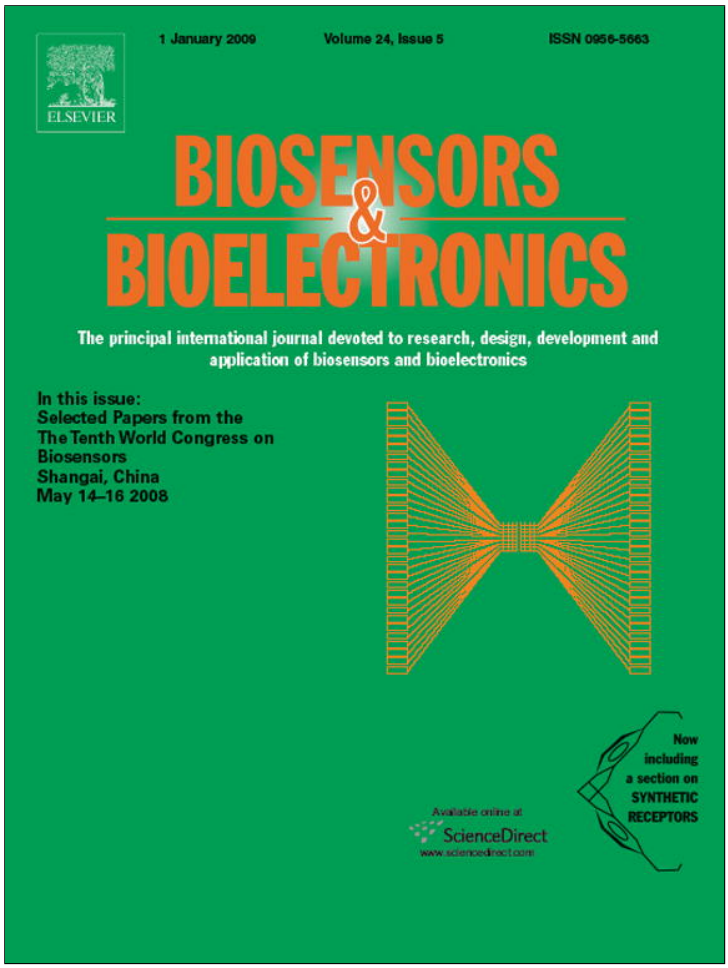

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Deposition of functionalized polymer layers in surface plasmon resonance immunosensors by *in-situ* polymerization in the evanescent wave field

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ABSTRACT

Traditionally, the integration of sensing gel layers in surface plasmon resonance (SPR) is achieved via "bulk" methods, such as precipitation, spin-coating or *in-situ* polymerization onto the total surface of the sensor chip, combined with covalent attachment of the antibody or receptor to the gel surface. This is wasteful in terms of materials as the sensing only occurs at the point of resonance interrogated by the laser. By isolating the sensing materials (antibodies, enzymes, aptamers, polymers, MIPs, etc.) to this exact spot a more efficient use of these recognition elements will be achieved. Here we present a method for the *in-situ* formation of polymers, using the energy of the evanescent wave field on the surface of an SPR device, specifically localized at the point of interrogation. Using the photo-initiator couple of methylene blue (sensitizing dye) and sodium *p*-toluenesulfinate (reducing agent) we polymerized a mixture of *N*,*N*-methylene-bis-acrylamide and methacrylic acid in water at the focal point of SPR. No polymerization was seen in solution or at any other sites on the sensor surface. Varying parameters such as monomer concentration and exposure time allowed precise control over the polymer thickness (from 20–200 nm). Standard coupling with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide was used for the immobilization of protein G which was used to bind IgG in a typical biosensor format. This model system demonstrated the characteristic performance for this type of immunosensor, validating our deposition method.

1. Introduction

Surface plasmon resonance (SPR) is a physical process that occurs when plane-polarized light irradiates a metal film under conditions of total internal reflection (TIR), (Maier, 2007). The energy from the incident photons is absorbed and converted into surface plasmons. By creating a state of total internal reflection (TIR) using an optical prism of suitable geometry, a surface plasmon evanescent wave (SPEW) is created at the interface between a metal (e.g. gold or silver) layer and the adjacent medium (Maier, 2007). The SPEW extends into the materials on either side of the metal film with the intensity of the SPEW depending on the angle of incidence θ (Maier, 2007; Zhang and Uttamchandani, 1988; Homola et al., 1999). Oscillations in the wave generated on the side of the metal opposite to the direction of the incident light (at the working interface) are very sensitive to changes in the local refractive index close to the surface, including those due to the adsorption of molecules. This effect can extend through materials bound to the working surface, allowing for binding events between receptor structures (antibodies, aptamers, etc.) bound to these materials and their ligands to be measured. Despite its non-selective nature, measuring only changes in refractive index, and its low sensitivity towards binding of small molecules, SPR has become a powerful and inherently flexible sensor platform when combined with various natural and synthetic receptors (Homola, 2003).

The introduction of sensing layers onto the working surface is traditionally performed by "bulk" methods, such as spin-coating or *in-situ* polymerization over the entire surface of the sensor chip. For example, Lavine et al. (2007) immobilized lightly cross-linked *N*-(*n*-propyl) acrylamide molecularly imprinted beads by spin-coating onto an SPR slide. The swelling properties of these beads were used to measure the concentration of the template theophylline. Gabai et al. (2001) used electropolymerization to deposit a boronic acid/acrylamide co-polymer film on gold SPR slides which were used to measure the concentration of glucose. Similarly Damos et al. (2005) electropolymerized ultra-thin films of (poly)methylene blue

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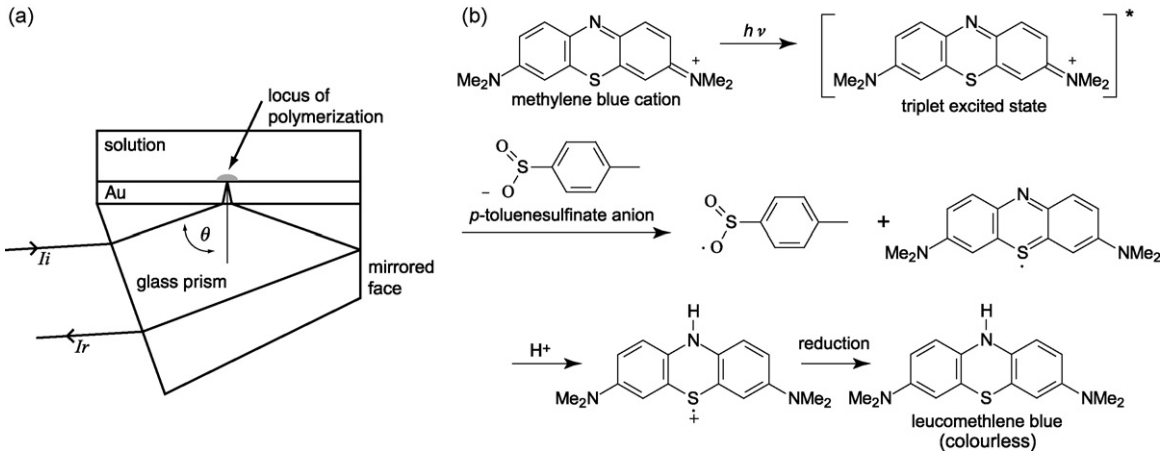

Fig. 1. (a) Schematic of the SPR optical prism configuration used, (b) scheme showing the photoreduction of the triplet excited state of methylene blue and formation of the bleached form of the dye, leucomethylene blue. It is likely that radicals derived from the *p*-toluenesulfinate radical are the active initiating species (Margerum et al., 1971).

and studied them using real-time electrochemical SPR. In these examples the area of the deposited sensing layer extends beyond the focal point of SPR, covering the entire slide. As the interrogation of the molecular interactions by the laser occurs only over a small portion of deposited material during any sensing process, this complete coverage becomes wasteful in terms of reagents. For expensive analytes such as peptides or aptamers this can rapidly become cost prohibitive for a commercial sensor system. Moreover, by limiting materials to the point of interaction only, an increase in sensitivity could theoretically be achieved, as capture of target analytes outside of the sensing zone should not occur. Any analyte present would only be able to bind at the point of interest, meaning binding events will not be "lost" by occurring outside this area. A third benefit lies in the option for miniaturization (interrogation at the point of interest only can lead to smaller devices, requiring smaller chips and fluidics).

To perform this specific placement of the sensing medium, we have used the SPEW itself to determine the site at which the material is deposited. The simplest way to envisage this is through a photo-initiated polymerization process, using the energy of the SPEW to drive the reaction *in-situ*.

In order to utilize the energy of the SPEW to initiate polymerization at the SPR sensor surface (Fig. 1a) and localize the polymer spot to the sensing zone, a photoinitiator with the appropriate wavelength sensitivity must be used. Dye-sensitization provides a convenient method for extending the wavelength of light used to initiate free radical polymerization far into the visible spectrum and even to the near infra-red (Oster and Yang, 1968). Phenothiazine dyes such as thionine and methylene blue have proved particularly useful at red-sensitization of aqueous-soluble monomers when employed as part of a photo-redox initiator couple, the second component being a mild reducing agent such as a diketone (Chaberek et al., 1965a,b), triethanolamine (Hsia Chen, 1965) or a salt of a sulfinic acid such as *p*-toluenesulfinic acid (Rust et al., 1969). An investigation of the mechanism of the latter system found that the triplet excited state of methylene blue was reduced by sulfinate ions and that the sulfinate-derived radicals were largely responsible for the polymerization of acrylamide, rather than semimethylene blue radicals (Fig. 1b) (Margerum et al., 1971).

In this paper we report the use of the photo-redox couple of methylene blue and sodium *p*-toluenesulfinate to initiate polymerization in an aqueous solution of *N*,*N*-methylene-bis-acrylamide and methacrylic acid. The energy required to initialize the reaction is supplied by the SPEW effect. The deposition was found to be controllable by switching the laser on/off, and by changing the relative concentrations of the components. As such we were able to alter the thickness of deposition. The polymer was used as a functionalized support for the immobilization of protein G, with the resulting biosensor being used to demonstrate the specific rebinding of immunoglobulin IgG (a typical model system). The system was shown to be highly reproducible.

2. Materials and methods

2.1. Materials

Methylene blue, sodium *p*-toluenesulfinate hydrate, acrylamide and methacrylic acid were obtained from Sigma–Aldrich (Gillingham, UK) *N*,*N*-methylene-bis-acrylamide, and sodium acetate trihydrate were purchased from Fisher Scientific (Loughborough, UK), disodium hydrogen phosphate and sodium dihydrogen phosphate were obtained from Acros Organics (Loughborough, UK). General purpose buffer (Biacore HBS-EP, containing: 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA and 0.005% (v/v) Surfactant P20) and amine coupling reagents kit (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinamide (NHS) and 1.0 M ethanolamine hydrochloride pH 8.5) were obtained from Biacore (GE Healthcare, Little Chalfont, UK). Protein G and IgG were purchased from Sigma (Gillingham, UK). All solvents were of HPLC grade and used as received.

2.2. Experimental set-up

An SPR instrument (NanoSPR-321, NanoSPR, USA), with ATR prism was set-up in the following manner. A glass prism with a refractive index of 1.6160 was selected as optimum for use with SPR in water, with a nominal ATR angle of 65°. A gold coated slide (20 mm × 20 mm × 1 mm), coated with a 45 nm layer of gold over a 5 nm layer of chromium (NanoSPR, USA) was mounted on the upper face of the prism with the gold layer uppermost. A thin layer of index matching fluid (NanoSPR, USA) was applied between the glass face of the slide and the prism. Care was taken such that no air bubbles were trapped at the interface and excess index matching fluid was cleaned from the optical face of the prism. A transparent block with microfluidic connections, separated by a silicone elastomer spacer (approx. 1 mm in thickness) was clamped to the gold-coated face of the slide. Within this spacer two cut-out areas defined chambers, each with its own microfluidic connections through the transparent block. These chambers therefore act as two independently operating flow channels, each interrogated with a separate light beam

from the instrument. The fluidic connections were attached to a peristaltic pump (Gilson, France), allowing liquid flow through the chambers and across the working face. Pure water and all buffers and solutions were degassed by sonication and/or sparging with argon before introduction into the flow system to avoid the formation of air bubbles on the sensor chip. Solutions were changed by exchange of the reservoirs containing the stock solutions under stopped-flow conditions. Data collection and instrument control was performed using the NanoSPR® application software running on a PC.

Calibration was performed by filling the solution cell with pure water (previously degassed by sonication), using the calibrate function of the NanoSPR® software. The SPR curve in water was recorded at the outset of each experiment by scanning the angle of the incident light beam (*Ii* in Fig. 1a). This is achieved by rotating the prism and solution cell assembly relative to the light source and detector, under software control. The light source was a diode laser emitting red light (GeAs laser, $\lambda = 670$ nm). Calibration was performed each time a new gold slide was fitted to the instrument.

2.3. In-situ polymerization

A stock solution of monomers was prepared comprising 0.1 g of *N*,*N*-methylene-bis-acrylamide and 0.9 g of methacrylic acid dissolved in 10 mL of water (9:1 ratio). A stock solution of methylene blue (10 mg mL^{-1}) in water was also prepared. For each polymerization experiment, two vials containing the monomer stock solution (0.5 mL) diluted with water (1.5 mL) were prepared. An equal amount of the methylene blue stock solution was added to each vial: 0, 5, 7.5, 10 or 15 μL, depending on the experiment, to give final dye concentrations of 0, 2.5, 3.75, 5 or 7.5 μg mL^{-1}. An aliquot (20 μL) of a freshly prepared solution of sodium *p*-toluenesulfinate hydrate in water (10 mg mL^{-1}) was added to one of the vials. The solution containing the sulfinate salt was protected from light by wrapping the outside of the glass vial with aluminum foil. Water (20 μL) was added to the second vial, which was identical therefore to the first, except for the absence of the sulfinate salt. Both solutions were degassed by passage of a stream of argon for at least 3 min shortly before use.

A calibration was performed, as described above, then the contents of the solution cell were replaced by flowing a portion of the solution containing monomers and dye only, and the SPR curve was again obtained by scanning the angle of the incident light. The angle of the light beam was then adjusted to the position of TIR, indicated by the minimum point of the SPR curve. This was achieved using the "measurement in slope" function of the NanoSPR® control software.

The light beam was temporarily blocked by an opaque obstruction (e.g. a piece of card) while the contents of the solution cell were exchanged for a portion of the solution containing monomer, dye and sulfinate salt. The dye and sulfinate constitute a photoredox couple, capable of initiating polymerization in red light. Removal of the blockage in the light beam allowed the SPR condition to be re-established at the gold surface and polymerization to proceed. The formation of a spot of polymer at the gold surface rapidly ensued. The SPR apparatus was shielded from stray sources of light during the polymerization process.

The deposition of polymer could be followed by the change in intensity of the reflected beam with time. The polymerization was limited by the penetration of the evanescent wave of red light and did not occur indefinitely, reaching a maximum thickness of film. Polymerization could be stopped earlier if necessary by removing the light source and flushing the monomer solution from the sample cell. No polymerization in solution was observed, the reaction being localized to the interrogation point. The excess unreacted monomer solution was removed from the solution cell and the polymer was washed with water until the SPR curve stabilized. The SPR curve was then recorded to demonstrate the presence, and calculate the thickness of the polymer layer (by refractive index, see below). Adjusting the parameters of the experiment (concentrations of the components, time of irradiation, angle of incidence of the laser light, etc.) affected the rate of polymerization and the size and thickness of the deposited polymer spot. This allowed a further level of control over the polymerization process.

2.4. Calculation of polymer thicknesses

The experimental SPR spectra of the polymer layers were fitted to the theoretical curves based on five-phase Fresnel calculations, using the Neelder–Mead algorithm of minimization (Beketov et al., 1998). The fitting procedure was based on modelling the SPR curve in a multilayer system, where each of the components: glass prism, Cr adhesion layer, Au layer, polymer and liquid environment were taken into account. Calculations of the scattering matrix for the multilayer system allowed the modelling of the form and angular position of the SPR curves with those obtained by experiment. The results of modelling allowed us to obtain approximate values for the complex refractive index of the polymer and its thickness.

2.5. Immobilization of protein G and analysis of specific interactions

In order to demonstrate the fabrication of a biosensor using a polymer layer as the immobilization material; protein G was coupled to a carboxylic acid-functionalized polymer and the specific interaction of IgG with the immobilised protein G was observed by SPR. This was selected as it is a generic system and has been well characterized. Coupling and assay conditions were based on those given in a Biacore® application note (Biacore Application Note). Polymers were formed, as described above, at a dye concentration of 2.5 μg mL^{-1}. After thorough washing with water, the available carboxylic groups of the polymer were activated with a solution of EDC (0.2 M) and NHS (0.05 M) for 10 min. The sensor surface was then flushed with phosphate buffer, pH 7.0 and the system was allowed to stabilize. Protein G, 10 μg mL^{-1} was added in 10 mM acetate buffer, pH 4.0 (ac. b) and allowed to react with the activated polymer for 10 min before flushing again with phosphate buffer, pH 7.0. A solution of ethanolamine was then added and allowed to react with any uncoupled NHS ester groups. This was followed by Biacore® HBS-EP mixed buffer solution, consisting of 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Surfactant P20 to wash away excess protein, the solution passing through the flow cell was then switched to 0.1 M HCl to remove non-specifically bound material, then back to acetate buffer, pH 5.0. Once the sensor response had stabilized, IgG, (200 μg mL^{-1}) in 10 mM acetate buffer, pH 5.0 was added. After 10 min 10 mM acetate buffer, pH 5 was passed through the cell. Once the sensor response had stabilized, 0.1 M HCl was flowed over the sensor surface to regenerate the polymer-immobilized protein G (Biacore Application Note). Finally the solution in the flow system was returned to 10 mM acetate buffer pH 5.0. The same experiment was run concurrently in the second channel, all steps being performed in the same manner except for the absence of protein G.

The above experiment was then repeated using stepped concentrations of IgG to study binding capacity of the immunosensor. Finally the IgG binding, desorption and regeneration steps were repeated in series to demonstrate the reproducibility and stability of the system.

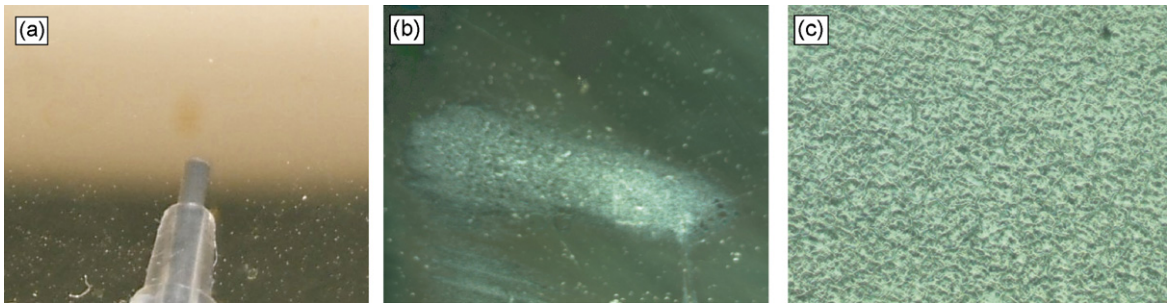

Fig. 2. (a) Photograph showing polymer spot on the gold-coated SPR slide, clearly visible with the naked eye, the width of the pencil lead is 0.5 mm. (b) Optical micrograph of a polymer spot, similar to that shown in (a). The spot is approximately 2 mm × 0.5 mm in size. (c) Close-up of the surface of the same polymer spot at 40× higher magnification.

3. Results and discussion

The choice of a photo-initiator system to selectively polymerize a mixture of monomers contained in the solution cell of an SPR sensor was determined by the wavelength of light used in the instrument chosen for this study. As the NanoSPR^{TM} platform allowed us easily to select and fix the angle of incidence as well as follow the sensogram during polymerization, this instrument was chosen for this work. The NanoSPR^{TM} uses a GaAs semiconductor laser (λ = 670 nm), therefore a red-sensitive photoinitiator was required. The photo-redox couple of methylene blue and sodium *p*-toluenesulfinate were selected as a water-soluble initiator, compatible with the laser wavelength used and known to be capable of initiating the polymerization of acrylamide and other water-soluble monomers (Rust et al., 1969). Initial experiments were conducted with solutions of acrylamide, *N*,*N*-methylene-bis-acrylamide mixtures to test whether polymerization occurred at the SPR position. This was indeed the case, not only was polymer formed on the surface of the gold slide, creating an image of the SPR spot, and nowhere else (on the surface or in solution) but the polymerization process was easily followed by the SPR sensogram, recorded during polymer formation. This allowed for both feedback on the progress of polymerization and the possibility to terminate polymerization at a predetermined point by blocking the light beam when a particular sensor response had been achieved. No specific steps were taken to ensure adhesion of the polymer to the gold surface. The bond between the polymer and gold appears to be quite strong and polymer spots were stable to washing and a range of chemical treatments. It is possible that adhesion is mediated through sulfur atoms present in the initiator components (Fig. 1b). Typical polymer spots on the gold surface can be seen in Fig. 2.

The thicker polymer spots were clearly visible to the naked eye, however, since the aim was to produce a platform for the immobilization of proteins and other receptor, very thin layers (ideally of the order of 10 s of nanometers) were required. In addition the polymer system chosen for our initial work lacked suitable functionality for protein attachment. Subsequent experiments were therefore performed with methacrylic acid solutions, containing 10% (w/w) of the cross-linker *N*,*N*-methylene-bis-acrylamide. Our initial experiments with acrylamide had shown that the thickness of polymer formed was determined by the concentrations of the components (dye, monomer and *p*-toluenesulfinate salt) of the polymerization mixture (results not shown). We therefore chose to vary the concentration of methylene blue in a series of experiments designed to follow the progress of polymerization with time, using the methacrylic acid system, described above. The results are shown in Fig. 3.

Fig. 3 shows the sensor response recorded at fixed angle during photo-polymerization. In the absence of methylene blue, no polymerization was observed, whereas in the presence of increasing concentrations of the dye a greater SPR response was seen. Curves showed typical polymerization behaviour; exhibiting a short induction period, followed by more rapid growth of the layer. It was evident, however, that the sensorgram curves rapidly reached a plateau within a few minutes, the level of which was dependent on the initial dye concentration (Fig. 3II). It is not clear why this behaviour is seen since dye and monomer were clearly not exhausted; the solutions over the gold slide still appeared to show a uniform blue colour and the same monomer concentration was used in all experiments. The most likely explanation is that the rate of initiation of polymerization will depend upon both the inten-

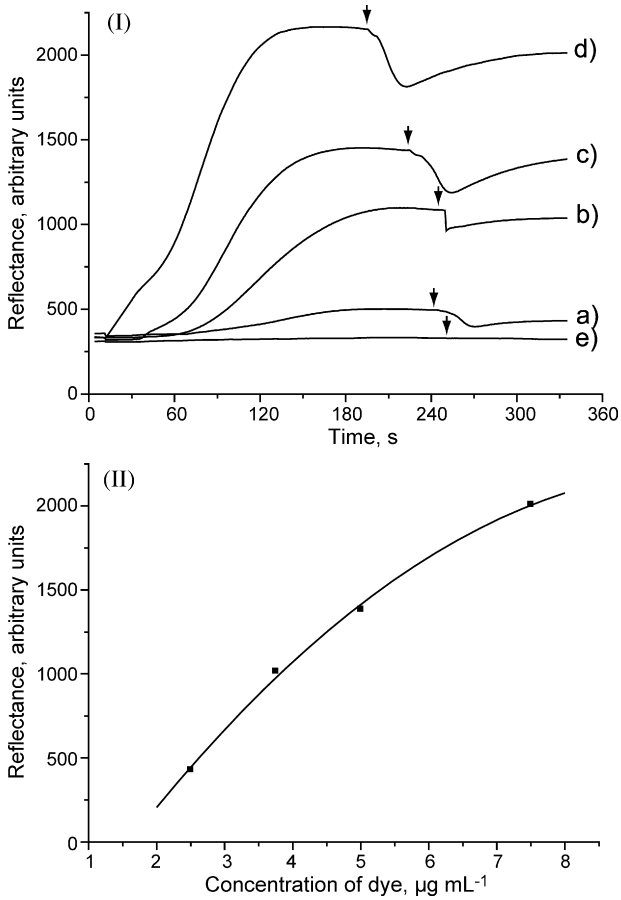

Fig. 3. (I) Time course of polymerization followed by monitoring the reflectance intensity for varying concentrations of methylene blue: (a) 2.5, (b) 3.75, (c) 5, (d) 7.5 μg mL^{-1} and (e) control experiment performed in the absence of dye. The arrows indicate the time when the flow cell was flushed with clean water. (II) Relationship between concentration of dye used and final stabilized (post-wash) reflectance intensity of the formed polymers. The line is for guidance only.

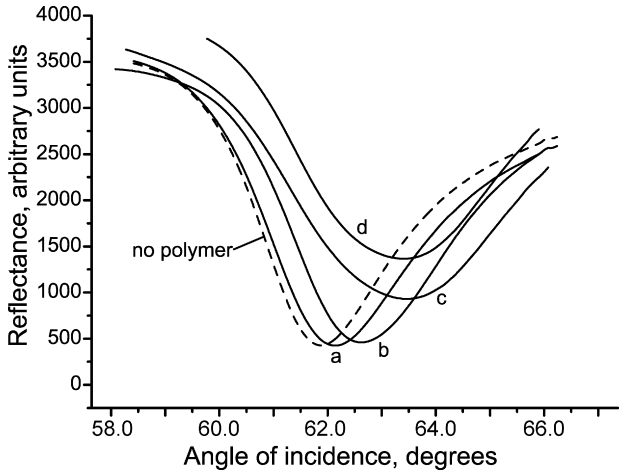

Fig. 4. SPR curves corresponding to the conditions used in Fig. 3I (curves a–d), the curve for bare gold (dashed line) was recorded with water alone in the flow cell, the curves a–d were recorded for water-filled cells following polymerization, the displacement of the SPR curve being due to the presence of thin polymer layers attached to the gold surface. Dye concentrations used were as follows: (a) 2.5 μg mL^{-1}, (b) 3.75 μg mL^{-1}, (c) 5.0 μg mL^{-1} and (d) 7.5 μg mL^{-1}. From an analysis of the SPR curves, the calculated refractive index of the polymer layers was estimated to be: real part: 1.345–1.348, imaginary part: 0.03–0.04. The calculated thickness of layers was: (a) 20 ± 5 nm, (b) 60 ± 10 nm, (c) 150 ± 20 nm and (d) 200 ± 30 nm.

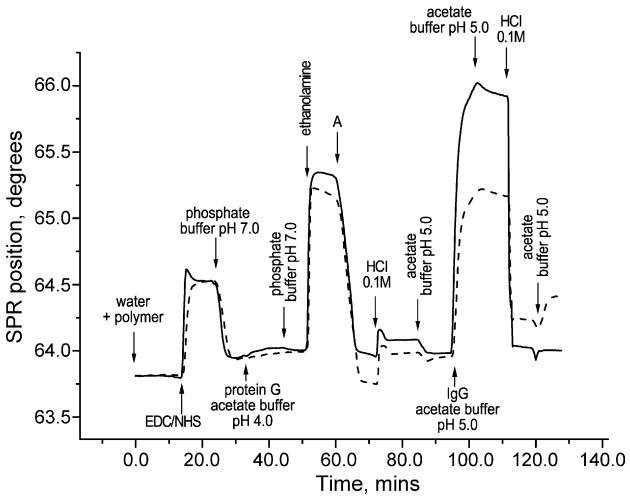

Fig. 5. SPR-sensogram recorded during chemical activation of a methacrylic acid-based polymer and the immobilization of the receptor protein (protein G), followed by detection of the binding interaction between the receptor protein and IgG, typical of the biomolecule interactions studied by SPR. The dashed line is the control experiment performed in the absence of protein G. Solution "A" comprised: 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P20.

sity of the evanescent wave, which rapidly falls off with distance from the gold surface, and the ability of the initiator to adsorb the energy of the incident light. The latter depends upon the dye concentration, therefore in order to produce a thicker layer; a higher dye concentration must be used.

The SPR curves, determined with water in the flow cell, corresponding to the experiments shown in Fig. 3Ia–d, can be seen in Fig. 4, curves a–d, respectively. It can be seen that with increasing dye concentration, a shallower curve is seen with progressive displacement to the right-hand side of the plot (higher angle) with respect to that for bare gold. Curve a in Fig. 4 (2.5 μg mL^{-1} dye) is only slightly displaced from that of the unmodified surface, whereas even at 3.75 μg mL^{-1} methylene blue concentration (Fig. 4, curve b), the effect is quite marked, potentially reducing the sensitivity of a sensor based on this layer. An analysis of the SPR curves allowed the refractive index of the polymer to be calculated. This was determined to be: real part: 1.345–1.348, imaginary part: 0.03–0.04. From the knowledge of the refractive indices, the layer thicknesses were estimated to be: (a) 20 ± 5 nm, (2.5 μg mL^{-1} methylene blue), (b) 60 ± 10 nm, (3.75 μg mL^{-1}), (c) 150 ± 20 nm, (5.0 μg mL^{-1}) and (d) 200 ± 30 nm (7.5 μg mL^{-1}). It appears therefore that adequate control over the extent of polymerization and consequently the thickness of the polymer film can be exerted simply through choice of the initial dye concentration, without any need to terminate the polymerization by blocking the light source.

Having demonstrated the formation of thin films of a carboxylic acid-functionalized polymer at the point of interrogation, we set out to demonstrate the utility of these films as a support for the immobilization of proteins in biosensors. A number of possible model systems, commonly used in SPR-based biosensors, were available to us (Yu et al., 2007). The protein G–IgG binding couple is often used a reference system for the Biacore® SPR platform (Newcombe et al., 2006), hence we elected to use this simple, but well studied model system. Polymers were formed using 2.5 μg mL^{-1} dye concentration, as described above. The activation of the acidic polymer with EDC/NHS, protein immobilization and blocking with ethanolamine was carried out according to a Biacore® application note for modification of their CM5 sensor chip with antibodies (Biacore Application Note). The same sequence of solutions was passed through the second channel of the NanoSPR,

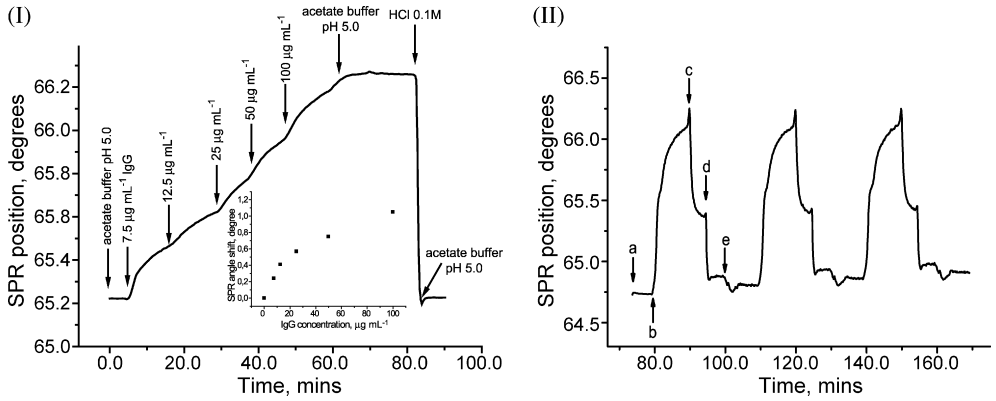

Fig. 6. (I) The SPR response for the protein G-modified cross-linked poly(MAA) matrix in response to the addition of increasing concentrations of IgG (in acetate buffer, pH 5.0). Inset: The same data represented in isotherm format. (II) Reproducibility of the sensor response using protein G-modified cross-linked poly(MAA) film over three cycles. The arrows mark the addition of the following solutions: (a) acetate buffer pH 5.0, (b) 100 μg mL^{-1} IgG in acetate buffer, pH 5.0, (c) acetate buffer pH 5.0, (d) 0.1 M HCl and (e) acetate buffer pH 5.0.

except that buffer alone was used in place of the protein G solution, to act as control. The sensorgram, following the change of SPR angle during the immobilization step and subsequent addition of IgG, is shown in Fig. 5. The working channel shows both a greater angular shift on binding IgG and more complete regeneration than the reference channel, where non-specific interactions are responsible for the response seen.

The concentration dependence of the biospecific reaction was determined by addition of successively higher concentrations of IgG in acetate buffer, pH 5.0, monitored by changes in the SPR angle. These data are presented in Fig. 6I and in isotherm form in the insert. Regeneration was again achieved by treatment with 0.1 M HCl. Reproducibility of the sensor response was also investigated over a number of cycles (Fig. 6II) with consistent results being shown on each cycle, suggesting that the protein is covalently bound to the polymer support and that neither the protein nor the polymer are dislodged by normal conditions of use. The results are comparable to those obtained using conventional immobilization methods (Newcombe et al., 2006).

4. Conclusions

This work has successfully demonstrated the in-situ polymerization of methacrylic acid, crosslinked with *N*,*N*-methylene-bisacrylamide using the energy of the plasmon resonance field created under conditions of total internal reflection at a gold surface. The red-sensitized photo-initiator couple, comprising methylene blue and sodium *p*-toluenesulfinate, was shown to initiate polymerization in the evanescent wave field, consistent with the wavelength of the light used to excite the plasmon. Polymer growth was confined to the position of interrogation of the gold surface by the laser spot with no polymerization observed elsewhere within the system (either on the gold surface, or in solution). Fine control of the thickness of the polymer film was demonstrated by adjustment of the dye concentration within the polymerization mixture. The resultant carboxylic acid-functionalized polymers were shown to be a suitable platform for protein immobilization following carbodiimide-mediated activation with *N*-hydroxysuccinimide. This was demonstrated for the model system protein G/IgG. While the system may still be further optimized, a working biosensor, with comparable performance to an industry standard, was constructed from a bare gold SPR chip within 2–3 h.

The use of the SPEW to initiate and control the production of a polymer support on a SPR chip is a novel process, and has a number of obvious benefits. Since the supporting layer is created by formation of a free radical-initiated polymer, a wide range of functionality can be introduced by varying the choice of monomer (including the use of custom-synthesized receptors and ligands modified with polymerizable groups) and other polymer components. The polymerization can be carried out under mild conditions and the photon energy used for excitation is relatively low, compared to UV irradiation for example. This could be important when using photo-sensitive ligands. By localizing the polymer at the position at which sensing takes place and not modifying the whole surface, it is possible to concentrate the recognition elements precisely where they are needed, provided the bare gold is treated to minimize non-specific adsorption.

It is important that the polymer layer thickness is not excessive, since the polymer adds to both the scattering and adsorption of light, both of which can degrade the sensor response. In our experiments we used the thinnest layer, since this material had the least effect on the SPR curve (see Fig. 4, curve a). It was not possible to see this polymer, either by naked eye or by microscopic examination; therefore the layers shown in Fig. 2 are not typical of those suitable for biosensor preparation. In all other cases, where higher dye concentrations than 2.5 $\mu g\,mL^{-1}$ were used, the SPR curve became broader and shallower as the dye concentration (and hence polymer thickness) increased, Fig. 4, curves b–d. Any change in polymer composition or in the optical set-up will require an optimisation of the polymer layer thickness to be performed to achieve the best response from the final sensor.

What is particularly interesting about this work is the direct use of existing optics to perform the formation of the polymer support. Combined with a suitable choice of monomers, the creation of a specific sensor platform can be performed remotely, without having to access the machine. This in turn reduces production costs and time, while giving operational flexibility. The potential for miniaturization exists on the device, as in theory the only part of a chip needed is that which contains the point of interest. As the optics used for the *in-situ* polymerization are the same as those used to analyse binding, the possibility of developing multisensor systems exists, with the in-built optics creating hundreds of different binding sites on the same substrate. This is possible as a "blanket" coating of the recognition element is not present, but is bound to specific points on the chip which can be located by Cartesian coordinates.

The results presented here clearly show that the performance of the present system is comparable to those currently available, however, by using this technique a higher effective concentration of sensing molecules can theoretically be achieved by limiting any binding to the point of interrogation. This would mean that rare or expensive receptors could be used more efficiently. This proposed increase in sensitivity is currently under study. Other ongoing work is aimed at the preparation of polymeric films at the surface of the SPR chip bearing an additional range of functionality. A further aim is to use the polymerization process itself as the means to introduce recognition properties, through the use of templates (molecular imprinting) as well as the creation of new biosensors.

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