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Ammonia Measurements in Mammalian Cell Culture Media with a Diffuse Reflectance-Based Fiberoptic Ammonia Sensor

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ABSTRACT

A solid-state, diffuse reflectance-based fiberoptic sensor is described for quantifying ammonia. This sensor is constructed by immobilizing chlorophenol red, a weak acid chromophoric indicator dye, in a microporous polypropylene membrane. A flow-injection analysis system is used to carry a 10-μL aliquot of the sample across the treated membrane. Ammonia in the sample diffuses through the air-filled pores within the membrane structure before reacting with the indicator dye. A reversible acid-base reaction between ammonia and chlorophenol red results in a measurable change in the reflectance at 560 nm. Response characteristics include a peak response within 20 s, a limit of detection of 0.2 ± 0.1 m*M* ammonia, and a dynamic range of up to 60 m*M* ammonia. The analytical utility of this sensor is demonstrated by measuring ammonia levels in 48 individual samples collected during the growth of PC3 human prostate cancer cells in a typical serum-containing growth medium. Accuracy of the proposed sensor is verified by a comparison of results from this sensor to those from a conventional enzyme assay.

Index Entries: Ammonia; sensor; fiberoptic; bioreactor; monitoring; mammalian cells.

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INTRODUCTION

Mammalian cell cultures are becoming widely used to make biotechnological products and to mimic in vivo behavior during fundamental studies. Generally, it is advantageous to maintain the cells in an optimum environment with sufficient nutrients and minimal waste products. Ammonia and lactate are cellular waste products that accumulate in batch mammalian cell cultures, and can inhibit cell growth *(1)*. Therefore, it is useful to monitor ammonia and lactate levels during cell cultivation in order to develop strategies for the reduction or removal of these species. The authors are interested in developing an ammonia-selective sensor capable of accurately following ammonia production in serum-based cell-growth media.

Commercially available ammonia sensors are potentiometric in nature, and suffer from long response and recovery times *(2)*. In addition, published methods for this sensor describe the need for relatively large sample volumes. Conventionally, glutamate dehydrogenase (GDH) is used to measure ammonia levels in samples collected during cell cultivation. In this enzyme method, the reductive amination of 2-oxoglutarate is selectively catalyzed by GDH in the presence of ammonia and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) *(3)*. The decrease in absorbance at 340 nm is measured and corresponds to the oxidation of NADPH. The magnitude of this absorbance change is proportional to the ammonia concentration in the sample. This method uses small sample volumes (100 μL), but requires a 15-min incubation period for each sample, and consumes expensive reagents.

Recently, various flowthrough systems have been reported for the determination of ammonia in bioreactor samples *(4–8)*. These systems utilize various ammonia detection techniques, such as potentiometric gas sensing *(4)*, *o*-phthaldialdehyde (OPA) derivatization with fluoresence detection *(5)*, gas diffusion with detection based on either the Berthelot reaction or a pH-indicator dye *(6,7)*, or membrane-introduction mass spectrometry *(8)*.

This article describes an automated, flowthrough, solid-state optical ammonia gas sensor specifically designed for measurements in serum-based mammalian cell-growth media. The reported system measures ammonia concentrations as low as 0.2 m*M* in 10μL samples. This sensor is constructed by immobilizing a weak-acid chromophoric indicator dye within the pores of a microporous polypropylene membrane. A bifurcated fiberoptic bundle transmits the incident radiation to the sensing membrane. This incident radiation strikes the indicator-containing membrane. A fraction of the diffuse reflected light is captured by the bifurcated fiberoptic probe and is directed to the detection optics for measurement. Chlorophenol red is used as the indicator dye. The analytical utility of this

system is demonstrated by analyzing a series of samples collected during the cultivation of PC3 human prostate cancer cells. These cells are grown in a modified serum-based RPMI-1640 culture medium maintained within either conventional tissue culture flasks or a high-aspect rotating wall vessel (HARV) bioreactor. The HARV reactor was developed by NASA to simulate microgravity conditions for cell growth *(9)*.

MATERIALS AND METHODS

Apparatus and Reagents

Reflectance measurements were obtained with a fiberoptic spectrometer equipped with a 100 W tungsten-halogen source (Oriel Model 66184, Oriel Instruments, Stratford, CT). Light from this source passed through an infrared (IR)-blocking filter and then a 560 nm interference filter, before being focused into one branch of a Oriel Model 77533 glass bifurcated fiberoptic bundle. The fiber bundle directed this light to the surface of the ammonia-sensitive membrane. A fraction of the light reflected off the sensing membrane was captured by the second set of optical fibers and directed to the detection optics. Intensity of the reflected light was measured by an Oriel Model 7070 photomultiplier tube (PMT) operating at 800 V. A Perkin-Elmer (Norwalk, CT) Lambda 1 UV-visible spectrometer was used for all reference ammonia measurements, based on the glutamate dehydrogenase (GDH) enzymatic assay (Sigma, St. Louis, MO).

Chlorophenol red (CPR) was used as purchased from Aldrich (Milwaukee, WI). Metricel polypropylene membranes were 100-μm thick, possessed a mean pore size of 0.1 μm, and were used as received from Gelman Sciences (Ann Arbor, MI). The RPMI-1640 tissue culture medium and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD). The bovine serum albumin (BSA) and the ammonia assay kit were purchased from Sigma. All other chemicals were reagent grade from common suppliers. All standard solutions were prepared by dissolving known amounts of ammonium chloride in freshly prepared 0.05 *M* phosphate buffer (pH 7.4).

Membrane Preparation

A procedure similar to that described by Sellien et al. *(10)* was used to immobilize the pH indicator in the polypropylene membrane. Briefly, 1 mg CPR was dissolved in 1 mL tetrahydrofuran (THF). A square (1 × 1 cm) of the microporous polypropylene membrane was dipped in the indicator solution for 5 s and air-dried under ambient conditions for 10 min. The membrane was then dipped in 0.1 *M* HCl to remove excess indicator, rinsed in deionized distilled water, and dried by blotting with a paper towel.

Sensor Construction

The flowthrough cell was constructed from a 2.5 × 2.5 × 4.5 cm Plexiglas® block. To create a sample well, a 3.5 mm id × 2 mm deep hole was drilled into one surface of this block. Sample flow lines were drilled with a 0.8 mm id at approx 35 degree angles from the sample well. A double-stick tape (3M, St. Paul, MN) ring with a 3.5-mm hole was used to hold the sensing membrane on the Plexiglas block, directly over the sample well. A glass bifurcated fiberoptic bundle having a 6.4 mm od, was fitted into a second Plexiglas block. The second block was securely fastened to the first with screws, directly over the ammonia sensitive membrane. A 2-mm air gap separated the fiberoptic bundle from the membrane, in order to prevent condensation on the fiberoptic surface. The flowthrough cell was oriented with sample entering in the bottom and exiting through the top, to prevent air bubbles forming on the membrane surface. The flowthrough cell is illustrated schematically in Fig. 1.

Sensor Operation

A Technicon proportioning pump (Technicon Instruments, Tarrytown, NY) was used to pump ammonia-free buffer (pH 7.4, 0.05 *M* phosphate buffer) through the flow-injection manifold. Teflon tubing (0.030 in. id, and 0.062 in. od) was used to contain the flowing solution. Ammonia standards or sample solutions were injected as 10-μL aliquots into the buffer carrier stream by using a Rheodyne (Rohnert Park, CA) model 7010 sample injector. A reagent stream of 0.1 *M* sodium hydroxide, also contained within the same type of Teflon tubing, was mixed with the carrier stream, to increase the solution pH prior to the ammonia measurement. A 50-cm reaction coil was placed between the mixing-tee and the ammonia sensor, to ensure complete mixing. The flow rates for the sample and reagent lines were each controlled at 1.0 mL/min.

The sensor output was recorded continuously as a function of time. Reflectance was calculated as log (I_0/I), where I_0 corresponds to the mean detector signal 20 s immediately prior to the sample entering the sensing unit, and I corresponds to the peak detector signal in response to ammonia in the sample.

Bioreactor Ammonia Measurements

PC3 human prostate cancer cells (ATCC CRL 1435) were grown in RPMI-1640 medium supplemented with 10% FBS, 2 m*M* glutamine, 10 m*M* HEPES, 100 U/mL penicillin, and 100 mg/mL streptomycin *(11)*. Cells were grown in a humidified incubator at 37°C and 5% CO_2, either in tissue culture flasks or in a HARV, with methods adapted from Prewett et al. *(8)*.

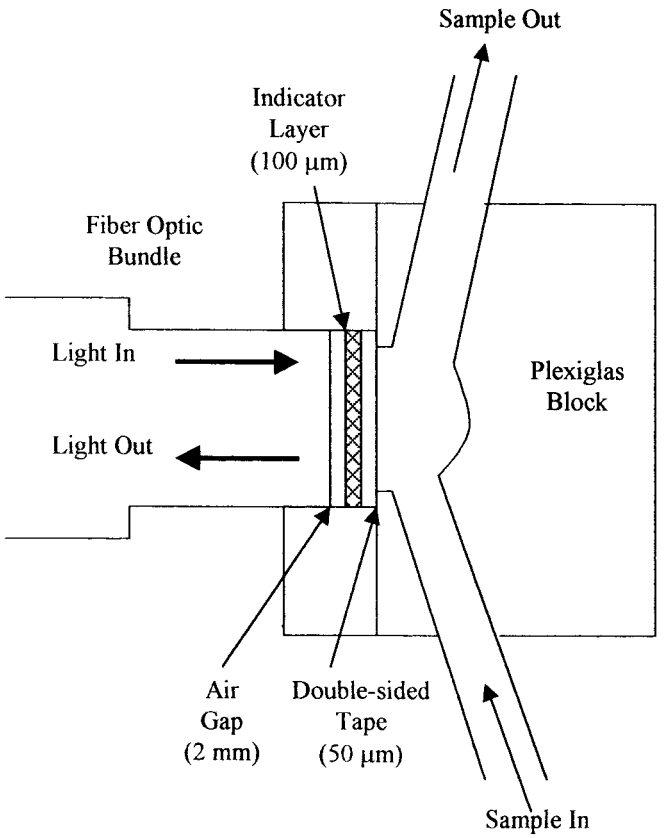

Fig. 1. Schematic diagram of the sensor geometry (not drawn to scale).

Briefly, the HARV was filled with 55 mL culture medium. Cytodex-3 microcarriers (Sigma) were added to provide a carrier concentration of 5 mg/mL. The medium was inoculated with 0.5×10^6 cells/mL, and samples were withdrawn periodically during the cell cultivation. Medium was also exchanged periodically in the HARV experiments. Cultivations in T150 tissue culture flasks were initiated with 1.0×10^6 cells each, and without microcarriers.

HARV samples were removed in 1-mL aliquots. Rapid sample treatment is required to reduce systematic errors caused by continual cellular metabolism during sample preparation. In this work, samples were centrifuged for 5 min, after which the supernatant was removed and immediately placed in a freezer maintained at −20°C. Tissue culture flask samples were collected directly from the supernatant above the cells, and these samples were placed immediately in a −20°C freezer. All samples remained frozen until just prior to the analysis, when samples were allowed to sit at room temperature until thawed. Each sample was ana-

lyzed by both the flowthrough sensor and the glutamate dehydrogenase enzymatic ammonia assay described above.

RESULTS AND DISCUSSION

The proposed ammonia sensor operates on the basis of gas diffusion *(12)*. Ammonia in the sample diffuses into the inner structure of a treated, microporous polypropylene membrane. The sensing chemistry corresponds to a thin layer of solid CPR coated along the surface of the polymer. Ammonia in the sample partitions from the aqueous layer into the polymer, where it reacts reversibly with the immobilized CPR. The color of this CPR coating changes, reversible in the presence of ammonia. Diffuse reflectance measurements of the CPR-coated membrane relates the extent of this color change to the concentration of ammonia in the sample solution.

The nature of the reaction between ammonia and CPR is detailed elsewhere *(12)* and is not the topic of this paper. Nevertheless, it is important to understand the basic function of the sensor components. The membrane is impermeable to liquid water, and, therefore, provides a means for isolating the measurement chemistry from the sample solution. The microporous structure of polypropylene membranes, coupled with the hydrophobic nature of the polymer and the surface tension of water, excludes liquid water from the inner regions of the membrane. The authors have established, by measuring the ionic conductivity across treated membranes, that this gaseous nature of the membrane is retained in the presence of a thin layer of CPR *(12)*, and have also established that the aqueous nature of the sample solution is completely isolated from the indicator chemistry, by exposing CPR-treated membranes to solutions of different pH. No change in the absorbance spectra of the CPR layer is observed when exposing the membranes to solutions with pHs from 4.0 to 10.0. Hence, the membrane fabrication method described above provides a sound, gas-permeable barrier for ammonia sensing.

The focus of this report is the ability of the proposed flowthrough sensor design to measure ammonia selectively and accurately in a typical serum-based mammalian cell culture growth medium. Response properties are examined over the millimolar concentration range expected for such mammalian cell cultures *(1)*. In addition, measurement accuracy is assessed for samples taken from common tissue culture flask experiments and the HARV reactor. These measurements demonstrate the utility of the sensor for following ammonia accumulation during cell growth.

Response Properties

Important properties evaluated include accuracy, precision, sample pH, hydrolysis of glutamine, and interference from soluble proteins.

Accuracy was evaluated by spiking a mixture of RPMI-1640 and FBS (10% v/v) with known amounts of ammonium chloride. The resulting ammonia concentrations ranged from 0 to 10 m*M*. A correlation plot of measured vs known ammonia levels was linear, with slope, *y*-intercept, and R^2-values of 0.97 ± 0.01, 0.2 ± 0.2 m*M*, and 0.990, respectively. No systematic bias was indicated in the distribution of points along the ideal unity line. The mean percent error for all 51 samples was 5%, and the standard error of prediction was 0.25 m*M*. Precision was assessed by repeatedly measuring ammonia levels in standards of known concentrations. The relative standard deviation for nine measurements was 3% at 1 m*M* ammonia and 3% at 10 m*M* ammonia.

Sample pH at the measurement site is critical in terms of both sensitivity and selectivity for ammonia. Measurement sensitivity is enhanced at high pHs, at which essentially all the total ammonia nitrogen is in the detectable form of ammonia (NH_3), as opposed to the nondetectable ammonium ion form. In addition, carbon dioxide in the sample can interfere with gaseous ammonia measurements performed at neutral pH, at which NH_3 and CO_2 co-exist. Again, high pH effectively eliminates interference from carbon dioxide by forming nonvolatile bicarbonate and/or carbonate anions *(13)*.

Sodium hydroxide is added to the sample stream, which increases the pH above 11.0. Equal flow rates for the sample and this sodium hydroxide reagent solution causes a 1:1 dilution of the sample. The effectiveness of the setup was evaluated by measuring the pH for a small volume of solution collected after the mixing coil. The solution pH was near 12.0, which indicates essentially 100% conversion to ammonia.

The possibility of forming ammonia from the nonenzymatic hydrolysis of glutamine was examined under the measurement conditions. The authors tested for evidence of significant amounts of ammonia formation from glutamine during the time between the introduction of sodium hydroxide and when the sample passed over the sensing unit. In this experiment, sensor results were compared for samples containing 1 m*M* ammonia with and without the addition of 10 m*M* glutamine. The resulting ammonia concentration values are 1.04 ± 0.04 m*M* (n = 6) and 1.07 ± 0.06 m*M* (n = 6), in the absence and presence of 10 m*M* glutamine, respectively. Given that glutamine levels typically do not exceed 4 m*M* under mammalian cell growth conditions, the authors conclude that interference from glutamine hydrolysis is negligible under these operating conditions of relative concentrations, flow rate, temperature, and pH.

A potential drawback of membrane-based sensors is the possibility of membrane fouling caused by surface adsorption of proteins. To test for this effect, ammonia was measured in buffer solutions that contained 10 g/L BSA. When the ammonia concentration is 1 m*M* in the absence or

presence of BSA, the predicted ammonia concentrations are 1.01 ± 0.06 m*M* (n = 5) and 1.0 ± 0.1 m*M* (n = 6), respectively. In all, the membrane was exposed to this high level of BSA for several hours. No signs of signal degradation were observed over the time-course of this experiment.

Ammonia Calibration Curve

Calibration curves for ammonia are generated by plotting the measured peak reflectance as a function of the sample ammonia concentration. Figure 2 illustrates the nonlinearity of such a calibration plot. This degree of nonlinearity is consistent with earlier models for fiberoptic ammonia sensors *(14)*, and has been reported for other solid-state optical ammonia sensors *(10,15)*. The calculated limit of detection (S/N = 3) is 0.2 ± 0.1 m*M*.

Stability of the calibration model was determined by verifying the calibration after every fifth sample during the analysis of a series of samples. The calibration model remained consistent for up to 12 h. Difference between measured and actual ammonia levels in a calibration standard remained less than or equal to 10% during this period. However, daily calibrations are necessary, and sometimes exceed 10% difference from one day to the next. Between-day variations in the calibration function vary in a random manner, and appear to be related to slight differences in alignment of the optical components, as opposed to an instability of the sensing chemistry. Indeed, a single membrane could be used continuously for periods longer than 6 mo, when properly calibrated.

The analysis time corresponds to the combination of the time required to reach the peak reflectance value and the time necessary to re-establish the baseline condition before the next sample can be introduced. A typical time profile is provided as the inset in Fig. 2. This plot reveals that the total analysis time is controlled chiefly by the recovery process. Typically, 45–130 s are required to re-establish the baseline condition, depending on the ammonia concentration. High ammonia concentrations require longer times for complete recovery. In general, the analysis time is 2.5 min per sample, which corresponds to a throughput of 24 samples per hour.

Ammonia in Bioreactor Samples

As described in Materials and Methods, samples were collected during the growth of PC3 human prostate cancer cells, and analyzed for ammonia. Samples were collected during six individual HARV runs and in six separate tissue culture flasks. In each case, ammonia levels were measured by the proposed optical gas sensor and the conventional GDH enzymatic procedure. In all, ammonia levels were measured in 30 HARV samples and 18 tissue culture flask samples. No sample workup was

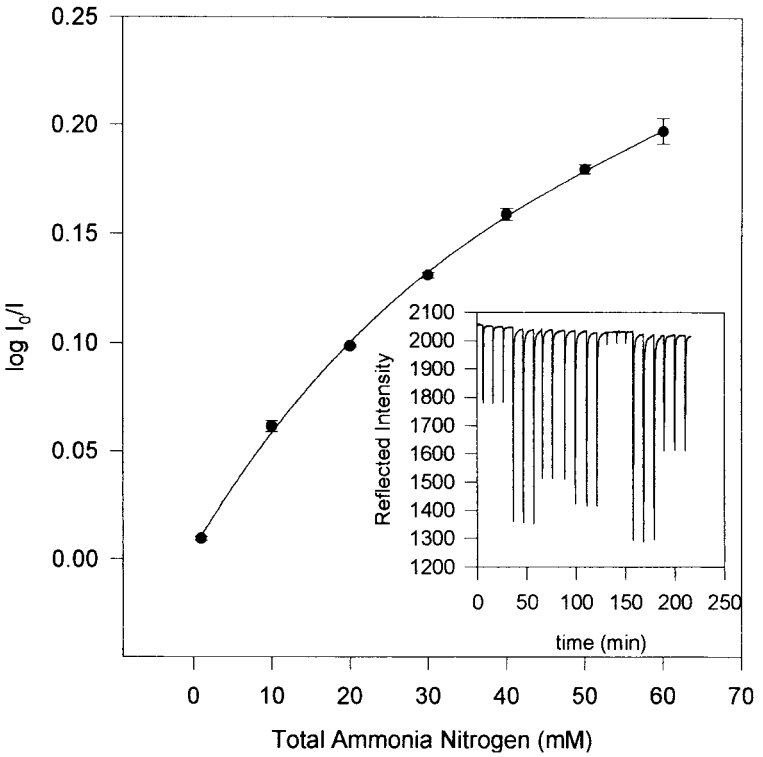

Fig. 2. Typical ammonia calibration curve. Inset shows the time-dependent response of the sensor following triplicate 10-μL injections of 10, 50, 30, 40, 1, 60, and 20 m*M* ammonia standards, respectively.

required for samples taken from the tissue culture flasks, because these samples could be directly injected into the system. For samples collected from the HARV reactor, cells were removed before injection. Values obtained from the proposed sensor were compared to those measured with the enzyme assay. The corresponding correlation plot is linear, with a slope of 1.05 ± 0.04, a *y*-intercept of 0.1 ± 0.3 m*M*, and an R^2-value of 0.931. Equivalent results from these two methods support the analytical utility of the proposed sensor for measurements in mammalian cell culture medium.

These analytical measurements can be used to follow the production of ammonia during the course of individual reactor runs. Figure 3, for example, shows the accumulation of ammonia during a typical tissue culture flask cultivation. In this particular experiment, ammonia accumulates over the first 80 h of cultivation, before cell growth subsides.

Ammonia toxicity is well known, and optimal bioreactor function demands control of ammonia levels within the culture medium. Two principal methods have been proposed for controlling the accumulation of

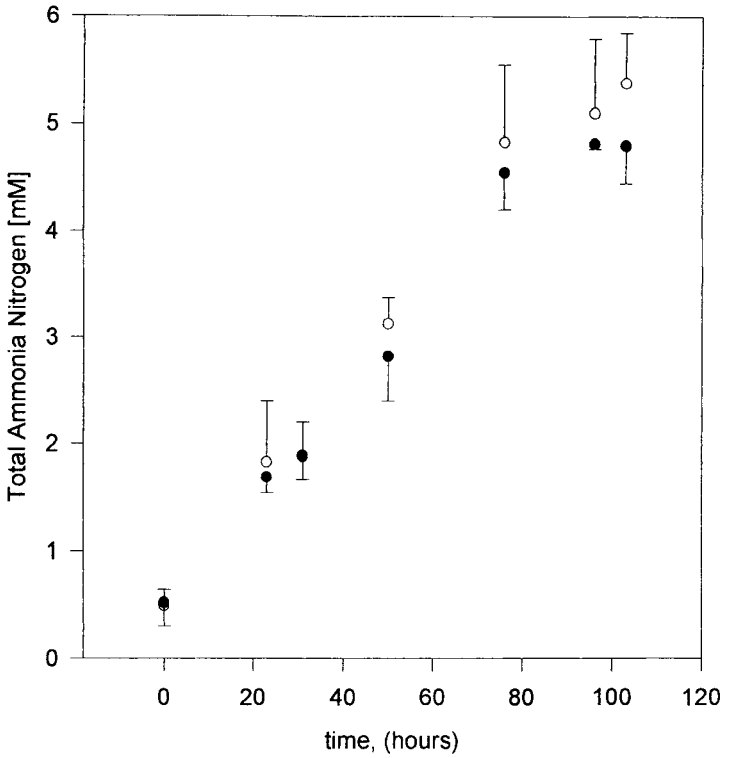

Fig. 3. Typical ammonia profile for a tissue culture flask cultivation. Ammonia concentrations were measured with the conventional GDH enzyme assay (solid circles) and the proposed flowthrough ammonia sensor (open circles). Error bars represent 95% confidence levels for three separate measurements with each method.

ammonia during cell growth: Control the rate of ammonia production by maintaining optimal levels of glucose and glutamine in the growth medium; and remove ammonia by gas dialysis, ion exchange, or electrodialysis *(1)*.

Figure 4 demonstrates the potential utility of the proposed sensing system to help maintain low ammonia levels in the HARV reactor. In this experiment, ammonia levels were monitored periodically, and the growth medium was replaced with fresh medium when ammonia levels exceeded 3 m*M*. The arrows in Fig. 4 indicate when the medium was exchanged. By exchanging the medium twice, the ammonia levels remained fairly constant from 50 to 270 h. A drop in ammonia is evident following the second exchange, when a measurement was made immediately following the exchange procedure. Although more work is necessary to confirm these findings, and to characterize cell integrity and growth patterns more thoroughly, these results are encouraging.

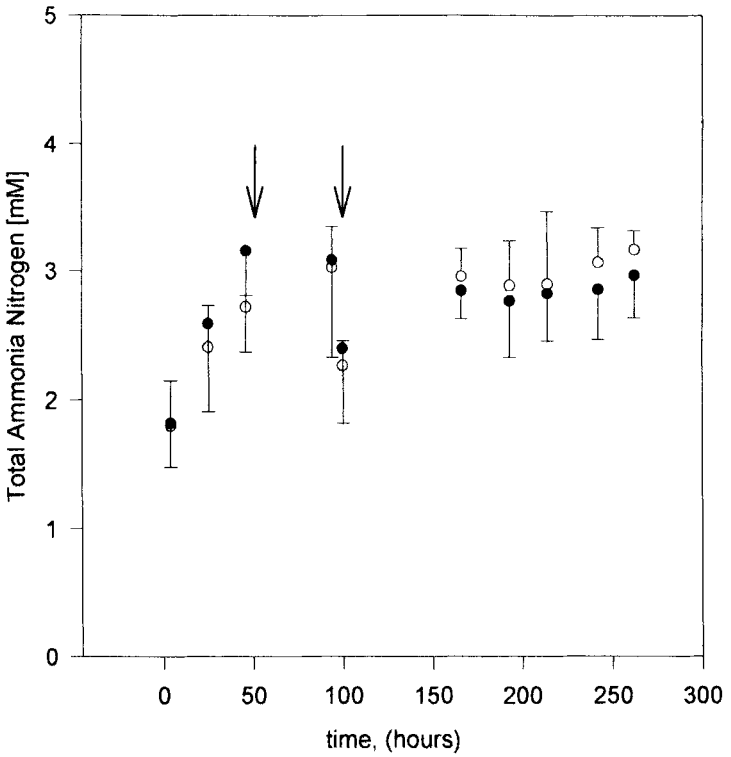

Fig. 4. Ammonia levels during a single HARV bioreactor run measured with the enzyme assay (solid circles) and the proposed ammonia sensor (open circles). Error bars represent 95% confidence levels for three separate measurements with each method. Arrows indicate when culture medium was changed (*see* text for details).

CONCLUSION

The proposed solid-state flowthrough ammonia sensor offers the advantages of small sample volumes, simplicity, sensitivity, accuracy, and long-term operation, which make it well suited for routine monitoring of bioreactor processes. The feasibility of using this sensor to control ammonia accumulation is demonstrated for the HARV bioreactor. Given the success of this sensor configuration, it may be possible to use this sensing chemistry to develop an on-line ammonia sensor capable of unattended, semicontinuous operation. Such an on-line device could provide the analytical information required for a feed-back control system.

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