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Short communication

An immunosensor with potential for the detection of viral antigens in body fluids, based on surface second harmonic generation

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Abstract

Field methods of assessing the immune status of animals are required to optimise vaccination programmes to control bovine viral diarrhoea (BVD) virus. An optoelectronic immunosensor was evaluated for the detection of viral antigens in a crude cell lysate in a pilot study. Binding of (BVD) virus antigen by two monoclonal antibodies immobilised on two different media (ELISA plate wells, and glass coverslips) was detected and quantified using the laser induced surface second harmonic generation (SSHG) technique. The results for both assays were correlated with an enzyme-linked immunoassay (ELISA) used for the diagnosis of BVD virus infection in cattle (ELISA plate; $R^2 = 0.86$, coverslips; Exp. 1; $R^2 = 0.75$, Exp. 2; $R^2 = 0.67$). The method will allow rapid detection of antigens in the body fluids of farm animals.

Keywords: Biosensor; Optoelectronic; Immunosensor; BVD; Bovine

1. Introduction

The financial cost of the failure to control bovine viral diarrhoea and associated fatal mucosal disease was estimated at £47 million/year to the cattle industry in 1986 (Bennett and Done, 1986). There is also a significant welfare cost in the distress of diseased animals. A field test to detect bovine viral diarrhoea virus (BVDV) in saliva or milk would allow the identification of asymptomatic infected cows within a herd and the efficacy of any vaccination regime to be closely monitored and assessed.

A method is required to detect directly the capture, by a specific antibody immobilised on a surface, of its antigen present in body fluids, such as milk, urine or saliva. The aim should be to have a test suitable for use on farms to remove the need for laboratory analysis.

Several one-step optical techniques have been used to detect and quantify the central reaction of the binding of antibody to antigen (Severs et al., 1993; Brecht et al., 1995). In this work we have used the laser induced surface second harmonic generation (SSHG) technique in a new non-labelled optical immunoassay to detect viral components in a crude white blood cell lysate.

Optical second harmonic generation is a second-order non-linear optical process, which converts two incident photons of frequency ω into a single emission photon of frequency 2ω. SSHG occurs when light of high intensity is incident upon the surface of, or interface between, media (Fig. 1). The SSHG originates from the field and structural discontinuity at the interface. The signal is strongly dependent upon the angle of the incident light and the detector, and the degree of molecular orientation and concentration at the surface. Thus, alteration of the molecules at the surface will alter the second harmonic signal. The technique has recently been developed for immunoassay applications (McStay et al., 1995).

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2. Materials and methods

2.1. Detection system

The surface second harmonic detector experimental set-up consisted of a Q-switched Nd:YAG laser (Spectron LS400), which provided an output at 1064 nm, with peak pulse power of typically 10^9 W (FWHM = 5 ns). The fundamental laser beam, incident at a variable angle (70°) from the normal of the sample surface, resulted in second harmonic signals ($\lambda = 532$ nm) being generated at the surface. The SSH generated radiated along the direction of the reflected fundamental incident laser beam. The collected light was passed through a monochromator to remove the fundamental and spontaneous light from the laser along with any other background light. Light passing through the monochromator was then passed through a 532-nm interference filter (FWHM 10 nm) and detected with a photomultiplier tube (PMT) (Thorn EMI 9524A). The output of the PMT was recorded via a digital storage oscilloscope (Tektronix TDS 620).

2.2. Antibodies and antigens

The monoclonal antibodies (MAb) Wb103 and Wb112 were a gift from Dr D. Paton, Central Veterinary Laboratory, Weybridge. Hyperimmune calf serum 2359 was obtained from a gnotobiotic calf, nasally infected six times with a noncytopathic viral strain Pec515nc. Following three further challenges with the same strain by intramuscular vaccination, using, respectively, Freund's complete adjuvant, Freund's incomplete adjuvant and QuilA adjuvant, serum was prepared from the blood. Viral antigen was prepared from leucocytes extracted from the whole blood of a cow persistently infected with BVD. The leucocytes were lysed, and the lysed cell suspension was taken as an antigen preparation, containing high levels of viral particles and viral proteins.

2.3. Coating procedure

Wells of rigid ELISA plates (Falcon, Becton Dickinson) were each coated (overnight 4°C) with 50 μl of a 50:50 mixture of a 1:500 dilution in carbonate buffer (pH 9.6) of MAbs Wb103 and Wb112. Glass coverslips 15 × 15 × 1 mm were coated by immersion and incubation in 200 μl of the same MAb solution, or in 200 μl of a 1:1000 dilution of MAb Wb112 alone.

2.4. Test procedures

Three experiments were performed. In experiment 1, a 1:5 dilution in phosphate buffered saline containing Tween 20 (0.5%) and normal pig serum (5%) (ELISA wash buffer, EWB) of the antigen preparation was serially diluted in twofold steps across MAb + ve rows and control (MAb − ve) rows of the ELISA plate, in EWB. After incubation (2 h, RT), the plate was washed in EWB then distilled water, air dried and read in the SSH detector. To obtain ELISA results for comparison, an identically treated plate was further incubated with hyperimmune serum, (1:250, 50 μl/well, 2 h/37°C) washed and bound secondary antibody quantified with peroxidase conjugated anti-bovine IgG and chromogenic substrate following the manufacturers instructions (Sigma).

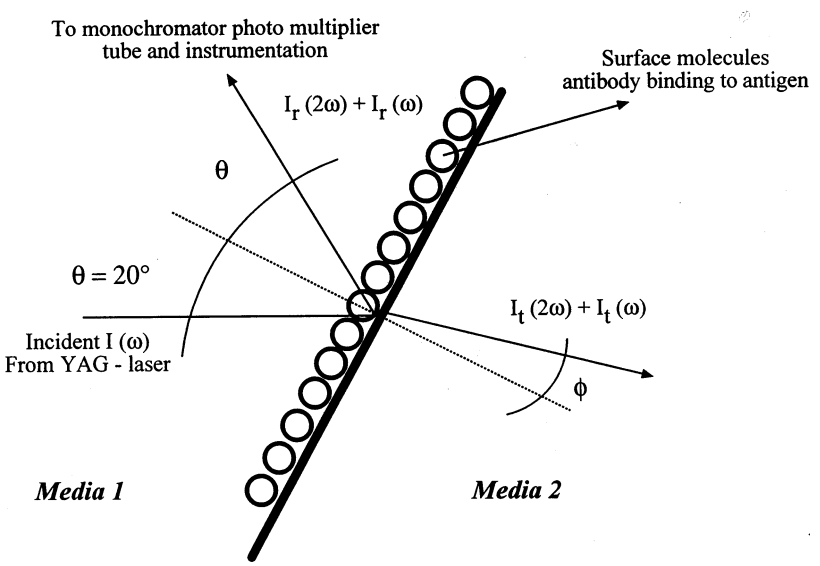

Fig. 1. Second harmonic generation from an interface between media. $I_r(\omega)$ and $I_t(\omega)$ are reflected and refracted fundamental beam, respectively. $I_r(2\omega)$ and $I_t(2\omega)$ are surface second harmonic signals generated by $I_r(\omega)$ and $I_t(\omega)$.

In experiment 2 MAb + ve and − ve coverslips were immersed in 200 μl of twofold dilutions of a 1:5 solution in EWB of the antigen preparation, incubated (2 h/37°C), and washed before reading. To confirm binding of antibodies and subsequently antigen to the coverslips, ELISA results were obtained by immersing the slips in secondary antibody, detection antibody and enzyme substrate solutions in turn, washing between each step. Stopped colour reactions (50 μl) were pipetted into ELISA plate wells for reading.

In experiment 3, coverslips were coated with MAb Wb112 alone, by incubation in 400 μl of 1:1000 antibody stock in coating buffer (4°C, O/N). The coverslips were gently washed in EWB, then one slip was placed in 200 μl of each of twofold serial dilutions, from 1:5 to 1:160 in EWB + 5% NPS, of virus positive or virus negative white cell antigen preparations, and incubated (RT, 3 h). After washing in EWB, three SSH readings were taken from each coverslip. Control coverslips not coated in MAb were also incubated in the positive and negative viral antigen preparations.

The dependence of the strength of the SSH signal on the incident laser power for several samples on the ELISA plates was examined. Within the range $0–3 \times 10^9$ W, the SSH signal was found to be linear with the square of the laser power. Above this power a non-linear effect was observed, due to laser-induced damage to the polypropylene of the microtitre wells. This effect was not seen with samples on the glass coverslips. The experiments were carried out with a laser power of 2×10^9 W. Each well to be read in the SSH detector was manually aligned with the laser beam, and the detector switched on. Readings (mV) were taken after 100 data acquisitions by the SSH detector. When the coverslips were used, they were cleaned on their reverse side, and clamped in a holder for reading.

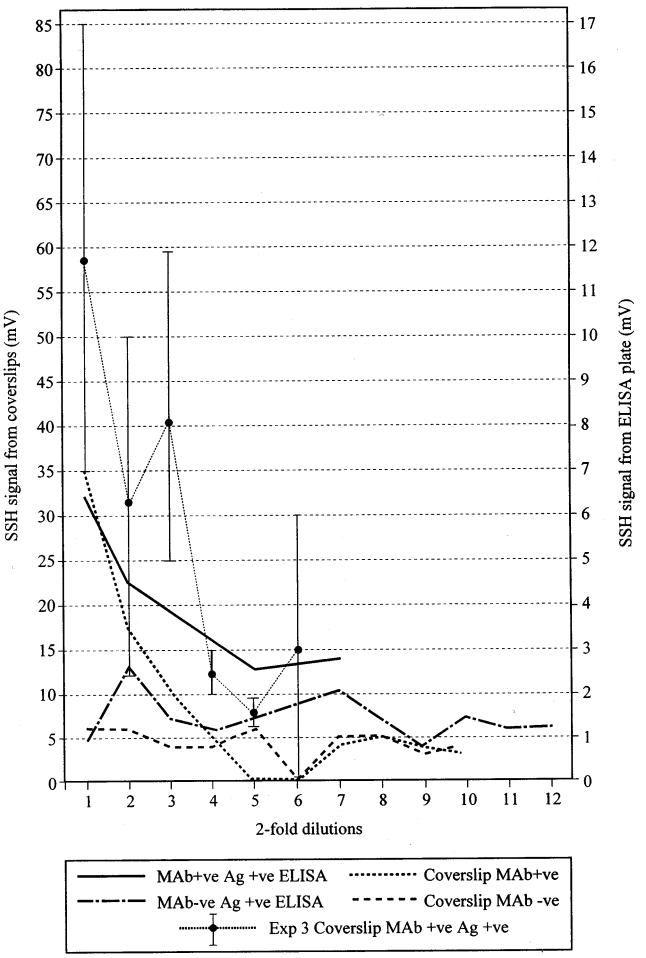

Fig. 2. Output of SSH signal generated by laser excitation of antigen captured by monoclonal antibodies immobilised on ELISA plates and glass coverslips.

3. Results

The highest antigen concentration produced higher SSH signal from test samples than from control samples. The signal decreased rapidly with antigen dilution in all SSH assays, more so than would be expected in the ELISA (Fig. 2). There was a correlation between the sets of SSH readings and the ELISA results obtained from the current test for infection with BVD. All the controls showed low generation of SSH signals.

Regression analysis showed that the results obtained from the capture assay for BVD on the ELISA plate using the SSH detector correlated well with the established ELISA assay ($R^2 = 0.86$). Also results from this ELISA correlated well with the ELISA results obtained with the MAbs immobilised onto glass coverslips ($R^2 = 0.96$). When capture assays on coverslips were read in the SSH detector, the correlation between the SSH results and the ELISA was: experiment 2, $R^2 = 0.75$, experiment 3; $R^2 = 0.67$.

4. Discussion

The monoclonal antibodies chosen to capture BVD antigen in this assay are currently used in an ELISA for BVDV in serum. MAb Wb103 recognises a 125-kDa viral serine proteinase NS2/3. This protein is only produced during viral replication and does not appear on the viral surface. MAb Wb112 recognises the major immunodominant glycoprotein E2, a component of the viral coat. Preparation of the viral antigen destroys the viral particles and so any change in SSH signal generated at different antigen concentrations is due to the binding of single molecules and not whole viral particles.

In experiment 1, antibodies were immobilised on rigid ELISA plates because of their reported antibody binding characteristics (McStay et al., 1995). However,

when an SSH signal was generated by the laser striking the wall of some wells of the ELISA plates the signal, combined with that generated from the antibody/antigen complex on the base of the well, produced spuriously high readings from these wells. Despite repeated washing of the plate with EWB it was possible that the sides of the well were inadvertently contaminated with antigen or antibody during pipetting. Results from the wells where this occurred were not included in the analysis.

Experiments 2 and 3 were carried out using MAb-coated glass coverslips as the support to avoid the problem of the laser striking the wall of the ELISA plate well. Confirmation of binding of antibodies to these coverslips, and subsequent antigen capture was by performing ELISA in parallel. The correlation of these results of this to the standard ELISA was good. A detectable difference between test and control samples was seen over a range of antigen dilutions, for experiments carried out using ELISA plates or coverslips as the antibody immobilising substrate (Fig. 2). The responses from the ELISA wells gave lower values of SSH and are plotted on the secondary axis for clarity. Non-specific binding of the target antigens was not seen in confirmatory identical ELISA assays, and the lower signal obtained from the control samples indicates that the signal seen from test samples is due to specific binding of the antigens.

Variations in the SSH signal may also have been due to uncorrected fluctuations in the laser power. In experiment 3, a reference signal was obtained as the SSH signal was read to correct for this. Using an uninfected white cell lysate as an antigen − ve control, low readings from MAb + ve and − ve, antigen − ve samples, and high and variable readings from MAb + ve antigen + ve samples were obtained. The variation in the readings taken from three points on the coverslip may have been due to uneven deposition of the MAb layer, and hence variation in the density of bound antigen molecules. The lack of further detection steps by secondary antibodies, as in the sandwich ELISA, means that specificity of the one-step assay is wholly dependent on the capture antibodies. The quality of binding of the capture antibodies to the support is clearly of vital importance to the further development of this technique but we have demonstrated feasibility with nothing more than a pipette. The SSH signal from the coverslips decreased rapidly as the antigen was diluted. The antigen used in these experiments is the same as the concentrated preparation used to produce the calibration curve in the current BVD ELISA, which requires concentration of the samples. The ability of the SSH detector to discriminate between successive dilutions of antigen, in the range found in concentrated field samples indicates its potential as a quicker test for BVD than the current ELISA. To detect viral antigen and other antigens of interest at physiological levels, and non-invasively, for instance in milk, the sensitivity of the detector needs to be increased. Many factors influence the signal/noise ratio in SSH generation, and it should be possible to increase the sensitivity by altering the incident angle of the laser, the concentration and mode of deposition of the monoclonal antibodies or the power of the laser.

The SSH has shown potential as a means of detecting viral antigens in samples. A glass support layer gave better results than an ELISA well but made the comparison with ELISA less direct. A number of tasks are necessary to convert this laboratory test into a field tool. The presentation and concentration of samples and reagents has to be optimised and automated and a simple method of calibration devised.

5. Conclusions

An SSH antigen capture assay was sensitive enough to discriminate between twofold dilutions of antigen in the same concentration range as the current ELISA test. The surface on which the antibodies were immobilised had an effect on the signal generated. A number of parameters (incident angle of the laser, deposition of the antibody and the antibody support) present many options for increasing the sensitivity and robustness of the technique. The SSH detector shows promise as a rapid method for detecting specific antigens in body fluids by detecting and quantifying directly the changes in protein conformation arising from specific antibody/antigen interactions.

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