



Use of antibody–hapten complexes attached to optical sensor surfaces as a substrate for proteases: Real-time biosensing of protease activity

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ABSTRACT

Fluorescent antibody protein (IgG) was attached to the surface of an integrated optical glass waveguide chip via specific binding to a covalently attached hapten and used as a substrate for the measurement of protease activities. Exposure of the optical chip to proteases resulted in digestion of the bound fluorescent antibody molecules and proportional decrease in the detectable fluorescence resulting from loss of fluorescence from the evanescent field. The bound fluorescent antibody protein was used as a unique universal protease substrate in which the combined biological activity and fluorescence signal were the basis of measurement. The action of proteases was monitored in real-time mode where the gradual decrease in evanescent fluorescence was recorded. The chip was regenerated by complete digestion of the antibody substrate by excess pepsin and recharged by incubation with a fresh sample of the labelled antibody. The biosensor was used to detect activity of several proteases including a bacterial protease preparation, Pronase E. The linear range of measurable Pronase E activity was from 0.03 to 2 units/mL. A measurement cycle took 40 min for samples with high protease concentration (≥ 0.5 units/mL), when the concentration of the protease was less measurement times up to 100 min were required. The method demonstrates the principle of a new mode of real-time biosensing of proteases. The modular integrated optical glass waveguide biosensor system used in this study is compact and controlled by a laptop computer and could easily be miniaturised and utilized as a true probe device for detecting proteases with potential applications in a wide range of areas including research, clinical diagnostics, biotechnology processing and food and detergent manufacturing industries.

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1. Introduction

Detection and measurement of proteases is used in research [1], diagnostics [2,3], biotechnology and in industrial application [4,5]. Proteases play critical roles in cellular processes and pathological mechanisms and are heavily exploited in industrial applications such as the food industry and detergent formulations [6–9]. The natural substrates for proteases are cellular proteins where the products (small protein fragment or amino acids) do not possess any easily detectable activity that is distinguishable from the

unprocessed substrates. The analytical protease substrates used currently are chemical constructs or genetically modified proteins. Synthetic substrates include fluorescence-labelled short peptides which offer varied degrees of specificity toward given proteases and therefore are of limited suitability for assaying specific proteases [10]. Hydrolysis of protein substrates labelled with fluorescent or chromogenic compounds produces smaller labelled fragments that must be separated from unhydrolysed substrate molecules before a meaningful signal can be measured [11–13]. However, fluorescence proteases substrates have a number of positive features including high sensitivity.

Rapid detection and continuous monitoring of protease activities in complex samples with optical biosensors would offer opportunities of direct analysis without the need for pre-test processing in laboratories by specially trained personnel. Such mode of analysis could significantly reduce analysis time and costs and make it possible to exploit convenient and practical biosensors as real-time monitoring of environmental samples and industrial processes as well as rapid analysis of clinical samples.

Abbreviations: 6HEX, 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein; NHS, N-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; APTES, 3-aminopropyltriethoxysilane; DAQ, data acquisition card; DMF, dimethylformamide; CC, cyanuric chloride; PBS, phosphate buffered saline; THF, tetrahydrofuran.

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Fluorescence-based methods are extensively employed in the protease analysis [10–12]. Improved fluorophores and light sources have recently been developed and, together with optical biosensing technologies, allow new and improved approaches for detection of proteases. Integrated optical biosensors based on fluorescence excitation by evanescent field have demonstrated improvement in sensitivity and specificity of detection procedures [14–16].

The availability of practical protease biosensor systems would offer new and interesting tools for clinical diagnostics of pathologies involving endogenous proteases [2,17,18] and protease toxins produced by pathogenic organisms [3,19] as well as in industrial process monitoring [4,5]. Sensing tools which allow for testing large arrays of proteases or substrates have recently been developed, as microarrays capable of analysing libraries of 10,000 peptides to determine the substrate specificity of proteases [20]. Gosalia et al. [21] describe a microarray sensor for the detection of multiple enzyme activities in human plasma based on fluorescent peptide substrates. Non-fluorescent biosensing approaches in detecting protease activities include impedance spectroscopy [22], microsphere resonators [23] and conductometry [24]. However, the above techniques involve relatively complex processes or instruments not suitable for rapid testing in diagnostic, environmental or industrial applications.

In this paper, we describe a novel approach to detecting activity of proteases based on the measurement of fluorescence excited by evanescent field on an integrated optics waveguide chip. The described method demonstrates the essential principles of an optical sensor system for measuring the activity of proteases using a measurable loss of biological activity of a natural protein substrate in a one-step direct procedure that does not require pre-treatment of samples or processing of post-reaction product mixtures prior to signal detection. In addition, the sensor system allows true continuous monitoring of protease activity and since the substrate and detectable signal are both associated with the sensing element, in principle the biosensor allows use as a probe.

2. Materials & methods

If not specified otherwise, chemicals and reagents were obtained from Sigma–Aldrich (Poole, UK) and Fisher Scientific (Loughborough, UK).

2.1. Reagents

Pronase E (protease XIV), a mixture containing the serine protease streptogrisin B and the metalloprotease griselysin extracted from *Streptomyces griseus*. Pepsin A, an aspartic protease isolated from the porcine gastric mucosa. The generation and affinity purification of sheep anti-cholic acid antibody are described in a separate report [25].

2.2. Labelling of anti-cholic acid antibody with 6HEX

25 mg of 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein (6HEX) (Anaspec, San Jose, CA) was dissolved in 0.5 mL acetonitrile and 0.5 mL 1,4-dioxane followed by addition of 0.25 mL of DMF. To activate the carboxylic group for coupling to the antibody 6HEX was converted to the succinimidyl ester derivative, 6HEX-NHS, which was formed by adding 4.53 mg of *N*-hydroxysuccinimide (NHS) and 9.73 mg of *N,N'*-dicyclohexylcarbodiimide (DCC) to the 6HEX solution. The mixture was left to stand for 16 h at room temperature. The antibody (IgG), was dialysed against 0.05 M NaHCO₃ (pH 8.7) for 3 h at room temperature, sterile filtered (0.2 µm) and the IgG protein content was quantified measuring the absorbance value at 280 nm. The amount of 6HEX-NHS to be added was calculated out of the dye labelling factor (ratio of the molecular weights multiplied

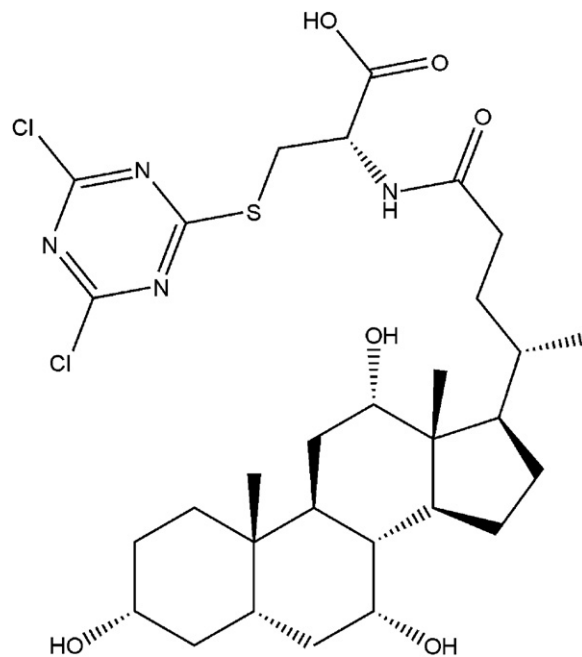


Fig. 1. Structure of the hapten linker molecule comprising of the antigen, cholic acid and the reactive group for covalent attachment, cyanuric chloride. Cysteine functions as a linker with the cholic acid bound to the α -amino group, cyanuric chloride bound to the thiol group and a free carboxylic acid.

by the desired molar ratio dye/antibody), the amount of antibody and by assuming 80% yield of the esterification, Eq. (1).

$$6\text{HEX-NHS (mg)} = \frac{\text{antibody (mg)} \times \text{dye labelling factor}}{\text{dye purity factor}} \quad (1)$$

The 6HEX-NHS was added in the dark to the antibody solution (19 mg in 10 mL) with stirring and the reaction mixture was stirred for 45 min at room temperature. The antibody–6HEX conjugate was dialysed against 1 L 0.15 M NaCl for 3 h at room temperature and then for 16 h at 4 °C followed by dialysis against 1 L phosphate buffered saline (PBS: 136 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄ at pH 7.4) containing 0.01% NaN₃ for 16 h at 4 °C to remove any unbound dye and equilibrate the conjugate in PBS. The dialysed conjugate was sterile filtered (0.2 µm), mixed with equal volume of glycerol, aliquoted and stored at –80 °C. The dye/antibody ratio of the conjugate was determined by reading the absorbance values at 533 and 280 nm (Lambda 25 spectrophotometer, Perkin-Elmer, Waltham, MA) and calculated according to Eq. (2):

$$(D/P) = \frac{[6\text{HEX}]}{[\text{Ab}]} = \frac{A_{533 \text{ nm}}/\epsilon_{6\text{HEX}}}{(A_{280 \text{ nm}} - 0.05 \times A_{533 \text{ nm}})/170,000} \quad (2)$$

2.3. Synthesis of the hapten linker complex

Cholic acid–cysteine–cyanuric chloride complex (Fig. 1) was prepared to enable covalent attachment of the cholic acid moiety (hapten) to either amine- or thiol-derivatised glass surfaces. Cholic acid-*N*-hydroxysuccinimide ester was prepared by adding 1.27 g of *N*-hydroxysuccinimide and 2.42 g of DCC to a solution of cholic acid (4.08 g) in 200 mL of tetrahydrofuran (THF). The mixture was left to stand at room temperature for 24 h before use. Cysteine (1.2 g) was dissolved in nitrogen-saturated 0.5 M NaHCO₃ solution (30 mL) and 5 mL of THF and the solution was cooled on ice for 30 min before 2 g of cyanuric chloride (2,4,6-trichlorotriazine) (CC) in ice-cooled 20 mL of acetone was added gradually whilst mixing the reaction mixture on ice. After stirring for 1 h on ice, the organic solvents were removed by rotary evaporation. The remaining suspension was

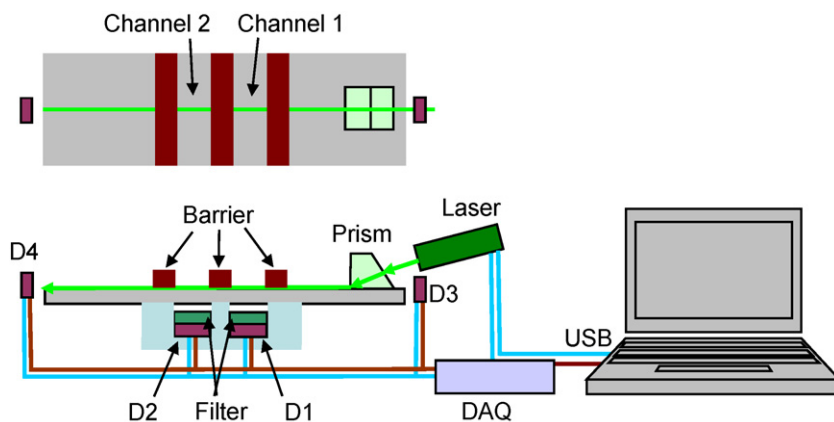


Fig. 2. Design of the detection system: top view of the sensor and front view of the complete system including a (laptop) computer, the data acquisition card (DAQ) and the optical components as labelled. D1, D2: high sensitivity photodetectors; D3, D4: low sensitivity photodetectors.

cooled on ice and carefully acidified to about pH 5 by the gradual addition of 5 M HCl, extracted with chloroform to remove unreacted cyanuric chloride and the aqueous solution was left standing in the cold where cysteine–CC product precipitated. The precipitate was isolated by filtration, washed with cold 0.1 M HCl and left to dry. To a solution of cysteine–CC (0.81 g) dissolved in 50 mL of 50 mM NaHCO₃ and 10 mL THF, cholic acid–*N*-hydroxysuccinimide ester (1.65 g in 136 mL of THF) was added gradually while mixing. The reaction mixture was stirred for 16 h, the organic solvents were removed by rotary evaporation and the suspension acidified to pH 6 and extracted with chloroform. The aqueous layer was left to stand in the cold where the product (Fig. 1) precipitated. The cholic acid–cysteine–cyanuric chloride complex was isolated by filtration, washed with cold water, left to dry and used for covalent attachment to treated sensor chips without further processing.

2.4. Fabrication of the waveguide

The sensing element was a 1 mm thick BK7 glass slide (VWR, Lutterworth, UK) with planar waveguide fabricated by dry Ag⁺ ion-exchange. A 200 nm thick Ag film was deposited on both surfaces of the glass by thermal evaporation. The sample was heated up to 180 °C and a 60 V potential was applied between the two surfaces for 18 min, during which Ag⁺ ions were diffused into the glass substrate from the positive voltage side. Remaining Ag was removed by acid (Aluminium Etch, Micro-Image Technology Ltd., UK) at room temperature and the sample was annealed at 300 °C for 1 h. This resulted in a single mode planar waveguide for the wavelength of 532 nm.

2.5. Amino-silanation of the waveguide glass surface

The waveguide chips were incubated with freshly prepared piranha solution (seven parts concentrated sulfuric acid plus three parts 30% hydrogen peroxide solution) 2 h at 80 °C and washed four times with water. The piranha treated glass was incubated with freshly prepared 5% solution of 3-aminopropyltriethoxysilane (APTES) in 90% ethanol for 1 h at room temperature, washed with water and ethanol and dried. The amino-silanated glass could be stored dry at room temperature until further use.

2.6. Covalent attachment of the hapten derivative to waveguide glass chips

The amino-silanated glass was incubated with the cholic acid–cysteine–cyanuric chloride complex dissolved in coupling

buffer (50 mM NaHCO₃, pH 9.5) to 0.5 mg/mL for 18 h at 44 °C. In this reaction, the dichlorotriazine moiety of the hapten derivative forms a covalent bond with free amine groups on the chip surface. The treated glass chip was washed three times with washing buffer (50 mM NaHCO₃, 100 mM NaCl, 0.05% Tween 20), three times with water and left to air dry at room temperature. The hapten-coated biosensor chips were prepared in batches and stored for several weeks at room temperature.

2.7. Detection system

Fig. 2 shows a schematic of the detection system. A 4 mW diode pumped solid state (DPSS) green laser at 532 nm (Photonic Products, UK), was powered through the USB port of a computer and modulated by a 32 Hz square wave signal generated by a data acquisition card (DAQ, National Instruments, Austin, TX). The laser beam was coupled into the waveguide through a prism and the evanescent tail of the guided laser beam extended into the layer of fluorescent molecules within close proximity to the waveguide surface thus exciting fluorescence. Two Si photodetectors (5.8 × 5.8 mm² detection area, Hamamatsu, Japan) with high sensitivity and low noise level (noise equivalent power 12 fW/Hz^{1/2}) were attached to the back of detection areas along guided wave propagation, thus forming a two channel detection system. A long-pass optical filter in front of the detectors blocked the 532 nm excitation wavelength and let the emitted fluorescence through. The DAQ card provided bias voltage to the photo detectors and digitised signals from the detectors with a 16 bit resolution. Controller software was developed which allows the user to set time intervals at which the laser would release a defined number of light pulses and the photo detector signals would be recorded. Fast Fourier transform (FFT) analysis was carried out by the software for each signal data channel to obtain their amplitude at the modulation frequency, thus minimising interference from background light and other noise sources. Two additional detectors were mounted, one near the input laser beam and prism and another at the end of the glass waveguide, to monitor input excitation laser power into the waveguide and remaining excitation laser output from the waveguide, respectively. The amplitudes from all channels were displayed and recorded over time for later data processing. The ratio of test channel and reference channel was also displayed. The data set could be exported into common software packages such as Excel for further analyses. One of the two measurement channels could be used as the reference channel and the other as test channel, thus eliminating the effect of change due to excitation laser power and other undesired environment drift.

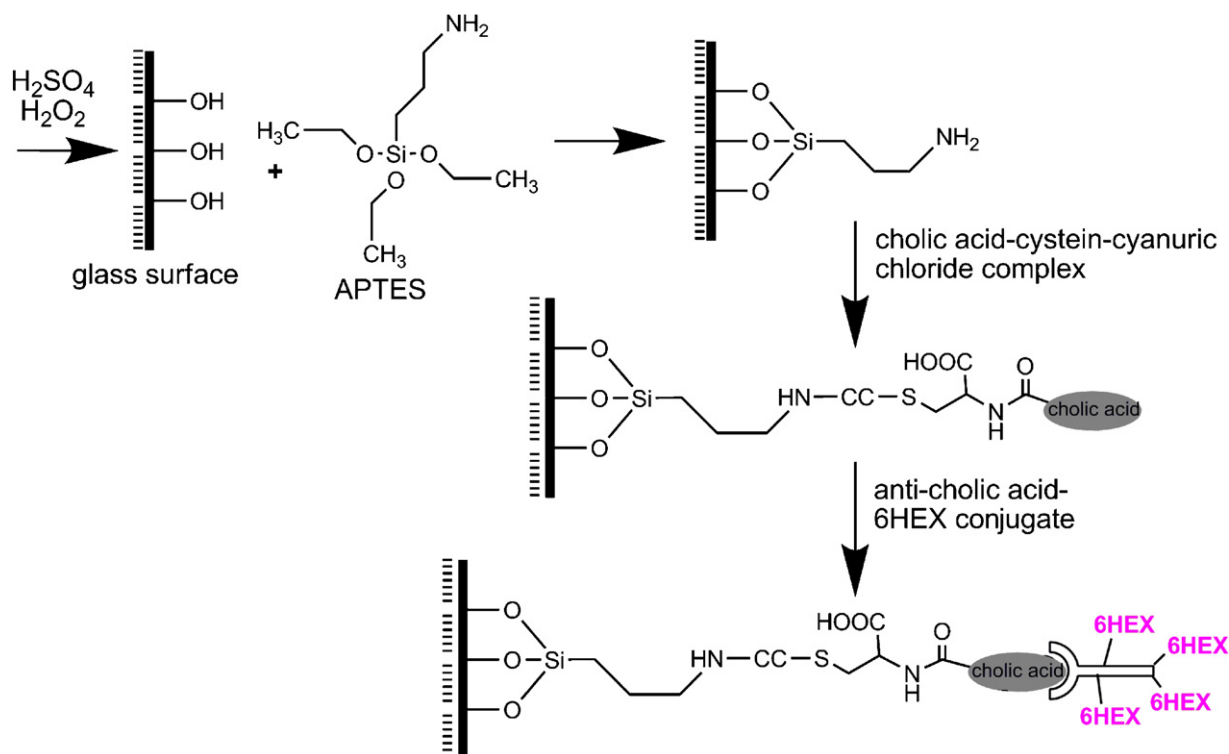


Fig. 3. Schematic of the biosensor: showing the chemical treatment of the waveguide glass chip for the covalent attachment of cholic acid–cysteine–cyanuric chloride via amino-silanation followed by loading of the chip with anti-cholic acid–6HEX antibody, the solid surface protease substrate.

2.8. Binding of antibody to the biosensor chip

On the surface of the hapten-coated waveguide chip a silicone separator (Stratech, Newmarket Suffolk, UK) was attached to form a well for incubating liquid samples. The chip was mounted on the detection system and the laser beam was coupled into the waveguide through a prism. The signals from both channels were measured simultaneously, and the interval between two measurement points was set to 2 min. One test channel was used as the reference to monitor the variation of system drift, including laser power fluctuations. The second channel was used as the test channel. 6HEX labelled anti-cholic acid antibody was diluted in antibody buffer (100 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 7.4, containing 250 mM NaCl, 0.5% gelatin (type A, porcine), and 0.1 g/L thimerosal) and filled into the well on the sensor surface. After 3 h incubation, excess antibody was removed by repeated washing with PBS until the fluorescent signal remained constant. An alternative protocol for loading the antibody–6HEX on to the chip was also used, where the binding step was carried out before mounting the chip onto the test bed. This alternative procedure allowed the treatment of several chips simultaneously and the storage of the loaded chip for up to 24 h showed no negative effect on performance.

2.9. Protease activity tests

Protease tests were carried out with chips loaded with the labelled antibody. The chip was mounted in the detection system, calibrated with assay buffer (20 mM Tris–HCl, pH 7.3, 100 mM NaCl, 5 mM CaCl_2 , and 50 nM ZnCl_2) and the signal recording was set up as described above. Varied concentrations of Pronase E in assay buffer were added and the change of fluorescence signal was monitored over time. The ratio of the decrease in fluorescence over time was calculated from these data post measurement.

2.10. Regeneration of the chip

The hapten-coated chips were regenerated by removing the remaining antibody and re-loaded with antibody–6HEX conjugates. After test experiments, any remaining bound labelled antibody was removed from the biosensor chip surface by incubating with excess pepsin (10 mg/mL pepsin in 50 mM glycine pH 2.5) for 3 h and subsequently washed with PBS. The regenerated chips were reloaded with fresh solution of labelled antibody as described above.

3. Results

3.1. Demonstration of the binding of antibody–6HEX to the waveguide surface

The chemical attachment of the hapten derivative (cholic acid–cysteine–CC) to the amino-silanated waveguide is illustrated in Fig. 3. The schematic shows the chemical treatment of the waveguide chip surface, amino-silanation and covalent attachment of the hapten derivative followed by binding of the anti-cholic acid–6HEX conjugate. The bound labelled antibody acts as the substrate for proteases. The synthesis of the hapten derivative to provide the bifunctional molecule comprising an hapten (antigenic function), cholic acid, and the 1,2-dichlorotriazine group as the reactive function involved use of cysteine as the backbone molecule. The derivative was designed as shown in order to enable efficient covalent attachment to amine as well as thiol groups. Silanation of glass can be carried out to provide either of the two functional groups. In addition, the free carboxylic acid function of the hapten derivative, which remains free during the coupling process, improved the solubility in aqueous solutions.

The binding of the labelled antibody to the hapten was followed as it happened by measuring the rise in the detectable

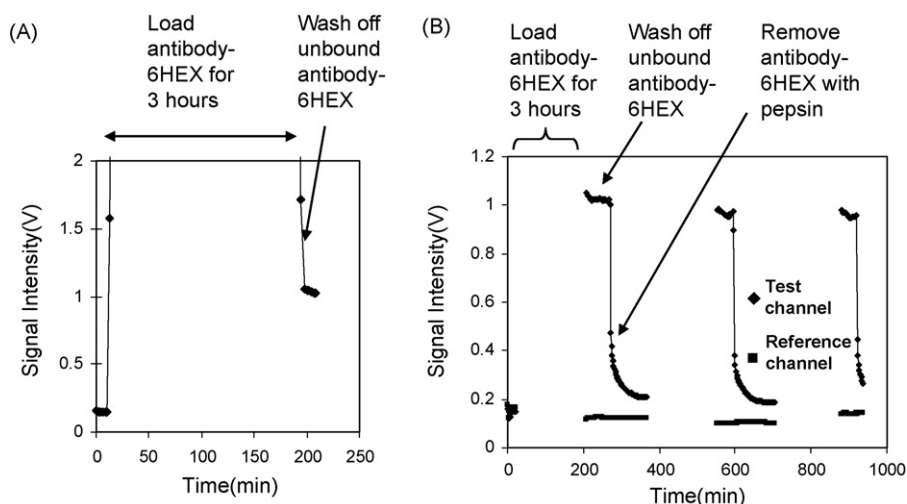


Fig. 4. Signal detection: (a) specific binding of the antibody to the glass surface after covalent attachment of the hapten showing the recorded signal before and during loading with antibody–6HEX, followed by washing unbound excess antibody–6HEX off; (b) a continuous experiment of 3 cycles of (re-)loading and proteolytic removal of antibody–6HEX on the biosensor.

fluorescence that appeared within the evanescent field at the surface of the hapten coated integrated waveguide chip. Fig. 4A shows recorded fluorescence signals over time during the loading phase of the hapten-coated sensor chip during incubation with the antibody–6HEX solution and subsequent washing steps. The signal gradually increased after bringing the antibody–6HEX solution in contact with the surface. After 3 h incubation, removal of the antibody solution and subsequent washing, the level of the detected signal fell slightly as the unbound antibody–6HEX was washed off. A strong fluorescent signal significantly above the initial background remained as a result of binding of the antibody–6HEX to the hapten-coated surface, thus bringing the labelled antibody conjugate, the protease substrate, into the evanescent field. During the optimisation phase it was found that a 3 h incubation period with antibody concentration of 70 $\mu\text{g/mL}$ achieved maximal loading. However, the vast majority of binding was observed to occur during the first 10 min of the incubation period.

3.2. Regeneration of the chip

A hapten-coated waveguide chip was mounted onto the detection system, and the signals from both channels were monitored continually at 2 min intervals. The loading with antibody–6HEX was carried out and monitored as described above. After washing the unbound antibody off, pepsin was added and left to digest the bound antibody–6HEX conjugates. Incubation with pepsin was continued until the fluorescent signal showed no further decrease. After washing to remove the pepsin and digestion products, a new cycle of loading the chip with antibody–6HEX followed by its removal was carried out. As shown in Fig. 4B, all three subsequent cycles showed similar strong fluorescence signals after loading, and dropping back to the same background level after pepsin treatment. The data demonstrated the stability of the covalently attached hapten to the steps involved, efficiency of removal of bound antibody, absence of build of background signal and the reusability of the protease integrated optical biosensor chip.

3.3. Fluorophore bleaching

Bleaching fluorescence signal was assessed after loading the biosensor surface with antibody–6HEX in both channels followed by continuous measurement for 16 h with laser pulses at every minute. The fluorescence signal level dropped to 78% of the initial

signals in channel 1 and to 82% in channel 2 in this time period (Fig. 5). This is equivalent to a decrease rate of 0.69% per hour (0.00011 min^{-1}) and 0.56% per hour ($0.000094 \text{ min}^{-1}$), for protease action monitoring carried out with 2 min intervals. The slightly smaller decaying rate in channel 2 may be due to a lower excitation optical power in that test cell.

3.4. Dose response analysis of a protease activity

The microbial protease Pronase E was used to demonstrate the effect of exposing the loaded sensor chip to a typical protease. These experiments revealed the relationship between the level of the detectable fluorescence signal and protease concentrations as well as the length of exposure periods required and the detection limits of the sensor system. Fig. 6A shows the fluorescence measurement obtained during a test cycle, including the pre-test loading of the chip, exposure to Pronase E and the complete removal of the remaining antibody with pepsin after the test to regenerate the chip. When the protease was added to the loaded chip, the fluorescence signal decreased as incubation continued. The hydrolysis of the bound antibody molecule resulted in disruption of the binding of the antibody to the attached hapten on the chip surface and lead to the removal of the fluorophore from the glass surface and out of the evanescent field. The data showed that the antibody–6HEX conjugate could be proteolytically detached from the surface of the waveguide with a typical microbial protease

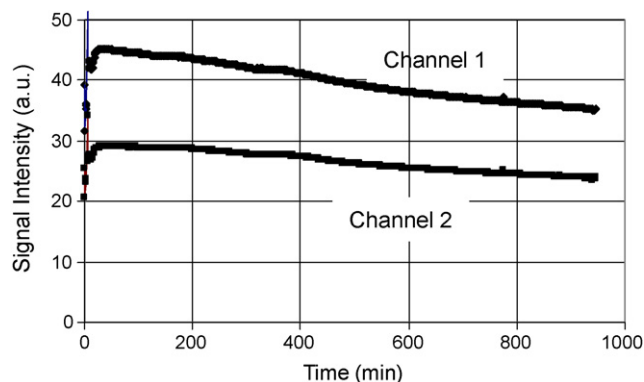


Fig. 5. Bleaching: stability of the fluorophore on the sensor surface over time with repeated laser pulses every 1 min.

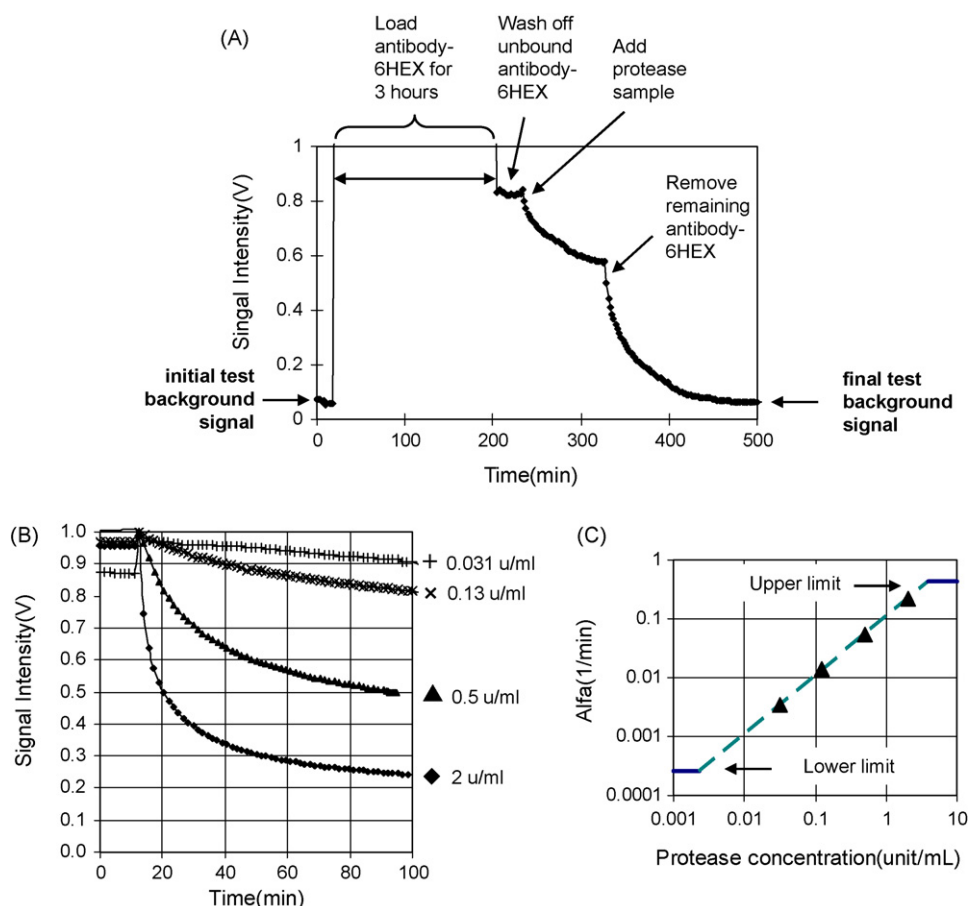


Fig. 6. Protease tests: (a) a typical cycle of loading the sensor with the antibody–6HEX, washing unbound material off, assaying with a protease sample and removing the remaining antibody–6HEX with pepsin to recycle the chip including the recording of the background signal at the beginning and end of test; (b) protease tests with concentrations of 2, 0.5, 0.13 and 0.031 units/mL, recorded signal intensity change with time; (c) linear fit of decrease of fluorescence over time Alfa (min^{-1}) vs. protease activity (units/mL) in log scale. Estimated upper and lower limit of detection are shown.

preparation, Pronase E. The findings demonstrate that decrease in fluorescence signal can be used as a direct measure of protease activity. The background signals due to residual excitation light were measured before loading antibody–6HEX and again after removing all remaining antibody–6HEX following the protease test, and subtracted from the test signal.

Dose response relationships of a range of protease concentrations were used to demonstrate the linearity and to assess the sensitivity of the sensor system. Pronase E concentrations ranging from 0.03 to 2 units/mL were tested and the change in fluorescence recorded over 90 min after adding the protease. Fig 6B shows the resulting graphs for four concentrations of Pronase E. With the highest protease concentration of 2 units/mL the fluorescence drops to 50% of the initial level within 10 min and declines to 25% after 90 min incubation. A significant decrease of fluorescence could still be measured with 0.03 units/mL Pronase E but after 90 min incubation time the signal had only decreased by 10%. There was no significant decrease in fluorescence if the loaded chip was incubated with buffer solution, demonstrating the stability of the hapten–antibody complex on the sensor chip surface in absence of proteases.

Whilst carrying out data processing, the ratio between data from the test channel and the reference channel was taken. Initial data points represented the background signal, and were subtracted from the whole data set. The maximum signal level of all tests was then normalised to 1 and the data set was used to fit into an exponential curve to determine the decaying time. When the signal decreased to very low levels, the data set diverts away from

an exponential function. In this case, only the initial part of the data set was used for fitting. The calculated rate of decrease in fluorescence over time Alfa (min^{-1}) derived from data set shown in Fig. 6B is plotted against protease activity (units/mL) and is shown in Fig. 6C. It clearly shows a linear relationship with a slope of $0.11 \text{ min}^{-1}/(\text{units/mL})$.

3.5. Stability of the system

The stability of the test system was assessed by measuring the background signal over time, which gave a variation of slope of $\pm 1 \times 10^{-5} \text{ min}^{-1}$, and by taking the ratio of data from the test and reference channels, thus eliminating interference from system drift. Fluorophore bleaching causes signal decrease independent of protease activity. The result of the bleaching test showed a signal decaying rate due to bleaching of about $1 \times 10^{-4} \text{ min}^{-1}$ for a measurement interval of 2 min. Due to the use of a low power laser in the detection system and the use of the more stable fluorescein derivative 6HEX, the effect of fluorophore bleaching was found to be minimal. Increasing the measurement interval time, which would be more in line with the intended use, further reduced the effect of bleaching and improved the detection limit, especially for low protease concentrations, where the decrease of the signal was slower and longer measurement times were required. The bleaching effect was compensated for by measuring the fluorescence in the loaded reference channel and taking the ratio of test channel data over reference channel data. The ratio of channel 2 over channel 1 (Fig. 5) gives a slope of $3.6 \times 10^{-5} \text{ min}^{-1}$, which is comparable to the sys-

tem variation of $\pm 1 \times 10^{-5} \text{ min}^{-1}$. In this demonstration biosensor construct, the measurement accuracy was significantly improved by using a reference channel, which is a monitor for changes in laser power or system drift.

4. Discussion

The study demonstrated the use of hapten-bound fluorescent antibodies as universal substrates for the detection and measurement of proteases. The use of fluorescent antibodies as substrates for proteases is of particular interest due to their natural susceptibility to digestion by a wide range of enzymes [26,27] and their versatility because antibodies with varied structures and specificity may be used. This is in contrast to the proteins commonly used as universal protease substrates such as casein and gelatin which provide excellent universal substrates but are devoid of any detectable biological activities and thus limiting their use in analytical formats. In this study we exploited the high affinity of antibody binding to respective hapten species. This enabled us to achieve two additional technically important and scientifically objectives. The labelled antibody was presented as a solid phase protease substrate where immobilisation was realized by specifically binding a layer of respective (cholic acid) molecules which were permanently attached to the glass surface by covalent bonding. The solid phase protease substrate is therefore a biologically active fluorescent protein. Only antibodies with intact functionally active binding sites will bind to the hapten and digestion of antibody polypeptide chains will lead to destruction of the binding site with loss of functional activity and subsequent loss of bound antibodies with concomitant decrease of fluorescence in the evanescent field. This direct link between protease action and the biological activity of the fluorescent substrate is a unique feature of the described assay. The described method allowed the construction of a regenerable integrated optical biosensor for proteases which combines a change of biological activity with a fluorescent signal. It is important to emphasise that high performance optical biosensor devices based on integrated glass chips are costly and cannot be proposed for single-use basis. Furthermore, it is not feasible to regenerate protease biosensors where the substrate is covalently attached directly to the sensor surface. Thus the regenerability of our biosensor construct provides a unique feature of practical importance. The biosensor can be easily stripped by pepsin digestion and re-loaded by incubating with a fresh sample of labelled antibody to regenerate a fully working device without loss of performance.

The sensor was shown to detect protease activities in a range of 0.03–2 units/mL with good linearity. The principal physical element of the system is a glass waveguide chip which is functionalised with a novel bifunctional molecule comprising of a reactive group for covalent attachment and an antigenic site for binding antibodies. The free carboxylic acid group in the linking cysteine (Fig. 1) increased the aqueous solubility of the complex and thereby enhanced reaction conditions during surface attachment.

Our results using Pronase E showed a good linear correlation between the decrease in the fluorescent signal and protease activity. This indicates that the biosensor construct may be employed as a measurement tool after calibration with appropriate enzymes.

In addition to quantitative measurements, the system also enables true continuous monitoring of protease action directly in real-time. This resulted from the full exploitation of the unique features of integrated optical device biosensing in conjunction with solid phase enzyme substrates presentation.

The developed protease sensor system has a noise level of 0.001 V and a signal level of 0.2 V. When the signal level is normalised to 1 V, the noise level is 0.005 V. If the system detects the signal decreasing by an amount of twice the standard deviation,

i.e. 0.01 V at 40 min, this is equivalent to a decaying time of $\text{Alfa} = 0.00025 \text{ min}^{-1}$, which corresponds to a protease concentration of 0.0023 units/mL. This would be the lower limit of detection. This limit can be reduced by increasing the measurement time. However, it was found that too long test times result in evaporation of the sample liquid, and hence a change of concentration. Covering the measurement area with a cover glass limited this effect and allowed for longer testing times. For high protease concentrations, the fluorophores are removed quickly and the fluorescence signal decreases rapidly. Several factors limit the measurement accuracy: test time interval, sample settling time and remaining fluorophore density on the surface. In the described setup, each test took less than two seconds, which determines the shortest possible time interval. But the time interval should be significantly longer than the time required for the sample to settle down on the sensor surface. For this reason, half a minute is a reasonable minimum time interval. As the fluorophore density decreases, the signal no longer follows an exponential curve. If the signal would decrease to 80% of the initial level, decaying time $\text{Alfa} = 0.44 \text{ min}^{-1}$ would let the signal decrease to 0.8 V at 0.5 min. On this basis an upper limit of detection of 4 units/mL can be estimated.

A label-free protease sensor based on optical microsphere resonators, with comparable to or even better than performance of surface plasmon resonator based sensor, showed a trypsin detection limit of approximately 10^{-4} units/mL within 15 min [23].

Similar “label free” detection techniques used in optical biosensors are based on detection of phase change of the bio-film, through combination of change of refractive index and change of film thickness, due to the binding of target molecules onto the sensor surface [28]. However, the combination of fluorescence signal, pre-loading of the biosensor with the labelled antibody substrate and the simple processing adds significant advantages to biosensor constructs such as the one described here for the detection and measurement of protease activities. The antibody protein provides a generic substrate for proteases with the distinct advantage of the convenience of immobilisation via hapten–antibody linkage which provides a regenerable biosensor.

The use of the linker molecule and cyanuric chloride as the reactive group allowed efficient attachment under mild reaction conditions and thereby the covalent attachment of sensitive biomolecules [29,30] and attachment of substances to highly structured surfaces such as integrated optical chip surfaces.

Cholic acid as an auxiliary hapten in conjunction with its specific and high affinity polyclonal antibody has been extensively exploited in our group [25]. The antibody can be purified in preparative quantities and labelled with fluorescent and non-fluorescent markers in good yield. The choice of a high-affinity antibody–hapten system to demonstrate the functioning and utility of the protease biosensor minimised the non-specific decrease of fluorescence, when adding buffer controls to the sensor surface. A small and insignificant signal decrease was observed under these conditions, which was expected due to the reversible nature of antigen–antibody interaction. The maximum detection range is limited when the protease concentration is so high that most of the substrate is removed immediately.

As the sensor is an integrated optical surface based detection system, sample volumes are not relevant as long as the whole surface remains in contact with the sample. This is important in monitoring where the biosensor in a probe configuration is required to be in an on-line contact with bulk volumes.

5. Conclusions

The study describes the foundations of a generic protease biosensor system, completely powered from a PC, that is low cost,

and potentially compact and portable. The demonstrated system exploits a number of remarkable features, both in the biological and physical components, to enhance the utility of biosensing techniques in protease detection and measurement. The study highlights the potential advantages of using biologically active fluorescent universal protease substrates in conjunction with integrated optical chips.

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