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A rapid nonradioactive sandwich immunoassay which utilizes biotin-streptavidin mediated filtration capture of immune complexes in conjunction with a silicon sensor was developed for the detection of virus. Using purified Newcastle disease virus as a model, the lower limits of detection (LOD) were determined for a number of immunoassay configurations employing both monoclonal and polyclonal antibodies. The LODs ranged from 1.3 ng/ml (sample volume of 100 μl) for an incubation of 60 min to 400 ng/ml for a 1 min incubation. The sandwich immune complexes were formed from one-step incubation of antibody and antigen. No 'hook' effects were observed over a wide range of analyte concentrations. The assays were easy to perform and required a total time equal to the incubation period plus about 5 min. The assay format is suitable for virus, bacteria and protein antigens. New assays can be developed and optimized readily, often within 1 day.

Key words: Newcastle disease virus; Streptavidin; Biotin; Filtration; Detection; Biosensor; Light addressable potentiometric sensor; Immunoassay

Introduction

The role of biosensors is increasingly important in detection technology. Biosensors combine the recognitive power of biological macromolecules with microelectronic signal transducers and provide a number of advantageous qualities such as high sensitivity and specificity, low power requirements, durability, and the capacity for miniaturization (Karube, 1987). When coupled with advances in immunoassay technology, biosensors provide a powerful tool for analytical biochemistry.

In this paper we describe a silicon sensor-based immunoassay for the detection and quantitation of virus in liquid samples. The reagent antibodies and the antigen were incubated together in a single step. The resulting sandwich immune complexes were filtered through biotin-embedded nitrocellulose membrane and immobilized on the membrane by means of biotin-streptavidin interactions (Green, 1975; Wilchek and Bayer, 1988). The presence of antigen on the membrane was detected by monitoring the enzyme activity associated with urease-conjugated antibodies in the immune complex. Monitoring was carried out by wetting the membrane with a solution of substrate, urea, and then placing the membrane in contact with the surface of a pH-sensitive light

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addressable potentiometric (LAP) sensor (Hafeman et al., 1988; Bousse et al., 1990).

Using Newcastle disease virus as a model we have determined the limits of detection for incubation times ranging from 1 min to 1 h. The sensitivity of the assay was found to be approximately equal to that of conventional microtiter assays which utilize polystyrene plates and a chromogenic substrate, however the total time required and effort expended were significantly reduced. A number of combinations of monoclonal and polyclonal antibodies were used for capture and detection, and although some differences in sensitivity were observed for the various combinations, all gave comparable results. Limits of detection for one-step assays of 1 h incubation were on the order of 2--20 ng/ml virus protein.

Materials and methods

Reagents

Bovine serum albumin (BSA), sodium dihydrogen phosphate, Tween 20, Triton X-100, urea, and goat anti-mouse IgG urease conjugate were obtained from Sigma Chemical Co. (St. Louis, MO) and used without any further purification. Streptavidin was obtained from Scripps Laboratories (San Diego, CA). It was reconstituted in distilled water to yield a stock concentration of 10 mg/ml. Hybridoma clones producing anti-NDV monoclonal antibodies were obtained under contract from the University of Alberta (Edmonton, AB). Monoclonal antibodies against NDV were purified from ascites fluid using high-performance liquid chromatography as described previously (Wong et al., 1992). Polyclonal antibody was purified from guinea pig serum using ammonium sulfate precipitation, followed by ion exchange chromatography. Anti-NDV was conjugated to urease using glutaraldehyde and was carried out by J.D. Biologicals (Brampton, ON). Antibody labelling reagents, *N*-hydroxysuccinimide esters of carboxyfluorescein and dinitrophenylbiotin, were obtained from Molecular Devices (Menlo Park, CA) and were used according to procedures described in the product literature. Nitrocellulose membrane filters (0.44 μm pore size) coated with biotinylated BSA, and anti-fluorescein urease conjugate were purchased from Molecular Devices. The antigen, NJ-La Sota strain of NDV, was obtained from the American Type Culture Collection (Rockville, MD), cultivated in the allantoic cavity of embryonated hen eggs (Hawkes, 1979), purified by sucrose gradient centrifugation (Fulton et al., 1988) and suspended in phosphate buffered saline pH 7.0. Protein concentrations were determined spectrophotometrically with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL)

Wash solution consisted of 150 mM NaCl, 10 mM phosphate buffer, pH 6.5, plus 0.2% Tween 20 detergent. The dilution buffer was the wash solution titrated to pH 7.0, containing 1% albumin and 0.25% Triton X-100. The substrate solution for the reader compartment of the LAP sensor was wash solution containing 100 mM urea.

Apparatus

The apparatus for the immunoassays was a commercially available LAP sensor, marketed under the name Threshold Unit. It was purchased from the manufacturer, Molecular Devices. The instrument was controlled by an IBM PS/2 model 30 microcomputer and custom designed software, supplied by Molecular Devices. The assay system was capable of processing (filtration, potentiometric sensing) eight samples simultaneously.

Immunoassay procedures

Fig. 1 provides the schematic representations of the sandwich immunoassays. For scheme A, the reagent solution consisted of 25 μl of urease conjugate (stock conc., 0.5 mg/ml), 50 μl of biotin conjugate (stock conc., 0.5 mg/ml) and 20 μl streptavidin (stock conc., 10 mg/ml) in 12 ml of dilution buffer. The reagent solution was allowed to stand at room temperature for 3 h. A volume of 150 μl of reagent solution was added to each 100 μl aliquot of NDV sample. The reagent and sample were mixed thoroughly and incubated at room temperature for the required length of time. At the end of the incubation period, 150 μl of the incubated reagent-sample mixture, was delivered to a well of the filter assembly of the Threshold Unit, filtered (200 μl/min) and rinsed with 0.5 ml wash solution.

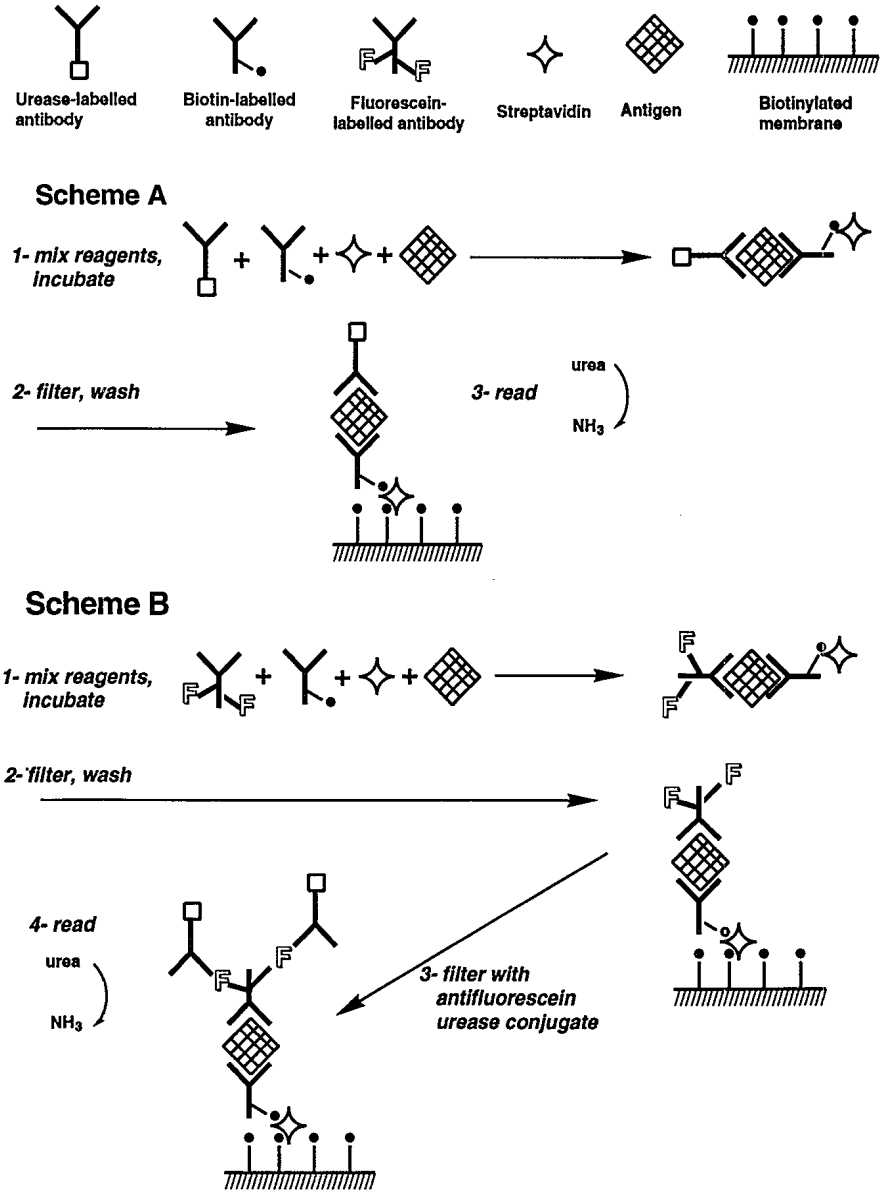

Fig. 1. NDV immunoassay. Scheme A: urease directly conjugated to anti-NDV. Scheme B: second antibody, anti-fluorescein urease conjugate. Scheme C: (not shown) anti-mouse IgG urease conjugate, analogous to scheme B, except using unlabelled mouse IgG anti-NDV and anti-mouse IgG urease conjugate as the second antibody combination.

The 150 μl aliquot contained 100 ng, 200 ng and 1.5 μg, respectively of urease conjugate, biotin conjugate and streptavidin.

For scheme B (Fig. 1), approximately 20 μl biotin-labelled and 20 μl fluorescein-labelled antibody preparations (typically 0.5 mg/ml) were added to 10 ml dilution buffer so that a 100 μl portion of the solution contained approximately 100 ng of each antibody. Aliquots (100 μl) of the diluted antibody solution and of streptavidin (10 μl, 0.1 mg/ml) were added to 100 μl of NDV samples suspended in dilution buffer, mixed, incubated for the required time, filtered through the nitrocellulose membrane and rinsed with wash solution (0.5 ml). After the rinsing step, 200 μl of anti-fluorescein urease conjugate (stock solution diluted 1 to 10 in dilution buffer) were filtered through the membrane containing the immune

complex. For scheme C, a similar procedure to scheme B was used, whereby a biotin-labelled guinea pig polyclonal anti-NDV and an unlabelled monoclonal anti-NDV and were incubated with the test sample. After the filtration and rinse, a volume of 200 μl of anti-mouse IgG urease conjugate (0.5 μg/ml) was added to each well and filtered at 200 μl/min.

After the filtration capture procedures, the membrane sticks with the immobilized urease-containing immune complexes were inserted into the reader compartment which contained the LAP sensor and substrate solution. A plunger pressed the membrane against the surface of the silicon sensor. The instrument was designed so that the spots on the surface of the membrane which contained immobilized immune complex aligned with the pH sensitive measurement sites on the surface of the LAP sensor. The data points were recorded and stored on the microcomputer using the manufacturer supplied software. The rate of change of pH with respect to time at the surface of the silicon sensor was monitored by the rate of change with respect to time of the surface potential as μV/s.

Results

Signal-to-noise ratio versus incubation time

In this work, the sandwich antibody-antigen complexes that constituted the products of the immunoreactions were derived from one-step incubations. In the reaction scheme of the assay (Fig. 1), the rate of change of pH vs. time, and hence the output signal of the LAP sensor, will depend upon the number of urease-containing antibody-antigen sandwiches immobilized on the membrane during the filtration capture process. Since the efficiency of the biotin-mediated filtration capture of streptavidin is high (approximately 95% under the present conditions) (Olson et al., 1990), the number of the urease-containing antibody-antigen sandwiches immobilized will be a measure of the number formed during the incubation period.

A series of immunoassay experiments was undertaken to investigate the effect of the incubation time upon the output response of the LAP sensor. The aspect of the response of interest was the signal-to-noise ratio (S/N) and its variation with respect to the length of the incubation time of the antibodies and antigens. The noise component of the ratio (N) was the background of the assay, that is, the output of the LAP sensor for the reagents alone, with no antigen. The signal component (S) was the output of the LAP sensor from a test sample of 500 ng/ml NDV (100 μl). For a given incubation time, four of the test sites of the capture membrane contained the NDV samples, the other four sites were reserved for the reagents alone (noise).

The test samples were incubated at room temperature for periods of time ranging from 1 min to 2 h and the values of S/N of the assays were plotted as a function of incubation time (Fig. 2). There was a continuous increase in S/N up to and including the longest incubation time (125 min). The value of S/N at 125 min was 7.6 which appears close to a maximum. For incubations of 1 and 5 min S/N, was found to be 1.5 and 2.2, or 20% and 29% of the signal obtained from an incubation of 125 min.

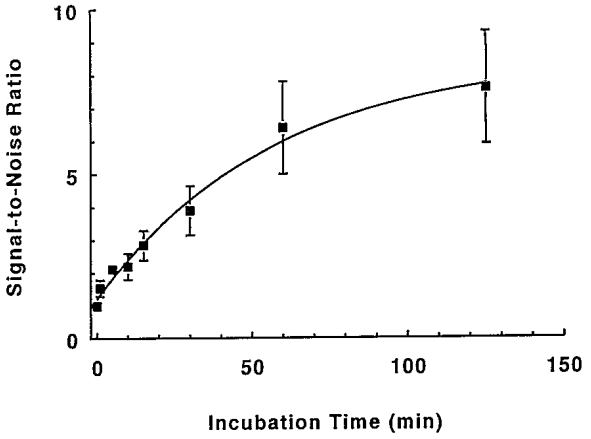

Fig. 2. The signal-to-noise ratio vs. incubation time for a LAP sensor assay (scheme A) of NDV. The signal component of the ratio was the sensor output for a 50 ng sample (500 ng/ml, 100 μl volume) of NDV; the noise component was the background, i.e., immunoreagents, no NDV. Each point represents the quotient of the mean signal ($n = 4$) and the mean noise ($n = 4$). The bars represent $\pm$sum of 1 SD of signal and 1 SD of noise.

The response of the LAP sensor to NDV

The response of the LAP sensor was monitored over a large range of NDV concentrations. At high concentrations of antigen, one-step immunoassays are generally susceptible to a 'hook' effect, whereby increasing antigen concentrations produce decreasing signals (Matsukura et al., 1971; Khosravi, 1990; Fernando and Wilson, 1992). This effect occurs when the antibody reagents are no longer in molar excess over the antigen and the increased amounts of antigen subsequently diminish the probability of the formation of antibody-antigen sandwiches. The results of a representative assay with NDV concentrations ranging from 380 ng/ml to 100 μg/ml are shown in Fig. 3. The output of the LAP sensor (μ/s) increased monotonically as a function of antigen concentration; there was no 'hook' effect observed in the assay although the slope of the curve (i.e., sensitivity of the assay) was reduced at the higher antigen concentrations. These results suggested that microgram quantities of virus are detectable at the high concentration end of the dose response curve and that a single reagent formulation can be used for a wide range of analyte concentrations. False negative responses of the LAP sensor due to large antigen excesses were considered unlikely.

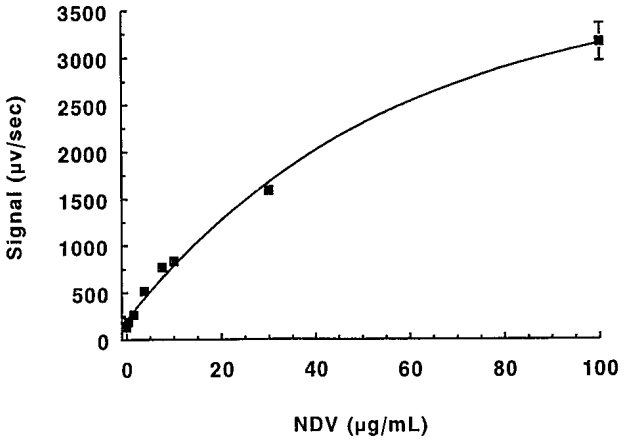

Fig. 3. The response (scheme A, 5 min incubation) of the LAP sensor to NDV. Each point represents the mean of three determinations. The error bars, where not masked by the data-point markers, represent ±1 SD.

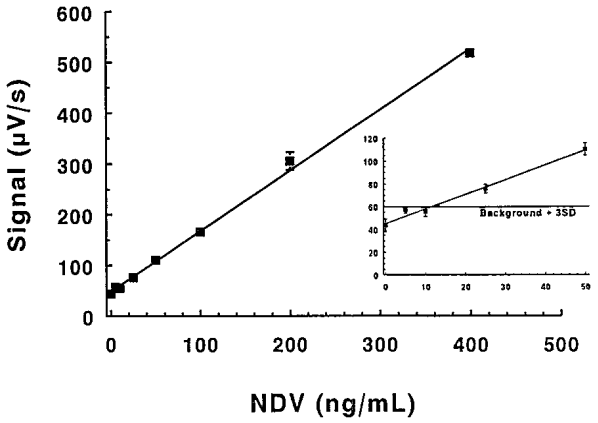

Fig. 4. NDV standard curve employing scheme A and a 60 min incubation. Each point represents the mean of four determinations. Error bars, where not masked by the data point markers represent ±1 SD. The inset shows an expanded 0–50 ng/ml region. The LOD, taken to be the intersection of the calibration curve with the background (0 NDV)+3 SD was 12 ng/ml (sample volume 100 μl).

Limits of detection of the LAP sensor for NDV

Urease-conjugated anti-NDV. The lower limits of detection (LOD) of the LAP sensor for NDV were determined at three incubation times (1, 5 and 60 min) using monoclonal anti-NDV directly conjugated with urease (see scheme A in Fig. 1). The results of a 60 min assay of NDV are presented in Fig. 4. The standards on the calibration curve ranged from 5 ng/ml to 400 ng/ml and were run simultaneously on a single capture membrane stick. Each data point represents the mean of three consecutive assays performed on the same day using the same reagents. The data, μV/s versus ng of antigen, was represented well by a linear plot. The error associated with the individual points was small: the coefficient of variation (CV), defined as the ratio of the standard deviation (SD) to the mean was about 6%. The LOD of the assay was 12 ng/ml. At the low concentration regime of the dose response curve, viz., near the limits of detection, the standard errors on the background were approximately equal to the standard errors of the samples. The LOD in this work was defined as a smallest sample having an output signal greater than the background plus three standard deviations of the background. Subsequent assays of NDV employing freshly prepared antibody and antigen solu-

tions, and identical reaction conditions provided LODs of comparable values. Assays of NDV carried out in a similar manner with incubation of 1 and 5 min (data not shown) gave LODs of 400 and 220 ng/ml, respectively (see Table I for a summary). The results of the 60 min assay compare favorably with a multistep sandwich chromogenic ELISA for NDV having incubation times of 1 h per step in which the LOD was about 10–20 ng/ml (R.E. Fulton, unpublished results).

Variations from day to day (that is, assay to assay) were observed in the slopes and the intercepts of the calibration curves were observed. The output (μV/s) depends upon the rate of hydrolysis of urea by the enzyme, urease, immobilized at the surface of the nitrocellulose membrane. Rates of hydrolysis are influenced by such factors as ambient temperature and pH of the buffers. Small day-to-day differences in these factors as well as the use of different stocks of conjugated antibodies may have combined to account for the observed variations. The day-to-day (or assay-to-assay) variation of the slope for a given incubation time was about 20% and somewhat higher for the background, about 50%. Despite the variations in slopes and background from day to day, the LODs were essentially constant ($\pm$25%).

Second antibody, anti-fluorescein urease conjugate. In place of the urease-conjugated anti-NDV described above, the same monoclonal antibody, labelled with fluorescein was employed (see scheme B, Fig. 1). Antigen and antibodies were incubated in a single step, filtered through nitrocellulose membrane, and washed with buffer. A solution of anti-fluorescein urease conjugate was then filtered through the membrane containing the immobilized immune complex. Using the monoclonal antibody combination of 55R3-biotin and 25R5-fluorescein, a LOD of 3.0 ng/ml was obtained. Similar values of the LOD were obtained using the reverse of the antibody pair, namely, 25R5-biotin and 55R3-fluorescein. Employing a single monoclonal, 25R5-biotin and 25R5-fluorescein, the LOD was only slightly higher, 6.3 ng/ml, for a 60 min incubation assay.

A polyclonal (guinea pig) anti-NDV was also used in this assay format (scheme B, Fig. 1) conjugated with biotin and with fluorescein. Under similar reaction conditions, a 60 min incubation assay yielded an LOD of 1.3 ng/ml (Table I).

Second antibody, anti-mouse IgG urease conjugate. In this configuration (see caption, Fig. 1) the biotin-labelled polyclonal anti-NDV described above was used as the capture antibody in conjunction with an unlabelled anti-NDV monoclonal (55R3). After the incubation, filtration and wash procedures, anti-mouse IgG urease conjugate was filtered through the membrane containing the immobilized immune complexes. The background of the assay was substantially higher than that seen in the other assays schemes but despite the magnitude of the background, it was stable from run to run. The LOD was calculated to be about 5 ng/ml (Table I). The source of the elevated background is probably a result of cross-reactivity between the urease-labelled anti-mouse IgG and the biotin-labelled guinea pig polyclonal. Nonetheless, the anti-species second antibody format allowed detection at levels comparable to the other formats. In principle, a better resolution may be obtained by incubation with an antibody pair that was less susceptible to cross-reaction. In this study, however, the overall selection of antibodies was limited.

TABLE I

SUMMARY OF THE LIMITS OF DETECTION FOR NDV AS A FUNCTION OF INCUBATION TIME AND ANTIBODY CONFIGURATION

Scheme [a]	Upper Ab [a]	Lower Ab [a]	LOD (60 min) ng/ml	LOD (5 min) ng/ml	LOD (1 min) ng/ml
A	55R3-u [b]	25R5-b [b]	12	220	400
A	25R5-u	55R3-b	18		
B	25R5-f [b]	55R3-b	3.0		
B	55R3-f	25R5-b	3.0		
B	25R5-f	25R5-b	6.3		
B	55R3-f	55R3-b	6.5		
B	pc-f [b]	pc-b	1.3		
C	55R3	pc-b	5.0		

[a] See Fig. 1 for antibody reaction schemes.

[b] u,b, and f indicate antibody labelled with urease, biotin, fluorescein, respectively; 55R3, 25R5 indicate antibody clones described in text; pc indicates polyclonal antibody.

Quantitation of NDV on the LAP sensor

The ability of the LAP sensor to provide quantitative analysis of NDV was demonstrated. Series of calibration standards and test samples were prepared and then assayed on the LAP sensor. There was good agreement between the measured values of the test samples and the actual concentrations. For eight different test sample concentrations of NDV in the range of 20–400 ng/ml, the mean difference between the measured and the actual amounts was about 9%. A linear regression analysis yielded a high correlation ($r^2 = 0.998$) between the measured and actual amounts (Fig. 5).

Effects of antibody concentration to output signal

The effects of varying the concentrations of biotin- and fluorescein-labelled antibodies in the one-step immunoassays were investigated for two antibody pairs. For the pair, 25R5-biotin/55R3-fluorescein, the signal-to-noise ratio increased with increasing amounts of fluorescein-labelled antibody (that is, lower *-b/-f* ratio). On the other hand, the pair, 55R3-biotin/25R5-fluorescein showed a slight increase in *S/N* with increasing amounts of biotin-labelled antibody (larger *-b/-f* ratio). The greater *S/N* ratios did coincide with somewhat smaller LODs, as summarized by the data in Table II. In both antibody pairs, it was greater amounts of the 55R3 antibody which provided greater *S/N* ratio. For the most part, the sensitivity of the assays was largely independent of antibody concentration (both total antibody concentration and *-b/-f* ratio) and a fixed amount of each antibody (about 100 ng per 100 μl of reagent) worked well in all cases. These findings were in keeping with the results of LAP sensor assays of protein and bacteria (H.G. Thompson and W.E. Lee, unpublished results).

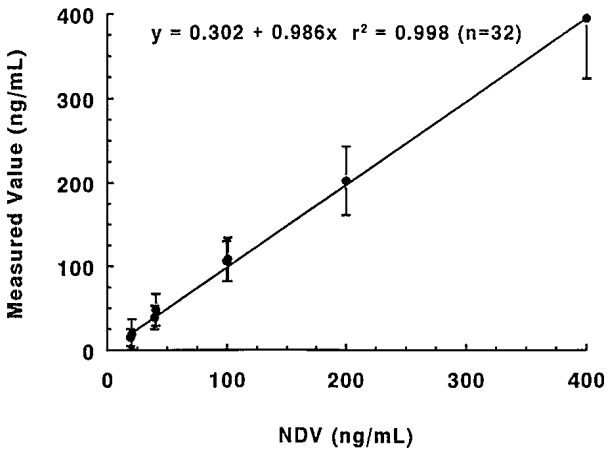

Fig. 5. Quantitation of NDV on the LAP sensor (using scheme A). Each point represents the mean ($n = 4$) of an independent determination of NDV concentration. The error bars represent ±1 SD.

TABLE II

EFFECTS OF ANTIBODY CONCENTRATION ON SIGNAL-TO-NOISE RATIO AND LOD

[b] [a]	[f] [a]	*-b/-f* [b]	*S/N* [c] (12.5 ng)	*S/N* [c] (0.5 ng)	LOD (ng/ml)
25R5	55R3	4/1	8.3		
25R5	55R3	2/1	10.2	1.1	6.3
25R5	55R3	1/1	7.9	1.2	4.2
25R5	55R3	1/2	13.8	1.2	2.7
25R5	55R3	1/4	17.2		
55R3	25R5	4/1	8.6	1.5	1.3
55R3	25R5	2/1	6.2	1.2	7.1
55R3	25R5	1/1	3.8	1.1	3.6
55R3	25R5	1/2	3.7		
55R3	25R5	1/4	3.9		
			S/N [d] (40 ng)		
25R5	55R3	1.5/1.5	2.4		
25R5	55R3	3/3	2.7		
25R5	55R3	8/8	2.6		

[a] Biotin-labelled and fluorescein-labelled monoclonal anti-NDV.

[b] Ratio (w/w) of biotin-labelled to fluorescein-labelled antibody; 1, 1.5, 2, 3, 4, 8 represent 25, 37.5, 50, 75, 100, 200 ng of labelled antibody, respectively, per 100 μl reagent solution.

[c] Signal-to-noise ratio (noise = blank assay; i.e., antibodies, reagents, no antigen) for 100 μl samples containing 125 and 5 ng/ml, respectively, of NDV; incubation time was 30 min.

[d] Signal-to-noise ratio (as above) for 100 μl samples containing 400 ng/ml of NDV, incubation time was 90 s.

Discussion

In this report we have described a sandwich immunoassay having a one-step liquid-phase incubation of antigen and antibodies followed by a biotin-streptavidin mediated filtration-separation. The overall assay time was governed by the time allotted to the incubation of the analyte and

antibodies to form the sandwich immune complex. Maximal sensitivity could be attained with incubations of about 1 h, however shorter incubations of 1 and 5 min were feasible, although the signal-to-noise ratios were reduced and limits of detection somewhat higher. In general, the total assay time was the incubation period plus about 5 min. The LAP sensor assay system yielded sensitivities comparable to conventional microtiter plate formats but the total time and the number of steps required was greatly reduced. Several combinations employing monoclonal and polyclonal antibodies were investigated in the development of the immunoassay. It was found that the reaction scheme using a second antibody anti-fluorescein urease conjugate provided smaller LODs and smaller CVs than did the scheme using detection antibodies directly conjugated to urease. The sensitivity of the viral assay presented here is comparable to the sensitivity of commercial radioimmunoassays for hepatitis B virus (AUSRIA, Abbott Laboratories).

The use of biotin-streptavidin mediated immobilization and fluorescein-anti-fluorescein mediated attachment of the detector enzyme, urease, allowed for generic capture and signal generation. Antibodies, or other macromolecular units that can be readily labelled with biotin can be easily immobilized via free streptavidin on the biotin coated surfaces (Wilchek and Bayer, 1988). In the present work, nitrocellulose membranes (0.44 μm pore size) were coated with biotinylated BSA which, in addition to providing immobilization, also helped reduce nonspecific protein binding to the surface. For samples containing particulate or cellular material, similar nitrocellulose membranes with 5 μm pore size are effective. For detection of the immune complex, the task of labelling the analyte-binding components with a small molecule such as fluorescein (and then purifying and characterizing the labelled product) is much easier than conjugating the antibody to a large-sized enzyme (and carrying out subsequent purification/characterization). The anti-fluorescein-urease conjugate need be prepared only once or obtained from a commercial source as was the present case.

The antibody labelling compounds used herein provide a simple method for measuring the molecular ratios of fluorescein or biotin to protein. These compounds aid in assay reproducibility when a new lot of antibody is employed. Fluorescein has intrinsic UV/Vis absorption at 490 nm and the incorporation ratio can be determined spectrophotometrically from the 490 and 280 nm absorbance of the labelled antibody preparation. The biotin molecule, itself, has no intrinsic UV/Vis absorption, however the biotin reagent employed (a *N*-hydroxysuccinimide ester) possesses a dinitrophenyl moiety, which although immunochemically inert provides a spectroscopic marker with absorption at 360 nm.

The anti-species assay employing an anti-mouse IgG urease conjugated second antibody, despite the high background signals, yielded LODs comparable to those using anti-fluorescein. Lower background would be obtained with antibody pairs having less cross-reactivity than guinea pig and mouse immunoglobulins. The utility of this approach is that the assay requires a single biotin-labelled antibody. At present, a variety of anti-species urease conjugates are available from commercial sources (J.D. Biologicals, Brampton, Ontario, Canada).

The monoclonal antibodies selected for this study were directed against different epitopes on the virus envelope, as determined by competitive fluorogenic ELISA (Wong et al., 1992). Thus in the reaction schemes employing both 55R3 and 25R5 antibodies, there was no competition between the upper (fluorescein-labelled) and the lower (biotin-labelled) antibodies. However when NDV was incubated with biotin conjugates and fluorescein conjugates of the same monoclonal, competitive binding on the virus surface did not effect the sensitivity significantly. The repetition of antigenic sites on the surface was great enough to allow the binding of sufficient numbers of biotin-labelled and fluorescein-labelled antibodies to effect both capture and detection. Similarly, the repetition of antigenic sites was a factor in minimizing potential 'hook' effects at high analyte concentration. Polyclonal antibody, when used as both the biotin-labelled and fluorescein-labelled binding component provided the smallest LOD values.

Assays typically contained about 100–200 ng each of biotinylated (lower) and upper antibody

and 1.5 μg streptavidin per test sample. These amounts provided an excess of antibody over NDV in the samples used for the calibration curves and quantitations, as well as a excess of streptavidin over the antibody-biotin conjugate. In this study and in other work employing the LAP sensor for bacteria and protein quantitation (H.G. Thompson and W.E. Lee, unpublished), it was found that the concentrations of the antibodies used in the one-step incubations were not very critical. Satisfactory working dilutions of the antibodies could be easily obtained.

The development of immunoassays for other antigens by the methods described herein can be achieved quickly. Starting with at least one high titer antibody, the preparation of the biotin and fluorescein conjugates, generation of dose response curves, antibody optimizations and limits of detections analysis can be achieved in about 8–10 h.

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