In addition, yeast-peptone medium is known to contain mediators,[7] and mediators such as flavins[11] (for example, Riboflavin, vitamin B_2) are often added to a medium directly[12,13] or indirectly in yeast extract.[1] Thus, it is always possible that cell disruption or media carryover can result in sufficient concentrations of mediators to produce measurable but low current. A low level of current production by microbes is, therefore, not sufficient proof that the microbes are exoelectrogenic, that is, that they can grow via exogenous electron transfer.

Experiments on MFCs and other bioelectrochemical systems, like all scientific experiments, require careful planning, proper controls, and a thorough analysis of data. The multidisciplinary nature of MFC experiments can sometimes make it difficult to establish minimum standards for experimental design (such as replicates) or electrochemical measurements. For example, wastewater treatment studies are often conducted with pilot or full scale reactors and thus lack separate controls due to the cost of the systems. Electrochemists evaluate chemical reactions and electrode performance using solutions (such as

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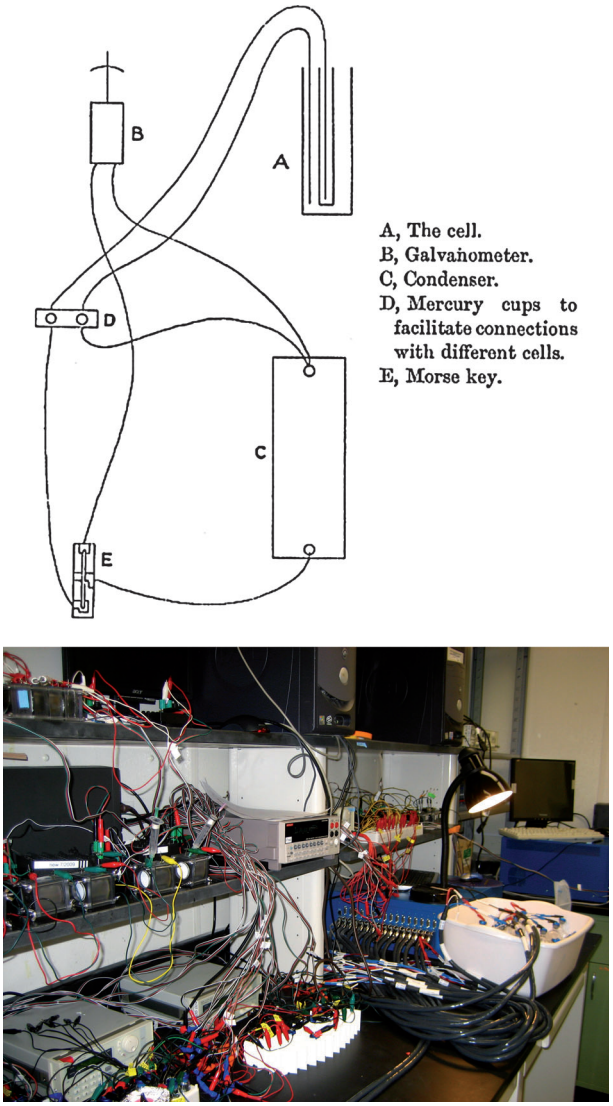

Figure 1. Comparison of past and modern day systems. Top: Experimental apparatus of Potter[1] showing the system components (but not reactor architecture). Bottom: A modern laboratory containing data loggers, a multichannel potentiostat, and various types of MFCs. (From the laboratory of the author).

perchloric acid) appropriate to understand mechanisms and choose scan rates in some tests that are appropriate for fast chemical reactions. However, these conditions may not provide results useful for understanding biological systems that are usually operated at neutral pH by using carbonate and phosphate buffers. The community of researchers studying electrode materials, microorganisms, and other factors that affect current generation in MFCs and other bioelectrochemical systems (BESs) must take care to obtain data under relevant conditions (such as neutral pH) to better understand the functioning of these complex systems. As noted in the examples below, full operational conditions and more complete description of the reactor architecture are needed to properly assess reactor performance. Careful planning and reporting can help provide data needed to better understand the operational results from these complex systems and help avoid misrepresentation of performance. The comments below generally apply to all types of BESs, although they are primarily directed at MFCs.

Experimental Design

Replicate reactors

In any experiment it is recommended that researchers use at least duplicate reactors to examine variability for the systems tested. This is especially true for MFCs, where high variability can occur among replicates. Many MFC studies have been conducted with only a single reactor,[14–17] whereas a few have used triplicate reactors[18–20] even though acclimation of the same inoculum in the same reactor can produce appreciable differences in initial power production.[21] Some researchers have recommended four or more replicates based on analysis of eight reactors,[22] but another study with triplicate reactors showed that there would have been no significant difference in the outcome if only duplicates had been used.[21] Based on experiments in my laboratory with many hundreds of MFC tests, duplicates should be sufficient for tests as long as the results are in relatively good agreement. However, without replicates, claims of changes or equivalent performance cannot be substantiated.

Steady state conditions, proper controls, and benchmarking

For closed circuit MFCs (treatment), stable current generation must be shown in continuous flow reactors. In fed batch systems, where the fluid is replaced after a cycle of operation (when the current drops below a certain level due to depletion of substrate) it is recommended that reproducible cycles of power generation be obtained (the same maximum power and fed batch cycle duration) over at least three fed batch cycles in duplicate reactors. Failure to demonstrate reproducibility means that the reactor is not sufficiently acclimated for stable power generation. Biological growth will occur in an MFC even if the circuit is open, resulting in chemical oxygen demand (COD) changes in the absence of current generation that vary for reactor types, inocula, and substrates. Therefore, when examining COD removal from a wastewater or microbial communities in MFCs,[22] it is important to run open circuit controls.

Metal corrosion can result in the generation of galvanic current by the anode. This is particularly a problem if using stainless steel or copper for the anodes. Copper is additionally a problem as it can be toxic to bacteria. In one study, there was a report of unreasonably high power densities (40 $W m^{-2}$) with a copper anode.[23] Abiotic controls can help to prove an absence of abiotic power generation, but as metals corrode the surface area can increase, so inactivating the anode (for example by heating) could help to demonstrate a lack of abiotic current generation after the anode has corroded.

When designing new electrodes or using new materials, it is important to benchmark system performance by using more conventional materials and to fully examine electrochemical

performance.[24] For example, tests of a new cathode catalyst on carbon cloth should be compared in side-by-side tests with cathode containing a Pt catalyst at typically used loadings[25,26] and not just a plain or otherwise modified electrode.[27] This benchmarking allows better evaluation of the cathode performance in relation to the best known materials.

Substrates

The amount of power generated varies with the substrate used in the MFC, with the highest power densities typically produced from acetate,[28–31] with variable and usually much lower power densities produced with different wastewaters.[32,33] It is, therefore, never acceptable to call an acetate or glucose solution a "synthetic wastewater", especially when the solution has a high conductivity ($>5\,mS\,cm^{-1}$) relative to that of actual wastewaters (typically $1\,mS\,cm^{-1}$) and a high buffer concentration. Such defined solutions should be referred to simply as the medium used in tests with the indicated main substrate. Also, if a wastewater is diluted, the conductivity should be maintained to avoid artifacts related to conductivity as opposed to substrate concentration.[34]

Coulombic efficiencies (CEs) with acetate and many pure compounds are usually much higher than those of wastewaters.[35–38] One main reason is the cycle time. Oxygen leaks into the reactor through the cathode, septa (if present), and gaskets at roughly a constant rate, and thus a longer cycle time means more oxygen has been provided to microorganisms for a given mass of substrate. The cycle time with acetate as a substrate is usually shorter than those with more complex substrates, reducing the time over which oxygen can leak into the reactors. In tests using only acetate at different external resistances, it has been shown that the CE substantially varies inversely with cycle time. Changes in CEs in one study were more affected by resistances, changing from 35–40% at lower current densities to 65–70% at higher current densities for a variety of separators.[38] Lowering the resistance increases current and decreases the cycle time, and therefore, decreases the mass of oxygen that has leaked in during the fed-batch cycle. Microbial communities can also change with current density and cycle time, likely as a function of the amount of substrate used by exoelectrogens compared to other processes, such as fermentation, methane generation, and aerobic growth using oxygen leaking into the system. Thus, community analysis of reactors with low CEs likely do not adequately capture the composition of the exoelectrogens compared to other microbes in the system. This may account for the much more variable microbial communities reported in earlier studies with low CEs[39,40] compared to later ones even when using simple substrates such as acetate or glucose.[21,32,41]

Catholytes other than oxygen

MFC experiments with electron acceptors such as ferricyanide and permanganate should be avoided, except in special circumstances, as they are not helpful for evaluating systems that can be scaled up for practical applications. Chemical catholytes such as ferricyanide can be useful when cultivating specific microbes[42,43] or perhaps evaluating anode materials. However, these chemicals add a chemical potential to the system and create conditions that are unrealistic compared to when oxygen is used as the catholyte, as it is known that oxygen leaking into the anode chamber can affect power generation in MFCs with closely spaced electrodes.[44] The anode potential can also be set by using a potentiostat, in which case the choice of a catholyte will be less critical.[45–48] However, the relative sizes of the cathodes and the materials used can affect the limiting current,[48] so this should be evaluated and considered in electrochemical tests. Solution conductivity can also affect cathode performance, and thus, it is important to consider the buffer and salinities used in electrochemical tests.[49,50]

Reporting Data on Reactor Conditions

Reporting power densities

Power should be reported in at least two ways: Per projected area of an electrode or membrane, and per total reactor volume. In early studies, power was normalized per anode area because it was presumed that the anode would limit power generation.[30,35,51–53] Now it is known that a membrane or separator placed between the electrodes (two chamber MFCs),[35,54] or the cathode (single chamber systems) usually limit maximum power densities.[55–57] Therefore, it is appropriate to normalize power to the membrane or cathode.[44,56] The anode area used may include one or both of the electrode sides, depending on the electrode orientation (suspended in solution or pressed against the reactor side). For an air cathode, a single cathode side used as the catalyst is usually placed only on one side (water facing side). Sometimes the net anode or cathode (liquid) compartment volume, or the volume of only one of the two chambers, has been used for normalizing power.[58] Although this gives a perspective of what the reactor can do on the basis of liquid volume, the environmental engineering convention is to use total empty volume (referred to as empty bed volume, or EBV) as the reactor size, and therefore, EBV should be used for BESs.

When reporting power densities, use significant figures, report standard deviations, and check units. For example, instead of reporting $841.45\,mW\,m^{-2}$ or $841.45\pm18.53\,mW\,m^{-2}$, write $841\pm19\,mW\,m^{-2}$ or $840\pm20\,mW\,m^{-2}$ as rounding to the tens units better reflects variability in these systems. Current is often normalized to cm^2 in the fuel cell literature, and to m^2 in MFC studies due to lower power densities. Unit errors during publication have also resulted in considerable confusion. In one paper, a unit error resulted in maximum current densities reported as $mA\,cm^{-2}$ rather than $\mu A\,cm^{-2}$.[59,60] This created unrealistic expectations relative to current densities in subsequent studies.

Electrode-specific surface areas

One of the most important factors affecting reactor performance is electrode density. Scaling up MFCs will require a high

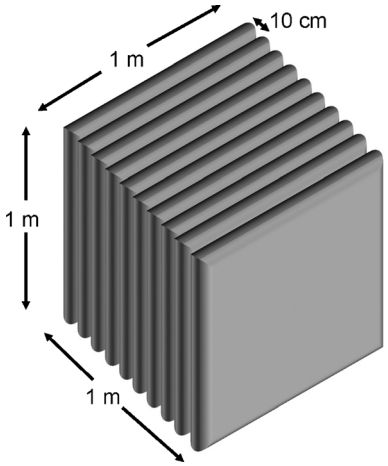

Figure 2. Example of module packing. A single anode-cathode pair 10 cm thick would produce a specific surface area of 10 $m^2 m^{-3}$ (based on one electrode with a projected area of 1 m^2).

density of electrode surface area to produce the maximum current or electrical power per volume. If we assume a modular design, then the width of a single electrode pair will define the electrode packing (Figure 2). Following the convention of normalizing power to only one electrode, the electrode surface area is represented only by one of the electrodes even though the total electrode surface will be larger. For example, if a single electrode pair with 1 m^2 each for the cathode and anode (projected area) is used in a 1 m^3 reactor, then the electrode density is 1 m^2 (cathode) per m^3 of reactor volume (1 $m^2 m^{-3}$). If each 1 m^2 electrode required 10 cm in width, then the electrode specific surface area would be 10 $m^2 m^{-3}$ (Figure 2).

The higher the electrode density, the better the reported volumetric power density may appear for conditions where power densities based on electrode-projected surface area are similar. For example, one early two-chamber MFC reactor had 22.5 cm^2 of anode (both sides) per total volume of liquid (anode and cathode), resulting in 4.5 $m^2 m^{-3}$ (two sides) or 2.3 $m^2 m^{-3}$ (one electrode side) for a power production of 0.039 $W m^{-2}$ (two sides of the anode) and 0.18 $W m^{-3}$.[61] Single-chamber designs eliminated the need for a large cathode chamber volume and a membrane and generally resulted in higher volumetric power densities due to the improved performance of an air cathode. For one single-chamber design with 29 $m^2 m^{-3}$ for the cathode, power was increased based on cathode surface area to 2.4 $W m^{-2}$ and 73 $W m^{-3}$ using a high surface area, graphite fiber brush anode.[56] Power densities were much lower in this same type of reactor using a membrane to create two chambers.[54] Using a flat anode (placed against the opposite reactor wall) produced less power per area of the cathode (2 $W m^{-2}$), but a higher volumetric power density by using less reactor volume (115 $W m^{-3}$, 29 $m^2 m^{-3}$).[62] Very high power densities were achieved by using much higher electrode specific surface areas. For example, Fan et al.[63] obtained a power density of 1.55 $kW m^{-3}$ (2.77 $W m^{-2}$) by using a small reactor (2.5 mL) with a cathode surface area of 280 $m^2 m^{-3}$, whereas Nevin et al.[64] produced 2.15 $kW m^{-3}$ (1.9 $W m^{-2}$) using an even smaller reactor (0.336 mL) with a higher cathode specific surface area of 1920 $m^2 m^{-3}$. Using these data, we can see that power on a volumetric basis increased in proportion to surface area over several orders of magnitude, whereas power per cathode area has been relatively flat (Figure 3). High electrode specific surface areas could be

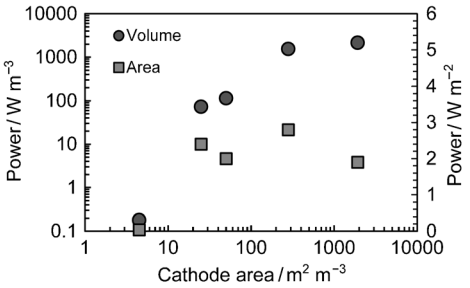

Figure 3. Power densities increase with electrode cathode specific surface area over several orders of magnitude, but do not vary appreciably when normalized to cathode projected areas larger than 10 $m^2 m^{-3}$.

a problem when using actual wastewaters due to the potential for clogging. Trickling filters used for domestic wastewater treatment, for example, tend to work well up to 100 $m^2 m^{-3}$ of plastic media surface area, but they can clog at higher specific surface areas.[65]

Aqueous solution chemistry

The solution conductivity affects power generation and, therefore, it should always be reported in a study.[66] If a wastewater is diluted, then it should be amended with a salt to maintain conductivity. It was shown that power scaled linearly with COD of a wastewater when it was diluted with water, but when the same conductivity was maintained through dilutions, the power changed with substrate concentrations according to Monod-like kinetics.[34] Changes in pH values should be reported for solutions before and after treatment if they appreciably change, as low pH values can limit power generation.[67]

Microorganisms

Biofilms in MFCs can be evaluated by using a variety of techniques. A recent review suggests that 16S rRNA gene clone libraries provide results generally consistent with operation (power generation in relation to abundance of certain bacteria) and possible synergisms among different types of bacteria in degrading fermentable substrates and complex wastewaters.[68] 16S rRNA gene finger printing analysis with denaturing gradient gel electrophoresis (DGGE) shows differences between communities, but gives us little insight into the predominance of different microbes in the biofilm. Pyrosequencing results have been found to be generally consistent with clone libraries,[21] and this approach provides an additional opportunity to probe more deeply into the community members, although

it is not clear what role numerically less abundant bacteria might play in current generation. Some exoelectrogenic bacteria can also be missed in biofilm analysis. For example, it was found in one study that a *Shewanella* species that was not identified in the clone library produced more power than a relatively abundant microbe based on isolates obtained from the same MFC.[69]

Pure culture work can provide great insight into levels of current generation by different microbes and the methods bacteria use to generate current. However, it is always important to check for culture contamination at the end of the experiment, for example, by using molecular techniques or microscopy in combination with culturing methods such as growth on agar plates.

Power production by mixed cultures can be improved through serial transfers from one MFC to another,[70,71] and long times are sometimes required in a single MFC to see increased power production associated with community changes.[15] It has become common among MFC studies to obtain inocula from other operating reactors. This works well if the same substrate and medium is used, but it might not work well for different substrates as the community may have become less diverse and therefore less able to efficiently degrade that new substrate. More standardized guidelines are needed on how much biomass can be transferred between reactors and how to best start up MFCs using mixed or pure cultures.

Polarization Data and Electrochemical Tests

Polarization data

There are several methods for obtaining polarization data, including changing resistors for different time intervals (single cycle and multiple cycle methods),[72,73] linear sweep voltammetry (LSV), and current interrupt.[74] When resistors are changed over a single cycle (single cycle method), sufficient time must be allowed at each resistance or the current, and therefore power, will be overestimated relative to that possible under steady state conditions. Reasonable times are 20–30 min per resistor, with longer times preferable to achieve steady state conditions, but substrate concentrations should not be reduced during the test to values that might affect power generation. The time at each resistance should always be reported in the study. In a procedure called the multiple cycle method, a single resistor is used for a full cycle. Additional cycles at the same resistance should show repeatable results.[75] Polarization data can be obtained by using LSV, but very slow scan rates of $\approx 0.1\ mV\,s^{-1}$ must be used. In one test, a scan rate of $1\ mV\,s^{-1}$ produced an apparent power density that was 80% larger than that obtained at $0.1\ mV\,s^{-1}$.[72] The current interrupt method was suggested as an approach for obtaining polarization data,[74] but it is not a commonly used method in MFCs, and there have been no studies that compare this approach to these other methods of obtaining polarization data.

Reference electrodes

The use of a reference electrode is critical to the study of electrode performance over time and the evaluation of the performance of new materials. For example, if a new cathode material is used, the reference electrode should be used to show that the anode potentials were not changed,[38] thus ensuring that differences between the reactors were due to the new cathode materials. The performance of a new electrode material should also be examined in standard three-electrode electrochemical tests, such as LSV and cyclic voltammetry (CV). When using a reference electrode, the potentials should be corrected to those for a standard hydrogen electrode or the appropriate conversion should be provided.[47] Reference electrodes should be placed as closely as possible to the working electrode and frequently examined for proper calibration. When using highly porous and distributed electrodes such as graphite fiber electrodes, measured potentials should be treated with caution. For example, in a long-term MFC test with graphite fiber brush anodes evaluated by using a reference electrode, measurements suggested that anode potentials had varied.[76] However, replacing the cathode fully restored power, suggesting that anode potentials were in fact constant over the 14 month study and incorrect values had been obtained with the reference electrode. When the same type of brush anode was used in a microbial electrolysis cell and the anode potential was set at a value identical to that produced when adding voltage to the system, the set potential result unexpectedly produced poorer results than the added potential results.[77] This suggests that the anode potential was not actually set at the indicated value.

Power overshoot

Two different types of power overshoot have been observed in power density curves: Type M and Type D.[75,78] In Type M, the maximum power is overestimated. This type of overshoot can be remedied by using a very slow LSV scan rate or longer times at fixed resistances. In Type D overshoot, the power density curve doubles back to lower current densities after a maximum power density is obtained. Type D overshoot appears in numerous studies[15,72,79–81] and is less easily explained or corrected. One approach for avoiding overshoot is to use the multiple cycle method rather than an LSV or single cycle method.[75] It has also been shown that pre-acclimation of the MFC to the lowest resistance that will be used in a polarization test can avoid overshoot.[78] Polarization data should be obtained in a way to produce a power density curve with sufficient data on both sides of the maximum power peak. Data should never be omitted that show overshoot (usually identified by less points than expected based on given methods or those reported in other power density curves).[82] Failure of the power density curve to reach a clear maximum can result in underestimation of maximum power.[75]

Additional Observations

There are many factors to consider when designing a new MFC experiment, ranging from inoculum, medium, and materials, to number of reactors and the length of the study. As the field advances, more techniques are becoming standardized through acceptance by researchers in different laboratories, but there is still a lack of agreement on many issues. In studies that look to advance these technologies for practical applications, there is now greater emphasis on examining conditions that are realistic for such applications. For example, that means avoiding addition of phosphate buffers to wastewaters or the use of expensive materials. Increased emphasis should be placed on architectures that can be scaled up from the laboratory to the field, and therefore, studies conducted using traditional "H-type" (two bottle reactors separated by a membrane) should be avoided in more applied studies, for example, in wastewater treatment. Such H-type reactors remain useful for more fundamental tests on bacteria and materials, but results cannot be directly translated to more applied conditions where reactor conditions affect performance.

Reviewers have an important role in ensuring only sound and credible studies are published and that errors are avoided and claims are based on sufficient data and sound science. Raising the level of the science is essential for any field, but it is often difficult for such an interdisciplinary and nascent field of BESs. It is critical to the further development of this field that sufficient data is collected to allow statistical analysis of the results and that the methods are adequately described. Attention to these issues will avoid the publication of results that are unreasonable or not reproducible.[23,83–85] As the field matures, many techniques and study conditions will become standardized, but for now greater caution is warranted when evaluating MFC protocols. Agreement on these issues will lead to more credible MFC results and the emergence of these MFC technologies from the laboratory into commercialization in perhaps many different applications.

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