Monitoring "Live" Cell Concentrations in Real Time

Using RF Impedance to Optimize Fermentation and Cell Culture Processes

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The productivity of any biological system is determined by the quantity of viable biomass present. Accurate measurement and control of the biomass within fermentors at both laboratory and industrial scales is therefore a major requirement. Many fermentation processes rely on estimating biomass off-line from frequent sampling throughout the process, but such methods suffer a number of disadvantages. They are time consuming to measure, subjective to human interpretation (e.g. microscopic cell counting and viability estimation), provide only a limited number of measurements during normal shift hours, and potentially introduce contamination.

An on-line system that is now considered as a true "off the peg" dedicated biomass instrument, particularly for industrial applications, is the Biomass Monitor from Aber Instruments, UK (**1–3**). The Biomass Monitor uses radio-frequency (RF) impedance and is based on the passive electrical (dielectric) properties of biological materials. It detects only those cells with intact plasma membranes and so gives values that correlate with viable biomass.

This is a report on current developments for monitoring fermentations by RF impedance. It includes the use of this instrumentation in cGMP production environments and discusses some recent applications of the technology to a range of cell types used in the bioprocessing industry.

PRODUCT FOCUS: PROTEINS, VACCINES, ANTIBIOTICS, ENZYMES

PROCESS FOCUS: FERMENTATION CELL CULTURE, SCALE-UP TO PILOT PLANT

WHO SHOULD READ: R&D, QUALITY CONTROL

KEYWORDS: FERMENTORS, PROCESS CONTROL, SCALE-UP, CELL CULTURE, BIOMASS, ON-LINE

LEVEL: INTERMEDIATE

PRINCIPLE OF MEASUREMENT

Cells with intact plasma membranes can be considered to act as tiny capacitors under the influence of an electric field. The nonconducting nature of the plasma membrane allows a build-up of charge. The resulting capacitance can be measured; it is dependent on the cell type and is proportional to the concentration of viable cells present.

A probe incorporating four electrodes is fitted into a standard fermentor port where it can be steam sterilized in situ. When fermentation or cell culture begins, the probe applies a low-current RF field to the biomass passing within 20 to 25 mm of the electrodes. Figure 1 shows a four-pin electrode producing a radio-frequency electric field and polarizing the viable cells. The nonviable and leaking cells

Biomass monitor 220 (Aber Instruments Ltd., UK) for real-time measurement of viable cell concentration. Photograph shows a cell culture application with a top entry electrode.

are effectively invisible to the detection system. The Biomass Monitor processes the signal from the probe to produce a highly accurate measure of the viable cell concentration.

The system is insensitive to cells with leaky membranes, evolved gas bubbles, cell debris, and other media components, so it detects only viable cells. Unlike conventional optical systems, the Biomass Monitor is not prone to fouling and is linear over a wide range of cell concentrations. It also measures very high biomass concentrations and operates in a wide range of media for measuring viable bacterial, yeast, animal, plant, and insect cells, either in free suspension or immobilized on inert carriers. The Biomass Monitor (model 220), released in the fall of 2002, was developed to incorporate a built-in multiplexer that allows as many as four bioreactors to be

Figure 1: Influence of viable and leaking cells under the influence of an RF field produced by a four-pin electrode probe. The leaking or dead cells are "invisible" to the probe.

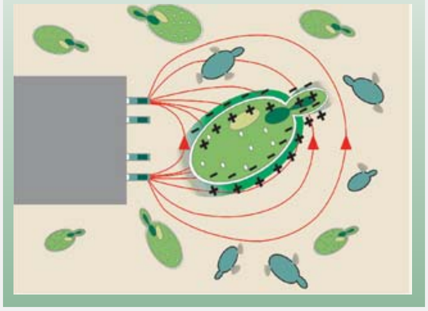


Figure 2: Biomass Monitor 220 (Aber Instruments, Ltd., UK) with 25-mm and 19-mm diameter probes. Materials of construction for the 25-mm probe: (1) probe body, 316 stainless steel; (2) neoprene sealing washer; (3) port O ring, FDA-approved silicone; (4) internal O ring, FDA-approved nitrile; (5) probe tip, Victrex PEEK approved to FDA 21 CFR 177.2415 for repeat food contact (meets the requirements of USP class VI); (6) electrode pins, 99.99% FDA-approved platinum.

monitored for viable cell concentration by a single instrument. The system can then be expanded with additional multiplexers to monitor as many as 34 fermentors.

Probes in cGMP Environments

Other probes for estimating viable biomass by RF impedance have traditionally used a high performance polymer matrix (usually a modified epoxy resin). The resin has performed well after repeated steam sterilizations or CIP and is suitable for most laboratory and pilot plant applications. However, for manufacturing in a cGMP environment, all the wetted materials must conform to FDA requirements. A probe was therefore specifically developed for use in this crucial application area.

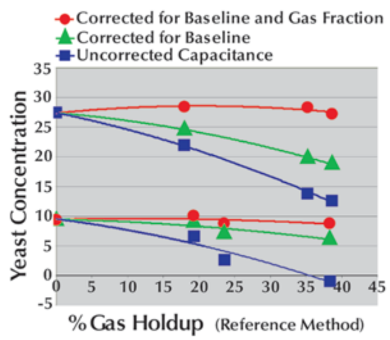
The main body of the probes used with the Aber Instruments Biomass Monitor 220 is composed of 316L stainless steel; the end holding the electrodes is made from the inert and dielectrically stable polyetherether ketone (PEEK) (Figure 2). PEEK is commonly used in the biopharmaceutical industry and has USP class VI and FDA 21 CFR 177.2415 accreditation for repeated food contact. The four electrode pins into the PEEK are sealed by a process designed to leave the pin fitting void free without requiring an O-ring. The junction between the stainless steel and PEEK is provided by an O-ring manufactured from an FDA-approved nitrile. Probe diameters of 25-mm and 19-mm in lengths of as much as 600 mm have been provided to date. The guaranteed life of a probe is one year, but tests have shown that probes can withstand more than 100 SIP (steam in place) or autoclave cycles.

Gas Hold-Up Measurement and Correction

Although the original Biomass Monitor is successfully used in many research, development, and production environments, one major source of uncertainty remains with data gained from on-line measurements. The agitation and gas hold-up regimes seen in many filamentous, bacterial, and yeast fermentations produce a homogenous but slightly varying mix of cells, media, and gas. The probe described above will "see" the viable cells that are within the radioelectric field, but this does not represent the true suspension of cells that would be measured in a degassed sample taken from the fermentor.

The newest Biomass Monitor (Model 220) incorporates a multifrequency measurement system that can deal with the majority of situations in

Figure 3: Using the Biomass Monitor 220 in highly aerated fermentors to allow a constant viable cell concentration with large changes in gas hold up.



fermentation. The instrument also can measure the actual gas hold up in the fermentation, providing customers with an additional useful measurement parameter (e.g. onset of foaming). The multifrequency system is used to calculate the true fraction of biomass present regardless of gas distribution. Figure 3 shows an example of how the

Figure 4: Monitoring animal cells grown in suspension and monitored for viable cell mass by using RF impedance. (DATA RELATES TO THE VIABLE CELL MONITOR 520 FROM ABER INSTRUMENTS, UK)

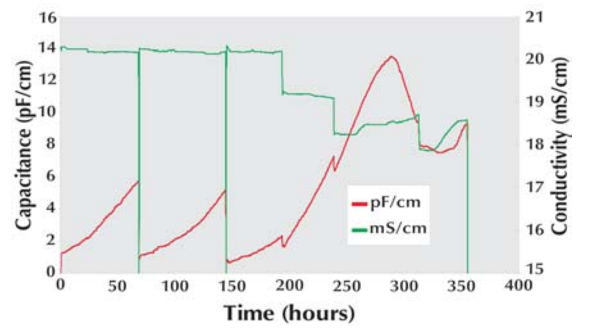
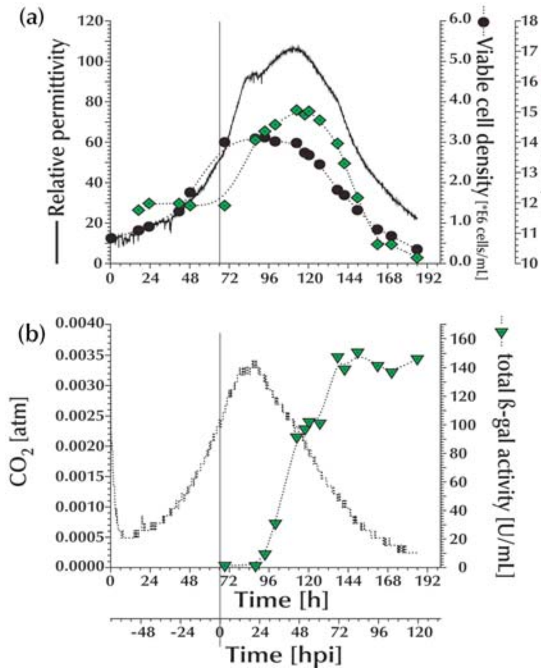


Figure 5: Monitoring the infections of Sf-9 insect cells with a baculovirus by RF impedance: (a) relative permittivity, viable cell density, and cell diameter; (b) CO_2 produced and total β-galactosidase with a vertical line at the time of infection and an additional *x*-axis for post time infection. (DATA COURTESY OF CNRC, CANADA)



The MYCELIAL fed-batch fermentations with partially soluble complex media are challenging applications in which many processes rely on indirect estimations of biomass.

Biomass Monitor 220 can be used to measure the concentration of viable yeast under varying gas hold-up conditions.

APPLICATIONS OF RF IMPEDANCE

In the following examples of the utility of the RF impedance probe, real-time traces of viable cell biovolume are expressed directly as units in either pF, pF/cm, or dielectric permittivity; or the pF value has been converted to read directly in viable cells/mL. A trace of the conductivity (mS/cm) is also shown, because the RF impedance instruments provide this value as an additional 4–20-mA output. The conductivity profile in real time is often found to be a valuable measurement in its own right, allowing an insight into some of the changes taking place during critical parts of fermentation.

The technology has been widely adopted in a number of key areas in bioprocessing. The mycelial fed-batch fermentations with partially soluble complex media are especially challenging applications in which many processes rely on indirect estimations of biomass. The original Biomass Monitor (model 214M) provides an accurate and reliable estimate of mycelial biomass during the entire process time. End users report "the data from the Biomass Monitor to be closer to physiological reality and easier to interpret than the traditional off-line methods of estimating biomass" (**4**).

The use of RF impedance technology to monitor animal and insect cells, whether in suspension or immobilized, has started in earnest over the past five years. The probe in this case provides an accurate and reproducible live cell count in real time, and both the Biomass Monitor and Viable Cell Monitor 520 (an RF impedance instrument dedicated to cell culture applications) are used in cGMP production-scale facilities. Applications include batch culture, in which an accurate on-line measurement is required under conditions where the cell viability can typically vary between 35% and 95% and where there can be a high degree of clumping. Under these conditions, providing an accurate viable cell count using traditional microscopic methods with a viability stain such as trypan blue can be prone to both inter- and intraoperator errors. Figure 4 shows a typical profile of animal suspension cells grown in two batch cultures and a fed-batch culture over 14 days in the same vessel. The large step changes in capacitance after 70 and 140 hours correspond to harvesting of the culture and replenishment of media. The small drops in capacitance after 190, 240, and 310 hours are due to dilution of the media during fed-batch culture. Decline of the viable cell concentration after 300 hours can be clearly seen.

Determining on-line the viable cell density of insect cells with the monitor can allow a baculovirus to be added at the most appropriate point of the growth curve for maximum production of recombinant protein (Figure 5). After the addition of baculovirus (indicated by the vertical line) to Sf-9 insect cells, the monitor can be used to track the progress of infection (**5**). In this case, the authors expressed the biomass as relative permittivity, a unit that can be derived from the capacitance measured by the monitor. There is an excellent correlation between the viable cell count and relative permittivity before the addition of the baculovirus. The relative permittivity continues to increase rapidly after infection. Because viable cell numbers (determined manually) are no longer rising, this must represent an increase in the diameter of the Sf-9 cells and therefore a successful baculoviral infection.

For animal cells attached to carriers, the Biomass Monitor provides a unique real-time estimation of the viable biomass. It replaces alternative off-line methods based on protein analysis or nuclear counts that only estimate total cell numbers. Examples of using the monitor include surface and macroporous microcarriers grown in a stirred suspension (**6**), porous glass beads in a fluidized bed (**7**), and porous disc carriers used in packed bed systems (**8**).

Figure 6 shows the application of capacitance to monitor CHO cells grown on microcarriers during long term culture runs at Genzyme Inc. (USA) for both the growth and harvesting phases. Figure 7 demonstrates the use of capacitance to monitor growth of Vero cells at Xenova (UK) on Cytodex 1 microcarriers with exchanges of media. Figure 8 presents data from a 20-day cultivation of baby hamster kidney (BHK) cells attached to macroporous microcarriers (courtesy of Novo, Denmark). Capacitance, conductivity, and the off-line cell counts are shown as a function of time. Samples were taken daily, and the cell numbers were estimated by crystal violet staining followed by manual counting of released nuclei in a hemocytometer. Vertical lines indicate the daily exchange of medium (the impeller is turned off to settle the cells). The off-line cell count on the macroporous carriers was unable to provide any meaningful data on the progression of the culture. By contrast, the Biomass Monitor provided valuable real-time information on the viable cell count. A small daily drop in capacitance signals indicates that essential nutrients are being depleted before the medium is exchanged.

Estimating the viable cell mass in processes with bacteria such as *E. coli* and *Streptomyces* (**9**) under highly aerated conditions is probably the most demanding application for any on-line cell mass sensor. On-line optical sensors are severely affected by gas bubbles under these conditions. The Biomass Monitor 220, with a multifrequency gas fraction correction, is therefore ideal for this application.

A great challenge is the on-line monitoring of aerated bacterial biomass below 1 g/L (e.g. for biodegradation). Figure 9 shows continuous growth of the bacteria *Variovorax paradoxus* DMSZ 4065 on phenol. The increase in biomass (1.1 g/L dry weight) initiated by enhanced phenol supply is clearly indicated by the capacitance trace from a Biomass Monitor incorporating the Model 220 technology with a new probe design optimized for compensating changes in gas hold-up during fermentation (10). Because the original Biomass Monitor 214M had a defined resolution of 0.5 g/L dry weight (this figure is cell size dependent), the latest Biomass Monitor technology clearly has a much improved resolution.

In fed-batch culture, concentrations as much as 100 g/L dry weight can be achieved in bacterial fermentations, and monitoring and control of feeding regimes have been linked to the capacitance signal. Figure 10 compares monitoring an *E. coli* fermentation by capacitance, optical density, and plate counts. The data in this case are normalized. Optical density (OD) measurement off-line is the standard practice at present for most fermentations, but in this case it merely shows a steady increase despite some major process events occurring as the fermentation is induced after 10 hours. By contrast, the capacitance trace reveals a series of events including the death of the most stressed cells between 10 and 18 hours and the increase in cell size as protein inclusion bodies swell. At the end of fermentation, the capacitance is measuring the concentration of viable but noncultivable bacteria in the fermentor.

The capacitance technology has also been adopted by many of the worlds' larger breweries as a control instrument for dosing the correct amount of live yeast at the start of each fermentation (**11**). The instrument (known as the Yeast Monitor) has in many cases superseded the traditional methods of yeast measurement and has led to greater process improvements in fermentation times and consistency with important cost savings. Other applications for the Yeast Monitor in brewing have been for optimizing yeast recovery and for monitoring viable yeast growth during propagation and fermentation. The data

Figure 6: Monitoring Chinese hamster ovary (CHO) microcarrier cultures during growth and harvesting phases over a three-month period using RF impedance.

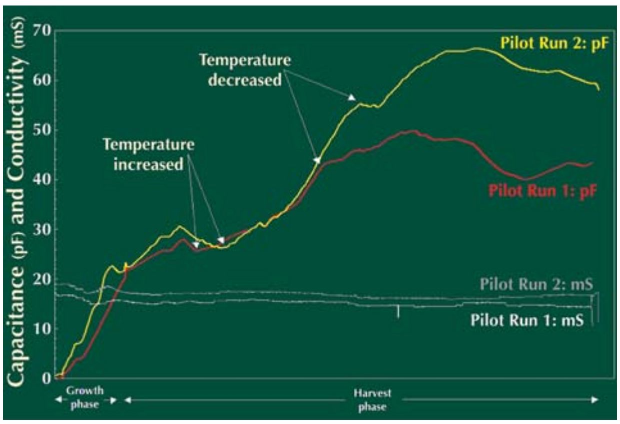


Figure 7: Monitoring the growth of Vero cells on Cytodex 1 microcarriers in a 20-L reactor using RF impedance. (Data courtesy of Xenova, UK, 2002)

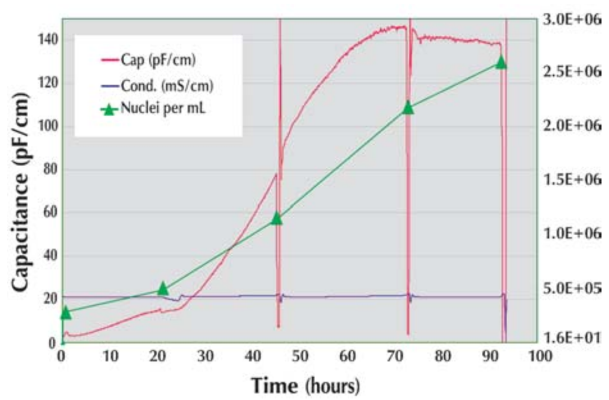


Figure 8: Monitoring the growth of baby hamster kidney (BHK) cells attached to macroporous microcarriers over a 20-day period using RF impedance. (DATA COURTESY OF NOVO NORDISK, DENMARK)

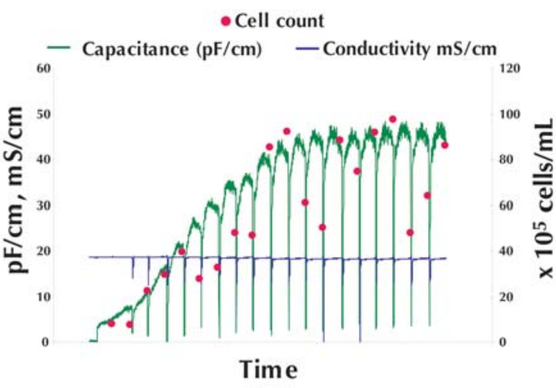


Figure 9: Monitoring small biomass concentrations of continuously growing *Variovorax paradoxus* with RF impedance.

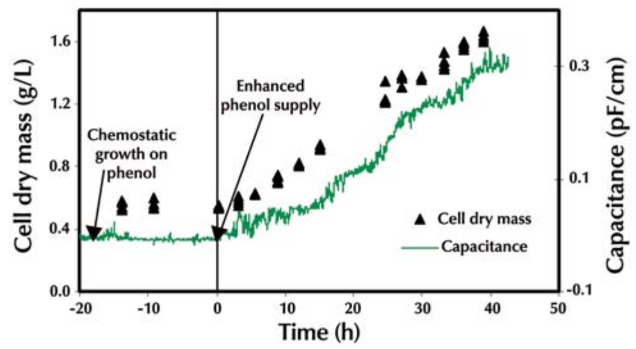


Figure 10: Monitoring an Escherichia coli fermentation by RF impedance, optical density, and plate counts in a pilot plant 2,000-L fermentor (units have been normalized).

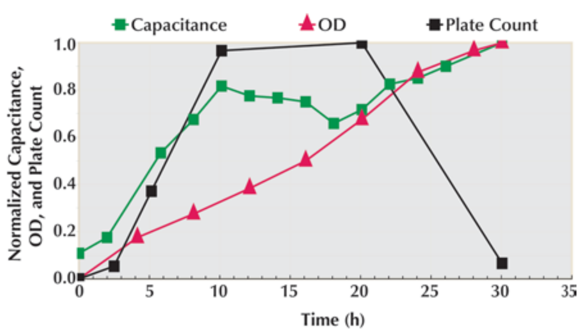
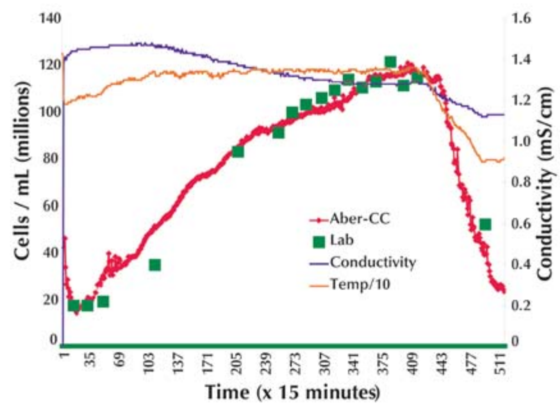


Figure 11: Monitoring a production yeast fermentation by RF impedance: capacitance has been converted to cells/mL.



in Figure 11 show how monitoring a production brewing fermentor with a Yeast Monitor can reveal a unique yeast growth and flocculation profile. The capacitance trace not only provides valuable information on the cell concentrations after seeding and transfer to the next stage, but a change in the profile will also provide valuable feedback on any upstream changes in the quality of the media (brewers' wort) feed.

GMP Applications

It is clear that RF impedance-based biomass measurements are generally accurate and reliable for determining viable cellular biomass on-line. However, of the several instruments that have been used to make this measurement, only the Biomass Monitor and its derivatives can be used for "off-the peg" applications, particularly in an industrial environment. Many of the earlier systems have been confined to research and development or processes that do not require validation because the probes were constructed of an epoxy-based resin. Now that probes for estimating viable biomass can be constructed entirely from wetted materials that conform to FDA requirements, opportunities open up to measure live cell mass on-line in cGMP facilities.

The use of multifrequency measurements with the latest generation of Biomass Monitors provides an effective method for measuring viable biomass during conditions of varying gas hold-up in a highly aerated fermentor. The gas hold up value can allow the effective surface area of gas exchange to be estimated on-line, a value that may provide useful information for more efficient control of impeller speed and aeration rate.

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