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A Novel Method for Monitoring Monoclonal Antibody Production During Cell Culture

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Abstract

We describe a new format for surface-based fluoroimmunoassays that allows detection of biomolecule interactions without separation steps. The bioactive layer was immobilized on the surface of a glass substrate covered with silver islands that provide optical amplification of the distinctive fluorescence signal from bound probes when compared to unbound probes. The technique used was phase-modulation fluorometry that allows sensitive detection of bound probes with a very short lifetime in the presence of excess free probes in solution. The new method was applied to assay monoclonal antibody production during cell culture. Excellent agreement was found between the new method and ELISA analysis of hybridoma cell culture samples. It is predicted that the near real time monitoring of protein products during bioprocessing will be possible with the described technology.

Keywords

metal-enhanced fluorescence; phase-modulation fluorometry; plasmon resonance; fluoroimmunoassays; monoclonal antibody production; near real-time monitoring

Introduction

Biosensor development for the diagnosis and monitoring of diseases, drug discovery, proteomics, and biotechnology are of interest as new technological advances with higher sensitivities and simplified sample preparation are always in demand. Due to the variety of instrumentation and fluorescent materials available, fluorescence-based biosensors are particularly versatile and widely employed. Of particular interest in medical diagnostics and biotechnology are fluoroimmunoassays typically derived from the coupling of an antibody-antigen binding reaction to a fluorescent signal (Hemmila, 1991; Soini et al., 1995; Yan and Marriott, 2003). Conventional methods to perform these types of assays include the standard enzyme-linked immunosorbent assay (ELISA) procedure (Goldsby et al., 2003). However, this method is time-consuming with no possibility of performing real-time measurements which

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are vital to optimizing the conditions during bioprocessing for optimal production. Also, ELISA has a higher degree of variability and requires a substantial amount of cell culture sample which makes the assays difficult during early development stages.

Most often these assays used to determine analyte concentration present in clinical and biological samples are intensity-based. Typically, the intensity-based approach of surface-based fluoroimmunoassays requires that any unbound probe be removed prior to signal readout. This is largely for two reasons: (1) the bound fluorescent probe usually has similar fluorescent properties compared to those of the free probe and (2) there is usually a large excess of the free probes compared to that of the bound probes. As a result, a washing step is required before analyzing the sample lengthening an already time-consuming assay.

Innovative approaches to obtain sensitivity enhancements with the potential for single molecule detection have begun to emerge from the nanotechnology field. Recently, several research groups have started to explore the application of metallic nanostructures for the detection of biomolecule interactions. The metallic nanostructures display unique optical properties due to a strong interaction between free electrons and an incident electromagnetic field. There are several approaches including resonance light scattering (RLS) (Fang et al., 2004; Wang et al., 2005), distance-dependent scattering properties of nanoparticles (Elghanian et al., 1997; Nam et al., 2003; Storhoff et al., 2004), or scattering and extinction properties of metal-dielectric nanoshells (Hirsh et al., 2003; Kumar et al., 2007). The properties of metallic nanoparticles and nanofilms have also been explored in combination with fluorescent probes. Plasmonic nanostructures in the form of metallic particles provide a means to greatly enhance fluorescence intensities and reduce lifetimes due to the increased excitation field and increased radiative decay rate (Gerber et al., 2007; Kummerlen et al., 1993; Lakowicz and Shen, 2002; Malicka et al., 2003a; Matveeva et al., 2007; Maliwal et al., 2003; Sokolov et al., 1998; Weitz et al., 1983). The near-field effect of fluorophore–plasmon interactions occurs when fluorophores are positioned within a distance of about 3–50 nm from the surface of metallic nanostructures. These effects are frequently known as metal-enhanced fluorescence (MEF) or surface-enhanced fluorescence (SEF). The decrease in fluorescence lifetime in the presence of metallic nanostructures results in better photostability of fluorophores because of less time for photochemistry to occur during the excited state of molecules. Also, the fluorophores are less prone to optical saturation (Geddes et al., 2003; Malicka et al., 2002). The large practical distance range where fluorophore–plasmon interactions occur is adequate to design surface-based assays with a biorecognitive capture layer and a suitable labeled biomolecule for detection. Several assay designs have been described using fluorescent probes and metallic nanostructures (Giakos et al., 2002; Malicka et al., 2003b; Matveeva et al., 2004; Mayer et al., 2001; Stich et al., 2001). All currently described approaches focused exclusively on using enhanced fluorescence intensity in the design of sensitive biosensors. To date, metal-induced decreases in lifetime have not been used for sensing applications.

In this report, we describe a new approach that employs the fluorescence intensity amplification of MEF through the generation of a distinct fluorescence signal from only probes bound to the bioactive surface. Two effects of fluorophore–plasmon interactions, enhanced intensity and decreased lifetime, are combined with phase-modulation fluorometry resulting in a technique (MEF-PM) that enables ultra-sensitive detection and the possibility for real-time monitoring of interactions between biomolecules. In principle, the large contrast between probes bound to the MEF substrate and free probes in solution provides an opportunity to measure the concentration of analytes without washing steps required for typical intensity-based fluoroimmunoassays.

Here we demonstrate the MEF-PM technique for quantification of monoclonal antibody produced during cell culture through a sandwich-based fluoroimmunoassay. The assay requires

immobilization of capture antibody specific for the target analyte on the MEF substrate. Following target binding, a fluorescently labeled detection antibody specific for the target must bind allowing a fluorescent readout to be performed. In this case, the MEF-PM technique allows the detection of bound target molecules without the washing out of unbound detection antibody.

Materials and Methods

Reagents

Immunoglobulin gamma-3 (IgG_3) standard was obtained from Pharmingen. The capture antibody (goat anti-mouse kappa, κ chain specific), enzyme-linked secondary ELISA antibody (goat anti-mouse IgG_3-alkaline phosphatase), and Cy5-labeled reporter antibody (goat anti-mouse IgG_3, γ_3 chain specific), were purchased from Southern Biotech (Birmingham, AL). The ELISA substrate, 4-methylumbelliferyl phosphate, was obtained from Sigma-Aldrich (St. Louis, MO). Buffer components and other chemicals were from Sigma–Aldrich.

Cell Culture

A SP2/0-based mouse hybridoma cell line (2055.5) (Rubinstein and Stein, 1988) secreted IgG_3 monoclonal antibody, specific for the *Neisseria meningitidis* capsularpolysaccharide (MCPS), was chosen as the model protein for our study. Samples with varying concentration were collected over 4 days from a 5 L bioreactor (Artisan, Waltham, MA) hybridoma cell culture. Cells were grown in a CD Hybridoma (Gibco, Carlsbad, CA): RPMI 1640 (Mediatech, Herndon, VA) (25:75) media mixture with 5% fetal calf serum (Hyclone, Logan, UT) and 3.5×10^{-4}% β-mercaptoethanol (v/v) (Sigma-Aldrich). Cells were counted using a hemocytometer with trypan blue (Sigma-Aldrich) staining for viability determination. Culture samples were clarified by centrifugation (500g) and supernatants were stored at −20°C until assaying for IgG3 concentration.

MEF Substrate Preparation

Silanized glass substrates, purchased from Sigma-Aldrich, were used for the deposition of silver islands. The wet chemical deposition method used has been described elsewhere (Lakowicz and Shen, 2002; Ni and Cotton, 1986). Briefly, silver island films were deposited on the substrate via the reduction of silver ions by D-glucose. On top of the silver islands, a silicone adhesive was placed with an array of wells, 2.0 mm in depth and 2.5 mm in diameter (Grace Bio-Labs, Bend, OR), that allowed for multiple samples (up to 30) to be investigated on the same substrate. The silver islands displayed an absorption spectrum maximum near 420 nm (optical density of about 1.5) which indicated that the particles were subwavelength size. In the small silver particle limit, the absorption maximum due to plasmon resonance is expected to be near 380 nm (Mulvaney, 1996). Wet chemical deposition results in a plurality of particle sizes and shapes as has been previously shown using atomic force microscopy with particle sizes up to 500 nm and thicknesses of 50–100 nm (Lakowicz and Shen, 2002). It has been demonstrated that the fluorescence enhancement strongly depends on the silver particle surface morphology in the case of silver islands and silver colloids deposited on a glass surface (Lukomska et al., 2004). The high density of the silver islands in the present study resulted in a uniform fluorescence enhancement over the entire glass slide allowing a multi-well IgG_3 assay to be performed using a single slide.

IgG_3 Immunoassay Using MEF

IgG_3 immunoassays were performed in a sandwich format. On the MEF substrate surface, capture antibody, IgG_3-κ was noncovalently immobilized by incubating in carbonate buffer, pH 8.0 for 1 h, followed with blocking solution of 1% fish gelatin in phosphate buffer, pH 7.4

at room temperature for 1 h. Similar results were obtained using immobilization overnight at 4°C. The blocking solution was used to minimize or prevent nonspecific binding of the IgG_3 and detection antibody IgG_3-Cy5 to the surface. Control samples with blocking solution verified that the binding of target IgG_3 to the surface was negligible. Two formats of sandwich immunoassays were prepared, sequential and "one step." In the sequential assay, 10 μL of IgG_3 in 1% fish gelatin was added to each well in several threefold dilutions and the samples were incubated in a humid environment for 1 h. After washing, 10 μL of reporter antibody, goat anti-mouse IgG_3 labeled with Cy5 was added to each well. Following the addition of the reporter antibody, the wells were covered with a coverslip and measurements were performed immediately (kinetics) and after 1 h incubation (end point). In the "one step" assay, the antigen and reporter antibody were premixed and then added in threefold dilutions to the substrate wells allowing for an incubation time of several hours.

Intensity, phase and modulation data were acquired using a modified frequency-domain fluorometer attached to a fluorescence microscope. The fluorescence measurements were performed using epi-illumination with excitation from a red LED (635 nm). The excitation intensity was modulated by applying a RF driving current to the LED (Sipior et al., 1996; Szmacinski and Chang, 2000). For system characterization we used sweep mode in the frequency range of 10–250 MHz and for sensing a single modulation frequency of 155 MHz was used. The background intensity was measured from a well with no antigen and no capture antibody. Figure 1 shows the schematic of the MEF-PM measurements where the fluorescence intensity, phase shift, and modulation are measured in the presence of the free probes. The bound probes are brighter and display shorter lifetimes compared to free probes in solution. For comparison, a surface-based assay on a glass substrate is also shown where washing out unbound probes is required prior to intensity readout.

IgG3 Immunoassay Using ELISA

Capture antibody was immobilized for 1 h in each well of a 96-well plate (Thermo, Waltham, MA). After washing by rinsing several times with washing buffer (0.1% tween-20 in PBS), the plate was blocked for 1 h with 1% fish gelatin in PBS. After washing, the standards and supernatant were serially diluted into the wells and incubated for 1 h. The plate was then washed and secondary antibody was added. After a final wash and rinsing with water, the plate associated enzyme was then allowed to cleave the substrate, producing a measurable fluorescent signal. Schematic of ELISA is shown in Figure 1 where the fluorescence signal is produced within a solution. Sample IgG_3 concentrations were determined by comparing their fluorescence curves to that from serially diluted IgG_3 standards of known concentrations by conducting parallel analyses.

Results and Discussion

Performance of IgG_3 Immunoassay Using Fluorescence Intensity

The fluorescence intensity-based calibration curves were generated using a standard antigen and serial dilution (similar to ELISA procedures). Following antigen binding in the wells and a washing step, the reporter antibody (4 μg/mL) was added to each well and allowed to incubate for 1 h at room temperature. After incubation, the fluorescence intensity from each of the wells was measured in the presence of free probes on the MEF substrates. A control experiment was carried out on bare glass. The results are shown in Figure 2. As was expected, because of the large intensity enhancement obtained on the MEF substrate (about 15-fold), it was possible to generate a calibration curve without any additional washing steps. For the assay on glass, generation of a calibration curve required washing out the unbound probes. Note that both calibration curves display a similar dynamic range for analyte concentration as can be judged by the midpoint values of 351.5 ng/mL for MEF and 274.9 ng/mL for the glass surface which

were determined from the fit of data to sigmoidal concentration-response relationships observed in immunoassays. The four-parameter logistic function was used which is recognized as the reference standard for fitting the mean concentration-response for immunoassays (Findlay et al., 2000). The function is defined by the equation

$$Y=D+\frac{A-D}{1+(X/C)^p} \tag{1}$$

where Y is the measured parameter, X is the analyte concentration, A and D are the responses at zero and infinite concentrations, respectively, C is the concentration resulting in a response halfway between A and D (mid point) and p is the slope parameter that typically is near 1.0.

The clear advantage of the MEF-based assay is that there is no requirement for a washing step prior to readout. Also, the enhanced intensity signals from MEF allow for more accurate measurements to be made. This increased accuracy and shortening of procedures is vital to performing measurements as rapidly as possible to allow for real-time monitoring of bioprocess samples and binding interactions. The possibility of real-time monitoring is of particular interest in biotechnology as in-processing testing is increasingly being used to provide the highest degree of assurance of product safety and efficacy as outlined in the FDA's Critical Path Initiative.

Performance of IgG_3 Immunoassay Using Phase and Modulation

While the intensity-based approach is simple, a lifetime-based MEF assay approach can additionally provide higher assay sensitivities. The interaction between fluorophores and surface plasmons of metallic particles causes the fluorescence lifetime to dramatically decrease many folds compared to free fluorophores in solution. Phase-modulation (or frequency-domain) fluorometry is widely used in research and has proven to be a sensitive technique to detect the presence of low signals from short lifetime fluorophores in the presence of substantially larger signals from longer lifetime fluorophores (Lakowicz, 2006; Szmacinski and Lakowicz, 1999). In the case of the MEF-based assays described here, this detection technique is desired because the bound reporter probes will have a dramatically reduced lifetime and higher intensities compared to unbound reporter probes which are not affected by the metallic particles.

In principle, for phase-modulation fluorometry, the excitation intensity light is sinusoidally modulated and the fluorescence lifetime is determined from the phase angle between the excitation and emission intensity and/or from the modulation of the fluorescence intensity relative to the excitation. The modulation frequency has to be within a certain range depending upon the lifetime of the fluorophore under investigation. For example, nanosecond range lifetimes require frequencies in the range of about 5 to 300 MHz. The relation between the lifetime and measured phase shift (φ), and the fluorescence modulation (m) for a single exponential intensity decay is given by the equations,

$$\tan(\varphi)=\omega\tau, \tag{2}$$

$$m=\left[1+(\omega\tau)^2\right]^{1/2} \tag{3}$$

where ω is the radial modulation frequency (ω $2\pi f$, f is the modulation frequency in cycles per second) and τ is the fluorescence lifetime. More complex equations describe the phase shift and modulation in the case of multi-exponential intensity decays (Lakowicz, 2006).

Phase-modulation intensity decays of Cy5 labeled reporter antibody when free in solution and when bound to the MEF substrate in the sandwich IgG_3 assay are shown in Figure 3. The average lifetime of the free probe is reduced about fivefold when the probe binds to the analyte in the sandwich format. The distance from the Cy5 to the silver surface is determined by the size of capture antibody, antigen and the reporter antibody which is labeled with Cy5. Despite the large size of the biomolecules (each approximately of 150,000 Da) used in the sandwich assays, a large difference in the phase shifts and modulations between the free and bound probes is observed. Consequently, this phase and modulation contrast between the free and bound probes, combined with the increased intensity from bound probes, creates a highly sensitive and accurate method for performing surface-based fluoroimmunoassays.

For sensing applications, measurement of the phase and modulation can be performed using a single modulation frequency (Szmacinski and Lakowicz, 1994). A modulation frequency of 155 MHz was selected for measurement of the phase shifts and modulations of Cy5-labeled reporter antibodies. The phase shift and modulation calibration curves for the IgG_3 assay are shown in Figure 4. The calibration curves were generated using two concentrations of the labeled antibody in solution (free probe), 4 and 1 μg/mL. By using a lower concentration of free probe (circles, Fig. 4), increased assay sensitivity was achieved as shown by the mid point values of the calibration curves in Figure 4. This is a unique feature of the MEF-PM method that allows tuning of the assay sensitivity by simple adjustment of the amount of labeled antibody in the solution. This is because the phase shift and modulation values depend on lifetime values and relative fluorescence intensities of free and bound probes. It is important to note that the phase shift calibration curve(s) are shifted towards lower antigen concentrations compared to the intensity measurements. For example, comparing the mid point value of 33.8 ng/mL for the phase shift (Fig. 4) to the value of 351.5 ng/mL for intensity (Fig. 3) results in nearly tenfold sensitivity improvement. Additionally, the modulation data is complementary to the phase shift providing increased accuracy and further extension of the analytical range of the assay. The responses of phase and modulation to IgG_3 concentrations are different in sensitivity because of different intensity/lifetime weighting. For example, Figure 5 shows that the sensitivity of the modulation-based assay is about twofold less than the phase-based assay. In fact, three parameters can be used together to determine the analyte concentration in biological samples (intensity, phase shift, and modulation) providing high accuracy and extended analytical range.

It is important to note that the data suggests that there is a small amount of nonspecific binding that is detected. The electrostatic immobilization and blocking procedures resulted in control samples without the antigen and samples with low antigen concentration (below 0.1 ng/mL) displaying similar signals. This indicates the presence of nonspecific binding of the detection antibody (IgG_3-Cy5) to the surface. Yet, our control sample with only blocking solution displayed a negligible fluorescence signal indicating that the nonspecific MEF signal could possibly be due to a very small fraction of the detection antibody displacing the capture antibody on the surface. To further minimize or prevent such binding, covalent immobilization of the capture antibody may be required. Despite the rudimentary immobilization method, the obtained sensitivity of the MEF-PM IgG_3 assay is comparable to that achieved via ELISA. Also, the presence of nonspecific binding does not compromise the performance of the MEF-PM assay for the detection of the relatively high concentration of IgG_3 produced during cell culture. In order to further improve the sensitivity of the MEF-PM based IgG_3 assay, the immobilization of the capture antibody and the blocking procedures will need optimization.

To compare the performance of MEF-PM based immunoassay to conventional methods, an ELISA IgG_3 assay was carried out using similar reagents and immobilization procedure as the MEF-PM method. The resulting sensitivity with phase shift measurements is practically the same for both methods as can be deduced from the similar mid point values shown in Figure 5. In this assay, we used the reporter antibody, IgG_3-Cy5, at a concentration of 0.25 μg/mL. Our approach of using different concentrations of reporter antibody was dictated by using the fixed thickness of the silicone adhesive of 2 mm which defined the thickness of the solution of free probes. However, the same tunability can be obtained at a fixed concentration of reporter antibody by varying the thickness of the free probe solution over the layer of probes bound to the MEF substrate. One can envisage a number of formats for the design of tunable cartridges that allow the user to adjust the assay sensitivity for fast measurements without exchanging the reagent with reporter antibody.

The comparable sensitivity obtained with MEF-PM to the ELISA assay demonstrates that the new approach is very promising. The ability to achieve faster results with similar sensitivity as ELISA could lead to near real-time monitoring of bioprocesses which is not possible with the ELISA technique.

One Step IgG_3 Immunoassay

Because the selected capture and detection antibodies bind to different epitopes of IgG_3, it is possible to reduce the immunoassay procedure to one step while eliminating any washing steps. In this case the samples with different IgG_3 concentrations were premixed with reporter antibodies and added into wells with pre-immobilized capture antibodies. Figure 6 compares calibration curves obtained using the one step and sequential immunoassays. The one step immunoassay results in moderately less sensitivity (about threefold) compared to the sequential assay (mid point for the sequential is 91.8 ng/mL and for the one step 226.1 ng/mL). This shift in sensitivity is likely due to steric hindrances to the binding of the reporter antibody-IgG_3 complex to the capture antibody on the MEF substrate. Nonetheless, these data demonstrate the substantial advantage of the MEF-PM method which allows for measurements without any separation and washing steps.

The possibility to detect the IgG_3 in one step will allow future monitoring of antibody production by sampling the bioprocess and performing the measurements in near real-time. This can allow in-process optimization of the bioreactor conditions to increase the yield. Also, it could allow for the early termination of bioprocesses that are not productive, thus saving the time and cost. Currently, this is not possible as the samples are acquired during the course of the bioprocess, stored and measurements are performed after the completion of the bioprocess because the ELISA measurements do not allow fast processing of samples.

Kinetics

Most fluoroimmunoassays do not allow for the monitoring of biomolecule interactions because of the difficulty in distinguishing between the fluorescence signals of unbound single biomolecules and those complexed. Two known detection modalities that allow monitoring of binding interactions between biomolecules in real time are fluorescence polarization and FRET-based immunoassays. However, both methods are restricted to the studies of low molecular weight molecules and display low sensitivities. Small biomolecules are labeled with fluorophores to fulfill the requirements for low fluorescence polarization prior to binding in polarization-based immunoassays. Similarly, FRET requires careful selection of two dyes and the short distance over which FRET occurs also limits the application to the detection of small biomolecules. The low sensitivity of both methods is due to the lack of signal amplification effects.

In contrast, due to distinct signals of the free and bound probes and relatively large distance between the metallic surface and fluorophore where fluorescence enhancement occurs, the MEF-PM method allows substantial expansion of its application to real time studies of biomolecule interactions. Figure 7 shows the binding kinetics between the detection antibody (goat anti-mouse IgG_3, γ_3-chain specific, labeled with Cy5) and the analyte captured on the surface analyte (IgG_3). The detection antibody was introduced into a well after the binding between the IgG_3 and the surface immobilized capture antibody (goat anti-mouse IgG_3, κ-chain specific) reached equilibrium (1 h incubation time). Interestingly, the binding occurred very fast with 90% of the signal change (response time of the sensing surface) achieved in about 9 min with intensity measurements and 3.5 min with phase measurements. The difference in response time between intensity and phase measurements is due to the high sensitivity of the phase shift to the small concentration of the bound probe with short lifetime. This fast response coupled with the lack of a washing step after addition of the probe results in an assay time of nearly 90 min less than that of the conventional ELISA technique. The binding affinity between IgG_3 and the capture and detection antibodies was observed to be high because there were no changes in intensity signal from the bound probes after dilution of free probe IgG_3-Cy5. The strong binding affinity observed in the IgG_3 sandwich assay and strong adhesion of the capture antibody to the silver island surface allowed for reproducible measurements after weeks of storing the assays at 4°C. This is another advantage over the ELISA assays which allow only one time measurements.

Analysis of Cell Culture Samples

In upstream bioprocess development, a number of experiments are carried out to optimize the manufacturing process and maximize the yield and product quality. There are many approaches to control the environmental conditions which are critical to cell growth and product formation such as the sensing of dissolved oxygen (Chotigeat et al., 1994; Hayter et al., 1992; Kunkel et al., 1998; Ozturk and Palsson, 1991) and pH (Hanson et al., 2007; Hayter et al., 1992; Muthing et al., 2003). While technology has enabled facile monitoring of dissolved oxygen and pH during cell culture, online quantification of protein production is challenging. Currently, the samples are collected during the bioprocess and analyzed later using standard ELISA procedures. However, the immunoassay procedure using ELISA is time consuming because it requires a multi step protocol, and thus, there is no possibility to perform real-time measurements. Also, while ELISA and MEF-PM use the same reagents, MEF-PM requires a significantly lower quantity of the reagents reducing the expense of the assay as compared to ELISA. We have shown above that the MEF-PM method provides the opportunity for a relatively fast and inexpensive method to monitor the concentration of proteins produced during cell culture in near real-time.

A series of cell culture samples were collected during the production of IgG_3. Post process analysis was performed using standard ELISA measurements on all the samples and on randomly selected samples using MEF-PM. The plots of serial dilutions of the standard and samples obtained using ELISA on the microplate are shown in Figure 8. Nine samples were collected at various time points during the culture. The results from the ELISA analysis using the four parameter logistic function are shown in Figure 10 and for selected samples in Table I. The concentration values increased from 5.64 mg/mL at the beginning of the process to 54.97 mg/mL after 96 h.

The analysis of selected samples using the MEF-PM approach was performed by comparison to a standard solution in a manner similar to that of ELISA. The selected samples were sample 1, 3 and 6 (Table I). Samples were analyzed using measurements of the intensity, phase shift and modulation. Figure 9 shows the phase shift standard curves and fitted curves for selected cell culture samples. The standard sample of 1 μg/mL was serially diluted in threefold

increments. We also used a standard with 10 μg/mL of IgG_3 to obtain the saturation baseline. This provided a complete knowledge of the response of the sensing system from background to saturating levels. Two different MEF substrates were used, one for measurement of sample 1 and sample 6 (Fig. 9a) and a second for measurement of sample 3 (Fig. 9b). Because the MEF substrates were from different batches of fabrication the calibration curves in Figure 9a are different than in Figure 9b.

The concentration of IgG_3 in the samples was determined from a mid point of the fitted curve with fixed values for the maximum and minimum phase shifts that correspond to the standard curve. Three parameters were used for calculation of the IgG_3 concentrations, phase shifts as shown in Figure 9, modulations, and intensities (plots not shown). Very good agreement between ELISA and MEF-PM values was found for the selected samples (Fig. 10 and Table I) using two different MEF substrates. The average values for ELISA were determined from duplicate samples, while the average values for MEF-PM were determined from triplicate measurements of intensity, phase shift, and modulation as shown in Table I for each parameter measured. The larger variations of the MEF-PM values compared to ELISA can be attributed to non-optimized MEF substrates.

Conclusion

In this report, we demonstrated the performance of the MEF-PM fluoroimmunoassay for the detection and monitoring of biomolecule interactions, specifically monoclonal antibody quantification through a sandwich assay. The MEF-PM approach is an excellent platform for surface-based assays that achieves sensitivity close to ELISA. The main benefit of such an approach for the IgG_3 assay is not in the sensitivity, but rather in the ability to shorten the time for the assay. Currently, the MEF-PM technique shortens the assay time by nearly 90 min. Future studies have been planned to optimize the immobilization procedures to provide the fastest possible assay times as well as to minimize or eliminate the small amount of nonspecific binding observed in the reported results. It is expected that due to the significantly smaller volume of the MEF-PM wells that the assay time could be significantly reduced to the order of an hour, thus allowing near real-time measurements to be performed directly from the cell culture without any storage of the sample.

The results for IgG_3 detection using a typical sandwich assay on MEF substrates showed that the bound probe is strongly enhanced by surface plasmons resulting in a large magnitude of phase and modulation signals and a broad detectable IgG_3 concentration range. The combined effects of enhanced intensity and decreased lifetime due to the fluorophore–plasmon interaction have the potential to further the development of ultra-sensitive assays with simplified biochemical procedures and the ability for real-time monitoring of biomolecular interactions. Further optimization in the preparation of MEF substrates, surface chemistry, and choice of fluorophore can be expected to enhance sensitivity to about 100-fold compared to the conventional intensity-based assays. Additionally, low-cost LED based phase-modulation instrumentation has already been demonstrated for similar measurements (Harms et al., 1999). These advances open the door for compact benchtop or handheld instruments similar to those routinely used for glucose monitoring in a bioprocessing lab.

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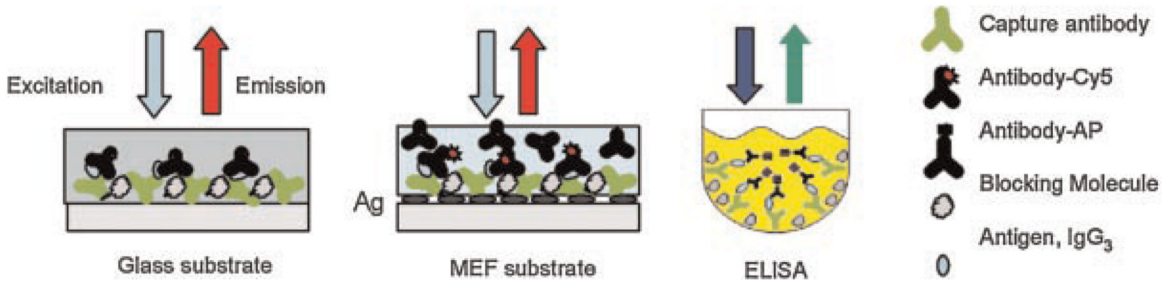

Figure 1.

Schematics of sandwich assays for the detection of IgG_3 on glass, MEF substrates (silver island film) and using ELISA. Note that detection of IgG_3 on glass and using ELISA requires washing out the unbound probes while on MEF substrates the readout is in the presence of unbound probes. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

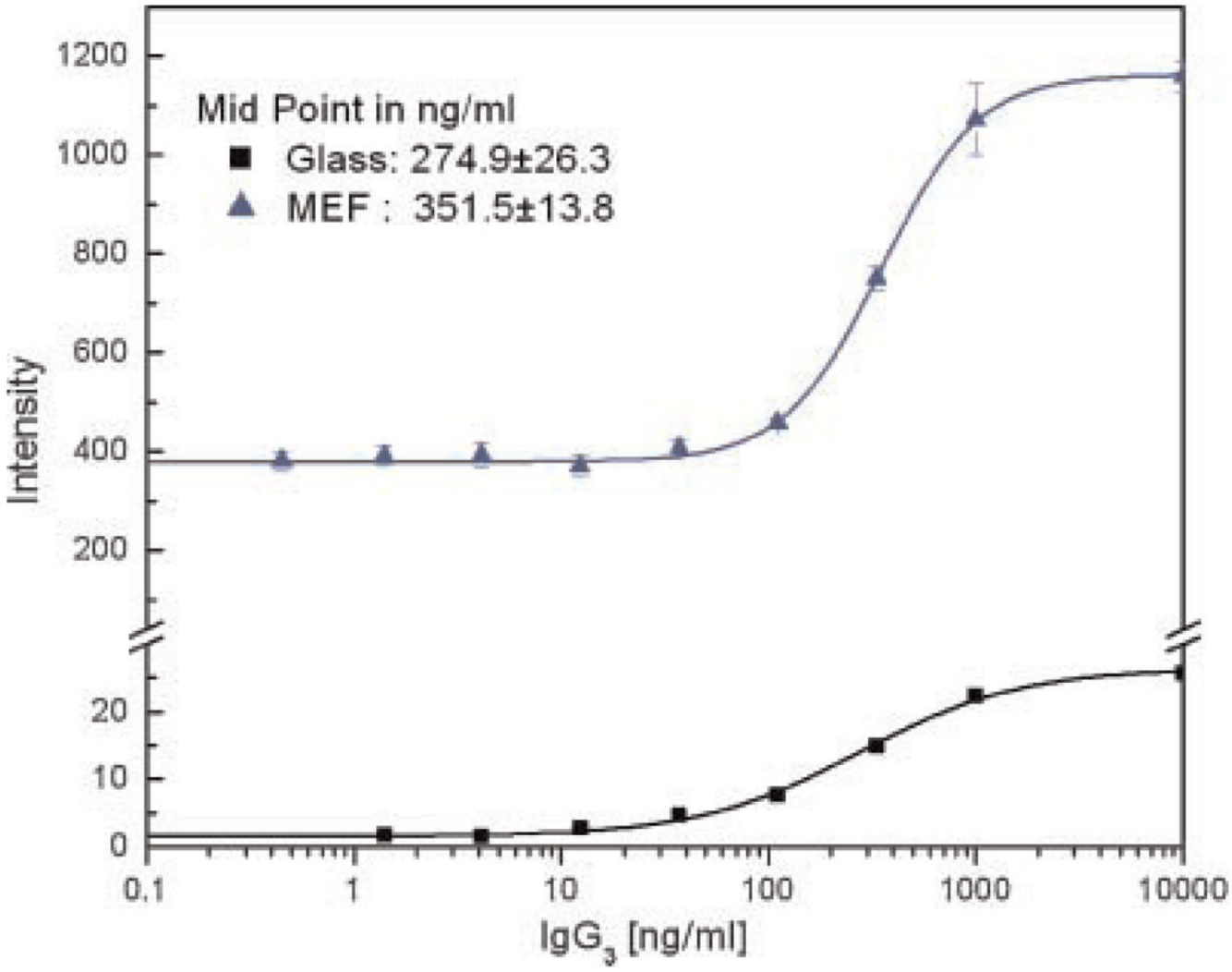

Figure 2.

Intensity-based calibration curves for IgG_3 immunoassays using glass and MEF substrates. The intensity readouts were performed in the presence of free probe (4 μg/mL) for the MEF substrate and after washing out for the glass. The intensity signals were corrected for scattered light and autofluorescence from the buffer and coated capture/blocking materials. Measurements for each well were based on the average intensity from at least three different spots within a well. [Color figure can be seen in the online version of this article, available at www.interscience. wiley.com.]

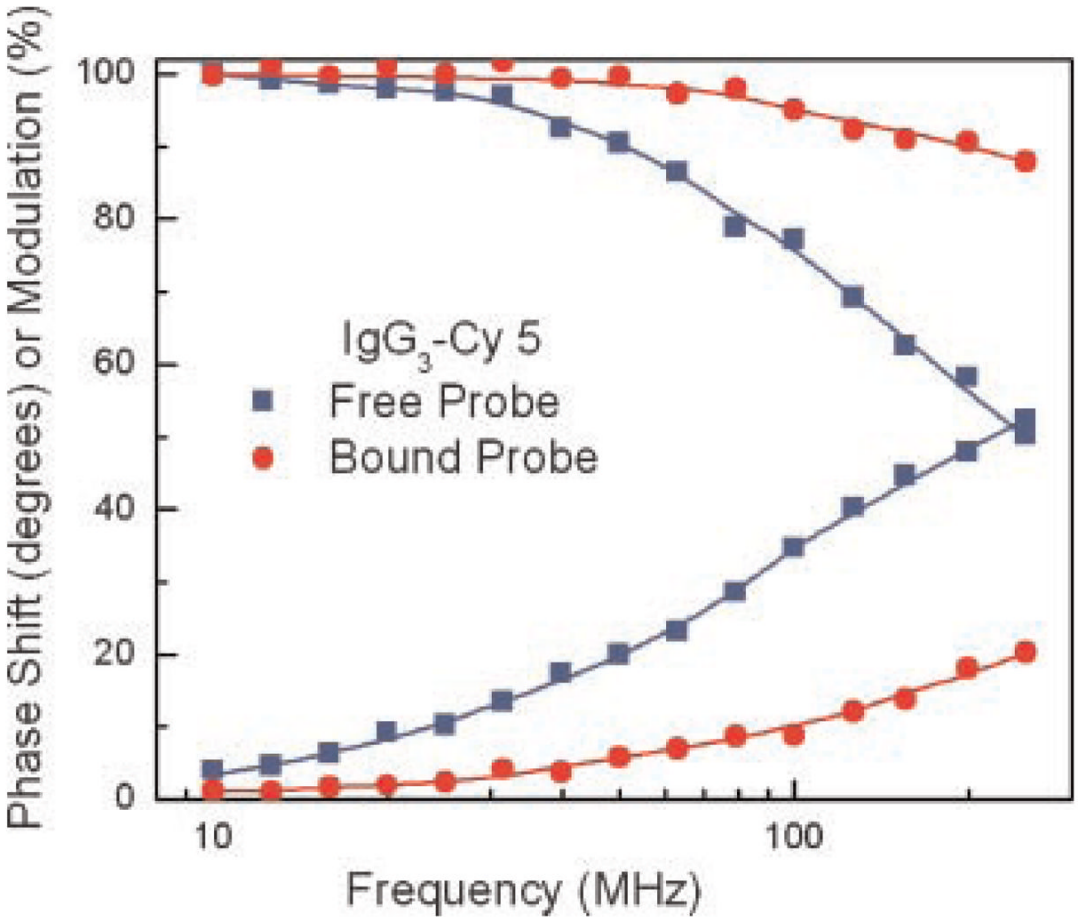

Figure 3.
Intensity decays of Cy5 labeled capture antibody when in buffer (squares) and when bound to MEF substrate. The excitation source was a RF modulated red LED at 635 nm. The average lifetimes ($\langle\tau\rangle = \Sigma\alpha_i\tau_i$ (Lakowicz, 2006)) are 0.87 and 0.17 ns for free probe and bound probes, respectively. [Color figure can be seen in the online version of this article, available at www.interscience. wiley.com.]

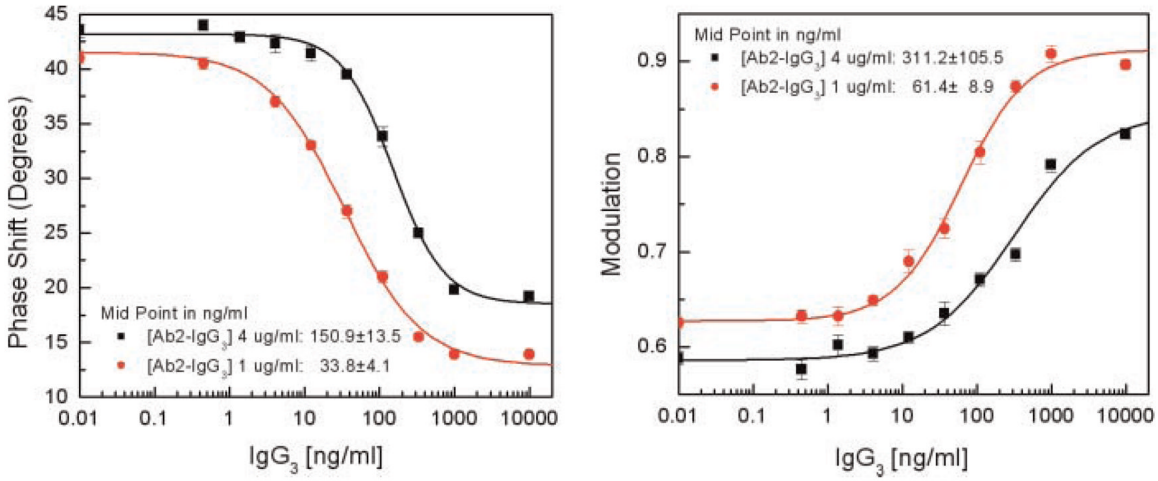

Figure 4.

Phase (**left**) and modulation (**right**) calibration curves for IgG_3 immunoassay at two concentrations of free probes, 4 and 1 μg/mL. The excitation was a red LED (635 nm) modulated at a frequency of 155 MHz. Data was fitted to the logistic function (Eq. 1). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

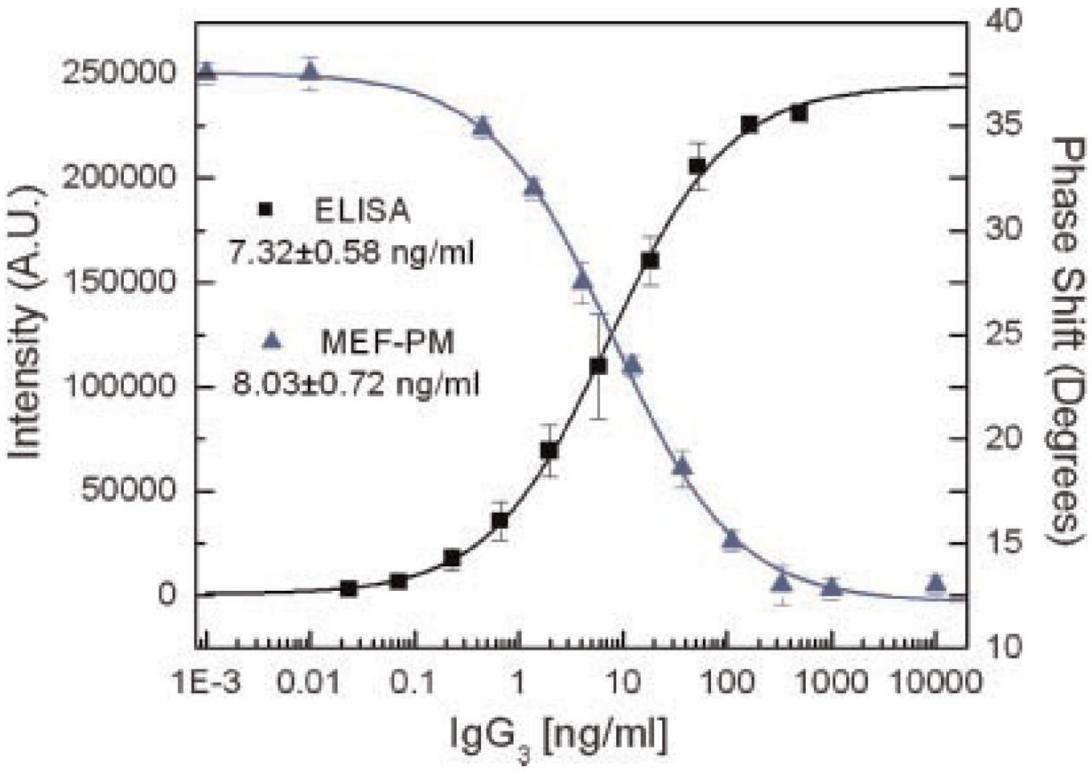

Figure 5.

Comparison of ELISA and phase calibration curves for the IgG_3 assay. ELISA and phase shift assays were performed using a threefold dilution from 500 and 1,000 ng/mL, respectively. An additional point was acquired for the phase shift at an IgG_3 concentration of 10,000 ng/mL. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

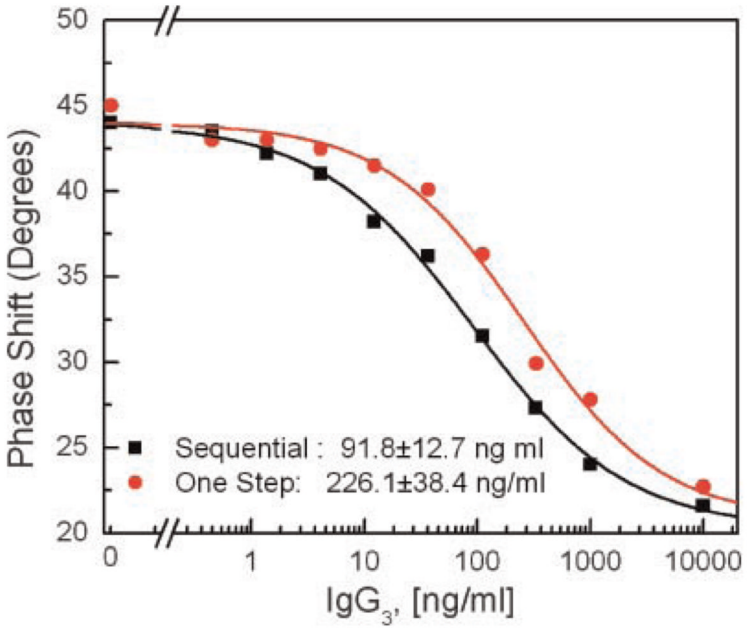

Figure 6.

One step and sequential IgG_3 immunoassays using phase measurements. The concentration of the detection antibody was 4 μg/mL, the same for both assays. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

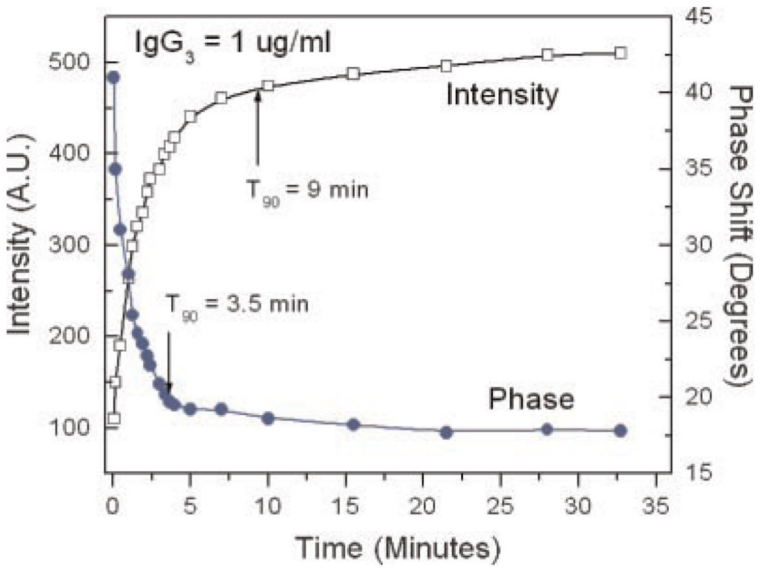

Figure 7.
Binding kinetics of the reporter antibody IgG-Cy5 to surface bound IgG_3. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

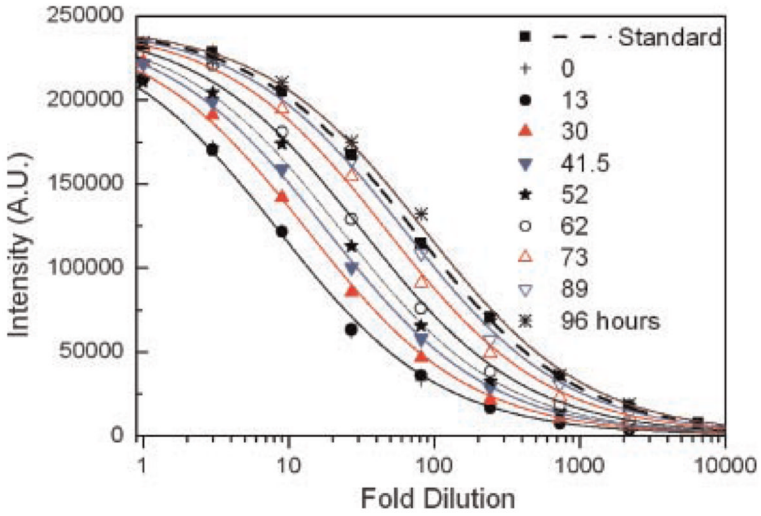

Figure 8.
Fluorescence of serially diluted (threefold) cell culture samples and IgG_3 standard. The standard calibration curve (squares) was obtained using an initial IgG_3 concentration of 500 ng/mL and fitted to the logistic function. Curves shift to the right with the age of the cell culture. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

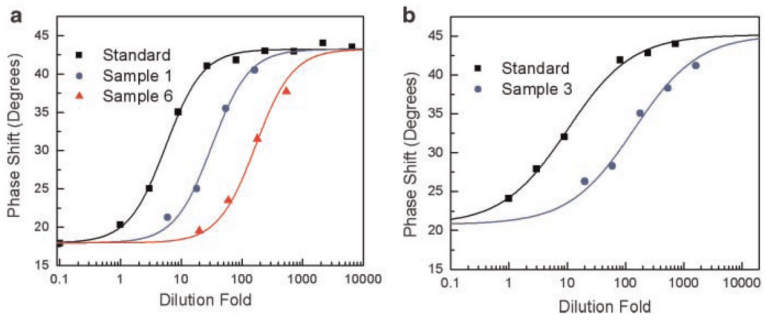

Figure 9.

Phase shift plots for standards and cell culture samples. The measurements of sample 3 were carried out on a different MEF substrate (b). The detection antibody concentration was 4 μg/mL. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

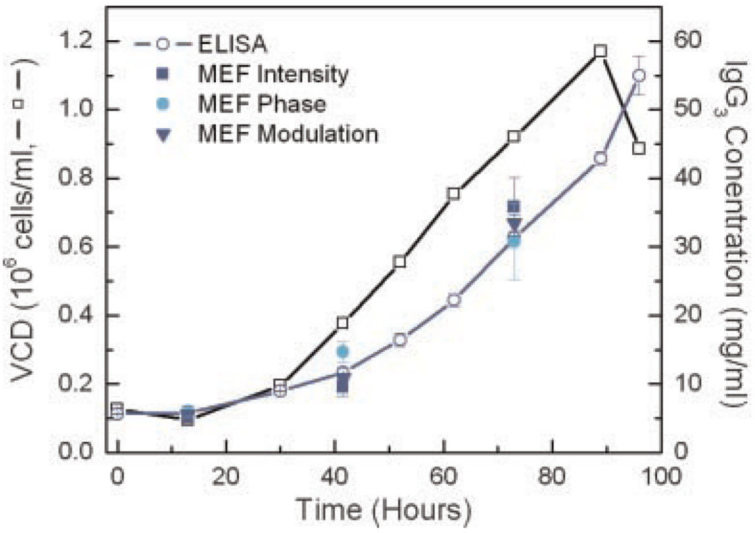

Figure 10.

Viable cell density (VCD) and monoclonal antibody production profiles for the cell culture. IgG_3 concentrations were determined with ELISA and through the averaging of the concentrations determined from each of the three parameters of the MEF-PM method given in Table I. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Table I

Concentrations of IgG_3 in cell culture samples determined using the multiple analysis parameters of the MEF-PM method and ELISA.

Sample #	**1**	**3**	**6**
ELISA	5.84±0.28	12.60±0.34	32.44±2.14
Intensity	5.18±0.73	9.55±1.23	35.83±4.28
Phase	5.97±0.71	14.72±1.53	30.77±5.60
Modulation	5.58±1.17	10.87±0.53	33.45±1.19