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Optical microchip array biosensor for multiplexed detection of bio-hazardous agents

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ABSTRACT

An optical waveguide array biosensor suitable for rapid detection of multiple bio-hazardous agents is presented. SpectroSens™ optical microchip sensors contain multiple spatially-separated waveguide channels with integral high-precision Bragg gratings sensitive to changes in refractive-index; selective surface-functionalisation of discrete sensing channels with different antibodies as bio-recognition elements enables selective multi-analyte biological detection. Interactions between target antigens in the test sample and respective surface-immobilised antibodies result in localised changes in refractive-index; the biosensor response manifests as increases in wavelength of light reflected from specific sensing channels. Multiplexed, label-free detection of 8 different biological agents, encompassing bacterial spores, vegetative cells, viruses and proteinaceous toxins has been demonstrated in real-time. Selective detection of *Bacillus atrophaeus* (BG) spores, *Escherichia coli* cells, MS2 viruses and ovalbumin (OVA) protein (simulant bio-hazardous agents) was first demonstrated as proof-of-concept; subsequently, detection of *Bacillus anthracis* (BA) spores (UM23CL2 strain), *Franciscella tularensis* (FT) cells (live vaccine strain), Vaccinia viruses (heat-killed) and ricin toxin (bio-hazardous agents) was proven. Two optical microchip sensors, each comprising 8 sensing channels were packaged into a single disposable cartridge allowing simultaneous 16-channel data acquisition. The specific antibody deposition sequence used in this study enabled detection of either 4 simulants or 4 bio-hazardous agents using a single consumable. The final device, a culmination of the multidisciplinary convergence of the fields of biology, chemistry, optoelectronics and microfluidics, is man-portable and inherently robust. The performance characteristics of the SpectroSens™ technology platform highlight its potential for exploitation as a 'detect to warn/treat' biodetector in security and defence operations.

1. Introduction

Rapid detection of pathogenic micro-organisms and toxins is prerequisite for an escalating number of applications, including bio-security and defence, medical diagnostics, environmental analysis, bio-processing and food safety. Over the last decade, there has been particular interest in the detection of bio-hazardous agents, since the 'anthrax events' (post 11th September, 2001) substantiated the threat of future incidents of bio-terrorism in both military and civilian environments (Inglesby et al., 2002). Conventional methods for pathogen detection are laborious, time-consuming procedures confined to specialised laboratories with expensive read-out instrumentation. Optical biosensors comprising target-selective bio-recognition probes are particularly well-suited to rapid, sensitive detection of biological agents in environmental samples. These have been receiving escalated interest in recent years for applications requiring 'on-site' analyses due to their portability, simplicity of operation, multiplexing potential and relatively low cost (Fan et al., 2008; Horvath et al., 2003; Kozma et al., 2011; Lazcka et al., 2007; Luchansky et al., 2010; Ramachandran et al., 2008; Rowe-Taitt et al., 2000; Taitt et al., 2008; Sai et al., 2010; Zourob et al., 2005). Whilst these devices exhibit numerous advantages over competitive systems, very few have successfully demonstrated detection of multiple biological targets using a single sensing chip (Rowe-Taitt et al., 2000; Taitt et al., 2008).

Recently, a novel integrated optical microchip sensing system (SpectroSens™), based on technology leveraged from the telecommunications industry, has been reported in its capacity for biological agent detection (Bhatta et al., 2010a, 2010b). SpectroSens™ optical microchip sensors comprise multiple

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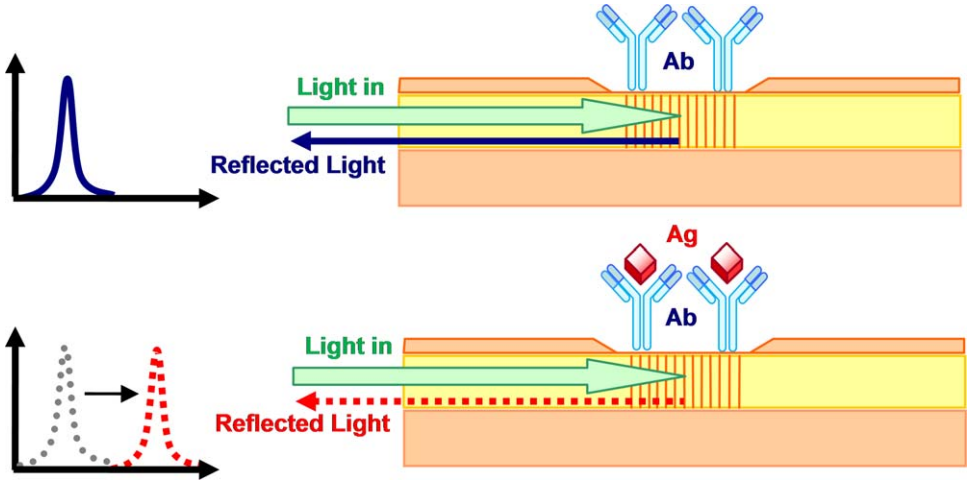

Fig. 1. Schematic illustration of the operating principle of a SpectroSens™ sensor for biological detection. Light travels through a waveguide within the microchip sensor to the Bragg grating, which reflects a precisely defined wavelength of light. Interaction of target agents with antibodies immobilised on the sensing region results in localised changes in refractive index, which manifest as changes in sensor reflected wavelength. Each chip contains multiple sensing channels operating independently enabling assay multiplexing.

high-precision Bragg gratings defined within spatially-separated waveguide channels, written into planar silica substrates using the direct UV writing technique described by Emmerson et al. (2002). Bragg gratings act as sensitive wavelength filters, reflecting light at precisely defined wavelengths as governed by the Bragg condition:

$$\lambda_{max} = 2\Lambda n_{eff}$$

where λ_{max} is the wavelength of light at which maximum reflectivity occurs, Λ defines the grating period and n_{eff} is the average refractive index of the waveguide's composite structure. Hence, changes in the immediate environment surrounding a particular grating result in measurable changes in the wavelength of reflected light from the corresponding waveguide channel. The sensor design results in a large penetration depth of the sensing light (>1 μm) into the sample liquid. SpectroSens™ sensors are designed to operate in the telecommunications window (1550 nm), hence commercial off-the-shelf sensor substrates and source/detector components are robust and ubiquitous, and remote sensing using fibre-optics is feasible. Functionalisation of independent sensing channels with different target-selective antibodies determines localised sensitivity to specific biological agents, enabling multiplexed detection using a single optical microchip sensor. The sensor response manifests as changes in reflection wavelength from specific sensing channels as a result of cognate antigen-binding to respective surface-immobilised antibodies (Fig. 1).

Real-time, label-free multi-analyte detection using the SpectroSens™ sensing platform is demonstrated in this communication through the detection of 8 different biological agents, comprising both simulant and bio-hazardous agents; these encompass all classes of biological targets, ranging from small proteinaceous toxins to larger particulate viral and bacterial (spore and vegetative cell) antigens.

2. Materials and Methods

2.1. Materials

All reagents were of analytical grade unless otherwise stated. Albumin from bovine serum (BSA) (electrophoresis grade), 3-aminopropyltriethoxysilane (APTES) (99%) and Dulbecco's phosphate buffered saline (PBS) (pH 7.4) were purchased from Sigma-Aldrich Company (Dorset, UK). Acetone (HPLC grade) and phosphate buffered saline with Tween (PBS-Tween) (×20 concentrate) were obtained from Thermo Fisher Scientific Ltd. (Leicestershire, UK). Bis(sulfosuccinimidyl)-suberate (BS^3) (2 mg microtubes) was sourced from Pierce, Thermo Fisher Scientific Ltd. (Leicestershire, UK).

Goat anti-mouse IgG polyclonal antibody (Fc-specific, affinity-purified) and goat anti-rabbit IgG polyclonal antibody (whole molecule, affinity-purified) were purchased from Sigma-Aldrich Company (Dorset, UK). Rabbit anti-sheep IgG polyclonal antibody (H+L specific) was purchased from Abcam (Cambridge, UK). Recombinant protein A/G from *Escherichia coli* (lyophilised powder) was obtained from Pierce, Thermo Fisher Scientific (Leicestershire, UK). Ovalbumin (OVA) protein (Grade V), *Bacillus atrophaeus* (BG) spores, *E. coli MRE 162*, bacteriophage virus MS2, *Bacillus anthracis* (BA) spores (UM23CL2 strain), *Franciscella tularensis* (FT) cells (live vaccine strain), Vaccinia viruses (heat-killed) and ricin toxin; rabbit anti-OVA polyclonal antibody (antigen-affinity-purified), rabbit anti-BG polyclonal antibody, rabbit anti-*E. coli* polyclonal antibody, sheep anti-MS2 polyclonal antibody, mouse anti-BA monoclonal antibody (DSTL110), mouse anti-Vaccinia monoclonal antibody (DSTL111), mouse anti-ricin monoclonal antibody (DSTL109) and rabbit anti-FT polyclonal antibody were provided by Defence Science and Technology Laboratories (Dstl) (Porton Down, Wiltshire, UK).

2.2. Instrumentation

SpectroSens™ microchip sensor outputs were monitored using a SIS:LabIIBio detector module (see Section 3.1) produced by Stratophase Ltd., UK, providing data at a refresh rate of 2 Hz, with a resolution of 1 pm. This instrument comprises the sensor light source and readout components, as well as a basic sample (liquid) delivery module. Introduction of test samples containing simulant agents was carried out using a QuadPump module (produced by Stratophase) comprising 4 computer-controlled 50 μl syringe pumps. Introduction of test samples containing bio-hazards was carried out using a standalone fluidic module (produced by BIRAL) comprising computer-controlled peristaltic pumps and inline degassers with automated purging and priming sequences (in anticipation of in-field use). Antibody deposition onto individual sensing channels within SpectroSens™ microchips was achieved using a microfluidic patterning system constituting an automated computer-controlled 8-channel peristaltic

pump and 2 × 8-channel polydimethylsiloxane (PDMS) patterning heads comprising 250 μm-wide, 100 μm-high microfluidic channels (produced by STRI, University of Hertfordshire), which were integrated into a dual patterning head docking-assembly (designed to enable both fluidic and optical connections with the test consumable) (produced by Stratophase and BIRAL).

2.3. Experimental

2.3.1. Sensor chip fabrication

8-Channel SpectroSens™ chips (20 × 10 mm) were fabricated by simultaneous writing of Bragg gratings and waveguides (10 μm in width) into three-layer silica-on-silicon substrates using the direct UV writing method described previously (Emmerson et al., 2002). The upper cladding directly above the sensor gratings was then removed using a microfluidic wet-etch procedure; subsequently, a high-index titanium oxide overlayer was deposited onto the etched surface to increase the sensitivity to refractive index (Sparrow et al., 2009).

2.3.2. Sensor surface functionalisation

SpectroSens™ microchips were cleaned using oxygen-plasma treatment (100 W, 5 min) with a Femto standard plasma asher (Diener Electronic GmbH). After cleaning, chips were immediately immersed in 4% (v/v) APTES in acetone and incubated for 1 h under agitation to allow formation of the silane layer. Excess APTES solution was removed by extensive rinsing of the chips with acetone, which were subsequently dried under nitrogen. The silane layer was left to cure at 110 °C for 1 h. Silanised microchip sensors were coupled with optical fibres (in order to deliver the source light and readout optical spectra) and stored until use.

2.3.3. Disposable test cartridge assembly

Two fibre-coupled, silanised SpectroSens™ microchips each containing 8 sensing channels, were mounted in a poly(methyl methacrylate) (PMMA) cartridge (120 mm (l) × 100 mm (w) × 10 mm (h)), such that both optical and fluidic connections were enabled from the same side of the consumable via a complementary docking assembly contained within the SIS:LabIIBio detector module (see Section 3.1, Fig. 2); this cartridge was also compatible with the patterning head docking assembly used for antibody deposition (see Section 2.2).

2.3.4. Multichannel antibody deposition/immobilisation

Antibody deposition onto individual sensing channels within SpectroSens™ microchips was achieved using the microfluidic antibody patterning system described in Section 2.2. Test cartridges housing two 8-channel SpectroSens™ chips were docked into the dual patterning head docking-assembly, which enabled individual sensing channels to be independently addressed with different agent-specific antibodies using the automated multichannel pump and allowed real-time monitoring of antibody deposition using the SIS:LabII. The specific antibody-patterning protocol utilised in this particular study involved patterning 4 sensing channels within each test cartridge with either 4 different simulant antibodies or 4 different bio-hazard antibodies, such that consumables were tailored for either simulant detection or bio-hazardous agent detection (as opposed to both) for practical purposes.

All 16 sensing channels within individual consumables were firstly primed with 1 M NaCl, 0.1 M sodium borate for 10 min to remove any residual organic material from sensing surfaces, before equilibrating in PBS, pH 7.4 until stable baseline sensor outputs were achieved for every channel (approximately 20 min). All 16 channels were then exposed to 5 mM BS^3 in PBS for 6.5 min in order to activate the silanised sensing surfaces, and washed in PBS for 2.5 min prior to addition of recombinant protein A/G (100 μg/ml, 2 min) for oriented antibody attachment. During consumable preparation for simulant detection, channels 3, 7, 11 and 15 were exposed to anti-sheep secondary antibodies, whilst all remaining channels were exposed anti-rabbit secondary antibodies (500 μg/ml, 2 min) for subsequent primary antibody attachment; channels 1, 5, 9 and 13 were then exposed to anti-OVA antibodies, channels 2, 6, 10 and 14 to anti-*E. coli* antibodies, channels 3, 7, 11 and 15 to anti-MS2 antibodies, and channels 4, 8, 12 and 16 to anti-BG antibodies (all at 500 μg/ml, 2 min). During consumable preparation for bio-hazardous agent detection, channels 1, 5, 9 and 13 were exposed to anti-rabbit antibodies (500 μg/ml, 2 min) for subsequent polyclonal antibody attachment, whilst all remaining channels were exposed to anti-mouse antibodies (500 μg/ml, 2 min) for subsequent monoclonal antibody attachment; channels 1, 5, 9 and 13 were then exposed to anti-FT antibodies, channels 2, 6, 10 and 14 to anti-ricin antibodies, channels 3, 7, 11 and 15 to anti-Vaccinia antibodies, and channels 4, 8, 12 and 16 to anti-BA antibodies (all at 500 μg/ml, 2 min). With both simulant and bio-hazardous agent consumables, all 16-channels were finally washed in PBS for 15 min prior to completing assembly of the consumable cartridge for subsequent assay testing.

2.3.5. Consumable flow-cell mounting

Assembly of the consumable cartridges containing antibody-patterned SpectroSens™ chips was completed by the addition of a dual flow-cell component comprising two silicone gaskets (12 mm (l) × 10 mm (w) × 0.5 mm (h)), one positioned above each microchip sensor (Section 3.1, Fig. 2d), and a single fluid inlet and outlet channel. Completed SpectroSens™ consumables were stored wet (in PBS, up to 48 h) until use.

2.3.6. Antigen detection assays

Consumable cartridges containing antibody-patterned SpectroSens™ chips were plugged into the SIS:LabIIBio instrument to enable fluid delivery and optical readout.

2.3.6.1. Simulant antigen detection. Using a single syringe pump of the Stratophase QuadPump module, the SpectroSens™ consumable (simulant antibody-patterned) was equilibrated in PBS-Tween (pH 7.4) at a flow rate of 20 μl/min until stable optical readouts were achieved across all 16 sensor channels (approximately 10 min). Individual simulant antigens were then introduced to the consumable (40-min incubation periods) in succession at the same flow rate, with intervals of PBS-Tween running buffer (10-min washing periods) between exposure to each antigen. The sequence of antigen addition was as follows: ovalbumin protein (100 ng/ml diluted in PBS-Tween), MS2 viruses (suspended at 2×10^{10} pfu/ml in PBS-Tween), *B. atrophaeus* (BG) spores (suspended at 9×10^6 cfu/ml in PBS-Tween) and *E. coli* vegetative cells (suspended at 3×10^7 cfu/ml in PBS-Tween). SpectroSens™ sensor outputs from the SIS:LabII:Bio enabled real-time monitoring of simulant antigen interactions across all 16 sensor channels simultaneously.

2.3.6.2. Bio-hazardous agent detection. Using the BIRAL automated fluidic module, the SpectroSens™ (bio-hazard antibody-patterned) consumable was equilibrated in PBS-Tween (pH 7.4) at a flow rate of 40 μl/min until stable optical readouts were achieved across all 16 sensor channels (approximately 15 min). Individual bio-hazardous agents were then introduced to the consumable (30-min incubation periods) in succession at the same flow rate, with intervals of PBS-Tween running buffer (30-min washing periods) between exposure to each antigen. The sequence of antigen addition was as follows: *B. anthracis* spores (suspended at 5×10^7 cfu/ml in PBS Tween), *F. tularensis* cells (suspended at 2×10^8 cfu/ml in PBS-Tween), Vaccinia viruses (suspended at 1×10^7 pfu/ml in

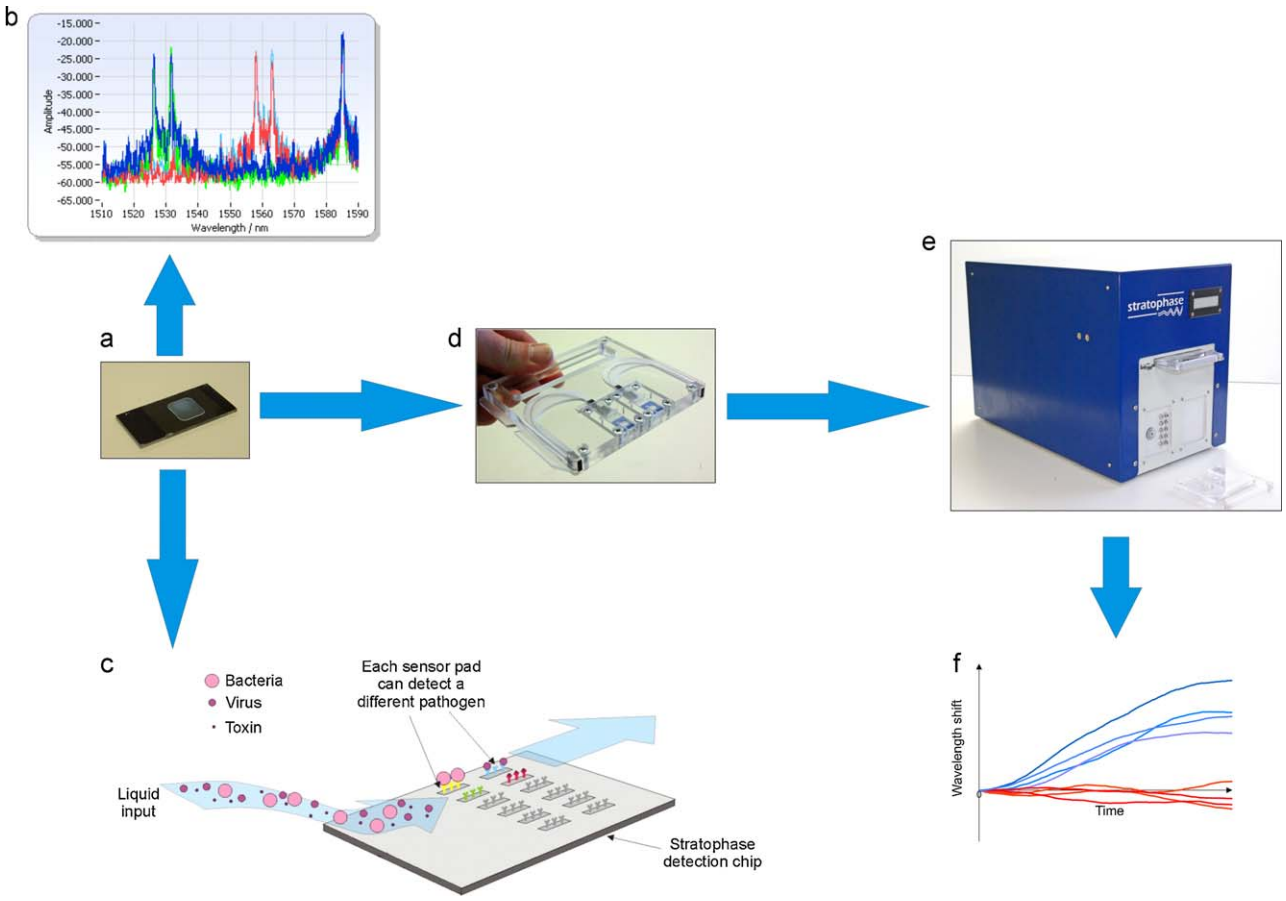

Fig. 2. SpectroSens™ technology: (a) 8-channel optical microchip sensor, (b) typical optical spectrum of a sensing channel, (c) schematic illustration of the principles of SpectroSens™ biological detection: target bio-hazards in the liquid sample are directly captured by specific antibodies immobilised on individual sensing channels, (d) disposable, plug-in consumable housing 2 × 8-channel microchips, (e) SpectroSens™ readout hardware and (f) sensorgram output highlighting changes in sensor reflected wavelength associated with selective antigen binding.

PBS-Tween) and ricin toxin (diluted to 250 ng/ml in PBS-Tween). Monitoring SpectroSens™ sensor outputs using the SIS:LabII:Bio enabled real-time bio-hazardous antigen detection across all 16 sensor channels simultaneously. All of this work was conducted within a Class II microbiological safety cabinet within a containment level II biological laboratory at Dstl.

3. Results and discussion

3.1. SpectroSens™ optical microchip sensors and readout system

The SpectroSens™ system is a result of the multi-disciplinary integration of the fields of optoelectronics, biochemistry and microfluidics, culminating in a robust, man-portable biosensing device, capable of multi-analyte bio-detection within minutes of exposure to target bio-hazards. Several configurations of SpectroSens™ optical microchip sensors have been fabricated using the direct grating writing method, examples of which are described in Sparrow et al. (2009). The multichannel SpectroSens™ chips used within this study comprised 8 independent sensing channels (Fig. 2a); a typical reflection spectrum from a single sensing channel is illustrated in Fig. 2b. Individual sensing regions were functionalised with different antibodies (either simulant or real-agent), enabling multi-analyte detection (Fig. 2c). To enable simultaneous 16-channel monitoring, two 8-channel SpectroSens™ chips were incorporated into a disposable test cartridge (Fig. 2d). Interactions between cognate antigens in the sample liquid and respective surface-immobilised antibodies were associated with localised changes in refractive index, captured by the SpectroSens™ detector (Fig. 2e) as changes in sensor reflected wavelength from specific sensing channels (Fig. 2f), indicating the presence of a particular target antigen.

3.2. Multi-channel antibody deposition onto SpectroSens™ microchip sensors

To impart sensor sensitivity to multiple biological targets, all 16 sensing channels within each SpectroSens™ consumable cartridge were individually addressed with different agent-specific antibodies as described in Section 2.3.6 (further details describing the microfluidic antibody-patterning apparatus will form the subject of a future publication). Surface-immobilisation of the detection antibodies whilst retaining optimal antigen recognition capabilities is critical to the performance of the biosensor. Functionalisation of the titania-coated SpectroSens™ microchips was achieved by exploiting the well-documented self-assembly of organosilanes on metal oxide surfaces (Liedberg and Cooper, 1998). Aminosilane-functionalised microchip surfaces were activated with BS^3 crosslinker for attachment of recombinant protein A/G in all 16 sensing channels; this was used to orient species-specific secondary antibodies via their Fc regions for subsequent target-specific antibody immobilisation. A schematic illustration of the bio-recognition layer is displayed in Figure S1 (supplementary material).

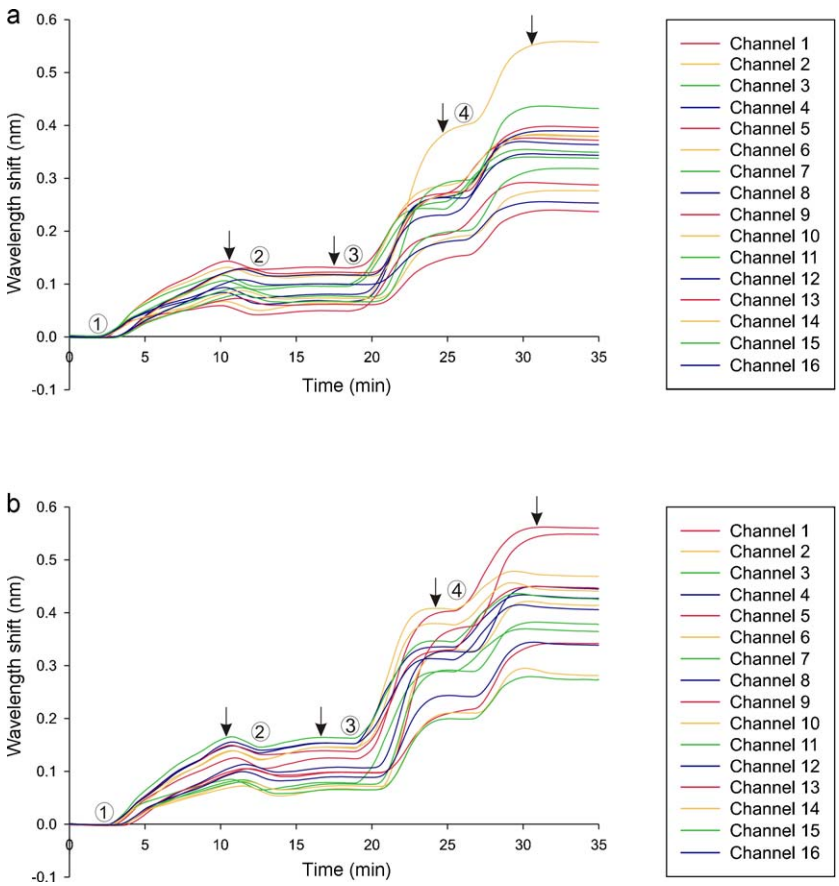

Fig. 3. Real-time SpectroSens™ sensorgram outputs monitoring 16-channel antibody deposition. Individual sensing channels within aminosilanised SpectroSens™ optical microchip sensors were addressed with different (a) simulant antibodies or (b) bio-hazard antibodies. The antibody deposition sequence was as follows: 1. BS^3 activation, 2. addition of protein A/G, 3. addition of anti-species secondary antibody, and 4. addition of target-specific antibody. Downward arrows represent introduction of PBS running buffer. (*Note*: data plots and figure legends can be visualised in colour online.)

Consumables patterned with simulant antibodies comprised anti-OVA antibodies in channels 1, 5, 9 and 13, anti-*E. coli* antibodies in channels 2, 6, 10 and 14, anti-MS2 antibodies in channels 3, 7, 11 and 15 and anti-BG antibodies in channels 4, 8, 12 and 16. Consumables patterned with bio-hazard antibodies comprised anti-FT antibodies in channels 1, 5, 9 and 13, anti-ricin antibodies in channels 2, 6, 10 and 14, anti-Vaccinia antibodies in channels 3, 7, 11 and 15 and anti-BA antibodies in channels 4, 8, 12 and 16. The SpectroSens™ detector enabled simultaneous monitoring of antibody deposition across all 16 sensor channels; real-time SpectroSens™ sensorgram outputs following the antibody immobilisation procedure are illustrated in Fig. 3. Target-specific antibody-capture is associated with a shift in sensor reflected wavelength of between 0.1 and 0.2 nm (Fig. 3, Step 4).

3.3. Real-time detection of simulant antigens (OVA protein, MS2 viruses, BG spores and E. coli cells)

16-channel SpectroSens™ consumables patterned with simulant antibodies were used to determine the presence of 4 simulant antigens by successive exposure to OVA protein (100 ng/ml), MS2 viruses (2×10^{10} pfu/ml), BG spores (9×10^6 cfu/ml) and *E. coli* cells (3×10^7 cfu/ml) in PBS-Tween (PBS-T) buffer in a flow-through format. SpectroSens™ responses demonstrating real-time, label-free specific detection of all 4 simulant antigens are illustrated in Fig. 4.

The sensorgram in Fig. 4a illustrates the SpectroSens™ responses associated with real-time capture of OVA protein (100 ng/ml) onto anti-OVA-functionalised sensing channels; these data demonstrate a significant increase in sensor reflected wavelength from all 4 specific anti-OVA channels within minutes of sample introduction (mean wavelength shift of ~0.002 nm in 5 min), in comparison with negligible changes in response from the anti-BG-functionalised channels presented as sensor controls. SpectroSens™ responses associated with real-time detection of MS2 viruses (2×10^{10} pfu/ml) on anti-MS2 functionalised sensing channels are illustrated in Fig. 4b. The sensorgram demonstrates a steady increase of ~0.002 nm in mean sensor reflected wavelength from specific anti-MS2 channels within 10 min of sample addition, relative to negligible changes in response from the anti-BG functionalised control channels. Fig. 4c displays the SpectroSens™ responses demonstrating real-time, specific detection of BG spores (9×10^6 cfu/ml); data from anti-BG-functionalised sensing channels and anti-MS2 channels (sensor controls) are presented. These data demonstrate that whilst a small degree of non-specific binding was observed on control channels, significant discrimination between specific and control channels was achieved (mean wavelength shift of ~0.002 nm) within 15 min of sample introduction. The SpectroSens™ surface mass sensitivity is estimated to be 5 pg/mm^2; this was obtained experimentally by SEM imaging of antibody-captured surface-bound spores (Bhatta et al., 2010a) and correlating captured spore mass (mass of 1 spore = 1 pg) to associated changes in sensor reflected wavelength (minimum $\Delta\lambda = 0.05$ pm). This mass sensitivity value is comparable to that of other state-of-the-art optical waveguide sensors (Kozma et al., 2011; Luchansky et al., 2010). Real-time identification of *E. coli* (3×10^7 cfu/ml) is demonstrated in the SpectroSens™ sensorgram displayed in Fig. 4d, which shows a steady increase in sensor

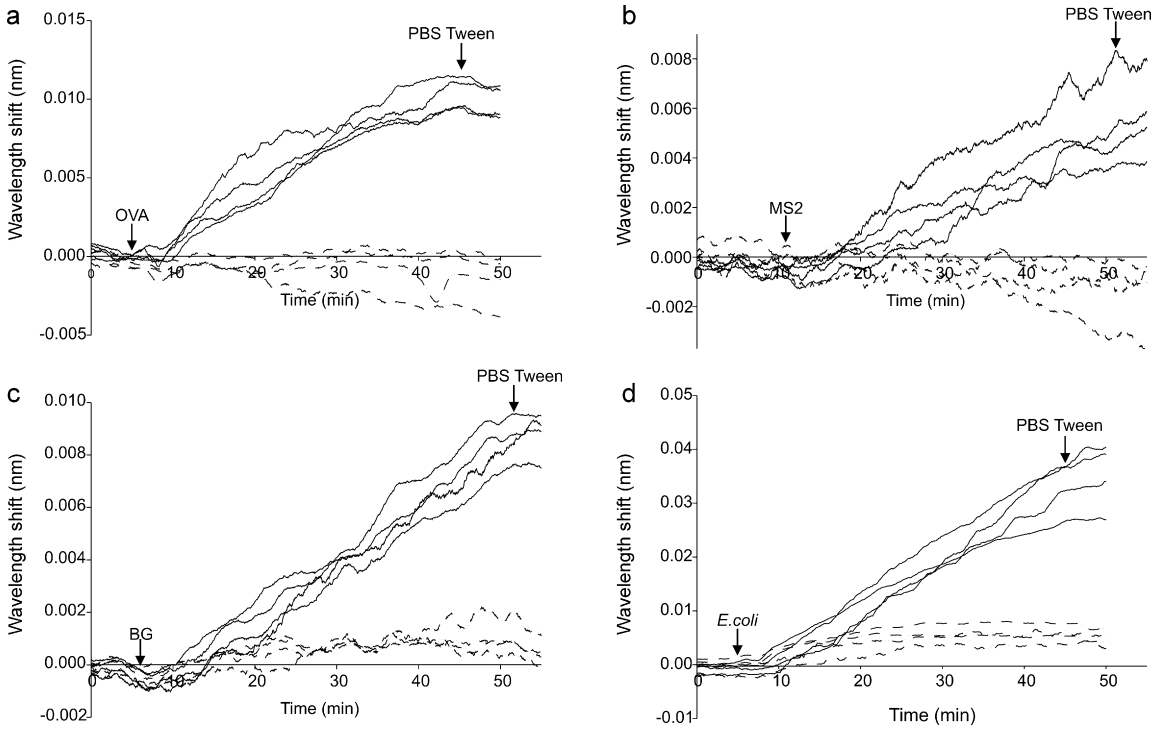

Fig. 4. Real-time SpectroSens™ sensorgrams demonstrating specific detection of 4 different simulant antigens: OVA protein, MS2 viruses, BG spores and *E. coli* cells. SpectroSens™ consumables patterned with simulant antibodies were exposed to (a) OVA protein (100 ng/ml), (-) anti-OVA channels, (–) anti-BG channels; (b) MS2 viruses (2×10^{10} pfu/ml), (-) anti-MS2 channels, (–) anti-BG channels; (c) BG spores (9×10^6 cfu/ml), (-) anti-BG channels, (–) anti-MS2 channels; and (d) *E. coli* cells (3×10^7 cfu/ml), (-) anti-*E. coli* channels, (–) anti-OVA channels in PBS-T under flow (20 μl/ml). Binding interactions across all 16 sensor channels were monitored for 20–25 min for each antigen before returning to PBS-T running buffer. *Note*: only 8 channels (4 specific channels, 4 control channels) are displayed on each graph for clarity.

reflected wavelength from anti-*E. coli* functionalised sensing channels that is significantly greater than the sensor response associated with non-specific binding onto anti-OVA control channels (presumably as a result of the relatively high antigen concentration in the test sample). Significant discrimination between specific *E. coli* channels and controls channels (~0.003 nm mean wavelength shift), however, was observed within 15 min of antigen exposure. All antigen detection sensorgrams illustrate that specific sensor responses observed subsequently plateau upon returning to PBS-Tween running buffer post-sample incubation, demonstrating that captured simulant antigens remain bound to sensing channels. Sensor response times towards the larger particulate antigens (bacterial antigens in particular) are inferior to those for protein detection for equivalent concentrations (100 ng protein equivalent to 10^5 cfu bacteria based on a mass of 1 spore of 1 pg). This is due to the delay associated with appreciable surface-capture of these large particles, which may be attributed to their relatively slow mass-transport rate and their need for attachment to multiple antibodies prior to firm adhesion to the sensing surface. Various performance-enhancement methodologies are currently being investigated to improve particulate detection.

3.4. Real-time detection of bio-hazardous agents (BA spores, FT cells, Vaccinia viruses and ricin toxin)

A single 16-channel SpectroSens™ consumable patterned with bio-hazard antibodies was used to determine the presence of 4 different bio-hazardous agents by successive exposure to BA spores (5×10^7 cfu/ml), FT cells (2×10^8 cfu/ml), Vaccinia viruses (10^7 pfu/ml) and ricin toxin (250 ng/ml) in PBS-T running buffer under flow. Fig. 5 illustrates the SpectroSens™ responses demonstrating real-time, label-free specific detection of all 4 bio-hazardous agents using a single test cartridge.

The sensorgram in Fig. 5a illustrates the SpectroSens™ responses associated with real-time detection of BA spores (5×10^7 cfu/ml) by anti-BA-functionalised sensing channels in comparison with responses from anti-Vaccinia channels as sensor controls. These data demonstrate a marked increase in sensor reflected wavelength from all 4 specific anti-BA channels, following an initial lag period (due to the time associated with appreciable build-up of spores at the sensor surface); whilst a smaller sensor response was also observed from control channels (indicating an element of non-specific binding), significant discrimination between specific and control channels (mean wavelength shift of ~0.003 nm) was achieved within 10 min of sample introduction. Real-time detection of FT cells (2×10^8 cfu/ml) is demonstrated in the SpectroSens™ sensorgram displayed in Fig. 5b, which shows a large increase in sensor reflected wavelength from anti-FT functionalised sensing channels (0.006 nm mean wavelength shift in 10 min), compared with negligible changes in response from anti-BA-functionalised sensor controls. SpectroSens™ responses associated with real-time, specific detection of Vaccinia viruses (10^7 pfu/ml) are illustrated in the sensorgram displayed in Fig. 5c; these data reveal that whilst a small increase in sensor reflected wavelength is observed across all sensing channels in the first 3 min (attributed to the bulk refractive index of the heat-killed virus sample; this is verified by the subsequent reduction in sensor response to the original baseline post-sample incubation), significant discrimination of sensor responses between anti-Vaccinia-functionalised channels and anti-ricin-functionalised control channels (0.003 nm mean wavelength shift) due to specific antigen-capture is observed within 10 min of sample introduction. Fig. 5d illustrates the SpectroSens™ responses demonstrating real-time of ricin toxin (250 ng/ml); these data show a steady increase in sensor reflected wavelength from anti-ricin-functionalised sensing channels (0.002 nm mean wave-

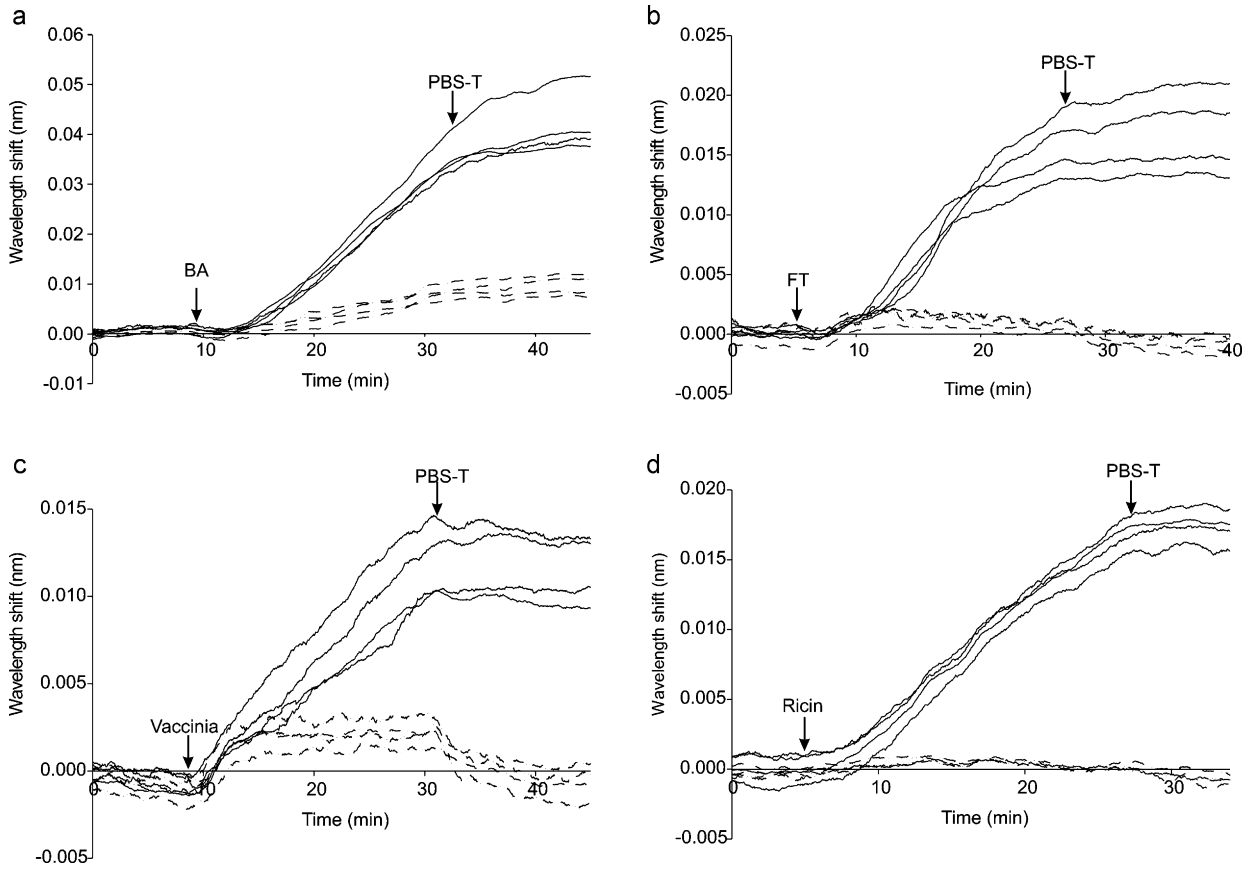

Fig. 5. Real-time SpectroSens™ sensorgrams demonstrating specific detection of 4 different bio-hazardous agents: BA spores, FT cells, Vaccinia viruses and ricin toxin. A single SpectroSens™ consumable patterned with bio-hazard antibodies were exposed to (a) BA spores (5×10^7 cfu/ml), (-) anti-BA channels, (–) anti-Vaccinia channels; (b) FT cells (2×10^8 cfu/ml), (-) anti-FT channels, (–) anti-BA channels; (c) Vaccinia viruses (10^7 pfu/ml), (-) anti-Vaccinia channels, (–) anti-ricin channels; and (d) *ricin* toxin (250 ng/ml), (-) anti-ricin channels, (–) anti-FT channels in PBS-T under flow (20 μl/ml). Binding interactions across all 16 sensor channels were monitored for ~30 min for each antigen before returning to PBS-T running buffer. *Note*: only 8 channels (4 specific channels, 4 control channels) are displayed on each graph for clarity.

length shift in 5 min), whilst negligible changes were observed from anti-FT-functionalised control channels. Analogous to the biodetector performance with simulant antigens, sensor response times were superior for ricin toxin detection compared with those for particulate bacterial detection (for equivalent concentrations). Antibody-patterned SpectroSens™ consumables were stored for up to 48 h before use, without significant deterioration of the sensor response.

On-going work is focused on improvements to sensor surface-chemistry for antibody immobilisation, with a view to achieving substantial increases in sensor signal to noise (S/N). To this end, the use of dendrimer molecules with abundant functional groups (Tomalia et al., 1985) in combination with long-chain PEG molecules and intermediary linkers exploiting the well-documented biotin–avidin interaction (Bayer and Wilchek, 1980) are examples of such approaches being investigated. The intention is to generate surface-coatings with a high density of antigen-binding sites and provide optimal steric interaction with antigens, whilst minimising non-specific binding of interferent materials.

The ability to perform multi-analyte detection on a single sensing surface demonstrates numerous advantages over performing multiple parallel analyses on different substrates; testing channels for one assay may be used as control channels for other assays, which can be used to correct for variations in sensor chip fabrication, surface-chemistry and sample delivery fluidics, thereby providing more effective cross comparison between experimental data and sensor controls. Ultimately, this sensor design opens up the possibility of simultaneous detection of multiple analytes, thus decreasing assay times when compared with sequential analyses.

3.4.1. Data processing algorithm

BIRAL's proprietary data-processing algorithm (VeroWarn biodetection software) was used to process the real-time SpectroSens™ data, in order to facilitate automatic detection of individual bio-hazardous agents using an agent-specific alarm, which was set to trigger above a pre-determined threshold (Fig. 6).

SpectroSens™ raw data is affected by factors that include, amongst others, time-related baseline drift, bulk liquid refractive index and non-specific binding events. As many of these factors are common to two or more sensor channels, the detection process was based on the extraction of features that were different from the general trends. Short term filtering was employed to remove sensor noise and this was followed by a continual normalisation process for the removal of fixed sensor channel offsets and time-dependent gains. Feature extraction was based on a channel by channel comparison of the normalised data to that of an average of the normalised data from selected channels. Alarm thresholds were set according to the variability of the difference between a given channel and its comparison set. An alarm was generated each time the sensor response from specific channels exceeded the threshold level for a given period of time (defined by the data quality).

Automatic detection of BA spores, FT cells, vaccine viruses and ricin toxin via alarm generation occurred between 12 and 15 min following sample introduction. Adjustment of the algorithm

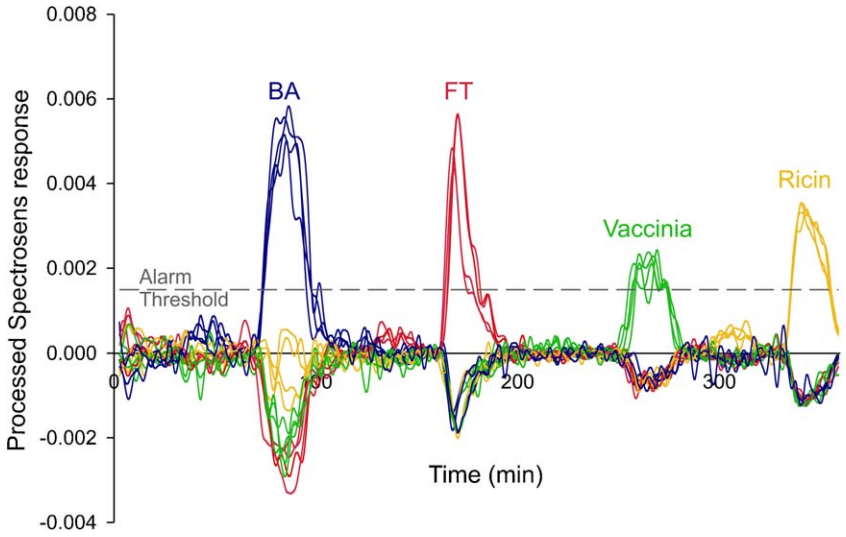

Fig. 6. Processed SpectroSens™ sensorgram with alarm threshold demonstrating consecutive bio-hazardous agent detection. SpectroSens™ data was processed using BIRAL's VeroWarn biodetection software which enabled identification of bio-hazardous agents by triggering an agent-specific alarm. Consecutive detection of BA spores, FT cells, Vaccinia viruses and ricin toxin was demonstrated using a single 16-channel SpectroSens™ consumable. ( ) Channels 4, 8, 12 and 16, ( ) channels 1, 5, 9 and 13, ( ) channels 3, 7, 11 and 15, and ( ) channels 2, 6 10 and 14. (*Note*: data plots can be visualised in colour online.)

parameters (e.g. alarm threshold, averaging period, etc.) may substantially decrease these response times.

4. Conclusions

SpectroSens™, a novel optical microchip sensing platform demonstrating multiplexed detection of a wide range of biological agents (encompassing pathogenic and simulant micro-organisms and toxins) has been presented. Real-time, specific detection of *B. atrophaeus* (BG) spores, *E. coli* cells, MS2 viruses and ovalbumin (OVA) protein (simulant agents) has been demonstrated using simulant antibody-patterned consumables, and multiplexed detection of *B. anthracis* (BA) spores, *F. tularensis* (FT) cells, Vaccinia viruses and ricin toxin (bio-hazardous agents) has been demonstrated using a single bio-hazard antibody-patterned consumable. SpectroSens™ responses manifested as increases in sensor reflected wavelength from specific waveguide channels within the optical microchip sensors as a result of cognate antigen-binding to corresponding antibody-functionalised sensing channels. Soluble protein antigens (ovalbumin and ricin) were most readily detected, with significant sensor responses to low antigen concentrations being observed within 5 min of sample introduction; confident detection of the larger bacterial and viral antigens within 10–15 min was demonstrated at (relatively) high antigen concentrations, due to the delay associated with appreciable capture of particulate antigens onto the sensing surface at low concentrations. SpectroSens™ consumables enabled simultaneous 16-channel data acquisition; their current design is easily adapted for simultaneous detection of 16 different biological agents by simple alteration of the microfluidic antibody delivery procedure to individually address independent sensing channels with 16 different agent-specific antibodies. These data illustrate the versatility of the man-portable and robust SpectroSens™ system for rapid detection of multiple biological agents of varying sizes, ranging from small soluble proteins (nanometres) to large particulate antigens (microns). Ongoing work is focused on investigating various performance-enhancement methodologies to improve both the sensitivity (particularly towards the larger particulate antigens) and reproducibility of the sensor response. The performance characteristics of the SpectroSens™ system highlight its potential for exploitation in security and defence operations, and in a variety of alternative applications including point-of-care/in-field medical diagnostics for human and veterinary health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.08.031.

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