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FIBRE OPTIC BIOSENSOR ASSAY OF NEWCASTLE DISEASE VIRUS

by

William E. Lee and H. Gail Thompson

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ABSTRACT

A fluorometric sandwich immunoassay for Newcastle disease virus (NDV) was developed using a fibre optic biosensor. Antibodies directed against NDV were covalently attached to the surface of silane-coated quartz fibres and were used to capture the antigen onto the optical element. Fluorescein-labelled anti-NDV served as the detector antibody. Assay times were approximately 10 min in duration with a limit of detection of 5 ng of purified virus. The biosensor detection system possesses several desirable characteristics such as a limited number of mechanical components, a reusable solid support and low baseline variation. These and other properties of the fibre optic biosensor indicate that it has the potential to serve as an on-line continuous monitoring device of an automated or semiautomated detection system. As a part of the fibre optic biosensor development, the surface coverage of the quartz fibres by capture antibody was characterized, and an enzyme immunoassay for NDV which employed quartz fibres as the solid support was undertaken. This work was conducted as part of a trinational (UK/US/CA) effort on the Biochemical Detector.

RÉSUMÉ

Un immunodosage fluorométrique de type sandwich faisant appel à un biocapteur à fibre optique a été mis au point pour le dépistage de la maladie de Newcastle. Des anticorps dirigés contre le virus de la maladie de Newcastle (VMN) ont été fixés par liaison covalente à des fibres de quartz enrobées de silane dans le but de capturer l'antigène sur l'élément optique. Les anti-VMN marqués à la fluorescéine ont servi d'anticorps détecteurs. La durée du dosage a été d'environ 10 min et la limite de détection se situait à 5 ng de virus purifié. Le système de détection par biocapteur possède plusieurs caractéristiques avantageuses comme un nombre limité de composantes mécaniques, un support solide réutilisable et une faible variation de base. Ces propriétés, entre autres, du biocapteur à fibre optique indiquent que ce dernier peut être utilisé dans un dispositif de surveillance en ligne continu dans un système de détection automatique ou semi-automatique. Dans le cadre de la mise au point du biocapteur à fibre optique, le degré de couverture de la surface des fibres de quartz par les anticorps de capture a été caractérisé et l'élaboration d'un immunodosage enzymatique pour la détection du VMN utilisant des fibres de quarts comme support solide a été entreprise. Ce travail a été effectué dans le cadre d'un effort trinational (R.-U./É.-U./Canada) sur le détecteur biochimique.

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INTRODUCTION

Advances in biotechnology have increased the threat of biological warfare and thus the requirements for detection of BW agents. As part of a collaborative research program amongst the United Kingdom, United States and Canada, directed towards producing a rapid BW detection system, a fluorescence-based fibre optic device has been investigated. In a survey of technologies, this device, incorporating a Fibre Optic Wave Guide (FOWG), was identified as being potentially useful as the sensor in a fieldable BW detector (1).

Fibre Optic Wave Guide - Principles of Operation

The index of refraction of an optical fibre is greater than that of the surrounding medium. As shown in Figure 1, when light passes through the length of the fibre, the portion of the light striking the interface at an angle greater than the critical angle, θ_c, undergoes total internal reflection. The light which strikes the interface at an angle less than the critical angle passes out of the fibre. The internally reflected light generates an electromagnetic wave in the surrounding medium in close proximity to the surface and is referred to as an evanescent wave. The extent of the penetration is dependent upon the angle of incidence, the refractive indices of the two media, and the polarization and wavelength of the light. The evanescent wave decreases exponentially with distance from the interface. The amplitude of the electric component of the evanescent wave (E) can be expressed as

$$E = E_o \exp-[2\pi(\sin^2\theta - n^2_{21})^{1/2} x/l]$$

where x is the distance from the surface of the fibre, E_o is the amplitude of the electric wave function at the interface, n_{21} is the ratio of the indices of refraction, l is the wavelength of the light. The angle, θ, is measured from the normal to the surface of the fibre. For the optical configuration used in the present device, the effective penetration was about 200 nm (2).

The component of the light that extends into the neighbouring medium is capable of exciting fluorescent molecules attached to the outer surface of the quartz fibre. Some of the emitted light from the fluorescent molecules re-enters the fibre and is reflected back through the core of the fibre toward the photodetector. Due to the limited penetration of the evanescent wave into the liquid medium, only fluorescent molecules at or near the surface (*ca.* < 200 nm) of the optical fibre contribute to the output signal. Fluorophores outside this envelope are not observable to any extent.

A FOWG instrument was designed by Hirschfeld and Block (3-5). The optical layout of the instrument is shown in Figure 2. The reaction chamber of the FOWG was a flow cell designed by the U.S. Army Chemical Research Development Engineering Centre, Aberdeen, MD (6). The design allowed the fibre to be centred in the flow cell along the optical axis of the FOWG. The fluidics were controlled by means of a peristaltic pump. The void volume of the flow cell with a quartz fibre in place was about 50 μL.

Immunoassay on Quartz Fibres

Antibodies have large binding constants and high specificities for select antigen molecules (7) and when immobilized on solid support can provide a capture system for these target molecules. Antibodies immobilized on surfaces, especially polystyrene microtitre plates, have been used extensively as capture devices in enzyme-linked im-

munosorbent assays or ELISA (8) and the physico-adsorption of antibodies to polystyrene has been studied (9). At the inception of this work the process of covalent immobilization of antibodies to silane-treated quartz was largely undefined. In order to characterize it, a comparison has been made between between ELISA on microtitre plates and an ELISA on quartz fibres. Capture antibody was covalently attached to segments (0.3 cm) of silane treated fibres. The fibres, thus prepared, were placed in the wells of microtitre plates and used as the capture surface for antigen. Addition of detector antibody, a conjugate of horse radish peroxidase, (Figure 3) followed by addition of a colour generating substrate completed the process. This approach allowed for a comparison of polystyrene and quartz surfaces.

For a fibre optic sensor to be effective, so too must the process of capturing antigen onto the optical element. By using the same reagents and carrying out an ELISA on quartz and comparing the sensitivity to that of an ELISA on polystrene microplates, a qualitative measure of the capture ability of the quartz surface could be obtained. Thus the sensitivities obtained from each medium should be comparable.

Fibre Optic Biosensor

The fibre optic biosensor combines immunochemical molecular recognition with opto-electronic signal transduction of the FOWG. Antibody directed against the target analyte was immobilized on the surface of quartz fibres. The fibres, when installed in the flow cell, provided the capture mechanism for the analyte. Fluorescein-labelled antibodies also directed against the analyte, were used to form a sandwich immune complex (Figure 3) within the evanescent zone on the surface of the fibre, generating a signal related to the amount of analyte to which the detector had been exposed.

The antigen used in this work was a BW simulant, a vaccine strain of Newcastle disease virus, an avian parainfluenza retrovirus. The strain of NDV employed, La Sota NJ, is Agriculture Canada approved for poultry innoculation and is suitable for release into the environment (10). Since the identification of the La Sota NJ strain of NDV as a tracer (11), that is, a live virus to be used in field trials, a number of studies using microtitre plates has been undertaken to determine the limits of detection and characterize the immunochemical properties (12-15). An assay for NDV using a light-addressable potentiometric (LAP) sensor, an immunochemically-based biosensor, has been reported (16).

The work described herein reports the development of an assay for virus using a fibre optic biosensor. In conjunction with the assay development, the surface coverage of quartz by antibody was characterized and an enzyme immunoassay for virus using horse radish peroxidase conjugated detector antibody and quartz solid support was studied.

MATERIALS AND METHODS

Fibre Optic Wave Guide Detection System

The FOWG was manufactured by ORD Inc. (New Salem, NH). The light source in the FOWG was a 4 W quartz halogen lamp and the photodetector, a Hamamatsu S1087-01 photodiode. The liquids were driven through the flow cell by means of a Cole Parmer peristaltic pump, model 7553-30 with two pump heads, 7013 and 7014. The output of the photodetector was connected to a Hewlett Packard model 7015B XY recorder for data collection.

Materials

Optical grade quartz fibres, having polished ends, were purchased from ORD Inc. and were used in the FOWG and as the solid support for enzyme immunoassays. Reagent grade acetone, methanol, chloroform and 3-aminopropyltriethoxysilane (APTES) were obtained from Aldrich Chemical Co. (Milwaukie, WI). Phosphate buffered saline (PBS) tablets, bovine serum albumin (fat free), fluorescein-labelled anti-guinea pig IgG from rabbit, anti-human IgG from goat, dimethyl sulfoxide, carbonyldiimidazole and fluorescein isothiocyante were obtained from Sigma Chemical Co. (St Louis, MO). Polystyrene Immulon 1 flat bottom microtiter plates were purchased from Dynatek Laboratories (Chantilly, VA). Purified La Sota NJ strain of NDV, purified monoclonal and polyclonal (guinea pig) anti-NDV, and a hydrogen peroxidase conjugate of the polyclonal anti-NDV were obtained from the Molecular Biology Group, DRES. The substrates for the horseradish peroxidase, H_2O_2 and 2,2-azino-di(3-ethyl-benzthiaolin) sulfonate (ABTS) were obtained from Kirkegaard and Perry (Gaithersburg, MD). ^{125}I-labelled anti-human IgG from goat was obtained from Dupont (Mississauga, ON). Wash solution consisted of 150 mM NaCl, 10 mM phosphate buffer, pH 7.2, plus 0.2% Tween 20 detergent. The diluent buffer was the wash solution containing 1% albumin.

Silanation of Quartz Fibres

Quartz optical fibres (1 mm x 6 cm, 40 in total) were placed in a 1^{c} x 90 mm glass receiver (14/20 joint). The fibres were washed three times with acetone, three times with water, and twice with nitric acid (15%). The fibres were covered with nitric acid (15%), a condenser was installed and the apparatus was placed in a Reacti-Therm heating module

(Pierce Chemical Co., Rockford, IL) with the upper portion of the receiver wrapped in cotton batting. The fibres were heated for two hours at reflux temperature. Afterwards, the fibres were rinsed thoroughly with water, then methanol, and dried in a convection oven for two hours at 100°C. Two boiling chips were added to the glass receiver and the fibres were refluxed for one hour in dry chloroform.

The fibres were rinsed three times with dry toluene. A 20% solution of APTES in dry toluene was added in sufficient amount to cover the fibres. The receiver tube was closed with a glass stopper and secured with metal springs. The vial was placed on a Nutator shaker and rotated gently overnight at room temperature. The fibres were rinsed repeatedly with dry toluene, then dried in the Reacti-Therm block under a stream of dry nitrogen. The coated fibres were stored in screw-cap polystyrene test tubes inside a desiccator.

Covalent Attachment of Antibodies to Quartz Fibres

Quartz fibres which had been previously treated with APTES were placed in glass test tubes (12 x 75 mm), about 10-15 fibres per tube, then 2 mL of 0.5 M carbonyldiimidazole in dimethyl sulfoxide were added to each tube. The test tubes were covered with parafilm and incubated for 2 h at room temperature; gentle agitation was provided by a Nutator shaker. The supernatant solution was then decanted. The activated fibres were first washed three times with dimethyl sulfoxide, then three times with acetone, and finally dried at room temperature under a stream of dry nitrogen for 15 min. Once activated, quartz fibres were never stored, they were always immediately coupled with antibody by the following procedure.

If the antibody had been stored in sodium azide or some other stabilizer, preservative or buffer which would react with the activated fibre, it was necessary first to purify the antibody prior to the linking reaction. A solution of the antibody was then prepared at a concentration of 100 μg/mL in PBS pH 7.4. The activated fibres were placed in polystyrene 12 x 75 mm snap cap test tubes (no more than 20 fibres per tube). Sufficient antibody solution to cover the fibres (0.5 mL) was added and the tubes were incubated at 4°C overnight with agitation provided by a Nutator shaker. After the coupling reaction was completed, the antibody solution was decanted and the fibres were washed three times with 0.1 M ethanolamine pH 8.5 and then were incubated with gentle shaking for 2 h at room temperature in the same solution. Finally, the antibody-linked fibres were washed three times with PBS. The fibres could be stored in buffer at 4°C for several days, or longer if the storage medium contained 0.02% sodium azide or merthiolate.

Conjugation of Antibodies with Fluorescein Isothiocyanate

The procedure used to conjugate anti-NDV IgG with fluorescein was similar to methods previously described (17). An aliquot of anti-NDV IgG (guinea pig) solution, 0.5 mL at 12 mg/mL, was diluted to 6 mL with 0.15 M sodium phosphate buffer, pH 9.0, to give a concentration of 1 mg/mL. A stock solution of fluorescein isothiocyanate (FITC), 1 mg/mL in 0.15 M sodium phosphate buffer, pH 9.0 was prepared. A volume of 10 μL FITC solution per mg of protein, in this case 60 μL, was added to the antibody solution. The pH was adjusted to 9.5 by the addition of 0.1 M sodium phosphate solution, pH 12.0. The reaction mixture was stirred for 1 h at room temperature while maintaining the pH at 9.5. The reaction mixture, about 10 mL total volume, was transferred to dialysis tubing and stirred overnight in a large flask containing 3.5 L of PBS. The PBS solution was changed and stir-

ring continued for an additional three hours. After dialysis, a uv/vis absorbance spectrum was recorded and a coomassie blue protein assay carried out. The A_{280} and A_{495} readings indicated a mole ratio of fluorescein to protein (F/P) of 2.3. The recovery of protein was about 85%. The FITC-conjugated antibody solution was stored frozen at -20°C in 1 mL aliquots.

Enzyme Immunoassay on Quartz Solid Support - Covalently Attached Capture Antibody

Quartz fibres previously treated with APTES were cut using a small bore tube cutter (Scientific Systems Inc., State College, PA) and sorted to 3 (+/-0.3) mm in length. Rubber gloves were used when handling the coated fibres. Capture antibody was covalently linked to the fibre segments according to above described procedures. The concentrations of capture antibody solutions employed were 3, 13, 50 μg/mL. Blocking of the fibres was achieved by three successive incubations with blocking solution (diluent buffer) at 37°C. The antigen, NDV, was reconstituted in diluent and incubated with the capture antibody-coated fibres for 1 h at 37°C. After incubation with NDV, the fibres were washed three times with PBS. Detector antibody was diluted 1/500 in diluent solution. Aliquots (200 μL) of diluted detector antibody were added to plastic test tubes containing the quartz fibres and the fibres were incubated for 1 h at 37°C. The fibres were washed several times with wash solution and then transferred to a 96-well flat bottom polystyrene microtiter plate, one fibre per well. Freshly prepared substrate, a mixture of ABTS and H_2O_2 solutions, was added, 200 μL per well, and the plate was placed on a Nutator shaker and allowed to develop at room temperature for 30 min. The absorbance of the reacted substrate in the wells was measured

in a microtiter plate reader (Flow Laboratories) at 405 nm. The presence of the fibres in the wells did not affect the absorbance readings if the fibres were positioned away from the centres of the wells.

Assays of NDV on the Fibre Optic Wave Guide

Assays on the FOWG employed two methods to introduce antigen to the detector system. In one method, fibres coated with capture antibody were set into polystyrene test tubes, one fibre per tube; 0.5 mL of diluted NDV solution was added to each tube; and the tubes were sealed with polystyrene caps. The tubes were placed on the platform of a Nutator shaker and incubated at room temperature for 5 - 10 min. After incubation the fibres were rinsed three times with wash solution. A single fibre was installed in the flow cell of the FOWG. The cell was flushed with wash solution for 1 min and a baseline output signal was obtained during the flushing procedure. After the baseline had been recorded, detector antibody solution was pumped through the flow cell and the resulting fluorescence signal was obtained by monitoring the voltage output of the photodetector with respect to time using the XY recorder. In the second method, the quartz fibres were incubated with NDV in the flow cell of the FOWG. An antibody-coated fibre was installed in the flow cell and a 0.2 mL aliquot of diluted NDV solution was injected by syringe into the cell. After 5 - 10 min incubation, the flow cell was flushed with wash solution for 1 min. A baseline was obtained while wash solution was flowing through the system, then the detector antibody was introduced by means of the pump and the signal was recorded with respect to time by the XY recorder.

RESULTS

Silanization of Quartz Fibres

Quartz is a clear hard material composed primarily of silicon dioxide, and like most silica-type glasses, is relatively chemically inert, save for extreme conditions. Such conditions are too harsh an environment to maintain protein in an active state and so direct reaction of protein to quartz is not feasible. In order to circumvent this difficulty, "inert" solid supports, such as quartz, which have surface hydroxyl groups can be reacted with aminoalkoxysilanes at elevated temperature under anhydrous conditions. The resulting covalently immobilized silane coating provides both a reactive amino function and a spacer arm between the biologically active protein and the solid support. This reaction scheme is summarized in Figure 4 whereby 3-aminopropyltriethoxysilane (**1**) was reacted with quartz to give the silanized surface **2**. Elemental analysis of the silanized quartz surfaces by X-ray photoelectron spectroscopy indicated that the coverages were approximately 45 - 50% of the surface area (18). Ellipsometry data gave a thickness of approximately 11 A, indicating that the coating was primarily monolayer silane (18).

Immobilization of Antibodies on Silanized Quartz Surfaces

Immunoglobulin G, like many water soluble proteins, possesses solvent exposed amino groups on lysine residues which are available for protein chemistry. The aminosilane coated quartz surfaces were reacted with carbonyl diimidazole (Fig. 4, surface 3) to yield an activated surface (**4**). Antibody solution was added to the activated surfaces to produce a covalently linked quartz-silane-protein surface (**5**).

In order to determine the optimum concentration of antibody to be used in the immobilization step, activated quartz fibres (surface 4) were incubated with mixtures of ^{125}I-labelled goat anti-human IgG and native goat anti-human IgG having a range of total protein concentrations of 1 - 10 μg/mL. The radioactivity levels of the antibody-coated fibres were determined in a scintillation counter and the data is shown in Figure 5. The amount of protein immobilized, measured as the radioactivity emanating from the fibres, initially increased with increasing protein concentration and reached a plateau in the range 7 - 10 μg/mL. This experiment was carried out using a ratio of 20/1 (w/w) of native to ^{125}I-labelled anti-human IgG. Immobilization experiments with ratios ranging from 10/1 to 1000/1 produced similar results, i.e., a plateau in radioactivity between 5 - 10 μg/mL (data not shown).

It was possible to calculate the extent of protein coverage from the initial radioactivity of the stock radio-labelled antibody, the radioactivity of the protein coated fibre, and the ratio of native to labelled antibody. By comparison of the radioactivity (cpm) of the protein-coated fibres (Table I) with that of a standard curve of ^{125}I-labelled antibody (Figure 6), the total amount of protein immobilized was calculated to be about 1 ng mm^{-2}.

Sample calculation: from the standard curve, Figure 6, the relationship between radioactivity and protein mass can be obtained, *viz.*, 1 cpm = 4.7 x 10^{-6} μg protein.

From Table I, row 2 we have a radioactivity of 505 cpm

Thus, 505 cpm = 4.7 x 10^{-6} μg cpm^{-1} x 505 cpm = 2.4 x 10^{-3} μg of ^{125}I-labelled protein.

From the table it can be seen that the radiolabelled protein comprised 5% of total protein. Therefore,

total protein immobilized = 2.4 x 10^{-3} μg x (100/5) = 4.7 x 10^{-2} μg.

The surface area of a fibre (20 mm x 1 mm dia.) = 63 mm^2

The coverage (mass protein per unit surface area) = $4.7 \times 10^{-2} \mu g \times (1/63\ mm^2)$

$= 0.75\ ng\ mm^{-2}$

Taking the molecular weight of immunoglobulin to be 160,000 (19) and the specific density to be 1.4 (20), the volume per molecule can be calculated to be

volume $= (1.6 \times 10^5\ g\ mole^{-1})/(6.02 \times 10^{23}\ molec\ mole^{-1} \times 1.4\ g\ cm^{-3})$

$= 1.8 \times 10^{-19}\ cm^3\ molec^{-1}$

For this analysis, if we assume a spherical shape for immunoglobulin, the radius then can be calculated to be 3.5 nm. Thus, for a two dimensional close packing array, each protein molecule would rest on the surface in a square with length of 2r (7 nm). The surface would give a total coverage of

$(1.6 \times 10^5\ g\ mole^{-1})/[6.02 \times 10^{23}\ molec\ mole^{-1} \times (2 \times 3.5\ nm)^2\ molec^{-1}]$

$= 5.4 \times 10^{-21}\ g\ nm^{-2}$

From Table I the average protein coverage was determined to be 0.94 ng mm^{-2} (or $0.94 \times 10^{-21}\ g\ nm^{-1}$), the amount of the surface covered by protein is

$(0.94 \times 10^{-21}\ g\ nm^2)/(5.4 \times 10^{-21}\ g\ nm^{-2})$

$= 17\%$

From photoelectron spectroscopy data, the surface coverage by silane was determined to be about 50%, thus the coverage of available surface by protein was approximately (17/50) 35%. In a similar experiment using noncovalent adsorption to the quartz surface as the method of immobilization of ^{125}I-labelled antibody, the coverage was determined to be 10 - 15 times less than for covalent fixation (data not shown).

The overall value of protein coverage was found to be 17%. The protein packing model used in the calculation most likely overestimates the amount of protein which can be covalently immobilized. Antibodies are not perfect spheres and a random attachment of the protein to the surface would not result in a close packing array. Thus the surface coverage obtained most likely represents a maximal amount for the proportion of silanized surface available. Silane coverages greater than those obtained here have been reported in the range of 80 - 90% (21). Coverage in this range would increase the amount of antibody immobilized by almost a factor of two.

Enzyme Immunoassay of NDV on Quartz Fibres

Polyclonal antibody directed against NDV was covalently immobilized on quartz fibres (1 mm dia., 3 mm length) according to the above described methods. The fibres were placed in the wells (one per well) of microtitre plates. Consecutive incubations of antigen (NDV) and detector antibody (anti-NDV-fluorescein conjugate) were carried out, substrate solution added, and the absorbance was determined at 405 nm. The results of an assay are shown in Figure 7. The lower limit of detection (LOD), taken to be the intersection of the background plus two standard deviations with the dose response curve, was about 0.5 ng (5

ng/mL for 0.1 mL sample). The detection limits were comparable to a colorometric microplate enzy e-linked immunosorbent assay (ELISA) of NDV (12). An assay for NDV employing capture antibody noncovalently adhered to quartz fibres was less sensitive, having a LOD of about 20 - 50 ng (Figure 8). The results shown in Figure 7 and 8 were representative of a number of immunoassays on quartz solid support that clearly demonstrated that covalently attached capture antibody gave lower detection limits.

These results indicated that quartz fibres having covalently immobilized antibodies were a suitable solid support for ELISA. The methods employed for preparing the fibres and attaching capture antibody were appropriate for use in a fibre optic immunosensor.

Response of the Fibre Optic Wave Guide

Aqueous solutions of fluorescein were introduced to the flow cell of the FOWG and the output signals were recorded as a function of the fluorescein concentrations (Figure 9). The detectable concentration ranged from about 10^{-8} to 4 x 10^{-6} M. The response to the aqueous fluorescein was used as a reference standard and a benchmark for determining the operating performance of the FOWG and the sensitivity of subsequent immunoassays. An experiment to determine the active area of the quartz fibre contained in the FOWG flow-cell was carried out. Guinea pig immunoglobulin was covalently immobilized on quartz fibres and a solution of fluorescein-labelled second antibody (anti-guinea pig IgG from rabbit) was incubated with the fibres in a test tube. Sections of the fibre of increasing lengths were treated with the second antibody solution. The output of the FOWG increased in an approximately linear fashion with the length of fibre treated with the fluorescein-labelled second antibody. The data indicated that the active portion of the optical fibre extended essentially along its whole length (Figure 10).

Immunoassay of NDV on FOWG

Capture antibody, polyclonal (guinea pig) anti-NDV, was covalently immobilized on the surface of optical grade quartz fibres. The fibres were incubated externally with aliquots (0.5 mL) of NDV in sealed polystyrene test tubes for 5 min and rinsed thoroughly with wash solution. A fibre was inserted into the flow cell of the FOWG, wash solution was pumped through the flow cell and, in the absence of detector antibody, a baseline tracing of the background fluorescence was obtained on an XY recorder. Detector antibody (fluorescein-labelled guinea pig anti-NDV) was then pumped through the flow cell. For the blank (no NDV) there was a small increase in the signal which corresponded to the fluorescein-labelled antibody passing through the evanescence zone of the flow cell (sample **A**, Figure 11). When the flow of detector antibody was interrupted and wash solution passed through the system, the output signal decreased to approximately its initial value. For sample **B**, a fibre incubated with NDV solution (17 ng/mL) was inserted into the flow cell. When detector antibody was pumped through the flow cell, there was a substantial increase in output signal. After the flow of detector antibody was interrupted and wash solution introduced, a small decrease in signal was observed. For fibres incubated in NDV solutions of 100 and 1000 ng/mL, the output signals increased accordingly upon introduction of detector antibody. Higher concentrations of NDV yielded larger output signals (voltage changes).

Plots of the rate of change of the output signal with respect to time as a function of NDV are more revealing. The slopes of the individual XY recordings (Figure 11), 1 min after introduction of detector antibody were plotted against added NDV as shown in Figure 12 to yield a pronounced dose response. A calibration curve of FOWG output as a function of added NDV is shown in Figure 13. If the lower limit of detection (LOD) is taken to be

the lowest amount of NDV which produces a signal two standard deviations above background, the LOD is about 5 ng. The overall slopes varied somewhat from assay to assay as experimental conditions were modified. In general, longer incubation times and greater concentrations of the detector antibody produced higher signals.

After a single run, only a fraction of the binding sites for antigen were occupied; the major portion of the available binding sites on the fibre remained vacant. It was possible to use a single fibre for repetitive assays of the same antigen provided low concentrations of antigen were present. Between runs, the flow cell was flushed first with wash solution for about 3 min., followed by a solution of 2 μg/mL guinea pig anti-NDV for 5 min. This was the same antibody used as the detector, without the fluorescein label and its effect was to block any free epitope sites on the antigen (NDV) captured during the previous run. Successive 200 μL aliquots of NDV solution were injected into the flow cell by syringe and incubated for 10 min. After the incubation, the flow cell was flushed with wash solution for 1 min to establish a new base line and then with detector antibody to produce the signal. The results of a repetitive assay on a single fibre are shown in Figure 14. The LOD for the assay was about 5 ng NDV.

DISCUSSION

The work described herein has outlined the development of an immunoassay for Newcastle disease virus on a fibre optic instrument. Optical grade quartz fibres were treated with an organosilane to permit covalent attachment of capture antibody. Surface characterization by X-ray photoelectron spectroscopy and radiolabelling indicated that there was a high degree of coverage by both the organosilane and the capture antibody. ELISA assays for NDV using quartz fibres as the solid support were carried out and the

sensitivity obtained was comparable to that of a microtitre plate assay (12). The overall procedures for the ELISAs on quartz fibres were labour intensive and did not have the convenience of the microtitre plate method. It was confirmed, however, the covalent immobilization of capture antibody was effective, and that a fibre optic biosensor could be used as a sensitive alternative to microtitre plate ELISA assays.

The main advantage of the fibre optic biosensor compared to the microtitre plate assay is that the flow-through design of the fibre optic sensor provides an on-line continuous monitoring detector. Thus, the sensor has the potential to be incorporated into an automated or semiautomated system. The advantanges of such a system include a limited number of mechanical components, a reusable solid support, low variation on the negative control baselines and the potential for multi-recognitive elements on a single surface. Miniaturization and durability are achievable through the use of solid state electro-optic components. Recent work on immobilized proteins (21) has shown that the stability of surface-bound proteins stored over aqueous buffer solutions can be greatly enhanced by means of phospholipid linking agents. With respect to on-line biosensors, such as the fibre optic device described here, the active surfaces could be expected to have a useful lifetime of many days. Other applications of fibre optic sensors include immunochemically-based devices for clinical, environmental and public health assessment and gene probe-based sensors for DNA hybridization assays.

In conclusion, the feasibility of performing immunoassays on a fibre optic wave guide instrument has been demonstrated. The limits of detection were about 5 ng of virus protein for a 15 min assay at room temperature. These values compare well with other immunoassay work using a fibre optic biosensor; for epsilon prototoxin and Venezuelan equi-

nine encephalitis virus, limits of detection of about 1 (22) and 15 ng (6), respectively, have been obtained. They also compare favourably with standard microtitre plate assays and with results obtained with a light addressable potentiometric sensor (16).

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Table I

COVALENT PROTEIN FIXATION TO QUARTZ FIBRES

Total protein conc μg/mL [a]	% ^{125}I labelled [b]	CPM (S.D.)[c]	Total protein per fibre (g)[d]	Protein coverage (ng mm^{-2})[e]
100	1	220 (50)	1.02×10^{-7}	1.6
100	5	505 (72)	4.7×10^{-8}	0.75
100	10	858 (204)	4.0×10^{-8}	0.63
50	5	530 (185)	4.9×10^{-8}	0.78
50	10	664 (67)	3.1×10^{-8}	0.49
25	1	237 (55)	1.1×10^{-7}	1.8
25	5	323 (134)	3.0×10^{-8}	0.48
10	1	194 (40)	9.1×10^{-8}	1.4
10	5	384 (111)	3.6×10^{-8}	0.57

a Labelled plus nonlabelled antibody

b Percentage by weight of ^{125}I labelled protein

c Mean counts per minute (standard deviation, n = 5)

d 20 mm x 1 mm dia. fibre, surface area = 63 mm^2

e Mean protein coverage 0.94 (0.51) ng mm^{-2}

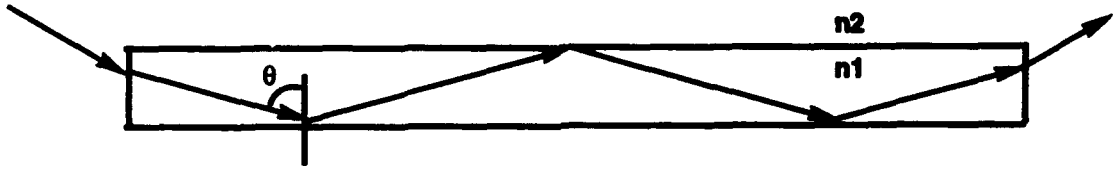

Figure 1. Total internal reflection of light in a quartz fibre. The refractive index of quartz (n_1) is greater than that of the surrounding medium (n_2). When $\theta > \sin^{-1}(n_2/n_1)$ total internal reflection will occur.

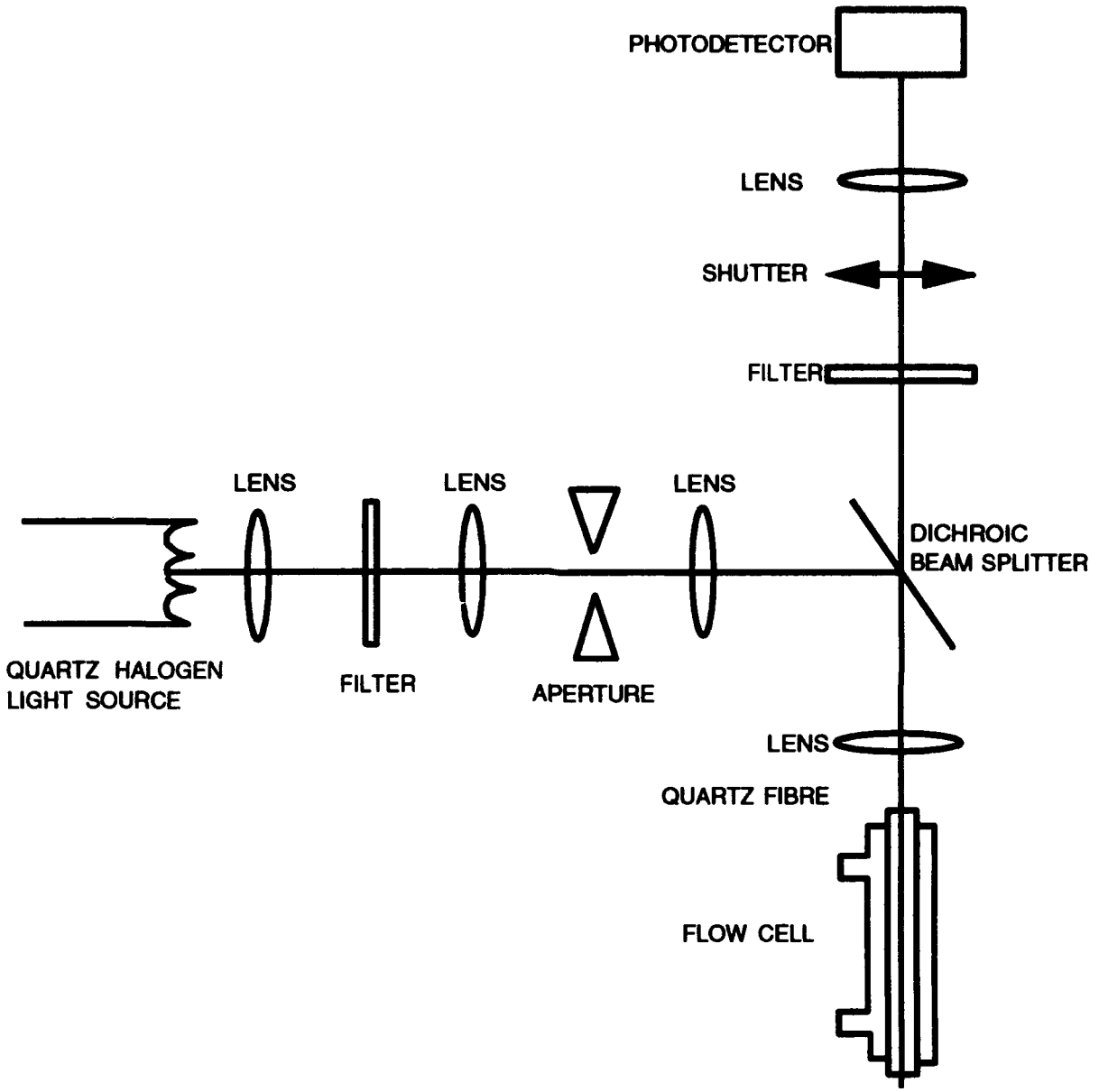

Figure 2

Optical layout of the Fibre Optic Wave Guide

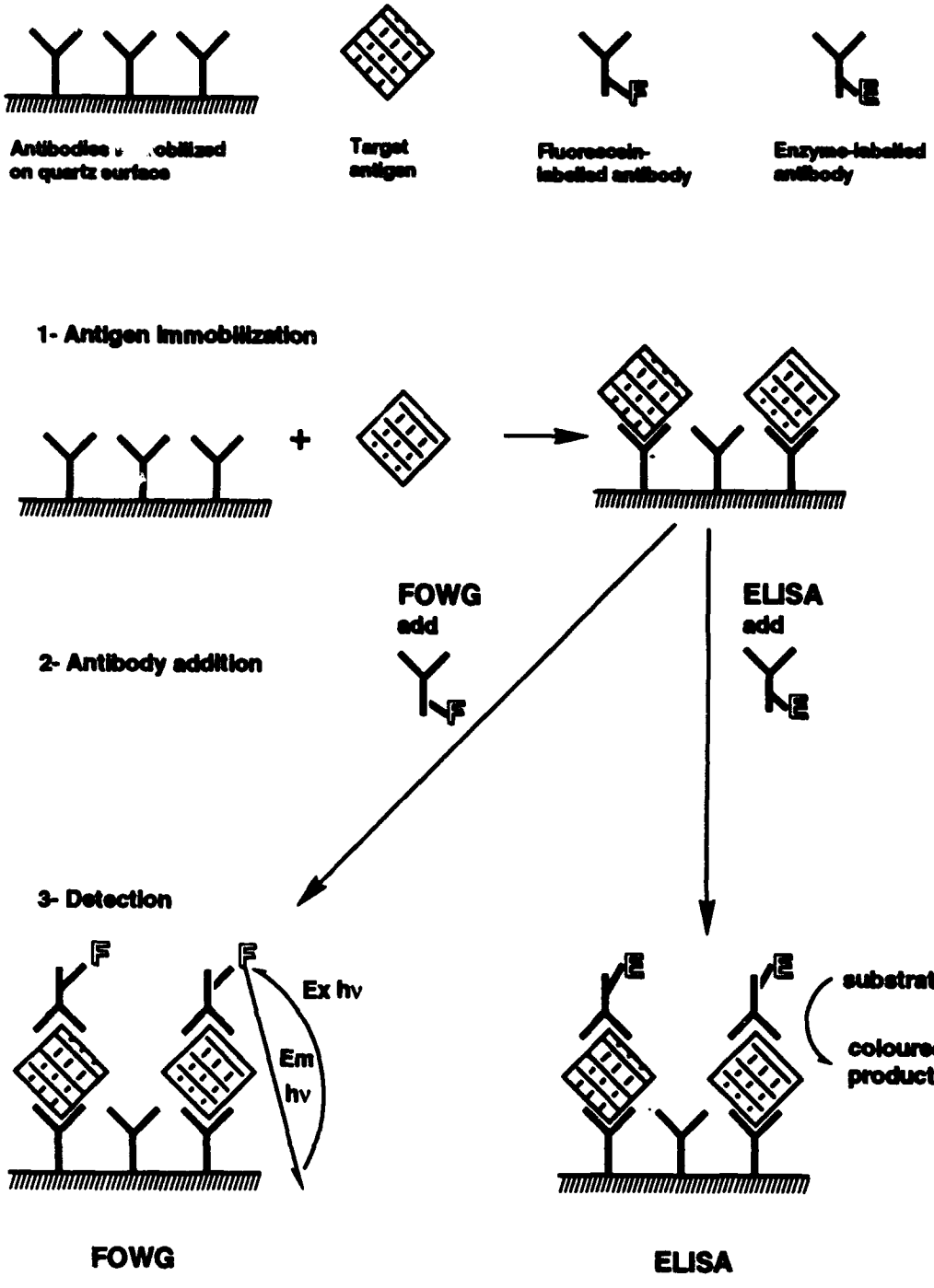

Figure 3. Reaction scheme for immunoassays in FOWG and ELISA employing quartz surfaces. The first step, capture of antigen, is common to both. For FOWG, fluorescein labelled antibody is added. A signal is generated through evanescent interaction of light with the fluorescein labels (Ex hν and Em hν represent excitation and emission photons, respectively). For ELISA, an antibody labelled with horseradish peroxidase is added. A signal is generated by the catalysis of substrate to form coloured products.

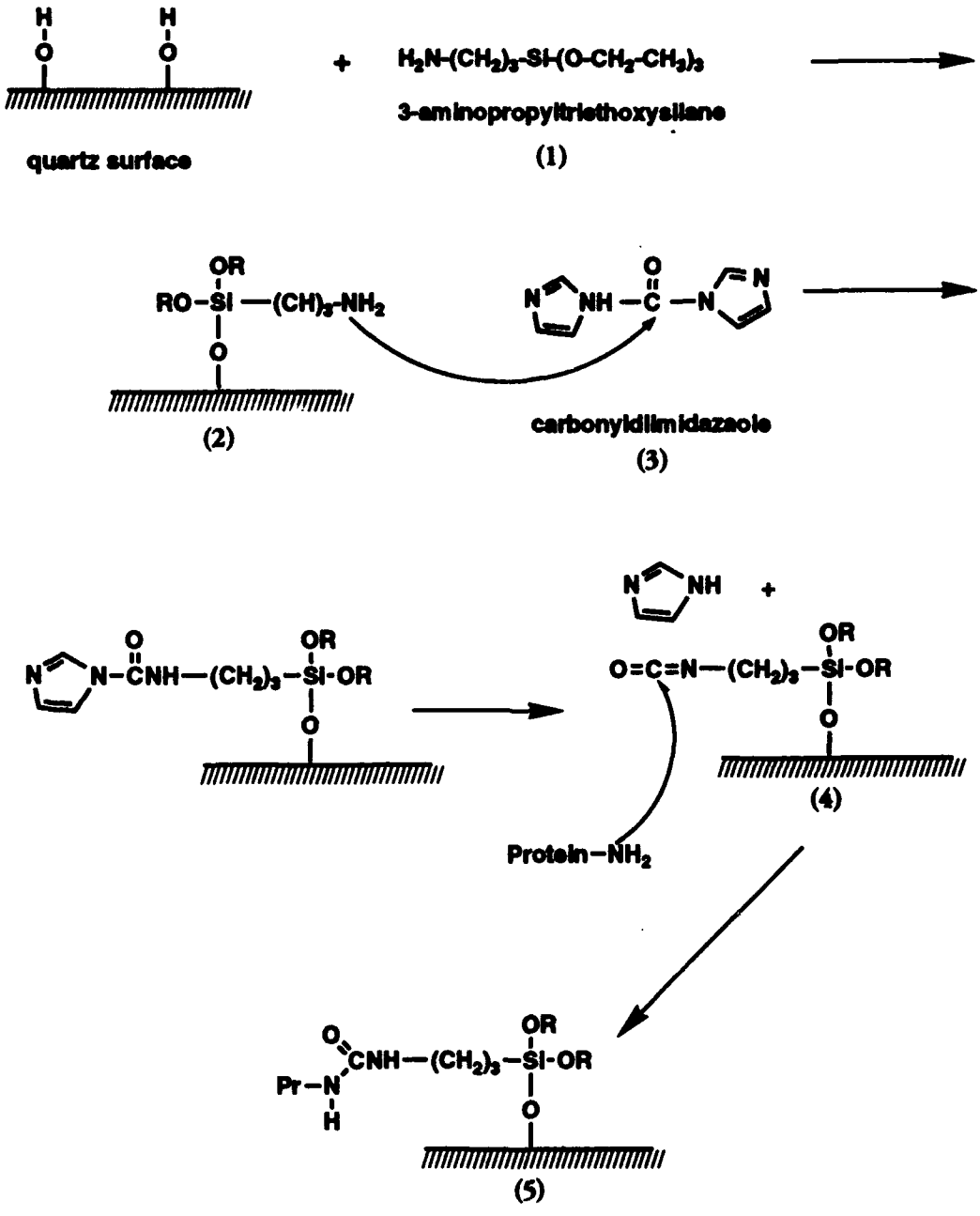

Figure 4. Reaction scheme of the covalent fixation of antibodies to quartz.

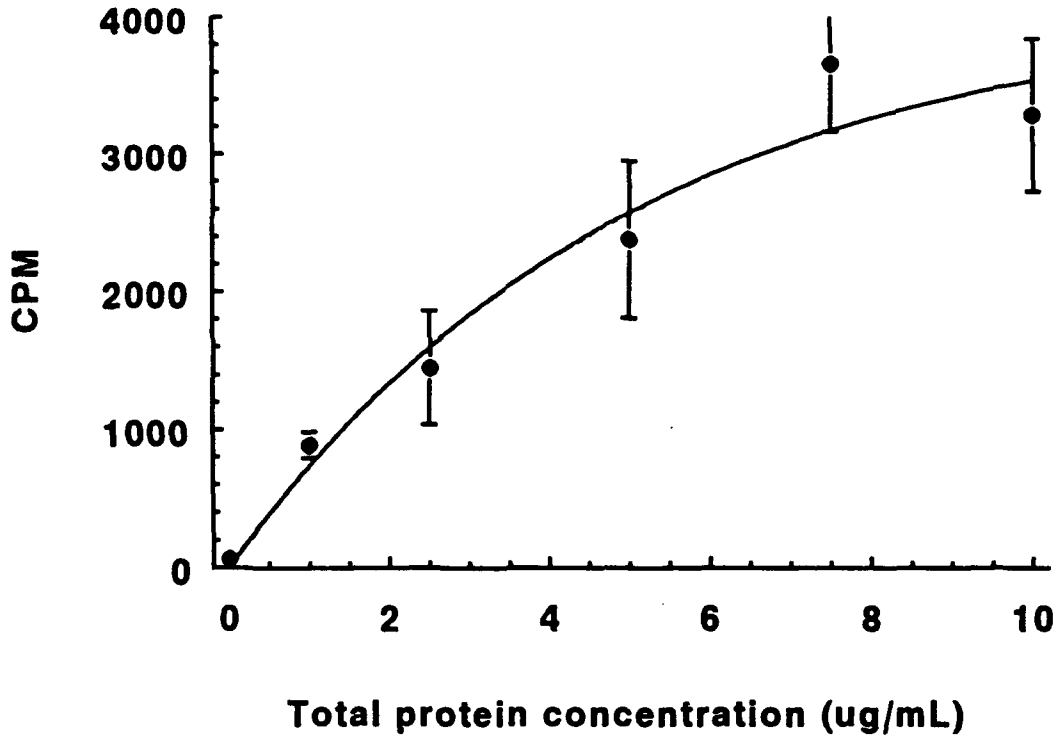

Figure 5. Radioactivity (counts per minute) of quartz fibres resulting from the covalent fixation of a mixture of native/^{125}I-labelled immunoglobulin G (20/1, w/w).

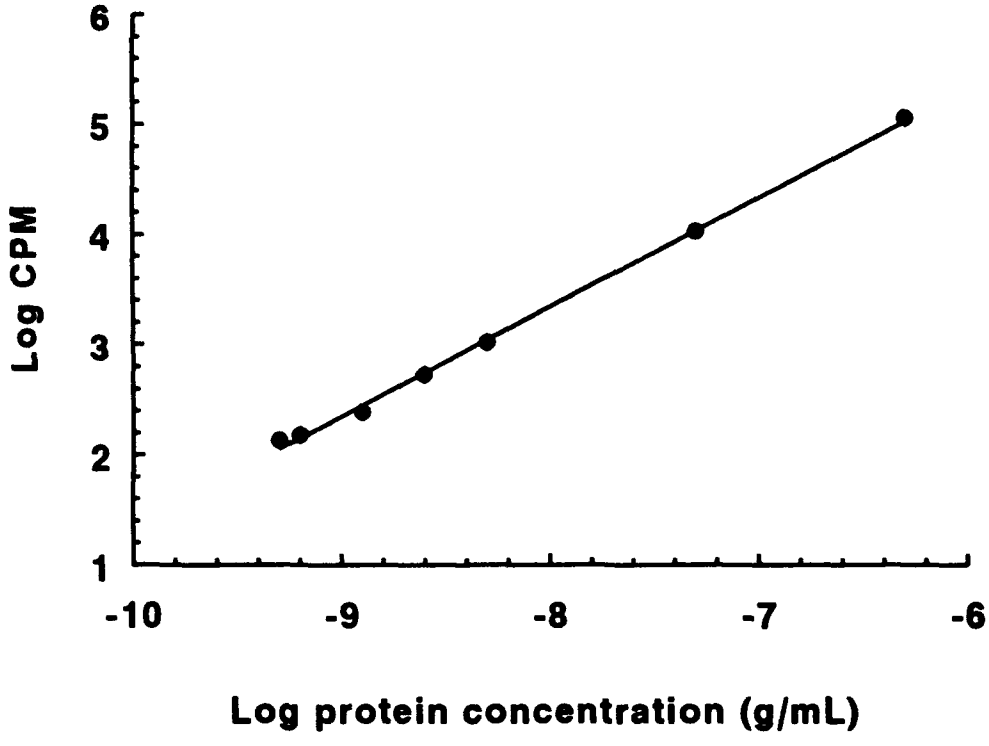

Figure 6. Standard curve of radioactivity (cpm) versus spiked samples of ^{125}I-labelled goat IgG. Aliquots (1 mL) of labelled antibody were delivered to vials and counted in a scintillation counter.

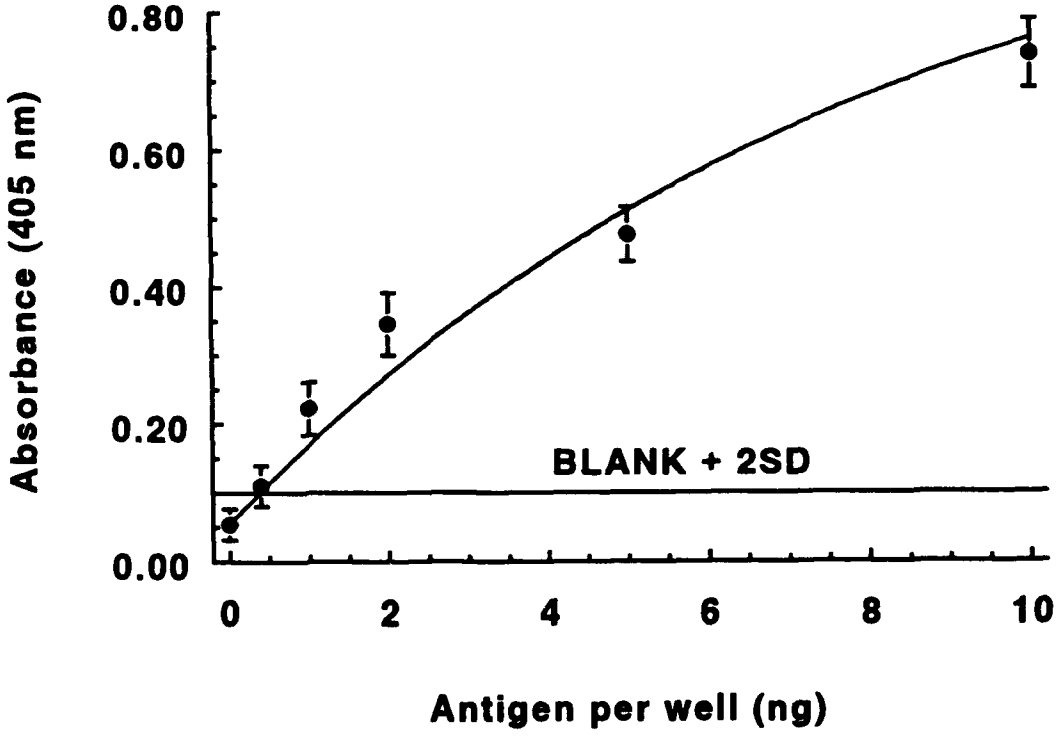

Figure 7. Enzyme immunoassay of NDV using quartz as the solid support. Capture antibody was covalently attached to the solid phase. The LOD was determined as the sample which produced a signal in excess of the background plus two standard deviations. For this assay the LOD was about 0.5 ng NDV in a total volume of 0.1 mL (5 ng/mL).

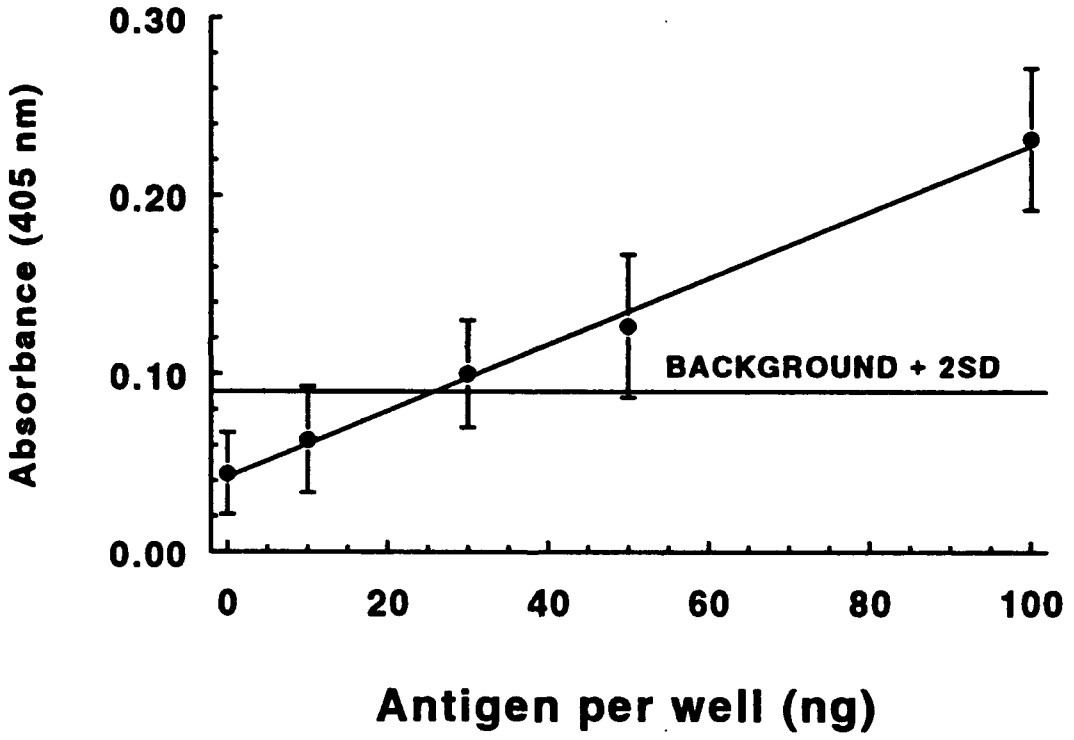

Figure 8. Enzyme immunoassay of NDV on quartz with capture antibody non-covalently attached to the solid phase. The LOD was determined to be about 50 ng in a total volume of 0.1 mL.

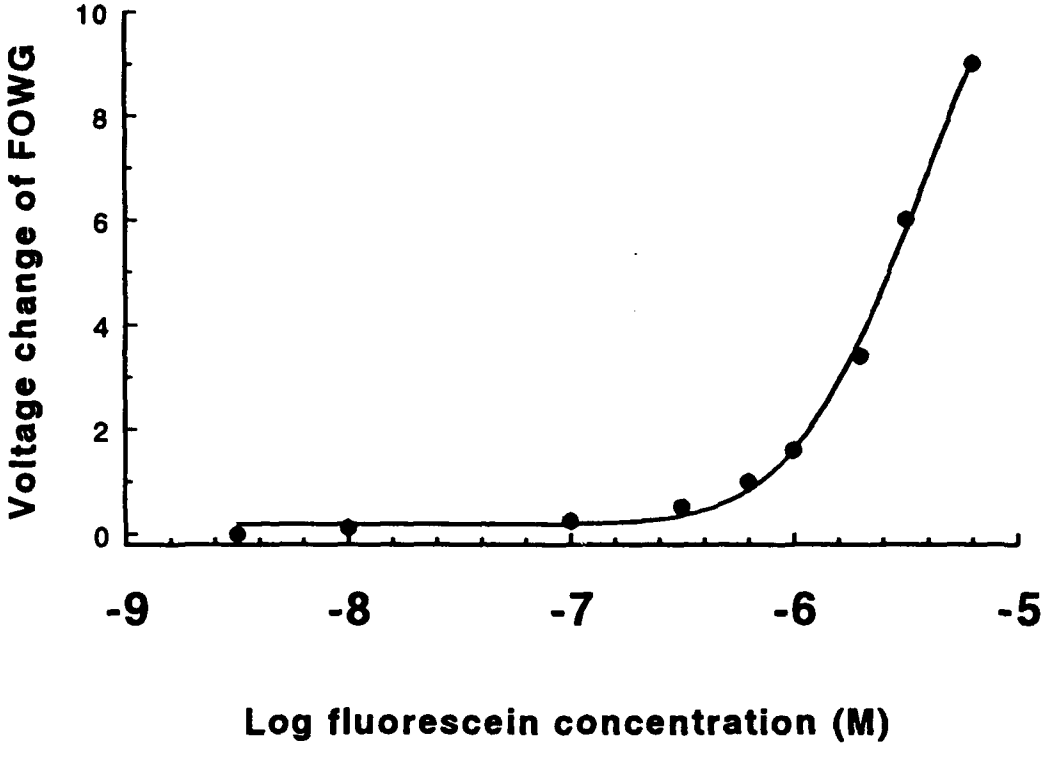

Figure 9. Response of the FOWG to aqueous fluorescein solution.

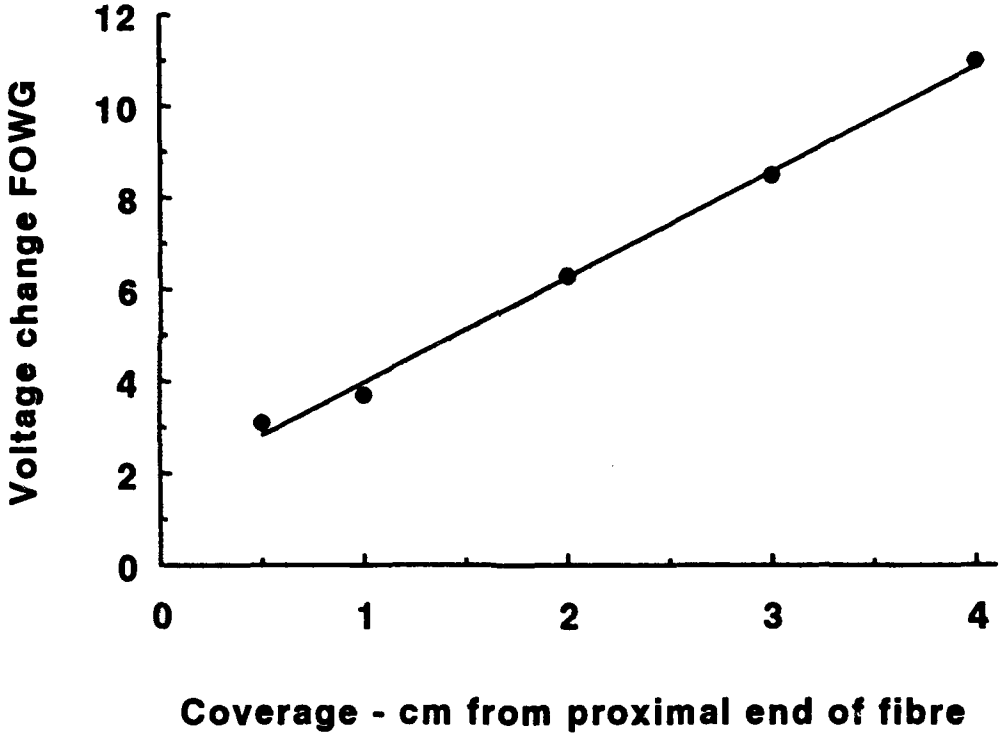

Figure 10. Determination of the active area of the quartz fibre in the FOWG. Fibres were covalently coated with guinea pig IgG and then incubated with fluorescein-labelled anti-guinea pig IgG (0.005 μg/mL) with varying lengths of the fibre exposed to the second antibody.

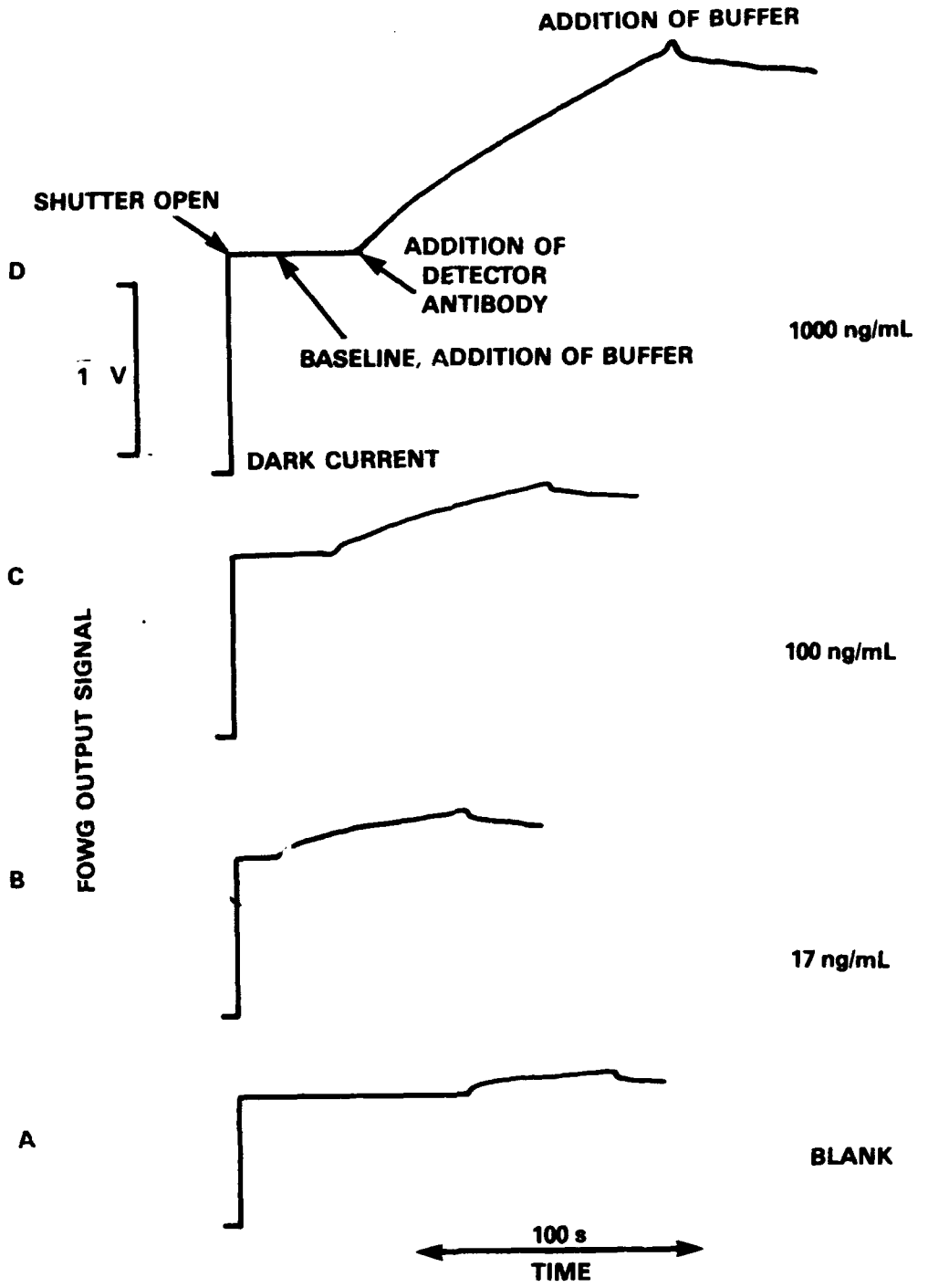

Figure 11

Sequential assay of NDV on the FOWG. A quartz fibre was incubated with NDV solution (0.5 mL) in a polystyrene test tube for 5 min at room temperature. After incubation the fibre was washed three times with PBS and placed in the flow cell of the FOWG. A baseline was recorded while flowing PBS through the system. Detector antibody solution (fluorescein-labelled guinea pig anti-NDV), 1 μl/mL, was pumped through the flow cell at rate of 1.2 mL/min. A separate quartz fibre was used for each run.

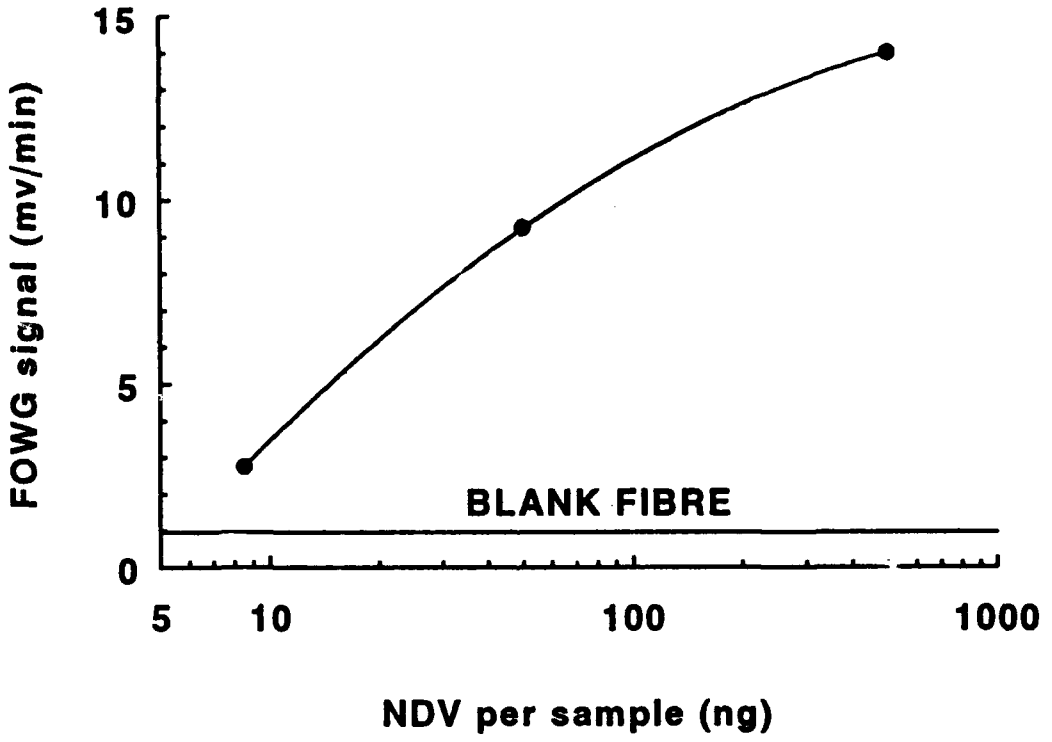

Figure 12. Plot of FOWG signal output versus NDV; the slopes of the tracings (mv/min) in Fig. 11 were determined 1 min after the addition of detector antibody. A separate fibre was used for each run. The horizontal line was the signal obtained from a blank fibre, reagents but no NDV.

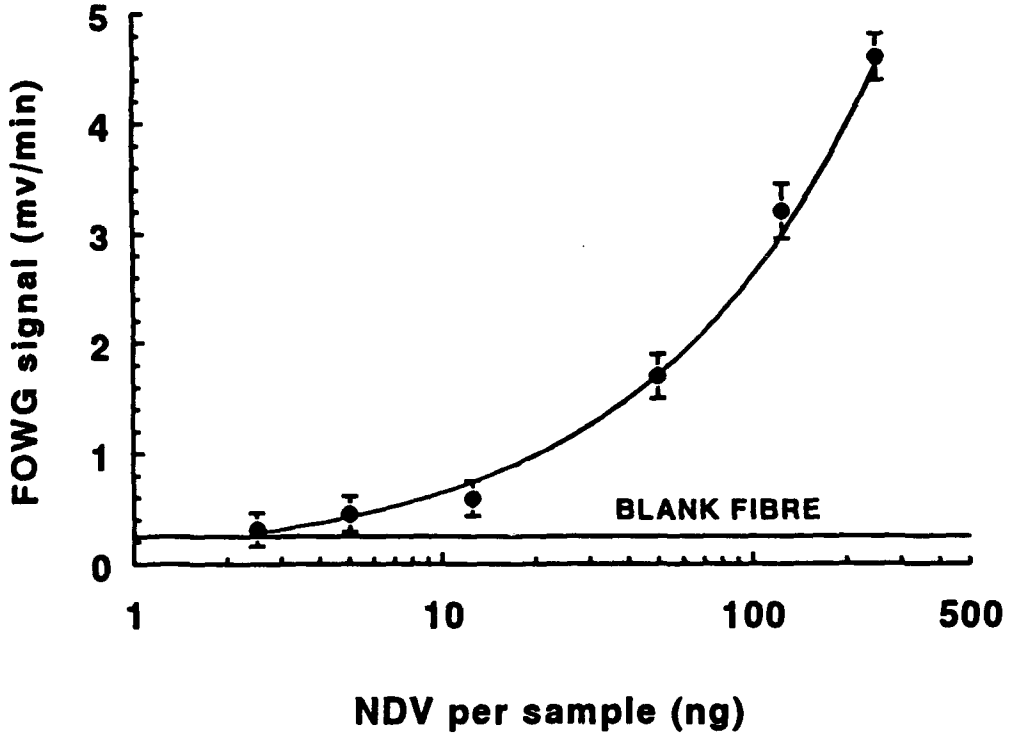

Figure 13. Plot of FOWG signal versus NDV. The slopes were determined 1 min after addition of detector antibody; 5 min external incubation, detector antibody concentration 1 μg/mL. The data points are the average of three runs; the bars represent the standard deviations. A separate fibre was used for each run.

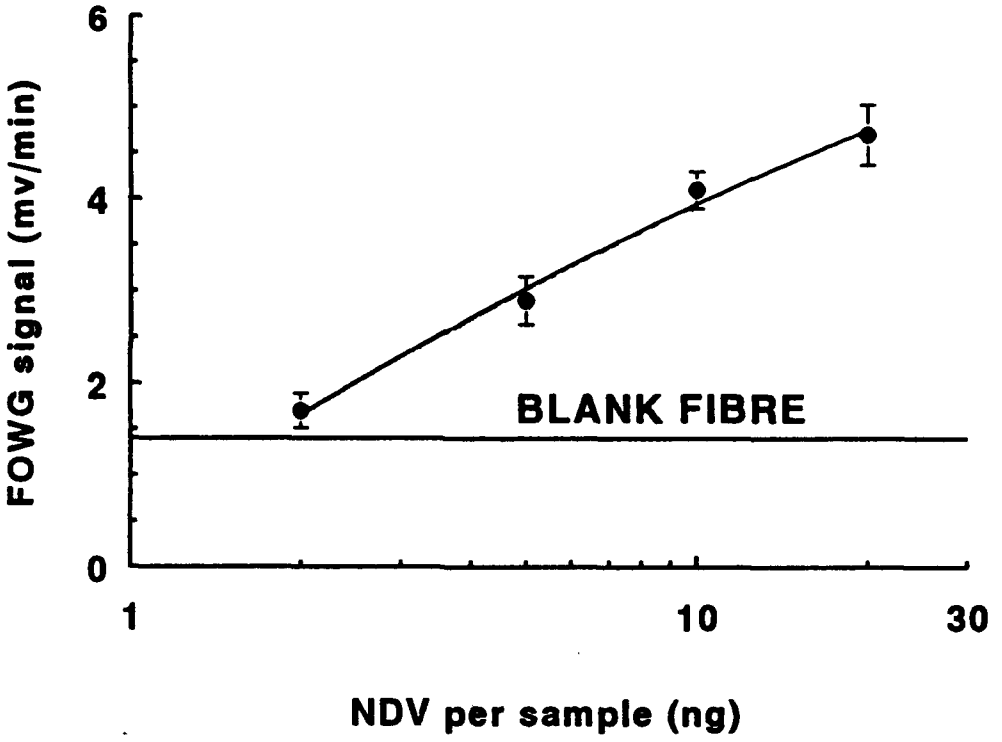

Figure 14. Plot of FOWG signal versus NDV carried out on a single fibre. Each data point is the average of three runs; the bars represent the standard deviations. The sample was incubated for 5 min in the flow cell; detector antibody concentration was 1 μg/mL.

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A fluorometric sandwich immunoassay for Newcastle disease virus (NDV) was developed using a fibre optic biosensor. Antibodies directed against NDV were covalently attached to the surface of silane-coated quartz fibres and were used to capture the antigen onto the optical element. Fluorescein-labelled anti-NDV served as the detector antibody. Assay times were approximately 10 min in duration with a limit of detection of 5 ng of purified virus. The biosensor detection system possesses several desirable characteristics such as a limited number of mechanical components, a reusable solid support and low baseline variation. These and other properties of the fibre optic biosensor indicate that it has the potential to serve as an on-line continuous monitoring device of an automated or semiautomated detection system. As a part of the fibre optic biosensor development, the surface coverage of the quartz fibres by capture antibody was characterized and an enzyme immunoassay for NDV which emp >yed quartz fibres as the solid support was undertaken. This work was conducted as part of a trinational (UK/US/CA) effort on the Biochemical Detector.

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