PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Fibre-Optic-Based Fluoroimmunosensors [and Discussion]

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Fibre-optic-based fluoroimmunosensors

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Remote sensing of chemicals can be performed using fibre optic chemical sensors that use immunochemical reagent phases. Exploiting the specificity of antibody–antigen interactions and the sensitivity of laser-excited fluorimetry, highly selective measurements of ultra-trace levels of chemicals can be performed remotely and *in situ* via fluoroimmunoassay techniques. In this work, heterogeneous assay protocols using immunobeads are implemented. A passive sensor that samples analyte by diffusion through a permeable membrane and is capable of a single analysis is described and used for the measurement of a naturally fluorescent compound. Subsequently, a regenerable sensor that can perform assay procedures in a repetitive fashion is described and characterized. The versatility of this sensor for performing remote measurements using a variety of established fluoroimmunoassay methodologies is discussed.

1. Introduction

Situations for which remote, *in situ* measurements of chemical concentration are preferred over traditional sampling with subsequent transport to the laboratory for analysis include (1) measurements of analytes present in hostile or not easily accessible locations, (2) continuous 'sensing' of analytes, and (3) situations for which traditional sampling procedures alter the concentration of the analyte. Fibre optics, which transmit light based on total internal reflection (Sepaniak *et al.* 1988), can be used to link spectroscopic instrumentation (e.g. light sources, photodetectors, etc.) to remotely located samples. Remote spectroscopic measurements exhibit high sensitivity if the measurement is based on laser-excited fluorimetry. Versatility and analytical selectivity are enhanced if the fluorescence signal is the result of the interaction of the analyte with a reagent that is immobilized at the sensing terminus of the fibre. Typically, this interaction involves reactions to form fluorescent products or the quenching or enhancement of the fluorescence of the reagent phase by the analyte. These sensors, commonly referred to as fibre optic chemical sensors (FOCSs), are often used to measure small molecules or ions such as H^+, O_2, and CO_2 (Seitz 1988). The feasibility of measuring large and macromolecules has been demonstrated using biologically significant 'affinity' reagent phases (Sepaniak *et al.* 1988).

As with conventional analyses, the attributes of high sensitivity and selectively, large dynamic range, and rapid response are important when using sensors. Since *in situ* measurements are generally performed without the advantage of analyte isolation via procedures such as extractions and chromatographic separations,

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excellent selectively is imperative. When investigating large analytes, another potential problem, slow transport to and within the reagent phase, can limit response rates. Additional desirable sensing characteristics, that are often not completely achieved in practice by the FOCSS that have been reported to date, include the capability of performing (1) remote, (2) *in situ*, and (3) continuous measurements.

The specificity of immune reactions, the availability of antibodies to many analytes, and the sensitivity of fluoroimmunoassays (FIAS), make the implementation of immunological reagent phases a logical extension of FOCS technology. We have conducted direct assays, of natural fluorophors (Tromberg *et al.* 1988) and competitive-binding (Tromberg *et al.* 1987) FIAS using 'fluoroimmunosensors' (FISS). The FIS generally used an analyte-permeable membrane to isolate antibody within a chamber at the terminus of a fibre optic and relied on passive diffusion to sample analyte. This sensor design was not capable of continuous use since, in operation, the limited amount of immobilized antibody is consumed through the nearly irreversible formation of immune-complex. Subsequently, we developed 'microscale regenerable biosensors' (MRBS) that combine the instrumentation used with the FIS with a capillary column-based reagent delivery system (Alarie *et al.* 1990). In principle, many different bioassay protocols can be performed remotely and repetitively with the MRB.

We illustrate herein the use of the FIS to measure benzo(a)pyrene tetraol (BPT), a naturally fluorescent metabolite of the carcinogen benzo(a)pyrene. High sensitivity and selectivity are achieved but the FIS response is relatively slow and continuous operation is not feasible. The reproducibility obtained when performing typical assay operations with the MRB is presented and the sensor is applied to the measurement of a large protein, rabbit-immunoglobulin G antibody (anti-rab-IgG). The ability to regenerate the MRB *in situ* is also demonstrated.

2. Experimental

The commercial sources of materials used in this work (e.g. immunochemicals and optical components) were given in our earlier cited papers. Detailed operational information was also presented in those papers. A brief description of the apparatus and procedures used in this work is presented here to provide continuity in the discussion of the results of these studies. The optical arrangement is shown in figure 1*a*. Fluorescence excitation is provided by a helium-cadmium laser for the BPT detection (325 nm) and an argon ion laser for the protein detection (488 nm). The proteins (see table 1) were purchased as fluorescein-isothiocyanate (FITC) derivatives. The laser beam was passed through a narrow hole, bored in the centre of a 25 mm diameter mirror, and focused onto the incident end of a plastic coated fused silica fibre. A shutter was used to reduce the duty cycle of the excitation and to minimize photodegradation (average laser power at the incident end of the fibre was less than 1 mW). Emission resulting from excitation of fluorophor at the sensing terminus of the fibre was partly collected by the same optical fibre and transmitted in a reverse fashion to the detector. The transmitted emission exited the fibre at a relatively large angle, was collimated by a lens, then reflected by the mirror and imaged onto the entrance slit of a monochromator (emission wavelengths were 400 nm and 520 nm for the BPT and FITC respectively). Photomultiplier (PMT) signals were then recorded using a picoammeter and strip chart recorder.

Critical to the performance of the sensor is the configuration of the sensing

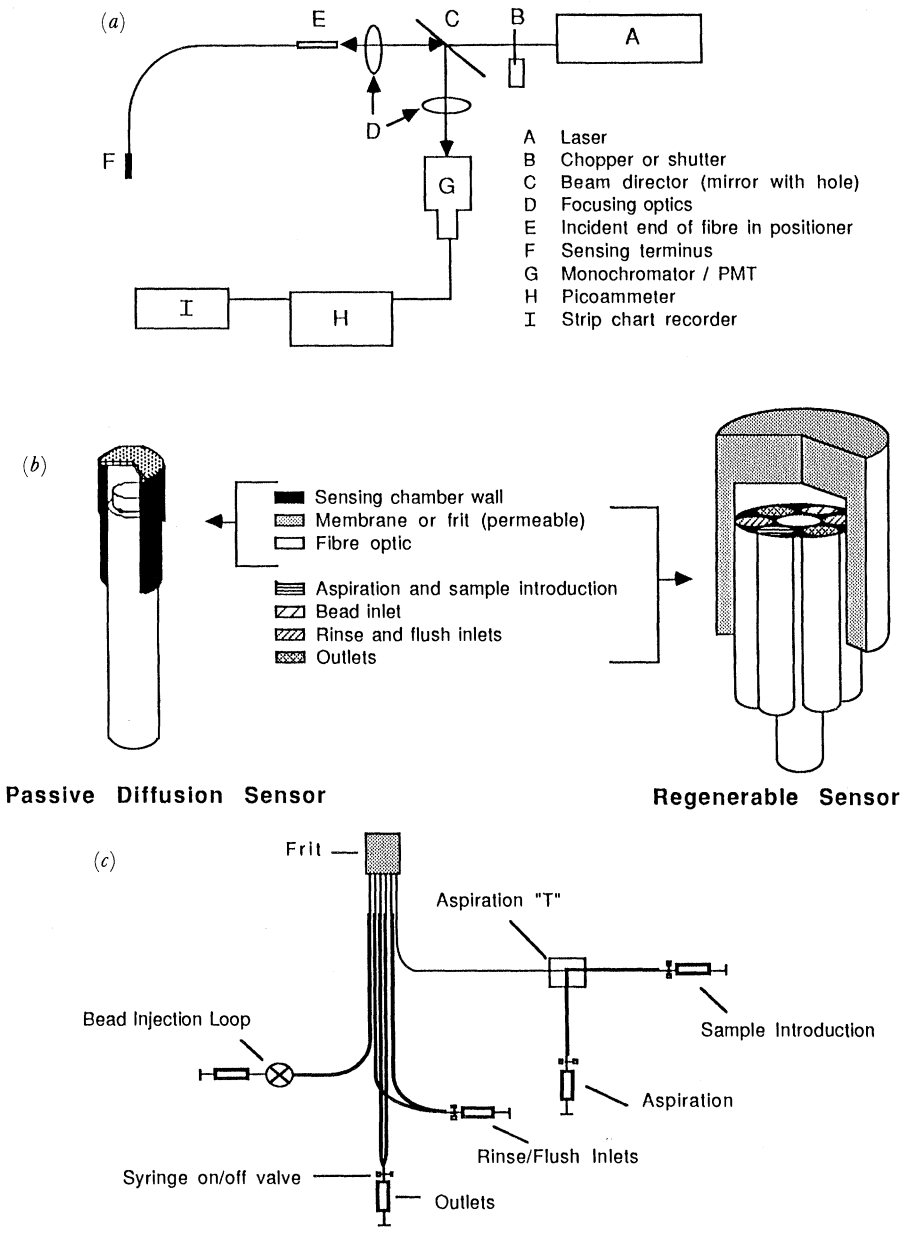

Figure 1. (*a*) Optical arrangement, (*b*) passive diffusion and regenerable sensing tip configurations, and (*c*) depiction of MRB capillary delivery system for fibre-optic-based fluoroimmunosensing.

terminus. The passive diffusion terminus shown in figure 1*b* consisted of a 140 nL chamber at the end of a 600 μm core diameter fibre. The chamber was created using heat shrink tubing and was terminated with a thin 10000 MW cut-off cellulose membrane. The chamber was filled with an anti-BPT immunobead slurry for each measurement (7 μm diameter silica with covalently bound Protein A-anti-BPT, 17 mg ml^{-1}, approximately 4 ng of anti-BPT per sensor fill) before immersion in the sample. When placed in a BPT solution, the analyte slowly diffuses into the chamber

and becomes trapped as bead-bound immune-complex. Details concerning the production of protein A immunobeads and the carbonyldiimidazole (CDI)-activated immunobeads used with the MRB (see below) can be found in a previous report (Alarie *et al.* 1990).

The construction and operation of the regenerable sensor terminus is more involved but is justified by its versatility and its ability to operate in a pseudo-continuous manner. Six 200 μm internal diameter (ID) capillary columns surround a 400 μm core diameter fibre. The sensing chamber is defined by a hollowed stainless steel frit (ID of 1.2 mm) that has a volume of less than 1 μl. Details concerning the construction of the MRB can be found elsewhere (Bowyer *et al.* 1990). In this work, a 50 μl volume of immunobeads (generally, 8 mg ml^{-1} of 7 μm diameter silica with covalently bound CDI-rab-IgG, approximately 4 μg of rab-IgG per sensor fill) is delivered *in situ* to the sensing chamber before each measurement.

The operation of the MRB depends upon the protocol for the FIA that is used. In this study, either the coefficients of variation (CVs) of isolated bioassay operations (e.g. delivering immunobeads, rinsing, or adding secondary fluorescent reagents) were performed or anti-rab-IgG-FITC was measured using a direct assay procedure. With reference to figure 1*b*,*c*, the steps for the anti-rab-IgG-FITC measurement involve (1) insertion in the sample solution, (2) aspiration of approximately 10 μl of sample (into a 30 cm length of capillary and past the 'T' shown in the figure) using a syringe, (3) delivery of immunobeads (see above) to the sensing chamber with the aid of an HPLC injection valve, (4) delivery of sample and then approximately 100 μl of rinse solution to the beads in the sensing chamber, (5) measurement of fluorescence signal, and finally, (6) flushing of the chamber. These steps can be performed in 10–20 min. An important distinction is that delivery and rinsing steps were performed with the outlet capillaries sealed (see valves in figure) such that flow is only through the frit with the immunobeads remaining in the field of view of the optical fibre, and flushing was performed with the outlets opened to facilitate removal of the beads. In some cases pumps are used to control delivery/rinse rates. A structurally similar protein, human-IgG, was used to demonstrate the analytical selectivity of the sensor. Unless otherwise stated, concentrations of the protein solutions were approximately 0.1 mg ml^{-1} (6×10^{-7} mol l^{-1}).

3. Results and discussion

(*a*) *FIS measurement of BPT*

The direct assay measurement of BPT was accomplished using the FIS (passive diffusion sensor) described in the experimental section. Excellent sensitivity, selectivity, and absolute limits of detection (LODs) were achieved. The response characteristics of the sensor are represented by the data plotted in figure 2. The temporal behaviour of the sensor, shown in figure 2*a*, demonstrates a large concentrating effect (signal enhancement) that can lead to excellent detectability if long incubations can be tolerated. The plot was obtained by placing the sensor in a 1.6×10^{-7} mol l^{-1} BPT solution. After 45 min the accumulation of immune-complex resulted in a signal/noise (S/N) enhancement of four over the no-antibody case and resulted in an LOD of 2.5×10^{-10} mol l^{-1}. In operation, the enhancement increases until saturation of the antibody occurs (several hours in the described experiment). At this point, the immune-complex concentration is approximately 1×10^{-9} mol l^{-1} (minimum detectable concentration of BPT in the sensing chamber) and there is no

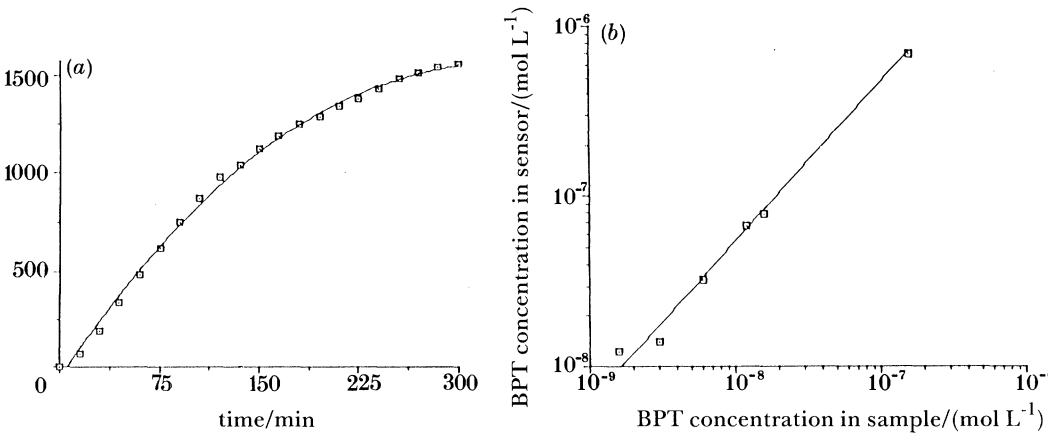

Figure 2. (*a*) Temporal response and (*b*) calibration plot for FIS measurement of BPT.

BPT chemical potential difference across the membrane (presumably chamber and sample uncomplexed BPT concentrations are about equal). Large amounts of immobilized antibody and large affinity constants (approximately 10^8 for liquid phase monoclonal anti-BPT used in this experiment) improve sensitivity.

Figure 2*b* is an adjusted calibration plot (Tromberg *et al.* 1988) that demonstrates linear response over approximately two decades in concentration. Five replicate measurements of the lowest concentration in the plot yielded a mean signal-to-noise ratio of 14 with a CV of 6.2. Despite the excellent analytical characteristics described above, this sensor suffered from several disadvantages such as (1) long response time, (2) only a single measurement can be performed, (3) high selectively could only be achieved by external dialysis, which limits its *in situ* capability, and (4) 'memory' effects when the membrane was used.

(*b*) *Evaluation of the* MRB

Heterogeneous FIAS can be performed remotely, *in situ*, and in a pseudo-continuous fashion with the MRB. The disadvantages cited for the FIS are largely eliminated by incorporating a means to regenerate the sensing chamber, and by sampling by aspiration rather than diffusion. The potential and versatility of the MRB for biosensing can be discerned by noting its operational similarity to that of the clinically popular Pandex Corp. instruments for performing bioassays (Jolley 1983). With the Pandex system, heterogeneous FIAS are performed using immunobeads that are mixed with sample and various reagents and placed into microtiter wells that are terminated with a frit. The solution in the well is drawn through the frit leaving the immunobeads, which are investigated by front surface fluorimetry. The microtiter wells are arranged on a titer plate that facilitates the rapid analysis of many samples. While multiple analyses are not possible with the MRB, repetitive measurements of a single sample can be performed *in situ* using protocols that resemble those used with the Pandex system. Moreover, the analytical advantages of FIAS, such as high sensitivity and selectivity, are potentially achievable with the sensor.

The results of an evaluation of isolated and combined assay operations is presented in table 1. The signals presented for the first two entries demonstrate that beads can be delivered to the chamber, using an injection valve, in a reasonably reproducible

Table 1. *Demonstration and reproducibility of bioassay operations*

operation	$X(N = 5)$ (signal or volume)	cv
delivery of FITC-labelled beads (6 μm)	510 nA	7.7
rinsing of beads (25 μl of water)	510 nA	7.7
delivery of FITC solution	25.3 nA	0.6
rinsing of FITC solution (25 μl of water)	3.8 nA	1.2
aspiration of IgG-FITC		
solvent–water	12 μl	7.5
solvent–(blood-serum)	13 μl	2.5
assay using rab-IgG immunobeads		
analyte–(6×10^{-7} mol l^{-1} anti-rab-IgG-FITC)	22 [13] nA	6.2
analyte–(6×10^{-7} mol l^{-1} human-IgG-FITC)	16 [15] nA	5.3

manner, and that with rinsing they remain in the field of view of the fibre. Immunobeads, labelled with a spectrally distinct fluorophor, could be used to normalize for bead delivery amounts. The third and fourth entries illustrate that liquid solutions can be delivered very reproducibly and rinsed through the frit. The 3.8 nA for the fourth entry represents the background photocurrent for the system. The cvs were based on five measurements and the magnitudes of the signals (determined by the concentrations of the reagents) have little significance in this study.

In an actual assay, accurate and reproducible aspiration of sample solution is critical, especially since there are no convenient procedures for normalizing for the volume sampled. Using the 'T' arrangement shown in figure 1*c*, it is possible to sample slightly different volumes while delivering a constant volume to the chamber. As shown in the table, the cvs in terms of volume of IgG-FITC delivered, are less than eight. The superior precision for the dense matrix (serum) may be due to less diffusion and transport into the outlet capillaries during the operation. The volumes were determined by expelling the contents of the aspiration capillary into a cuvette, diluting to 1.0 ml, and comparing the resulting absorbance, measured with a conventional spectrophotometer, with a previously established calibration plot. The ability to rapidly sample large analytes is a unique and potentially important advantage of the MRB.

The protocol for the measurement of anti-rab-IgG-FITC, described in §2, was used to demonstrate that complete assays can be performed with the MRB with reasonable reproducibility. The numbers in brackets are the mean background levels for the respective experiments. The ability to selectively measure immune-specific analyte is clearly evident, as the human-IgG-FITC signal-to-noise ratio is statistically insignificant, while for anti-rab-IgG-FITC, $S/N \approx 7$. The signals were acquired after approximately a 100 μl rinse and were completed in about 10 min.

The calibration plot shown in figure 3 was obtained in a similar fashion. The nonlinear response at the high concentration end is probably due to saturation of those immunobeads that are exposed to analyte during sample delivery. Because of non-ideal flow patterns in the sensing chamber, not all of the beads are exposed. The limit of detection ($S/N = 2$) is 5×10^{-8} mol l^{-1}, which corresponds to 5×10^{-13} moles in the 10 μl sample. While this detectability is good, it should be possible to improve the limit of detection and, thereby, extend the linear dynamic range of the

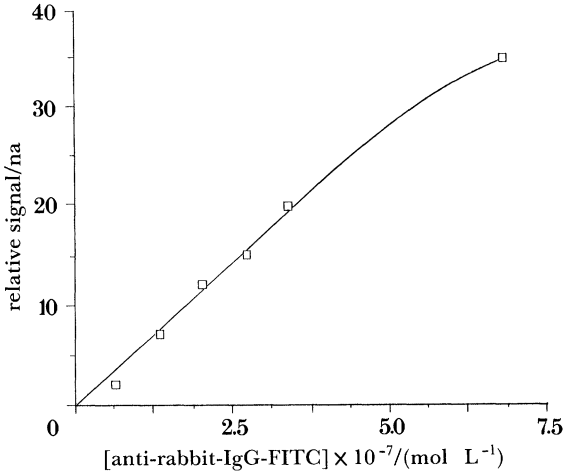

Figure 3. Calibration plot for MRB measurement of anti-rab-IgG-FITC.

calibration plot at the low-concentration end, by improving the flow patterns in the sensing chamber. In particular, the current design uses a frit that is thinner at the walls than at the end (see figure 1*c*). Thus the flow of solvent and, hence, beads is toward the walls of the chamber and out of the field of view of the fibre. Future work will address this problem and experiments are in progress to evaluate the MRB for both competitive-binding and sandwich FIAS.

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Discussion

G. S. Wilson (*University of Kansas, U.S.A.*). The immunoassay can certainly be used for environmental analysis. Most assays of small molecules such as pesticides and herbicides will require the use of a competitive assay. This assay has the intrinsic property of short dynamic range to that it may be necessary to adjust concentrations of reagents to permit operation in a linear domain. This may not be easy for the novice. Nevertheless, such assays should be useful for screening purposes.

M. J. Sepaniak. Professor Wilson's comment concerning environmental analysis using competitive-binding FIA using fibre optic sensors is certainly valid. It is worth noting that while our early passive diffusion sensors were not capable of *in situ* measurements by competitive-binding protocols, our more recent work with the regenerable sensor that was described at the meeting can be adapted for such measurements (albeit not a simple matter), and we are currently investigating this.

W. J. Albery (*University of Oxford, U.K.*). I was interested in Dr Sepaniak's analysis to discriminate between kinetic and membrane control. We have the same situation in a membrane enzyme electrode. If we can, we prefer to have the system under membrane as opposed to kinetic control since then the system is more reproducible. Does he pursue the same strategy?

M. J. Sepaniak. Professor Albery's comment concerning the improved reproducibility for membrane kinetic controlled sensors is certainly reasonable (although we have not attempted any studies to validate). With regard to our pursuing strategies to accomplish such kinetic control, it has not been necessary since our particular membrane-based sensors were under such control. With regard to our most recent work with regenerable sensors, this kinetic consideration no longer exists (although reproducibility is still a critical concern that we are addressing with current work).

L. de Galan (*Unilever Research Laboratory, The Netherlands*). What is the current and expected state of the art for the immunoassay determination of small molecules, e.g. pesticides, PAHs, etc.?

M. J. Sepaniak. The remote sensing of naturally fluorescent molecules is relatively simple. In addition to the BPT work described in this paper, we have explored the determination of naturally fluorescent compounds such as aflatoxins and the antitumor drug doxorubicin. The detection of non-fluorescent small molecules requires a competitive-binding protocol and, as stated above, this complicates matters when the described sensing instrumentation is used. Nevertheless, we are attempting to develop the MRB for competitive-binding FIAs. The lack of reliable commercial antibodies for small molecules hinders that development.