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Proceedings of SPIE - Advanced Environmental, Chemical, and Biological Sensing Technologies VIII, 2011 / VoDinh, T., Lieberman, R.A., Gauglitz, G. (ed./s), vol.8024, pp.1-8

Enzyme detection by surface plasmon resonance using specially engineered spacers and plasmonic labelling

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ABSTRACT

Surface Plasmon Resonance (SPR) is a powerful label free optical biosensing technology that relies on the measurement of the refractive index or change of mass in close vicinity of the sensor surface. Therefore, there is an experimental limitation in the molecular weight of the molecule that can be detected and consequently small molecules are intrinsically more difficult to detect using SPR. One approach to overcoming this limitation is to first adsorb smaller molecules onto the sensor surface, and to follow this by using their higher molecular weight antibodies counterparts which ensure the specificity (and are easier to detect via SPR due to their higher weight). Although this has been demonstrated with some success, it is not applicable in every case and some biomolecules such as enzyme are still difficult to detect due to their specific reactivity (enzymatic reaction). In this paper, we present a powerful new method that utilises specifically engineered spacers attached on one end to the sensor surface and on the other end to a nanoparticle that behaves as a plasmonic label. These spacers are design to specifically react with the biomolecule to be detected and release the (relatively large) plasmonic label, which in turn results in a measurable SPR shift (which is much larger than the shift that would have been associated with the binding of the relatively small biomolecule). As a proof of concept, this approach was used within a recently developed new form of SPR optical fibre sensor which relies on the measurement of the re-emitted light by surface scattering of the plasmonic wave rather than transmission through the fibre was used to detect an enzyme. Here trypsin (25kDa) was successfully sensed. This molecule is involved in both intestinal and pancreatic diseases.

Keywords: Surface Plasmon Resonance, Optical Fibre, Enzyme, Biosensors

1. INTRODUCTION

Surface Plasmon Resonance (SPR) is a powerful analytical method that detects local change of refractive index at the interface between a thin metallic layer deposited onto a dielectric material and the surrounding environment [1, 2, 3]. Since the SPR transducing mechanism relies on refractive index changes, SPR sensing has an intrinsic limitation in terms of the molecular weight of the molecule to be detected [4, 5, 6]. In order to be able to perform analytical biosensing measurements of low molecular weight molecules, typically below 30kDa, strategies have been developed which consist of either immobilising the low molecular weight target molecule non-specifically onto the sensor surface first and then binding specifically the corresponding antibodies to the target molecule [7], using conformational changes of macromolecules, such as maltose binding protein and tissue transglutaminase upon analyte binding [8] or by using large particles that increase the local change of refractive index, therefore behaving like a plasmonic label [9]. In the later case, 1000 fold increase of the detection sensitivity has been observed.

Proteins such as enzymes are known to have a major role in many biological processes. Enzymes, like trypsin, are for example involved in gastric functions and can be used as a biomarker for some intestinal and pancreatic diseases [10], while other enzymes are found in higher quantities in cancer patient [11, 12]. Here, we demonstrate a new form of enzyme sensing that makes simultaneous use of SPR and fluorescence sensing. The transduction mechanism relies on the utilisation of especially engineered spacers that are selectively cleaved by the enzyme to be detected. As a proof of

Advanced Environmental, Chemical, and Biological Sensing Technologies VIII,
edited by Tuan Vo-Dinh, Robert A. Lieberman, Günter Gauglitz, Proc. of SPIE Vol. 8024, 802402
 · doi: 10.1117/12.883835

principle, trypsin which is a clinically relevant enzyme [10] was selected. One end of the spacer is attached to the sensor surface while a macromolecule, a quantum dot in this particular case, is attached to the other end of the spacer. Upon cleaving of the spacer by the enzyme, the macromolecule is released, which results in a significant decrease of the local refractive index and consequently a shift of the plasmonic resonance.

A new type of SPR fibre sensor, recently reported in the literature [13], has been used for this demonstration. While most commercial and experimental SPR sensors rely on either the measurement of the reflectivity of a metallic coating deposited on a dielectric [1, 2, 3] or spectral characterisation of light transmission through an optical fibre [14, 15], this new form of sensor exploits the re-emitted light from the plasmonic wave that occurs due to surface scattering [16, 17]. As shown in the Figure 1, a broadband light source is coupled into the fibre while the light re-emitted by the plasmonic coating is collected using optical fibres located within the sensing regions by an "evanescent light collection apparatus". The emission spectrum is resolve using a spectrometer. This rather simple but versatile architecture enables the user not only to assess the spectral position of the plasmonic resonance but also to perform fluorescence sensing as a secondary confirmation of the SPR sensing [13].

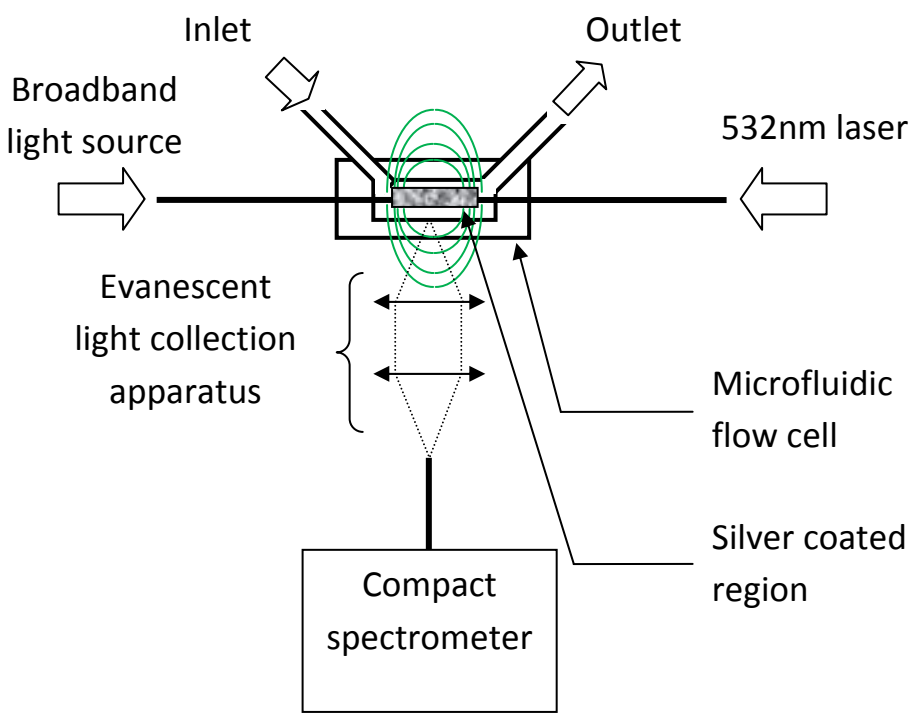

Fig. 1: Representation of the SPR fibre sensing architecture: an optical fibre comprising a silver-coated sensing regions embedded into a microfluidic flow cell. A broadband source and a 532nm laser are coupled into the fibre for the generation of the plasmonic wave and fluorescence sensing respectively. A fibre positioned adjacent to the sensing region collect both the SPR and fluorescence emission.

2. MATERIALS AND METHODS

2.1 Enzyme synthesis and characterisation

A 7nm long petptide chain (spacer) which contains a specific functional group that can be cleaved specifically by the chosen enzyme (trypsin) was synthesised by applying fluorenyl methyloxycarbonyl (FMOC) chemistry on 128 mg Fmoc-L-Ala functionalized Wang Resin (Auspep) at a loading level of 0.98 mmol/g. Peptide synthesis was carried out on the microwave peptide synthesizer (CEM Liberty 1, CEM PepDriver Version 3.5.5). 5 equivalent of each amino acid was reacted with 0.5 M HATU (5 equiv.) and 1 M DIPEA (5 equiv.). Each coupling cycle was 35 min. The spacer was cleaved from the resin using TFA/TIS/H2O (9.5:2.5:2.5). The crude product was precipitated with cold diethyl ether.

HPLC: Linear gradient of 90 % acetonitrile/0.01 % TFA in water from 0 to 45 %. t_r = 18.5 min; LCMS *m/z* for $C_{63}H_{101}N_{26}O_{18}$ + H ($[M+H]^+$) calcd 1509.8.

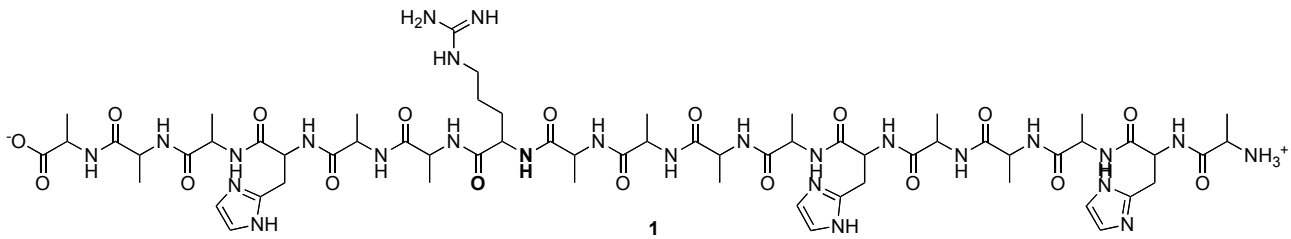

Fig. 2: Chemical structure of the spacer

All Liquid Chromatography Mass Spectroscopy (LCMS) spectra were recorded on Finnigan LCQ Ion Trap mass spectrometer. Electrospray conditions were as follows: needle potential, 4500 V; tube lens, 60 V; heated capillary, 200 °C, 30 V; sheath gas flow, 30 psi. Dimethylformaide, dichloromethane, diethyl ether, trifluoroacetic acid (TFA), triisopropylsilane (TIS), piperidine were received from Merck, diisopropylethylamine (DIPEA) was received from Sigma Aldrich and HPLC grade Acetonitrile was received from Ajax Finechem. Peptide coupling reagent 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) as well as all amino acids were purchased from GL Biochem (98% purity). All chemicals were used as received. Figures 3 (a) and (b) show the LCMS spectra of the spacer after purification and after cleaving with trypsin respectively. It was found experimentally that the molecular weight of the purified spacer was 1509.7 g/mol, which is consistent with the theoretical mass (1509.8) calculated from the structure. Upon exposure of the spacer to trypsin, the spacer is cleaved in two fragments as shown in the Figure 3 (b) of 555.1 and 1061.4. Need to put some concluding comments here

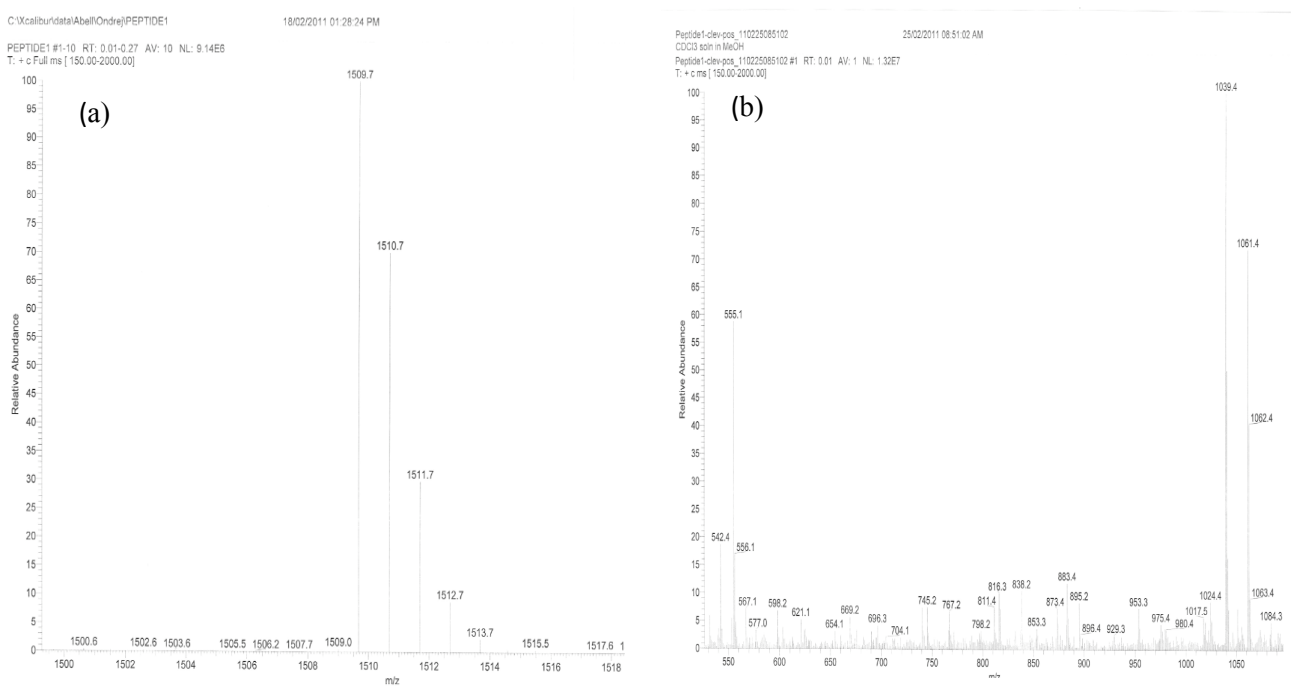

Fig. 3: LCMS spectra of the spacer (a) before and (b) after cleaving with trypsin

2.2 Silver electroless plating

This chemical coating technique allows metallic silver to be deposited onto the fibre surface without requiring specialised equipment while providing the opportunity of coating multiple regions of the same fibre simultaneously.

Moreover it has been previously shown that the silver coating deposited using this chemical technique has the same optical properties as a silver film deposited by thermal evaporation [18]. The Tollens reaction (also known as the silver mirror reaction) consists of adding a solution of silver ammonium to a reducing agent, usually a sugar such as glucose, in order to produce silver nanoparticles that can subsequently be attached to a substrate [19]. The preparation of the Tollens reagents starts with the precipitation of a silver nitrate solution (20mL of 0.24mol/L $AgNO_3$), into silver oxide nanoparticles using potassium hydroxide (40uL of 0.25mol/L KOH) according to Eq.1. This produces a brown precipitate in the initially transparent silver nitrate solution. Then ammonia (3mol/L) is added drop by drop to dissolve the silver oxide and produce a transparent silver ammonium complex (Eq. 2).

$$2\ AgNO_3 + 2\ KOH \rightarrow Ag_2O\ (s) + 2\ KNO_3 + H_2O \quad (1)$$

$$Ag_2O\ (s) + 4\ NH_3 + 2\ KNO_3 + H_2O\ (l) \rightarrow 2\ Ag(NH_3)_2NO_3 + 2\ KOH \quad (2)$$

The reducing agent was made up of a 1:2 mixture of methanol and glucose (1.9 mol/L,) solution and added in equal parts to the silver ammonium solution, then mixed using a magnetic stirrer. Once the reducing agent is added to the silver ammonia solution, a metallic silver coating is produced (Eq. 3).

$$CH_2OH(CHOH)_4CHO + 2Ag(NH_3)_2 + 3OH^- \rightarrow 2Ag(s) + CH_2OH(CHOH)_4CO_2^- + 4NH_3 + 2H_2O \quad (3)$$

After coating, the fibres were rinsed in de-ionized water and then air-dried.

2.3 SPR sensor fabrication

The SPR sensor was fabricated from a simple unstructured optical fibre made of F2 Schott glass with a refractive index of 1.62 and a diameter of 140 μm. The fibre was produced in-house by extruding a bulk glass sample purchased from Schott into a rod [20] and drawing this into a fibre on a soft glass fibre drawing tower. The fibre was coated with a polymer with a refractive index of 1.52 yielding a numerical aperture (NA) of 0.56. A short section (~ 4mm) of the fibre was stripped of this polymer coating, and coated with silver using the Tollens reaction to form the sensing region, as described in Section 2.2. Following the silver deposition, the sensing region was placed into a microfluidic flow cell moulded in PDMS (polydimethylsiloxane (Sylgard 184; PDMS) from Dow Corning Co.) and hermetically sealed unless stated otherwise. Two PEEK capillaries were then inserted into the flow cell and used as inlet and outlet to allow passage of test solutions through the flow cell.

2.4 Sensor surface functionalisation

Polyelectrolytes (PE) (Poly(allylamine hydrochloride) (PAH), MW ~15,000 Da, poly(sodium 4 styrenesulfonate) (PSS), MW ~70,000 Da received from Sigma-Aldrich) were used to functionalise the surface of the silver coated region of the second sensing region to provide reactive groups that can be subsequently used to immobilise the spacers onto the sensor surface. All injections were performed at an infusion rate of 10 μl/min for 15 min. The wavelength of light emitted from the sensing region was monitored during each step of the surface functionalisation processes and spectra collected. Positively charged PAH (2mg/mL in 1M NaCl solution) and negatively charged PSS (2mg/mL in 1M NaCl solution) were deposited alternately onto the sensor surface using the layer by layer deposition technique described elsewhere [21], ending with a PAH layer (PAH/PSS/PAH) which provides amino groups for immobilisation of the spacer. In between depositing each layer the sensor was rinsed extensively with deionised water. To immobilise the spacer, a solution containing equal volumes of freshly prepared 1M NHS, 1M EDC (N-hydroxysuccinimide (NHS), 1-Ethyl-3-[3-dimethylaminopropyl], carbodiimide hydrochloride (EDC), and ethanolamine hydrochloride (EA), 1 M, were obtained from Biacore as a part of the amine coupling kit) and 1mg/mL spacer was injected into the flow cell, incubated for 30 min and any excess active ester formed by the coupling reagent that did not react with the antibodies was neutralised with an equal volume of 1M ethanolamine. Following immobilisation of the spacer, quantum dot with carboxylic function (100nM) (received from Invitrogen) were covalently bound to the free end of the spacer using EDC/NHS following the same procedure.

2.5 Optical setup

In the SPR configuration shown in Figure 1, light from a high intensity fibre coupled light source (Thorlabs – OSL1) and from a 25mW, 532nm laser (Crystal Laser) were coupled into the fibre samples using aspheric achromatic lenses (Thorlabs C390TM-A). The evanescent field emerging from the silver coated region was collected using a microscope objective positioned directly under the sensing area and directed into a compact spectrometer (Ocean Optics, QE65000) using another optical fibre (Ocean Optics).

3. RESULTS AND DISCUSSION

3.1 SPR fibre sensor sensitivity

To determine the sensitivity of the sensor, the flow cell surrounding the silver coated region was filled with solutions containing increasing concentrations of glycerol (glycerol, > 99%, was obtained from Wako Pure Chemical Industr.) with refractive indices ranging from 1.33 to 1.47. Figure 4 (a) shows the spectra recorded for these different refractive indices; the evolution of the position of the emitted SPR peak can be clearly seen. After performing background subtraction, corresponding to a spectrum recorded without any refractive index liquid around the sensing region, an autocorrelation function was used to calculate the wavelength shift as function of the refractive index, as shown in the Figure 4 (b).

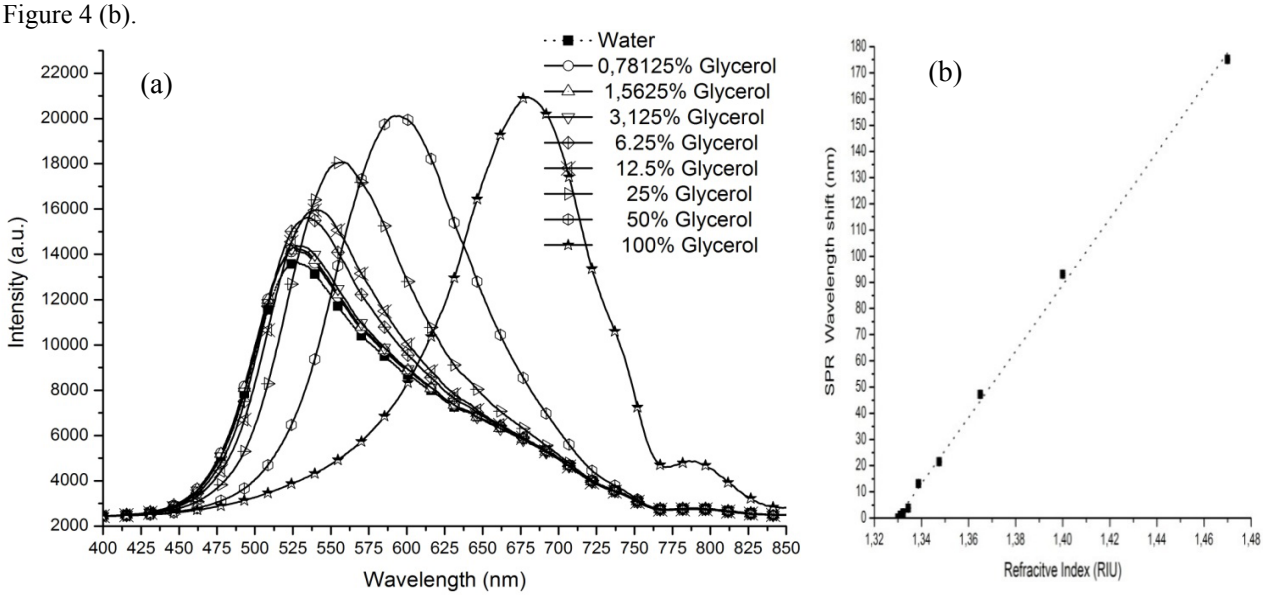

Fig. 4: (a) Evanescent field spectrum acquired in different refractive index conditions using the SPR fibre sensor and (b) SPR wavelength shift measured as function of the refractive index.

In those conditions, the sensitivity calculated from experimental data was 7.9×10^{-4} RIU. This is of comparable magnitude to the first SPR fibre sensor reported in the literature by Jorgensen & Yee (2.5×10^{-4} - 7.5×10^{-5} RIU) [15]. Unsurprisingly, there was no improvement of the sensitivity observed depending on the characterisation of the SPR signal (collection mode vs. transmission reported by Jorgensen et al. [15]) since sensitivity primarily depends on the dielectric function of the silver and the optical fibre characteristics. The Full Width at Half Max (FWHM) of the SPR peak varies between 45nm for the lower range of refractive index tested to about 80nm for the highest refractive index range. Assuming that 1/50 of the FWHM can be detected in terms of the SPR wavelength shift (corresponding to the spectral resolution of a compact spectrometer), it implies that the smallest RIU change detectable is at best about 1.6×10^{-5} RIU. It is important to note that significant improvement of the resonance full width at half maximum should be possible using smaller core fiber designs [22], since such fibres have fewer modes that can satisfy the coupling condition with the plasmonic wave.

3.2 Demonstration of enzyme detection

The sensor was prepared for the specific detection of trypsin as described in the materials and methods section (Figure 5(a). Evanescent field spectra (SPR) were recorded after each deposition step (Figure 5(b)) and fluorescence spectra of

the attached quantum dots onto the sensor surface were also recorded before and after cleaving of the spacer (Figure 5 (c)). The evaluation of the evanescent field spectra were performed as described previously, using an autocorrelation function to calculate the wavelength shift between each spectra after subtraction of a background signal corresponding to the scattering of the white light source (Figure 5 (d)).

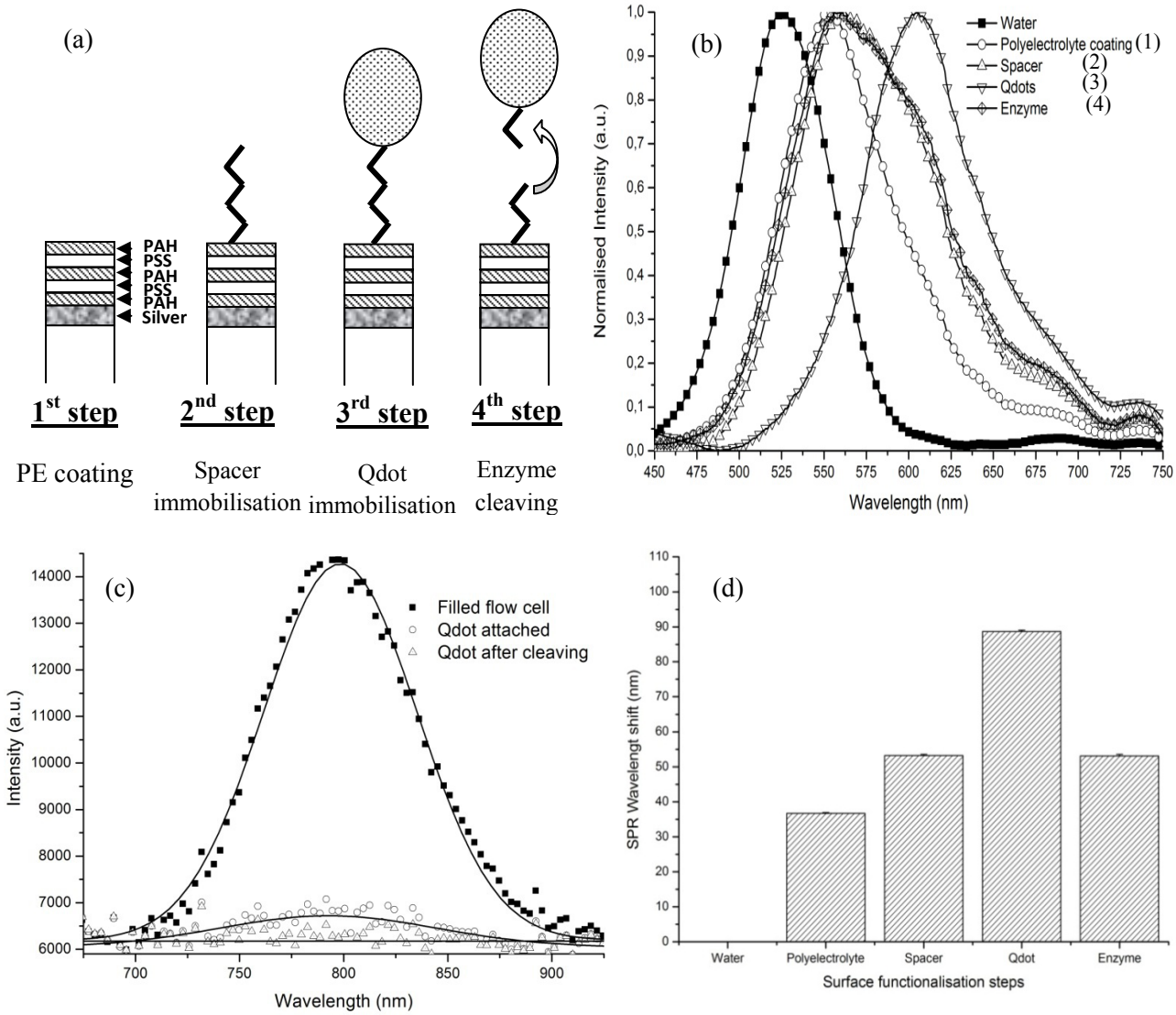

Fig. 5: (a) Representation of the surface functionalisation steps for the detection of trypsin and (b) SPR spectrum acquired after each step of the surface functionalisation. (c) Fluorescence spectra acquired from the Qdots immobilised onto the sensor surface before and after cleaving and (d) SPR wavelength shift measured after ech step for the surface functionalisation process, the SPR signal in water was used as a reference.left part of vertical axis 5c is cut off

Although the molecular weight of the spacer is small (1.5 kDa), it can be seen from both Figure 5 (b) and (c) that the SPR wavelength shift increases from 35nm to 50nm upon the immobilisation of the spacer onto the sensor surface which confirms the immobilisation of the spacer. This high wavelength shift occurs as a result of the cross-linking of the spacer onto the sensor surface. As can be seen by considering the chemical structure of the spacer (Fig. 2), this structure presents one carboxylic function and two amino groups, one of these amino groups being part of a larger functional group specifically recognised by the enzyme. The carboxylic function was intended to be used for covalent binding to the sensor surface coated with the PAH coating, which provides amino groups. However, since during the immobilisation step, the spacer is mixed in solution with the amine coupling reagent, the succinimide ester is free to

react with any amino groups in solution or on the sensor surface. Although this situation is not ideal because instead of a single monolayer, a 3D gel of spacer molecules was deposited onto the sensor surface which makes it difficult to quantify properly the amount of spacer molecules subsequently cleaved by the enzyme, this approach was used as a means of confirming that the spacer was indeed immobilised onto the sensor surface. Note that a variation in the surface functionalisation process, using another polyelectrolyte that provides a carboxylic function (such as poly-acrylic acid) would have solved this issue but it would not have been possible to detect the presence of the spacer onto the sensor surface. Following the immobilisation of the spacer, carboxylic functionalised Qdots were again covalently bound to the free amino groups of the spacer. The resulting SPR wavelength shift is about 35nm. Fluorescence measurements of the immobilised Qdots onto the sensor surface were performed during the immobilisation with the flow cell filled with the Qdots solution and after rinsing (Figure 5(c)). Gaussian fitting of the fluorescence spectra was used to calculate the area under the fluorescence spectra and consequently estimate the surface density of bound Qdots (~38 fmol/mm^2). A 100μg/mL solution of trypsin, dissolved in PBS buffer (Phosphate buffered saline (PBS) was received in the form of tablets from Sigma-Aldrich and dissolved in deionized (DI) water yielding a pH of 7.4.) was injected into the flow cell after the Qdots immobilisation, at 5μL/min (150μL). Then the flow cell was rinsed with PBS buffer and both SPR evanescent field and fluorescence spectra recorded. As can be seen in both Figures 5 (b) and (d), the SPR signal shift to shorter wavelengths indicates a release of material from the sensor surface. This was confirmed using the fluorescence spectra (Figure 5 (c)), where the signal from the Qdot coating vanished. Unsurprisingly the entire spacer coating was not removed since the cross-linking prevented the enzyme to reach the functional group to be cleaved as the SPR wavelength position did not came back to the position right after the PE coating but after the spacer coating.

CONCLUSION

We have demonstrated that enzymes can be detected using a new SPR fibre sensor that enables the measurement of both the SPR signal as well as the fluorescence signal of a labelled molecule. This particular approach, which relies on the utilisation of especially engineered spacers that are specifically cleaved from the sensor surface by the target enzyme along with the utilisation of large fluorescent macro particles such as quantum dots proved to be an efficient way to detect smaller biomolecules than can be detected using more traditional techniques in which molecules are cumulatively added to the sensor surface. Although the detection limit of this approach has not yet been assessed, the direct detection of enzymes with our particular SPR fibre sensor is already beyond the range of the molecular weight of detectable molecules. Another motivation for this work was also to find a method to regenerate the surface of the SPR fibre sensor. One can imagine that an antibody can be attached to the free end of the spacer instead of a quantum dot. Once the sensor has been used and the epitope of the antibodies are occupied by their respective antigen, the spacer could be cleaved, releasing the used antibodies from the surface while another layer of antibody could be re-attached to the regenerated surface.

ACKNOWLEDGMENTS

T. Monro acknowledges the support of an ARC Federation Fellowship. The authors acknowledge Dr. Ondrej Zvarec and Prof. Andrew Abell from the University of Adelaide for their valuable input concerning the peptide synthesis as well as the support of DSTO Australia for this work.

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