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ประเภททุนต่อเนื่อง

ชื่อโครงการวิจัย

(ไทย) เซ็นเซอร์สำหรับตรวจโปรตีนก่อแพ้ในถุงมือยาง: การพัฒนาพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลของถุงมือยางแอลเลอจีโปรตีนเป็นวัสดุจดจำในควอตซ์คริสตอลไมโครแบลานซ์ และ อินเตอร์ดิจิตอลเซ็นเซอร์

(อังกฤษ) The rubber glove-allergen protein sensor: Development of rubber glove allergy protein imprinted polymer for the use as a sensing element in quartz crystal microbalance and inter-digital conductometric sensor

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บทคัดย่อ: ในงานวิจัยนี้เป็นการศึกษาการตรวจแอลเลอเจนโปรตีน Hev b1 จากน้ำยางพาราโดยใช้การพิมพ์พื้นผิวของพอลิเมอร์ที่อยู่บนควอซ์ทคริสตัลไมโครบาลานซ์ (QCM) ทรานซ์ดิวเซอร์ ทำให้สามารถตรวจวัดสารโดยสามารถหลีกเลี่ยงวิธีการตรวจวัดด้วยวิธีการติดฉลาก โดยได้เตรียมพอลิเมอร์ด้วยวิธีโคพอลิเมอร์ไรเซชันของเมธาคริยลิคแอซิด และไวนิลพัยโรลิโดน-ไดไฮดรอกซี่เอธิลลีนบิสอะคริยลิเอไมด์ในตัวกลางน้ำ ทำให้สามารถเลี่ยงการเสียคุณสมบัติของโปรตีน และทำการเคลือบพอลิเมอร์พิมพ์ประทับรอยโมเลกุล บนผิวของ QCM อิเล็คโตรด ทำให้ได้เซ็นเซอร์ซึ่งให้การความถี่ของการตอบสนองที่ขึ้นกับความเข้มข้นของโปรตีนจากน้ำยางพาราในช่วง 10 ถึง 1000 ไมโครกรัม/ลิตร กับ detection limit ที่ 1 ไมโครกรัม/ลิตร นอกจากนี้พอลิเมอร์ที่พิมพ์ประทับรอยโมเลกุลแสดงความสามารถในการจดจำสูง และมีการเลือกต่อตัวพิมพ์ Hev b1 โปรตีน และสามารถแยกโปรตีนก่อแพ้ Hev b1 จาก lysozyme, ovalbumin และ bovine serum albumin ด้วยค่า selectivity factor 2 ถึง 4 และ rubber elongation factors ให้ค่า selectivity factor เท่ากับ12 และในการตรวจวิเคราะห์ของเซ็นเซอร์ในการตรวจแอลเลอเจนโปรตีน ให้ค่า recovery เท่ากับ 97-105 ± 5% เซ็นเซอร์นี้ให้ความไวสูงในการตรวจวิเคราะห์และมีความน่าเชื่อถือในการตรวจโปรตีนก่อแพ้จากน้ำยางธรรมชาติแม้แต่ตรวจวัดโปรตีนก่อแพ้ที่มีแมททริกซ์จากสารสกัดถุงมือยาง

Abstract Detection of allergen protein Hev b1 was performed using an imprinted surface layer combined with a quartz crystal microbalance (QCM) transducer, allowing label-free measurements. Copolymers made from methacrylic acid-vinylpyrrolidone-dihydroxyethylene bisacrylamide are soluble in aqueous solution and devoid of denatured protein. When deposited as a coating onto QCM, such materials lead to sensors showing frequency responses clearly dependent on the concentration of the latex protein (10 to 1000 μg L^{-1}). An excellent dynamic range of the quartz crystal microbalance sensor response is observed, with a detection limit of 1 μg L^{-1}. The functionalized polymers showed a high recognition with good selectivity for the template. They can be applied to distinguish latex allergen Hev b1 from such analogues as lysozyme, ovalbumin and bovine serum albumin, with selectivity factors of two to four, and the response of the rubber elongation factors by an astonishing factor of twelve. The ability of this analytical procedure to detect allergen proteins gives a recovery of 97-105 ± 5%. This sensor provides a fast and reliable response to natural rubber latex protein, even in real matrices obtained by extracting rubber gloves.

Keywords Rubber elongation factor · Allergen protein · Molecularly imprinted polymers · Surface imprinting · Quartz crystal microbalance

Introduction

The complex machinery of protein biomolecules arises from their supramolecular nature [1], that has been naturally selected in a conformation that allows it to perform a particular task. Proteins do not function as static entities but engage in functional motions and interactions both within and between molecules; this allows them to achieve their function. As structures become elucidated significant insights are provided that could not be acquired from the examination of amino acid sequences alone, and an emerging view is that the knowledge of structure alone may not, in many cases, be sufficient for assessing the chemical nature of their natural function. Templated polymerization of proteins on polymers, for recognition, provides enormous possibilities for the study of structure, conformation, assembly and physicochemical properties of proteins [2, 3]. Selectivity achieved from templating a polymer material with a protein of interest can also be used for separation and chemical sensor applications [4, 5]. Molecularly imprinted polymers (MIPs) are biomimetic materials used for sensitive imprinting conditions, such as the selective detection of various chemical compounds and biomolecules [6, 7]. Most molecularly imprinted systems created for protein recognition contain components of the synthesized polymer in highly crosslinked polymer networks, and thus there are few methods available for extraction of these proteins from complex matrices. A surface imprinting approach has been suggested by Mosbach and coworkers [8]. In this study, surface imprinting for the recognition of proteins provides the advantages of fast rebinding kinetics. It is possible to construct an optimum adsorption site by self-organization, so the method requires a functional monomer with high interaction with proteins. A number of different approaches have been proposed for imprinting proteins on polymers in order to solve the problems of template removal and slow protein migration during the rebinding process. Other techniques have also evolved to produce the recognition sites of proteins for polymers, including epitope imprinting [9] and imprinted polymer arrays

[10], protein crystal imprinting [11] and protein imprinting that employs grafting a polymer brush [12].

The rubber elongation factor Hev b1 is a protein with an 138 amino acid sequence that belongs to the REF (rubber elongation factor)/SRPP (small rubber particle protein) family, and contains a HLA-DR4Dw4-binding motif [13]. Hev b1 has been identified as a 14.6 kDa water-insoluble protein on rubber particles of natural rubber latex (NRL), and may be part of the rubber biosynthesis machinery that plays a role in rubber elongation in *Hevea brasiliensis*. To date, more than thirty significant allergens in NRL have been described, and Hev b2, Hev b5, Hev b6 and Hev b13 together and Hev b6 with Hev b1 or 3 are common allergen in health care workers and patients with spina bifida [14]. Hev b1 was demonstrated to be the dominant allergen in manufactured latex gloves [15]. When exposed to allergenic proteins, allergic reactions to rubber latex can occur, particularly among healthcare workers, even causing death through anaphylactic shock. Chemical processes may reduce the amount of natural rubber latex proteins, and while not completely hypoallergenic, do provide lessened exposure to latex allergens. Numerous studies have mostly investigated the immunology of rubber allergy proteins [16, 17]. Hev b1, recognized as severely allergenic, is of great concern to allergic persons and hence for biomedical chemists who are performing research regarding new detecting methods. Besides, determination of the functional structure and resulting physicochemical properties of the allergen detection of the remnants of industrial additives in NRL finished products, which are responsible for the allergic reactions, would be of great biomedical interest and have advantageous implications.

Many of the quantitative methods for measuring the total protein content of the allergenicity of NRL products have several drawbacks. The Buret method is relatively insensitive to amino acid composition since it relies on reaction with peptide bonds. This method involves the reaction of compounds containing several peptide bonds with Cu^{2+} under

alkaline conditions to form a violet-coloured species. The sensitivity of the Buret method can be increased by the addition of the Folin-Ciocalteau reagent which is the basis of the celebrated Lowry assay [18]. Protein levels in gloves are usually determined using a modified Lowry assay (ASTM D5712) [19]. However, the disadvantages of the Lowry assay are that it takes longer and the colour is unstable – it depends on the tyrosine and tryptophan content, and mercapto-compounds interfere with the assay. The Bicinchoninic acid assay (BCA) method is very popular; it relies on the production of Cu^{+} which in this case is converted into a violet-colored substance by reaction with bicinchonate [20]. NRL glove protein levels can also be assessed using immunological assays such as the LEAP latex ELISA for antigenic proteins (LEAP), enzyme link immuno sorbent assay (ELISA) or RAST (radioallergo sorbent test) inhibition assay. LEAP has a chemical interference by surfactant chemicals in latex glove product, which tends to decrease the amount of glove protein binding and thus result in underdetection of protein antigens. The ELISA-inhibition latex antigen test can reduce the variability and surfactant interferences inherent in the LEAP assay, and thus is a sensitive and reproducible method for determination of rubber latex allergenic proteins and can be standardized as an approved ASTM Standard Test Method (ASTM 1999) [20]. In most cases, detection of allergen protein is based on LEAP and ELISA [18, 21]. These assays use anti-NRL antibodies to detect the antigenic protein. However, none of the above assays demonstrate consistent quantification of known NRL allergens or total proteins. All have practical limitations in their sensitivity, repeatability, and/or precision. The commercial diagnostic test kit, based on the enzyme immunometric assay technique with the use of specific monoclonal antibodies can also be used to determine the specific allergen levels in the gloves [22].

In the present work, the molecular imprinting technique provided the possibilities for fabrication of artificial receptors with predetermined selectivity for recognition by employing

the proteins of interest as binding-site-forming templates. For this, we applied a protein stamp incorporating a surface-imprinted thin layer on a transducer of a quartz crystal microbalance (QCM) sensor, whereby the natural counterparts and geometric conformation are transferred from a templating protein to a polymer network. A quartz crystal microbalance (QCM) measures a mass by detecting the change in resonance frequency of a quartz crystal resonator [23]. The resonance is disturbed by the addition or removal of a small mass at the surface of the acoustic resonator. Frequency measurements are easily made to a high precision; thus it is easy to measure mass densities down to a level of below 1 ng/cm^2 [23]. The QCM is a simple, cost effective, high-resolution mass sensing technique based upon the piezoelectric effect. This technique provides a wide detection range: from small molecules or polymer films to much larger masses deposited on the surface. These can be complex arrays of biopolymers and bio-macromolecules. We aimed to produce the sensor devices to determine allergen proteins in the complex matrix of NRL glove. The measurement of allergen protein with MIP-based QCM sensor may also provide a means for determining the biofunctional structure and physicochemical properties at a nanoscale level of the target protein described above. After adsorption on a gold electrode, the binding of the proteins was measured with a QCM. According to Sauerbrey's equation the frequency decreases with the increase of mass on the electrode, and this allows for the examination of hevein latex proteins interacting with the surrounding environment.

Here, we have prepared a Hev b1 surface-imprinted polymer coating onto the QCM, and developed a QCM sensor assay for the quantitative detection of rubber latex allergen protein Hev b1 with high sensitivity and selectivity. Hev b1 (remaining in the finished product of gloves) was used as the template molecule for imprinting on the polymer. Moreover, we have investigated the influence of polymer components and experimental parameters for an effective sensor response of the prepared MIP thin-films in aqueous media.

We have developed and evaluated the cross-selectivity of Hev b1 protein molecularly imprinted polymers that are specific for particular binding species. Hev b1 protein derived naturally was employed as the other protein template. In addition, we determined the analytical characteristics of the MIP-based QCM sensor for detection of the allergenic proteins in spiked samples and applied the Hev b1 assessment by the newly developed MIP-QCM sensor method to real-life samples.

Experimental

Chemicals and materials

Acrylamide (ACM), 1-vinyl-2-pyrrolidone (NVP), methacrylic acid (MAA), *N,N′*-methylene bisacrylamide (MBA), *N,N′*-(1,2-dihydroxyethylene)bisacrylamide (DHEBA), sodium dodecyl sulfate (SDS), Triton X-100 and Tris-HCl were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Janssen Chimica (Geel, Belgium). Lysozyme, ovalbumin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rubber latex was obtained from commercial rubber latex, in Songkla, Thailand, which was chilled in an icebox, brought to the laboratory and processed at a temperature of 2-4 °C. Cellulose dialysis bags with a MWCO of 3,700 Da were purchased from Sigma (St. Louis, MO, USA). All solvents used were of analytical-reagent grade.

Protein extraction

All other allergenic proteins extracted from latex mattresses made from rubber tree latex are termed the blanket (L). The extractable Hev b1 protein in medical gloves is termed the blanket (G). Hev b1 (14.6 kDa), Hev b2 (35 kDa) and Hev b3 (23 kDa) were isolated from rubber latex by a protocol similar to that described previously [24]. Briefly, fresh rubber

latex was centrifuged in a L-100 XP Beckman high-speed centrifuge at 60,000 g at 4 °C for 45 min, and the aqueous phase (the C serum) was removed. The small rubber particles present in fresh latex were obtained from zone 2 of the rubber cream after centrifugation for 2 h. These rubber particles, that contain relatively large amounts of Hev b1 (L) and Hev b3 (L) protein, were washed, resuspended in 30% sucrose, and recovered by centrifugation. The proteins on the surfaces of the rubber articles were then solubilized and extracted by adding an equal weight of detergent, composed of 0.1% Triton-X 100 and 1% SDS, to the rubber cream. The mixture was centrifuged to obtain the supernatant, which contained solubilized Hev b1 (L) and Hev b3 (L). Crude protein was dialyzed against several changes of distilled water and lyophilized. The protein extract was purified by gel permeation chromatography on a Sephacryl™ S-100 column (1.5 x 64 cm, Pharmacia) using 50 mM phosphate buffer, pH 7.4, containing 0.1% SDS and 0.15 M NaCl at a flow rate of 1 mL/min. Protein elution was monitored by measuring the absorbance at 280 nm. The collected fractions were concentrated and analyzed by 15% SDS-PAGE with Coomassie staining. The sequence of the allergenic proteins was confirmed using an Ultraflex III MALDI TOF/TOF (Bruker Daltonik, Bremen, Germany) mass spectrometer by using data processing in the MALDI-MS Data Explorer software [25]. The MALDI-MS data were blasted against the Mascot proteomics database. Mascot Score Histogram Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores were found to greater than 70% ($p<0.05$). In addition, the collected bottom fraction was washed three times with isotonic buffer and recovered by centrifugation. The rubber particles in the bottom membrane fractions were extracted with a mixture of 50 mM Tris-HCl, pH 7.4, and 0.2% Triton X-100. The crude extract was centrifuged to obtain the supernatant containing Hev b2 (L). Proteins in the collected fractions were precipitated by addition of ice-cold acetone and recovered by centrifugation. The sample was dialyzed through a cellulose dialysis bag against distilled water, and lyophilized and purified as described above.

Hev b1 (G) was obtained from natural rubber latex gloves, as described previously [24]. Briefly, the gloves were cut into small pieces and extracted with 0.2 M phosphate buffer pH 7.4 containing 0.5% SDS. The mixture was centrifuged and the crude filtrate was dialyzed with several changes of distilled water. The extracted protein was purified and analyzed in the same way as for the proteins from natural rubber latex. The protein was lyophilized for use as purified Hev b1 (G) for surface imprinting of proteins onto polymers.

QCM

The imprinting was performed on quartz crystal microbalances with two-electrode geometry. One electrode is incorporated with the imprinted polymer layer and the other one is a blank control electrode of the non-imprinted control polymer, which was prepared with the template molecule omitted. The former is used as a measuring electrode and the latter is a reference for eliminating unspecific effects such as temperature changes or changes in viscosity. Dual electrode geometries were screen printed (brilliant gold paste, GGP 2093 – 12%, from Heraeus, Germany) onto quartz discs (10 MHz, AT-cut, 15.5 mm diameter) and burned-in at 420 °C overnight. Electrodes oriented toward the aqueous phase were grounded and 5 mm in diameter, whereas electrodes oriented to the gas phase were 4 mm in diameter, as previously reported [26].

Sensor layers

Molecularly imprinted and corresponding non-imprinted polymer film layers coated on the QCM electrodes were created following a common protocol for MIP synthesis [27]. Monomers were MAA, NVP and ACM at an 8:2 mole ratio, in respect to crosslinker to crosslinker 2 mole as the monofunctional monomeric reagents. In a difunctional

polymerization process, 6 mol of MAA was added to 2 mol of NVP or ACM, or 6 mol of ACM was added to 2 mol of NVP, along with the crosslinking monomer (2 mol). Their molecular structures are shown in Fig. 1.

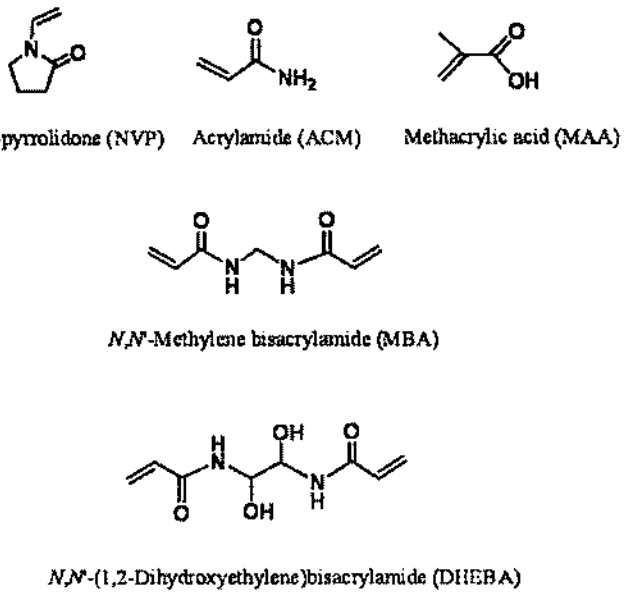


Fig. 1. Chemical structures of functional monomers and crosslinking monomers used in this study.

A typical monomer solution preparation would consist of dissolving 0.7 mmol of the chosen monomer together with 0.15 mmol of crosslinker in 800 μL of distilled water as a porogen solvent. The initiator (1% w/w) was then added to the above solution. The mixture was sparged with nitrogen for 30 s to remove any dissolved oxygen. For preparation of the MIP films, polymer coatings with different monomer solutions were polymerized up to the gel point in a water bath at 70 °C for 15 min. The viscous pre-polymer was spin-coated onto a QCM electrode at 3,000 rpm just prior to surface imprinting. The procedure for loading of proteins onto the stamp was as follows: the glass substrate for the protein stamp was cleaned with detergent and solvent, and a 5 μL sample of 100 μg mL^{-1} protein in 0.1 mM phosphate buffer solution (pH 7.4) was spread on the glass substrate and dried in an incubator for 2 h at 25 °C. The final stamp was pressed into the pre-polymerized coating localized on the

electrodes of a QCM. Polymerization was carried out at room temperature (25 ± 1 °C) overnight under UV light at 254 nm in a nitrogen atmosphere. Both templates and stamps were washed by incubation with distilled water at 40 °C for 30 min, and the residual adhered protein template washed off with three portions of 100 mL of distilled water at room temperature (25 ± 1 °C).

AFM measurements

Atomic force microscopy (AFM) measurements were performed on a Veeco Nanoscope IVa system with Veeco SNL-10 silicone tips with a spring constant of 40 N/m and an onset pressure corresponding to a differential signal of 1 V on the photo diode. The topography and phase images of the protein stamps and polymer films were obtained by contact mode AFM at a scan rate of 1 Hz under ambient laboratory conditions. The thickness of the films was determined by scratching with a needle and measuring the depth of the scratches using an AFM.

QCM assays with imprinted polymers

The coated quartz electrode was mounted in a self-made measuring cell of 250 µL volume and thermostatted at 25 °C. Home-built oscillator circuits and self-programmed frequency readout and processing software were used. Oscillator frequency was measured by means of an HP 53131A frequency counter, and the responses of the oscillator circuit were checked by means of a network analyzer (HP 8572C) to record damping and phase spectra. Sensor experiments were performed using a stopped-flow system. The coated QCM devices were first stabilized in 0.1 mM phosphate buffer solution (pH 7.4), and a steady resonant frequency was obtained at room temperature (25 ± 1 °C). The frequency shift responses of QCM with

the prepared imprinted and non-imprinted polymers were measured when exposed to Hev b1 (G) solution at an initial concentration. The experimental parameters (background solution, medium pH, polymerization temperature) that enable the optimal interaction of Hev b1 (G)-MIP with the template species were identified with regard to the response to Hev b1 (G), using the optimized polymer at the initial concentration of the analyte. The sensitivity of the Hev b1 (G)-MIP-based sensor was measured as a function of the changes in frequency shift of the polymer upon exposure to Hev b1 (G) with concentrations ranging from 10 to 2000 μg L^{-1}. The cross-reactivity of the imprinted polymers was examined by comparing the frequency shift response of protein analogues to the frequency shift response of the template. The protein template was considered to give 100% selectivity and all other proteins were related to this value. Every experiment was repeated three times at room temperature (25 ± 1 °C).

Analysis of latex extracts and gloves

Quantitative analysis of the target analyte in the extracts of the latex articles and glove samples was achieved by a Hev b1 (G) sensor using optimized sensor conditions. A latex extract was obtained from commercial rubber latex in Songkla, Thailand, from which protein was extracted according to the procedures mentioned above. A latex examination glove was cut into small pieces and assayed for the target analyte by the QCM method. The crude proteins from rubber latex and glove extracts were analyzed by a MIP-QCM-assay protocol. For this purpose, 1 g of the solid powder (or 4 g of the glove extracts) spiked with Hev b1 (G) standards was extracted according the procedure previously described for Hev b1 (G). Spiked samples of allergen protein (250, 500, and 1000 μg L^{-1}), used in recovery studies, were prepared by adding the appropriate volume of a Hev b1 (G) standard solution to an extract of a matrix sample from a latex glove, resulting in a final volume of 15 mL. A

extract of a matrix sample from a latex glove, resulting in a final volume of 15 mL. A calibration curve was prepared by dissolving Hev b1 (G) in 0.1 mM phosphate buffer, pH 7.4, to obtain solutions having Hev b1 (G) of between 10-1000 μg L^{-1}, and then comparing them to that measured for Hev b1 (G) in the spiked samples. Every experiment was carried out in triplicate on any particular day of experimentation.

Results and discussion

Protein stamp and imprints

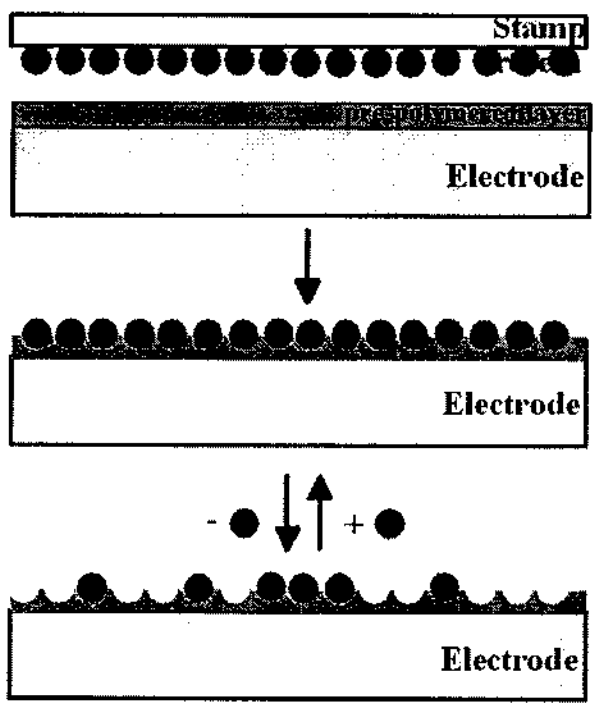


Fig. 2. Schematic representation of the surface-imprinted polymer deposited onto a gold-coated QCM electrode for recognition of a protein.

The schematic representation of a preparation of allergen proteins surface-imprinted polymer coating on a QCM electrode is depicted in Fig. 2. The initial evaluation was examined with a selective layer fabricated by the prepolymerization of MAA-NVP-DHEBA. Polymer based

on (MAA-NVP-DHEBA) copolymer can be prepared in aqueous solution and initiated radically, which can be devoid of denatured protein. Thus, the Hev b1 (G) stamp was aligned and brought into the spin-coated pre-polymer layer by pressing with constant force throughout the stamp. The pre-polymerized materials were polymerized photochemically in the presence of a radical initiator. After washing, the template proteins have been eliminated from the templated (MAA-NVP-DHEBA crosslinked) polymer structures, but retain imitating geometrical features of Hev b1. The oriented Hev b1 (G) patterns on the stamp were observed at different protein contents. The development of compact islands of 3 nm height after drying and attributed to an aggregated monolayer, were observed on the stamp surface when the concentration of the protein was applied as described in the "Experimental" section. The packing density and orientation of Hev b1 (G) on drying can be seen in Fig. 3a. The lateral width of an island of dry Hev b1 (G) on the glass substrate is 135±10 nm. The Hev b1 (G) column-like islands are most likely assemblies that maintain their adsorbed orientation on drying. Fig. 3b shows the surface of a non-imprinted polymer layer that has been synthesized from the same monomer mixture as the MIPs. Spin coating, in this case, leads to flat surfaces and surface roughness of the layers of approximately of 30 nm. After washing, the template proteins have been eliminated from the templated (MAA-NVA-DHEBA crosslinked) polymer structures, but retain imitating geometrical features of Hev b1 (G). Fig. 3c shows the topographic AFM image of gold electrodes coated with a Hev b1 (G)-MIP film layer. The difference toward the NIP material is clearly shown: the surface roughness is higher and reaches a level of 70 nm, with the diameters of individual hollows of about 130 nm. The size of the original Hev b1 (G) are retained in the polymer matrix; this is a strong indication of surface imprinting.

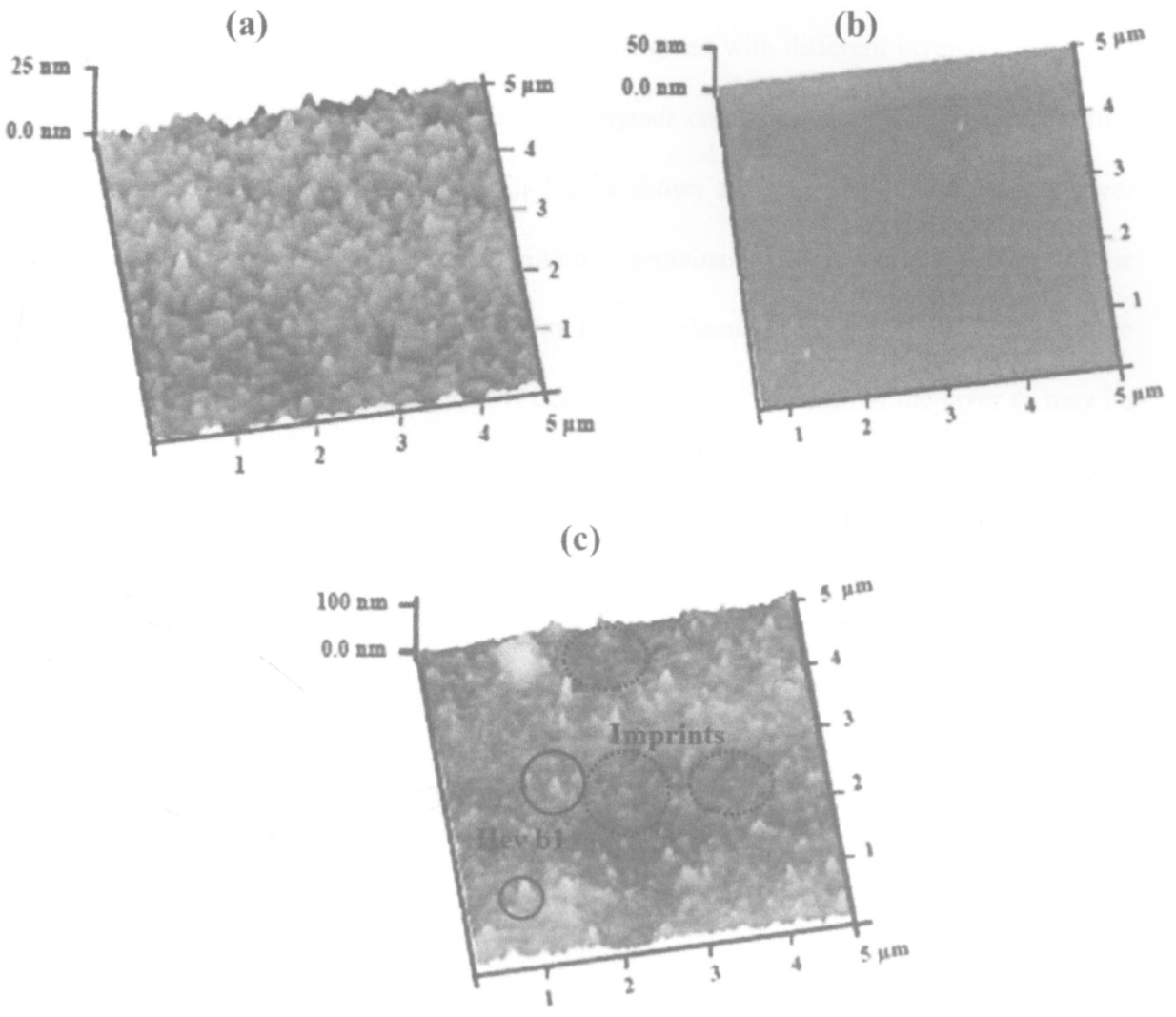


Fig. 3. Topographic AFM images of (a) Hev b1 (G) self-assembled on a surface, (b) non-imprinted polymer, and (c) Hev b1 (G) surface-imprinted of poly(MAA-*co*-NVP-*co*-DHEBA) deposited onto a gold-coated QCM crystal. Positions of imprints sites are marked by dashed circles and continuous line circles are cavities containing a Hev b1 (G).

Effect of layer thickness

In this study, the effect of the layer thickness on the frequency shift response of the sensor upon exposure to Hev b1 (G) (2 mg L^{-1}) was investigated with different layer-heights of the imprinted (MAA-NVA-DHEBA crosslinked) polymer coated on the QCM electrodes in a range of 80-240 nm. The sensor plot in Fig. 4 shows an increase with increasing layer thickness of the MIP film due to the adsorption of proteins. The response frequency of the sensor reached a plateau level at about 160 nm layer thickness of the MIP film. The possible explanation for this fall-off in binding may be conformational changes of the layer or may be due to irreversible binding between the polymer and the template due to geometrical restrictions and probably to high numbers of hydrogen bonds.

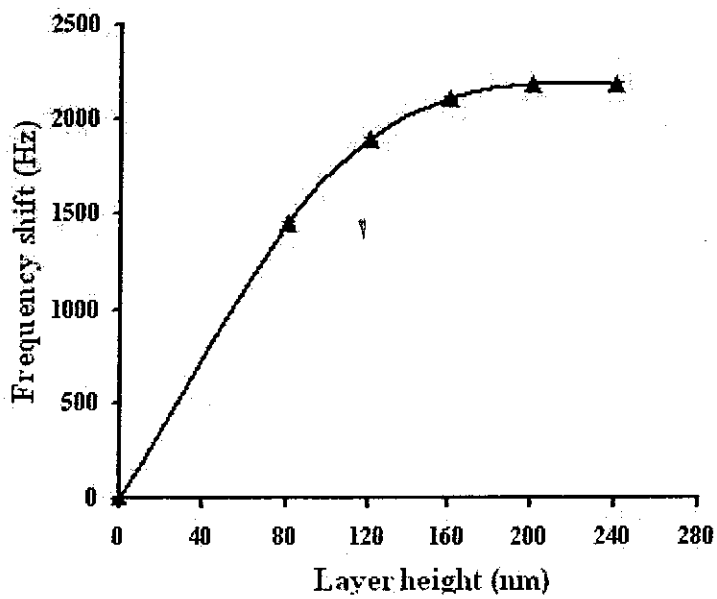


Fig. 4. Effect of imprinted layer height on frequency shift of Hev b1 (G)-imprinted coated QCM exposed to Hev b1 (G) at a concentration level of 2 mg L^{-1}.

Optimization of polymer compositions

To achieve highly selective recognition of the target analyte, the influence of polymer components on the sensor response (frequency shift) of Hev b1 (G)-MIP-based QCM with a

layer height of 200 nm was studied, using corresponding non-imprinted polymer film layers as a reference channel. Hev b1 (G)-imprinted sensor thin-films on QCM were prepared by using a single functional monomer or combinations of two different functional monomers with a fixed crosslinking concentration. DHEBA and MBA were chosen as crosslinking agents for surface imprinting of Hev b1 (G) via a non-covalent approach, as they could minimize the non-specific interaction forces between protein and polymer in order to achieve reasonable adhesive properties of the polymer from the solvent. As shown in Fig. 5, a MAA monofunctional polymer gives a frequency shift response to Hev b1 (G) higher than the NVP monofunctional polymer or even higher than the ACM monofunctional polymer, prepared using DHEBA as a crosslinker. For the monofunctional polymers with a molar ratio of 8:1, an excess of the functional monomer with respect to the template yielded higher non-specific affinity. Generally the proper molar ratios of functional monomers to template are very important to enhance the specific affinity of polymers and the numbers of MIP recognition sites. High ratios of functional monomer to template results in high non-specific affinity, while low ratios produce fewer complications due to insufficient functional groups. So, the difunctional polymers were studied for sensor effects on QCM measurements, by combining the MAA, or ACM with another functional monomer at a molar ratio of 6:2. QCM with MIP prepared with MAA-NVP monomers gives a high frequency response for Hev b1 (G) with an astonishing minor unspecific effect with respect to other MIPs (Fig. 5). The polymer functionality of the MAA-ACM copolymer is likely to interact with protein via hydrogen bonding, that leads to a high sensor effect similar to that of a monofunctional MAA polymer but with selectivity factors of less than 2 toward Hev b1 (G). When ACM is combined with NVP monomer, the effect of either the MIP or NIP thin-films on the QCM sensor was lower than that of a single ACM monomer. Polymer functionality of the ACM-NVP copolymer is neutral in an aqueous solution, providing much less frequency response of the imprinted sensor and a very low selectivity. A considerably higher mass-loading capacity can be

achieved with the MAA-NVP copolymer, which is a polymer capable of not only highly selective recognition of the target analyte but also having good kinetic parameters. The piezoelectric frequency response improvement and optimization of the imprinted polymer film layer can be accounted for by the functionality and hydrophilic nature of such a functional monomer. In this case, the functional monomer MAA provides electrostatic interaction and a greater amount of hydrogen monomer bonding in the polymer networks. Owing to the strong hydrogen bond forming capability of NVP, it is able to enhance mechanical properties and surface wetting properties of a polymer that stabilizes the inclusion of protein in the MIP and stabilizes the protein conformation after attachment [28].

When the selectivity of the Hev b1 (G)-MIP prepared with MAA and NVP functional monomers with DHEBA and MBA as crosslinkers was determined in QCM experiments, the DHEBA in the polymer matrix produced a high selectivity for the response to Hev b1 (G) by a factor of 12, compared with MBA in the polymer which obtained a selectivity factor value of only 3 (data not shown). The superior binding capacity and effective recognition properties achieved by the optimized imprinted polymer, in which DHEBA provides an –OH surface on the polymer, can be attributed to the greater polarity and higher wettability of DHEBA-rendered protein preferentially included in MIP [27, 29]. The imprinted polymer from crosslinked poly(MAA-NVP-DHEBA) displays a high efficacy of recognition for the target analyte; therefore this polymer has been used for investigation in the following experiments.

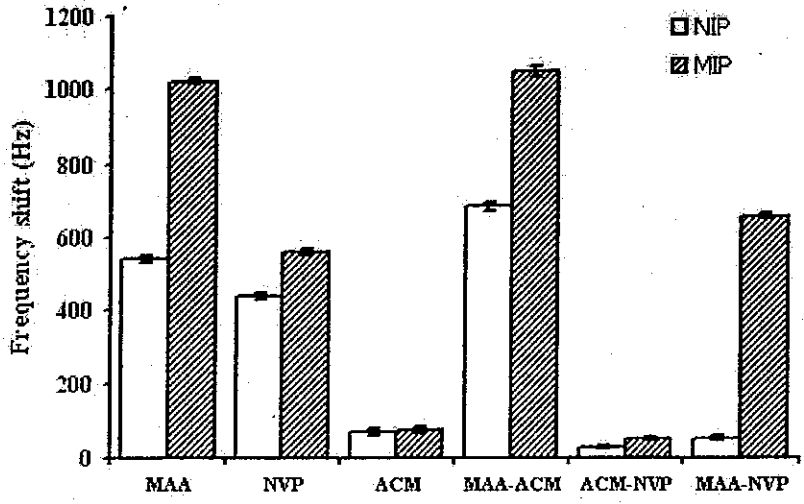


Fig. 5. Effect of the polymer components for a MIP- or NIP-modified QCM sensor on the frequency shift responses to Hev b1 (G) at a concentration level of 500 μg L^{-1}.

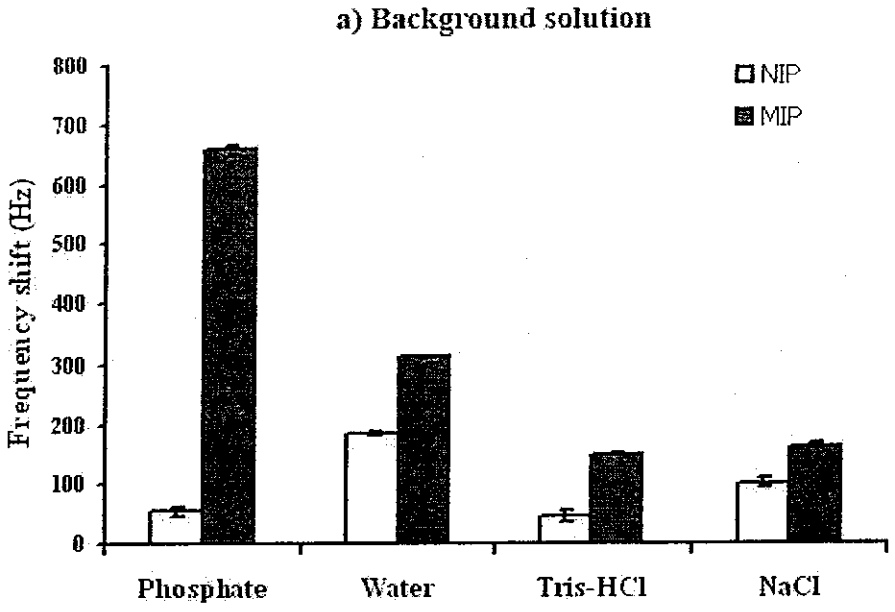
Optimization of QCM sensor measurement

The effect of the experimental parameters (such as background solution, pH of the medium and polymerization temperature) was examined further so as to provide optimized parameters for selective recognition of Hev b1 (G) in aqueous media, with a view to developing a Hev b1 (G)-MIP-QCM sensor suitable for determination of the latex allergy proteins in a glove sample. Fig. 6a shows the effect of the solution media on the frequency shift response of Hev b1 (G) (500 μg L^{-1}) to MIP-modified and non-imprinted polymer (NIP)-modified QCM electrodes when exposed in water, pH 7.4 buffer (0.1 mM), Tris-HCl buffer (0.1 mM), and NaCl solution (0.1 mM). The frequency shift of the imprinted sensor coating from pH 7.4 buffer was as high as 660 Hz, and was twice as low from distilled water. The frequency response of Hev b1 (G) for the imprinted polymer was low for both NaCl and Tris-HCl buffer solutions. In addition, the non-specific adsorption of protein in water was high compared to that in NaCl solution and the other two solvents. The pH 7.4 buffer, which was used as the preparation phase of the imprinted layer, was found to be a better solvent for selective

rebinding of Hev b1 (G) to MIP, as the optimized sensor signal was achieved in this solvent with a very low non-selective signal. It is possible that in water and NaCl solutions the denaturation of protein occurred due to a pH change and/or a salting out effect of Na^+ and Cl^- ions; whereas in Tris-HCl buffer, the binding interaction of MIP and protein might be inhibited by conjugation of amine-based crosslinkers [30]. The pH medium had a large effect on the frequency response of Hev b1 (G) on Hev b1 (G)-imprinted and non-imprinted sensors (see Fig. 6b), with a low pH (pH 5) leading to a much lower frequency response of Hev b1 (G) on the sensors than when the sensors were exposed to protein at a high pH (pH 6-8). At a lower pH value, electrostatic interactions of positively charged lysine residues of the Hev b1 (G) protein can occur. This favors intramolecular interactions that change the three-dimensional shape of the protein molecule. Also, at acidic pH, renaturation was achieved and this affects the binding motifs on the latex protein [31]. These effects cause a decrease of adsorption interactions between protein and polymer, and lead to loss of the frequency signal at a low pH value (pH 5). The binding property of Hev b1 (G) to the MIP artificial receptor was optimized at pH 7.4, which is explained by the success of memorizing the spatial features and bonding preferences of the template, because of a highly stabilized protein conformation and no collapse of the MIP matrix.

The influence of the polymerization temperature on the frequency shift response of the sensor exposed to Hev b1 (G) (500 $\mu g\ l^{-1}$) at 25 °C was investigated over a temperature range of 25-75 °C during the polymerization process. The frequency response of the imprinted sensor was reduced at a polymerizing temperature above 25 °C because of the template/polymer exothermic interaction (Fig. 6c). In addition, the frequency shift of the corresponding non-imprinted coating gradually increased with an increasing polymerization temperature from 25 to 75 °C. The binding of the analyte at NIPs (without polymerization template) is overall endothermic. During the polymerization of the layers, NIP interaction

sites are minimized at high temperatures. The interactions of the protein and solvent molecules inside and outside the backbone of the polypeptides are greater for NIP, and thus endothermic. Temperature has a complex effect on monomer-template and polymer-template interactions. In the previous study a complex behavior of the imprinting effect with the change of polymerization temperature was observed. This enabled a good selectivity for template being achieved in the binding groups of the resulting materials [32]. The selective sensor response of the optimized imprinted polymer for the present study was upon polymerization the highest at room temperature (25 °C). A different Hev b1 orientation prevails on the MIP material surfaces under different polymerization conditions. On the other hand, the dehydrated polar DHEBA chains result in an interfacial surface property modulation of the polymer-protein attachment. Thus, the surface imprinting and protein rebinding process with the optimized conditions may cause the increased possibility of hydrogen bonding contacts between the protein surface and the binding sites on the interfacial surface of the MIP.



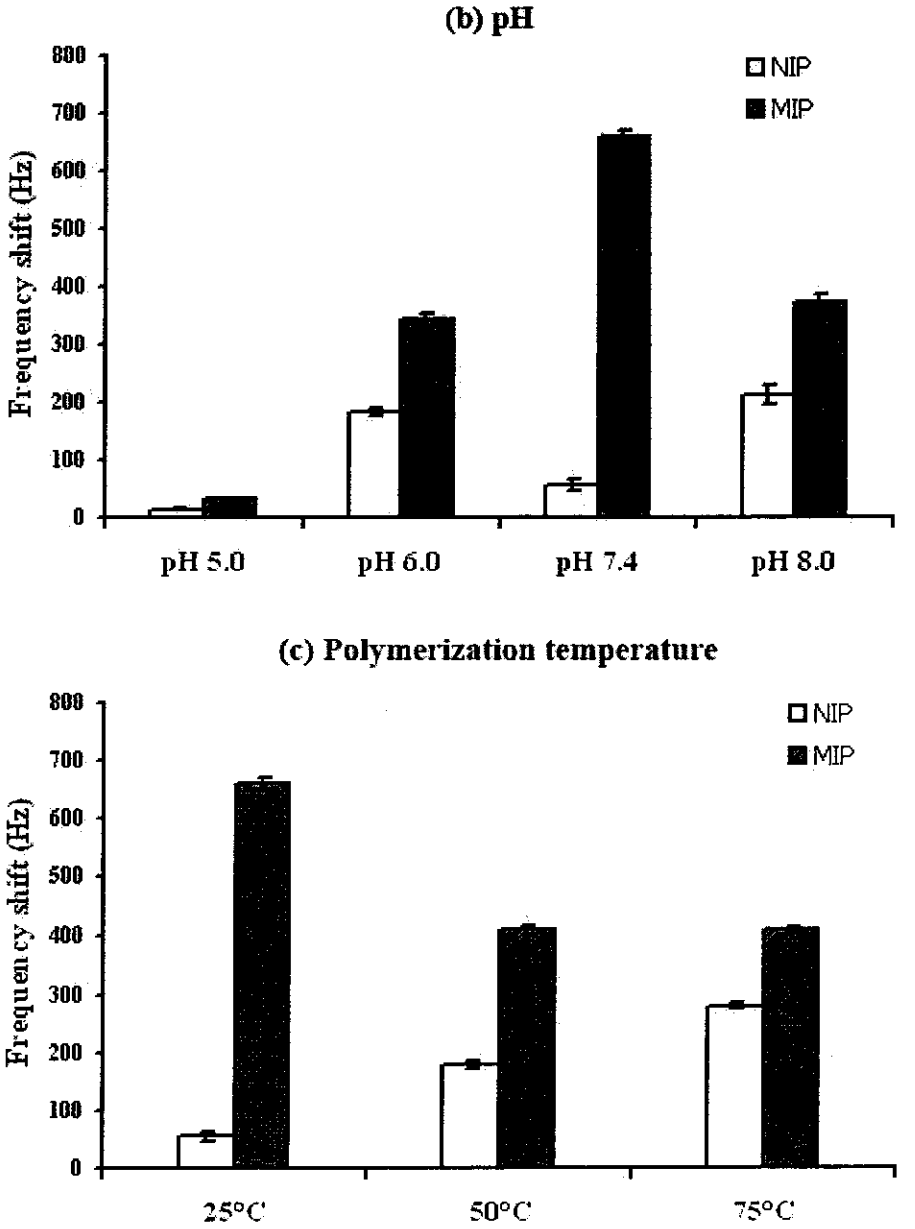


Fig. 6. Effect of (a) background solutions, (b) medium pH, and (c) polymerization temperature on the frequency shift responses for non-imprinted and Hev b1 (G)-imprinted (MAA-NVP-DHEBA-polymer) coated QCM, with QCM measurement on 500 μg L^{-1} of Hev b1 (G).

Moreover, the frequency shift due to protein adsorption of the MIP layer and the NIP reference on the QCM sensor was examined as a function of time upon exposure to the addition of 10 μg L^{-1} Hev b1 (G) in phosphate buffer, pH 7.4. It appears that the normal baseline of a MIP-based sensor changes to 110 Hz upon the addition of Hev b1 (G) solution

(Fig. 7). This can be explained by the protein readily interacting with high-affinity binding sites in the cavities of MIP that have a low surface free energy and the binding energy stabilizes the template protein as in the behavior of a monolayer thin polymer film. Subsequently, the frequency signal of the sensor reaches a new baseline, and appears as a plateau peak (see Fig. 7) that may be interpreted as the re-orientation of Hev b1 (G) in the conformational spaces of cavities on the molecularly imprinted polymer material surfaces. On the other hand, the frequency shift to Hev b1 (G) for the non-printed sensor turns out to be as low as 8 Hz; this is because there is no specific site on the polymer surface that can geometrically include the protein. This indicates that there is very low unspecific adsorption of the Hev b1 (G) proteins on the polymeric material. These results demonstrate the binding ability of Hev b1 (G) via non-covalent interaction with the imprinted polymer, and the regulation of protein orientation by the active binding site in imprints.

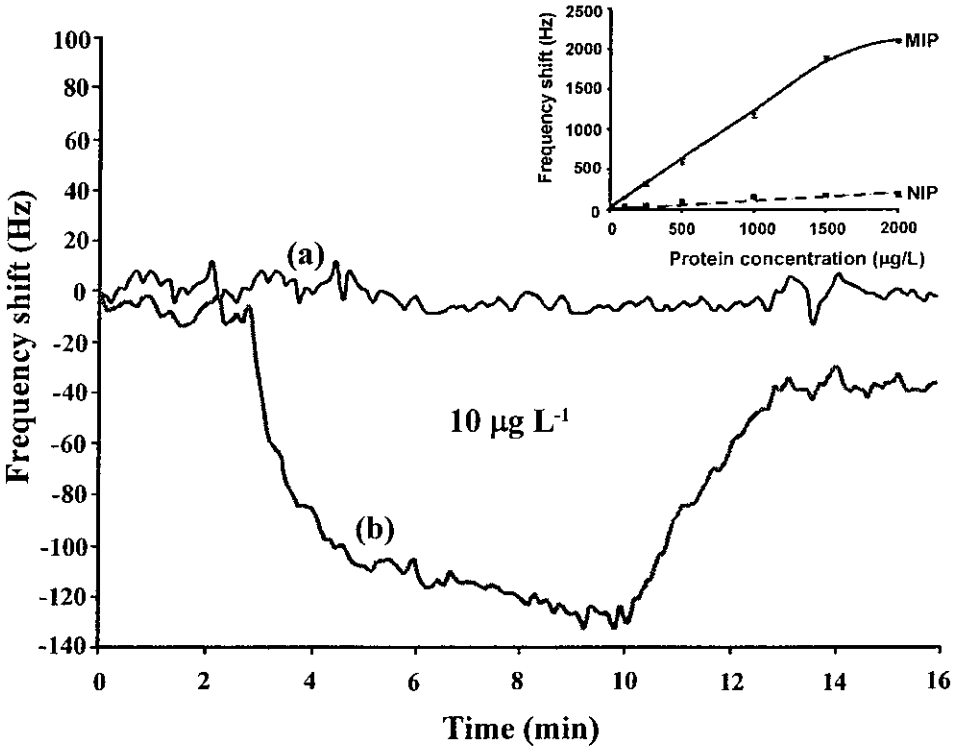


Fig. 7. Signal response of 10 MHz stopped flow QCM with (a) non-imprinted and (b) Hev b1 (G)-imprinted (MAA-NVP-DHEBA-polymer) coated electrodes at a concentration level of 10 μg L^{-1}. At the top right is sensor characteristic of NIP and Hev b1 (G)-MIP coated QCM.

Measurements were carried out in 0.1 mM phosphate buffer solution (pH 7.4) at room temperature.

The concentration dependence of these relatively small effects achieved on the reference electrodes can be used for verification during cross-selectivity measurements (see Fig. 7, top right), for which it is necessary to choose the appropriate concentration. The mass response generated by the selective layers is dependent on the analyte concentration, showing a typical frequency shift response for the MIP-QCM electrode when having been exposed to increasing Hev b1 (G) concentrations from 10-2000 μg L^{-1} (Fig. 7, top right). Until about 1500 μg L^{-1} the sensor response increases linearly with analyte concentration. Above this range, the dynamic behavior shows a leveling off and thus the onset of a plateau. One explanation could be that above this concentration of Hev b1 (G) all available selective cavities are occupied and hence increasing the amount of proteins in the sample, will not further increase the sensor response. This also confirms that any possible non-specific adsorption of a double layer of proteins did not form on the sensor surfaces leading to an additional Sauerbrey effect.

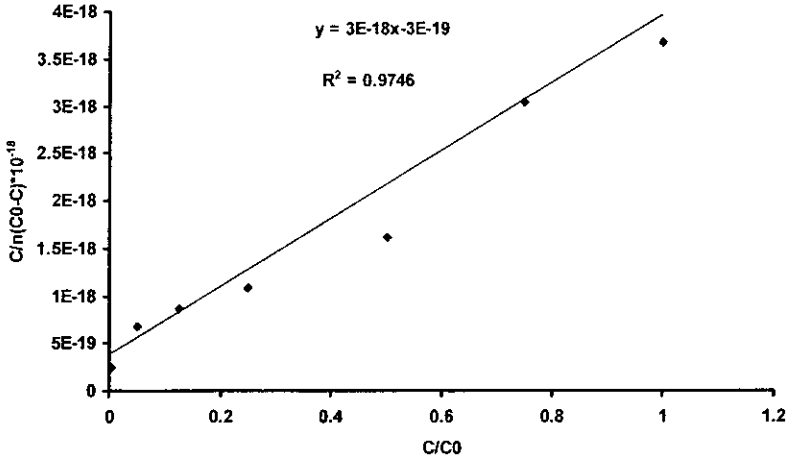


Fig. 8. Lineared BET adsorption isotherm of Hev b1 (G) on MIP at 25°C, according to ref. [33] (correlation coefficient of linear regression, $R^2 = 0.97$).

The sensitivity of the QCM was 1.04 Hz/ng. At the highest concentration measured, the overall mass effect is 2.1 KHz, that corresponds to 2.52 x 10^{17} Hev b1 (G) proteins bound to the sensitive layer. The number of adsorbed Hev b1 (G) proteins in the sensor layer was found to be 3.03 x 10^{17} (Fig. 8). The number of the allergenic proteins reaches 82% of the active sites in the selective layer, as determined by a Brunauer-Emmet-Teller (BET) analysis method [33].

Specific binding of Hev b1 protein surface-imprinted polymers

The interaction of the protein with the imprinted polymer can determine the efficiency of the biofunctional protein organization, and control of the physicochemical properties of the protein. The MIP, in turn, can be used to tailor this interaction. Molecularly imprinted polymer sensor layers selective for non-native and native Hev b1 have been synthesized under the same protocol of synthesis. In the present study, the evaluation of the synthesized MIP system as selective recognition elements has been further explored through sensitive QCM measurements using non-imprinted polymers as a reference channel. The cross-selectivity of Hev b1 (G)-MIP and Hev b1 (L)-MIP with templates Hev b1 (G), Hev b1 (L) and their analogues was studied, with measurements performed under controlled conditions. The value of the cross-reactivity, obtained from the signal responses of the MIP on the sensor for the analogue relative to that for the template, is shown in Fig. 9. Clearly, in both cases the imprinted sensors result in a substantial frequency shift response when exposed to the respective template. The non-selective adsorption of all proteins binding to a non-imprinted polymer is much lower than that on an imprinted polymer due to the lack of selective recognition on the non-imprinted references.

The imprinted polymers exhibit greater cross-reactivity for proteins related to Hev b1 than for non-Hev b proteins; while Hev b1 (L)-MIP has a higher cross-reactivity toward Hev

b (L) proteins, to a much greater degree than an analogous MIP obtained by Hev b1 (G) as the templating protein. Although Hev b1 (G), Hev b1 (L) and lysozyme have the same geometrical dimension (14 kDa), at the same concentration level they lead to distinct sensor responses of the Hev b1 (G)-MIP-modified-QCM. The closely related structural protein Hev b1 (L) generates a signal approximately 44% of that obtained when exposed to the template. With the non-related protein lysozyme, the cross-reactivity value governing selective recognition, is much lower (about 12%), and thus is about four times larger than the non-selective responses on the non-imprinted sensor materials, since it is a hydrophilic protein and its conformation is more globular. This result confirms the difference in conformation between Hev b1 (G), Hev b1 (L), and lysozyme. Thus, it was proven that the shape selectivity of proteins by the MIP is important in the selection of proteins for recognition.

Both the Hev b1 (G)-MIP and Hev b1 (L)-MIP based receptors provide a high selective recognition of Hev b2 (L) and the closely related protein Hev b3 (L), while non-related proteins such as ovalbumin and BSA, which are different in size and shape, have a less selective recognition interaction. The cross-reactivity shown for Hev b3 (L) may arise from the fact that the Hev b3 protein-associated latex allergy shares a 47% sequence identity with Hev b1 at the amino acid level, and the two molecules have been shown to share IgE epitopes [16]. However, there appears to be cross-selectivity between Hev b1 (G) and Hev b1 (L) because of their similar functional group and dimension of protein. Hence, it can be concluded that the Hev b1 (G) molecularly imprinted polymer has a selective recognition to a template with a geometrical fit and functional group interaction that depends on the conformation of the protein. It is noted that Hev b1 naturally interacts with the specific binding sites created for the non-native protein recognition, showing a 50% reduction in cross-reactivity to the template protein. This indicates that the substrate protein, bound to the "active site" of its chaperone proteins in a non-native conformation, has been made use of in

the MIP-QCM sensor method. Interestingly, the recognition site generated by Hev b1 (L) leads to the greatest selectivity for Hev b1 (L) (the template) as well as Hev b1 (G). The difference of signal response for Hev b1 (G) on the imprint of Hev b1 (G) and Hev b1 (L) is ~13% to the case of Hev b1 (L) on Hev b1 (L) imprint. This indicates that it is possible that non-native Hev b1 undergoes re-orientation onto the surface near or adjacent to the active site of MIP, is specifically able to bind species of naturally derived Hev b1. This may be because non-native Hev b1 protein refolds to a stable state in the Hev b1 (L)-imprinted cavity to its native conformation.

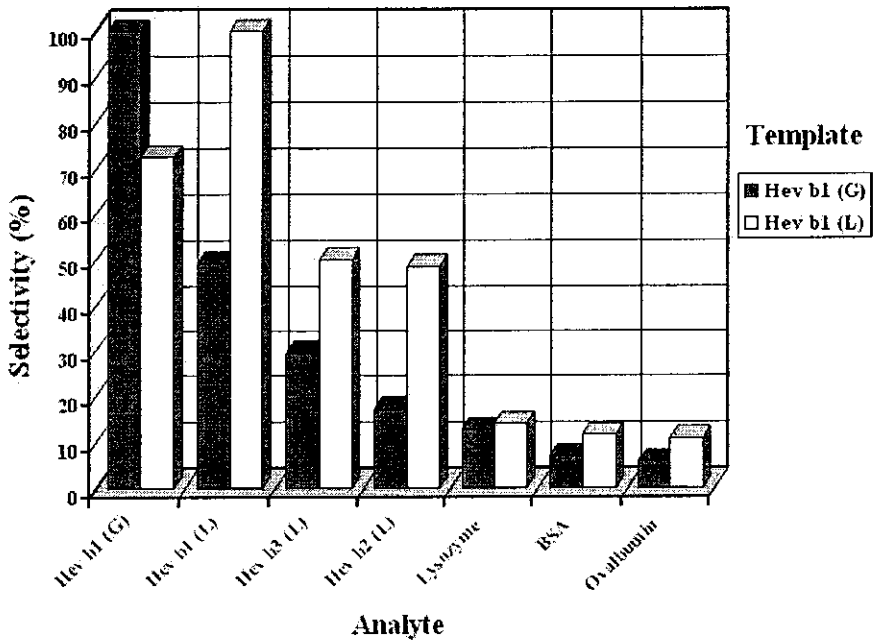


Fig. 9. Selectivity profiles of the surface-imprinted polymers for Hev b1 (G), and Hev b1 (L) templates from QCM experiments at an analyte concentration level of 500 μg L^{-1} in phosphate buffer (pH 7.4).

QCM assays using Hev b1 (G) surface-imprinted polymer

Using Hev b1 (G) surface-imprinted polymer as a selective layer in QCM is important for determination of Hev b1 in the extracts of latex articles and finished products of gloves. We developed a MIP-QCM sensor-based assay for measuring Hev b1, and studied its analytical performance. As a label-free detection method, QCM allows for a rapid and simple

continuous Hev b1 assessment. The assay covered a linear range of 10 to 1000 μg L^{-1} (R^2 > 0.996). The analytical detection limit, calculated using a signal-to-noise ratio of 3, was 1.0 μg L^{-1}. The more sensitive Hev b1 (G) surface-imprinted polymer was applied to glove extracts. The principal intention of the MIP-QCM sensor is to minimize the matrix effects of real samples, owing to the selectivity of the Hev b1 (G) imprinted polymer. Initially, evaluation of the matrix effect on QCM measurement was carried out; and secondly, a study was performed on the extraction efficiency of Hev b1 (G) from spiked glove samples to verify the accuracy of the QCM-based assay of Hev b1 (G). For this purpose we used an extract of matrix sample from a latex glove spiked with a standard solution of Hev b1 (G) at concentration levels of 250, 500 and 1000 μg L^{-1}. The method showed high selectivity and good precision and compatibility with the biological complex matrix. Recoveries were in the range of 97 to 105%, with RSD values between 3 and 6.5. The measurement of latex allergens in real samples was performed by the developed MIP-QCM assay method. The amount of latex allergens in natural rubber latex mattresses and latex gloves were found to be 1822 ± 41 and 22 ± 4 μg/g of rubber material, respectively – results similar to or lesser than those reported by other authors [34, 35, 36]. The amount of latex allergens in natural rubber latex mattresses and latex gloves were found to be 1822 ± 41 and 22 ± 4 μg/g of rubber material, respectively – results similar to or lesser than those reported by other authors [34, 35, 36]. The amount of latex protein penetration through human skin into the body depends on the skin conditions. Little protein penetration (less than 3%) was observed into or through intact skin and up to 40% of Hev b1 penetrate abraded skin [37]. Smaller levels of protein exposure will reduce the risk for sensitization and elicitation of symptoms. Although the threshold latex protein level for sensitization is unknown, the potential for allergenicity is markedly decreased when is the level is below 100 μg/g [38]. Most NRL-allergic patients show a positive skin prick test reaction when the sum of the four allergens, Hev b1, Hev b3,

Hev b5 and Hev b6.02, exceeds 1 μg/g [22]. The sandwich ELISA for the detection of Hev b1 concentrations in rubber latex and latex products had a detection range of 125-4000 μg L^{-1} (detection limit of 1.25 μg of Hev b1/g of rubber) [35] compared with 10-1000 μg L^{-1} for the MIP-based QCM assay (detection limit of 1 μg L^{-1}). Moreover, the MIP can undergo at least 50 generation steps without losing recognition ability.

Conclusions

This paper presents the development and optimization of a surface imprinted polymer for detection of the rubber elongation factor (Hev b1) in natural rubber latex and latex gloves. Surface imprinting allows for the recognition of proteins based on shape selection and the positioning of functional groups that can memorize Hev b1 protein in a molecular recognition process. Combining functionalized materials with mass-sensitive chemosensors such as the quartz crystal microbalances (QCMs) is a powerful and label-free tool for chemical sensing purposes, allowing distributed online measurement of allergen proteins. Polymers as sensor coatings enable a variety of modifications of composition and functionality, ensuring optimum modification of the sensitive surface to the analyte. Hev b1 surface-imprinted onto methacrylic acid-vinylpyrrolidone-dihydroxyethylene bisacrylamide polymer materials produces an excellent and reversible frequency response toward Hev b1, leading to sensitive responses as low as 10 μg L^{-1}. Additionally, the sensor shows high selectivity. The selective layers can distinguish between the template and others (i.e. lysozyme, ovalbumin, and bovine serum albumin), showing the geometrical and functional selective fitting of the imprinted cavities of the resulting polymers. The MIP material has conformational memory. The surprising distinct orientation of the non-native protein Hev b1 arises from interaction of the binding group ordering with a preferred conformation due to a gain in entropy on the

molecularly imprinted polymer material surfaces produced with native Hev b1 as a templating protein. The results of the present study may provide a better insight into the structure and conformation arrangements in the supramolecular structure and physicochemical properties of the latex allergen Hev b1, that has been implicated in life-threatening immunological reactions. Additionally, the results demonstrate that QCM sensor measurements using MIP as a recognition material can be used to screen and detect Hev b1 in such complex matrices as manufactured rubber latex gloves.

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References

1. Elizabeth H, Bromley C, Channon K, Moutevelis E, Woolfson DN (2008) ACS Chemical Biology 3:38-50
2. Shi H, Tsai W-B, Garrison MD, Ferrari S, Ratner BD (1999) Nature 398:593-597
3. Turner NW, Jeans CW, Brain KR, Allender CJ, Hlady V, Britt DW (2006) Biotechnol Prog 22:1474-1489
4. Bossi A, Bonini F, Turner APF, Piletsky SA (2007) Biosens Bioelectron 22:1131-1137
5. Lieberzeit PA, Dickert, FL (2008) Anal Bioanal Chem 391:1629-1639
6. Haupt K, Mosbach K (2000) Chem Rev 100:2495-2504
7. Wulff G (1995) Angew Chem Int Ed Engl 34:1812-1832

8. Yilmaz E, Haupt K, Mosbach K (2000) Angew Chem Int Ed Engl 39:2115-2118

9. Nishino H, Huang CS, Shea KJ (2006) Angew Chem Int Ed Engl 45:2392-2396

10. Takeuchi T, Goto D, Shinmori H (2007) Analyst 132:101-103

11. Matsunaga T, Takeuchi T (2006) Chem Lett 35:1030-1031

12. Zdyrko B, Ooy O, Luzinov I (2009) Biointerphases 4:FA17-FA21

13. Turjanmaa K, Alenius H, Mäkinen-Kiljunen S, Reunala T, Palosuo T (1996) Allergy 51:593-602

14. Kurup VP, Sussman GL, Yeang HY, Elms N, Breiteneder H, Arif SA, Kelly KJ, Bansal NK, Fink JN (2005) Clin Mol Allergy 3:1-9

15. Palosuo T, Mäkinen-Kiljunen S, Alenius H, Reunala T, Yip E, Turjanmaa K (1998) Allergy 53:59-67

16. Banerjee B, Kanitpong K, Fink JN, Zussman M, Sussman GL, Kelly KJ, Kurup VP (2000) Mol Immunol 37:789-798

17. Yeang HY, Cheong KF, Sunderasan E, Hamzah S, Chew NP, Hamid S, Hamilton RG (2006) J Allergy Clin Immunol 98:628-639

18. Lowry OH, Rosebrough NJ, Farr AL, Randel RJ (1951) J Biol Chem 193:265-275

19. Tomazic-Jezic VJ, Lucus AD (2002) J Allergy Clin Immunol 110:S40-S46

20. Smith PK (1985) J Anal Biochem 76:150-158

21. Beezhold DH, Kostyal DA, Tomazic-Jezic VJ (2002) Methods 27:46-51

22. Palosuo T, Alenius H, Turjanmaa K (2002) Methods 27:52-58

23. Ballantine DS Jr, White RM, Martin SJ, Ricco AJ, Zellers ET, Frye GC, Wohltjen H, (1997) Acoustic wave sensors, theory, design and physical-chemical applications, Academic press, San Diego, USA

24. Chen Z, Cremer R, Posch A, Raulf-Heimsoth M, Rihs HP, Baur X (1997) J Allergy Clin Immunol 100:684-693

25. Marchetti-Deschmann M, Allmaier G (2009) J Mass Spectrom 44:61-70

26. Dickert FL, Hayden O, Bindeus R, Mann K-J, Blaas D, Waigmann E (2004) Anal Bioanal Chem 378:1929-1934

27. Hayden O, Haderspöck C, Krassnig S, Chen X, Dickert FL (2006) Analyst 131:1044-1050

28. Bühler V (2005) Polyvinylpyrrolidone excipients for pharmaceuticals, Springer-Verlag, Berlin Heidelberg, Germany

29. Lin HY, Hsu CY, Thomas JL, Wang SE, Chen HC, Chou TC (2006) Biosens Bioelectron 22:534-543

30. Ahmed H (2005) Principles and reactions of protein extraction, purification, and characterization, CRC Press, Boca Raton, FL, USA

31. Wagner B, Krebitz M, Buck D, Niggemann B, Yeang HY, Han KH, Scheiner O, Breiteneder H (1999) J Allergy Clin Immunol 104:1084-1092

32. Dickert FL, Achatz P, Halikias K (2001) Fresenius J Anal Chem 371:11-15

33. Jenik M, Schirhagl R, Schirk C, Hayden O, Lieberzeit P, Blaas D, Paul G, Dickert F (2009) Anal Chem 81:5320-5326

34. Deborah JS, Katrina C (1995) Anal Biochem 229:278-281

35. Yagami A, Suzuki K, Saito H, Matsunaga K (2009) Allergol Int 58:347-355

36. Raulf-Heimsoth M, Sander I, Chen Z, Borowitzki G, Diewald K, van Kampen V, Baur X (2000) Int Arch Allergy Immunol 123:236-241

37. Hayes BB, Afshari A, Millecchia L, Willard PA, Povoski SP, Meade BJ (2000) Toxicol Sci 56:262-270

38. Hepner DL, Castells MC (2003) Anesth Analg 96:1219-1229

Running Title 2:

Rubber elongation factor-imprinted polymer coated on interdigitated capacitance electrodes for determination of rubber latex allergen proteins in products such as rubber gloves

Abstract

Molecularly imprinted polymers for detecting Hevein latex allergen proteins (Hev b1) in rubber gloves were designed as recognition materials on interdigitated capacitance electrodes. The recognition for rubber latex allergen was generated by applying methacrylic acid and vinylpyrrolidone mixed functional monomers, directly copolymerizing together with an extract of Hev b1 obtained from rubber gloves used as the template molecule matix from a shared solution that was coated onto the interdigital capacitive electrodes. Imprinted cavities on the polymer surface mimicking the surface functionality and configuration of the protein served as recognition sites for re-binding after removing the template by washing out. The sensor materials reversibly and selectively bound the template in the aqueous phase. They could distinguish between Hev b1 isolated from rubber latex of the rubber tree and that present in the extract from the glove samples. The artificial layer favored binding with the templating allergen by a factor of 4 compared to the competing one. Different hevein latex allergenic proteins isolated from natural rubber latex from the rubber tree (Hev b1, Hev b2 or Hev b3) and non-related proteins as lyzozyme, ovalbumin and bovine serum albumin were distinguished by the sensor material, depending on the dimension of these proteins with selectivity factor values of between 2 to 4. The sensors can detect Hev b1 in amounts that ranged from 10 ng ml^{-1}-0.8 μg ml^{-1} within minutes and with a detection limit of 10 ng ml^{-1}. Moreover direct measurements can be made on simple extracts from the complex matrices such as manufactured rubber latex gloves so avoiding any time consuming sample preparation.

Keywords Molecularly imprinted polymers, Interdigital capacitive transducer, Rubber elongation factor, Allergen protein

บทคัดย่อ:

พอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลสำหรับใช้ตรวจโปรตีนก่อแพ้ในน้ำยางที่ตกค้างในถุงมือ ถูกนำมาใช้ออกแบบวัสดุจดจำบนอินเตอร์ดิจิเตทคาแปซิแทนต์ (Interdigitated Capacitance) การจดจำโปรตีนก่อแพ้จากยางพาราถูกสร้างขึ้นมาโดยใช้เมธาคริลิค แอซิด และไวนีลพัยโรลิโดนเป็นฟังชันนีลมอโนเมอร์ผสมและทำการโคพอลิเมอร์ไรซ์กับไดไฮโดรซีเอธีลีน-บิส-อะคริลามายด์ เป็นตัวเชื่อมส่วนรอบๆ Hev b1 ที่สกัดได้จากถุงมือยางซึ่งอยู่ในสารละลายของสารเตรียมพอลิเมอร์บนอินเตอร์ดิจิตอลแคแปซิแทนซ์อิเล็คโตรดรอยพิมพ์ประทับบนผิวของพอลิเมอร์จดจำหมู่ฟังชันที่ผิวและรูปร่างในสามมิติของโปรตีนจะทำหน้าที่เป็นตำแหน่งของรอยจดจำสำหรับการจับกับเทมเพรทในตัวกลางที่เป็นน้ำ พอลิเมอร์ที่พิมพ์ประทับรอยโมเลกุลสามารถแยกระหว่าง Hev b1 ที่สกัดจากน้ำยางของต้นยางพาราและ Hev b1 จากตัวอย่างถุงมือยางพอลิเมอร์วิเคราะห์ชอบ Hev b1 จากถุงมือที่เป็นเทมเพลตด้วยค่าของการเลือกจดจำมากกว่า 4 เมื่อเปรียบเทียบกับ Hev b1 จากน้ำยาง นอกจากนี้โปรตีนก่อแพ้ต่างๆ ที่สกัดได้จากน้ำยางธรรมชาติของต้นยางพารา (Hev b1, Hev b2 และ Hev b3) และ non-Hev b1 เช่น lyzozyme, ovalbumin และ bovine serum albumin สามารถถูกแยกได้ด้วยวัสดุเซ็นเซอร์ที่เตรียมได้นี้ด้วยค่าการจดจำระหว่าง 2 ถึง 4 เซ็นเซอร์ชอบที่จะตรวจ Hev b1 ในช่วงความเข้มข้น 10 ng/ml - 0.8 μg/ml ภายในเวลา 2-3 นาทีด้วยค่าความเข้มข้นต่ำสุดในการตรวจ (limit of detection) เท่ากับ 10 ng/ml นอกจากนี้ยังสามารถทำการตรวจวัดโดยตรงโดยปราศจากการเตรียมตัวอย่างที่อยู่ในสารประกอบที่ซับซ้อนอย่างเช่นถุงมือยางได้อีกด้วย

Introduction

Allergies to natural rubber latex (NRL) proteins is a well-recognized health problem among sensitized persons [1]. Healthcare workers are frequently exposed to natural rubber latex products such as gloves, used preferentially as a protective barrier. In addition to the healthcare setting, rubber gloves are commonly used in other industries such as food and beverage, manufacturing, and also in domestic settings for protective or hygienic purposes. The production process for natural rubber gloves, involves high temperatures and various chemicals are applied during the different process stages as well as during the washing steps of downstream operations. However some proteins will be retained in the product. The increasing demand for rubber based gloves has also led to a decrease in processing time, and this in turn has led to an increase in retention of latex proteins. Different manufacturers of gloves use different methods, standards, and processing times. There is a 3000-fold difference in the latex contents of ten different brands of gloves [2]. A method for the rapid and inexpensive screening of rubber latex allergy proteins is needed to reduce the risk of developing allergies from latex proteins and to quantify the amount of specific allergens found in NRL finished products, such as gloves. The approach presented here takes advantage of interdigitated capacitance (IDC) electrodes coated with man-made "antibodies" [3] that can react with and detect remnants of allergenic proteins present in the complex matrix of manufactured latex rubber gloves.

A number of IgE-reactive proteins have been identified in natural and processed latex products. Alergenic polypeptides (Hev b) of natural rubber latex (NRL) from the rubber tree, *Hevea brasiliensis* that elute from surgeon's glove and other manufactured rubber products can sensitize exposed individuals and elicit severe hypersensitivity reactions. There are 13 known Hevea latex allergens, Hev b1 through Hev b13. They differ in size, shape, and charge [4]. Though most Latex-allergic patients are sensitised to more than one Latex

allergen, Hev b1 (also called the rubber elongation factor [REF]), is a major latex allergen with an extremely high involvement in sensitizing patients to latex allergies [5,6]. Hev b 1 is found mainly on large rubber particles (>350 nm in diameter) derived from the fragmentation of Hev b 3 (24 kDa), found mainly on small rubber particles (average diameter, 70 nm) is recognized by IgE from patients with spina bifida and latex allergies. The other Hevea allergens that have been shown to be clinically relevant to NRL allergies and detected in the final NRL products with no changes to their allergenicity are Hev b3 (22-27 kDa rubber particle-associated protein), Hev b5 (16 kDa acidic NRL protein) and Hev b 6.02 (mature hevein). Although sensitization does not always lead to an anaphylactic reaction, continued exposure to latex will increase the possibility of such a reaction. Occupational exposure to latex is common among health care workers such as nurses in intensive care units who need to change gloves 50-100 times per day. The risk of latex sensitization or latex allergy can be minimized by decreasing the amount of skin contact as a route of exposure to protein allergens. Latex sensitivity and latex-related symptoms of latex allergy can also be reduced by changing from protein powdered gloves to low-protein, powder free gloves.

NRL industries require a method that is rapid and easy to handle, like the proposed sensor using a biomimetic polymer, for detecting rubber allergen protein within minutes. Such a sensor system would guarantee rapid analysis without the necessity of larger numbers of operating personnel. In most cases, detection and quantifying of specific rubber allergenic proteins are based on latex ELISA for antigenic protein (LEAP) and an enzyme linked immunosorbent assay (ELISA) that use anti-NRL antibodies to detect the antigenic protein. The production of a special antibody is expensive, however, and acts as a one-way test. A molecularly imprinted polymer (MIP) involves the polymerization of a highly crosslinked macromolecular matrix in the presence of a template or target analyte. MIP materials represent artificial antibodies and offer fully artificial, robust and highly selective materials.

Therefore, they can replace a natural receptor as a molecular recognition agent in biosensor applications and for separating small molecules or biomacromolecules [7-9]. In this study, an interdigital capacitive (IDC) transduction system was modified with imprinted materials made by cross-linking copolymerization of an appropriate monomer with a cross-linker in the presence of the template Hev b1, a model for Hevein latex allergenic proteins. After template removal the recognition site is formed within the developed sensitive layer and allows for specific sorption of the protein template on the MIP sensors and provides sufficient capacitive performance. Combining rationally structured polymer layers with a capacitance transducer has proven to be a highly promising approach for designing chemical sensor systems in a variety of analytical applications [10-12]. The main advantages of inter-digitated, capacitive immuno-sensors are inexpensive production, low sample consumption, a label-free detection mechanism and a fast and reliable measurement. The detection mechanism of the sensor system as used in this study is based on the change in the dielectric constant of the inter-digitated capacitance using the following equation [13-14]:

$$C = \frac{\varepsilon_0 \varepsilon_r A}{d} \qquad (1)$$

where, ε_r is the dielectric constant of the medium between the plates, ε_0 (permittivity of free space) = 8.85419 pF/m, A the area of the plates and d the distance between them. However, a capacitive sensor cannot discriminate between the measured signal from a specific or non-specific interaction.

The transducer has to be sensitive to a conformational change of the analyte binding site or to changes in charge distribution around this site when the analyte binds. It is therefore important to design the surface in such a way that it ensures a higher specificity for the analyte than for a non-specific binding.

In this work, we present a novel approach for a fast and reliable method for screening and detection of the specific allergen Hev b1 in gloves based on selective layers prepared via solution-based imprinting as recognition materials on an IDC transducer in an aqueous phase. Copolymerization of methacrylic acid and 1-vinyl-2-pyrrolidone functional monomers and *N,N′*-(1,2-dihydroxyethylene) bisacrylamide as a cross-linker was carried out on the IDC electrodes using distilled water as a porogen solvent. The influence of the degree of crosslinking on the imprinting effect was examined after the binding was measured by IDC techniques. We investigated technological parameters (i.e. frequency, medium pH, NaCl concentration) for the sensor in terms of the capacitive response of MIPs on the IDC sensor. The cross-reactivity of the selective layer to the template and structurally closely related proteins was examined using the IDC techniques. Subsequently, the newly developed Hev b1-MIP-IDC sensor method was used to determine the presence of hevein latex allergen proteins in the glove samples at the optimized sensor conditions.

2. Experimental

2.1 Materials

Acrylamide (ACM), 1-vinyl-2-pyrrolidone (NVP), methacrylic acid (MAA), *N,N′*-(1,2-dihydroxyethylene)bisacrylamide (DHEBA), sodium dodecyl sulfate (SDS), Triton X-100 and Tris-HCl were from Aldrich Chemical Company (Milwaukee, WI, USA). 2,2′-Azobisisobutyronitrile (AIBN) from Janssen Chimica (Geel, Belgium). Lysozyme, ovalbumin and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). Rubber latex was obtained from a commercial rubber latex manufacturer, in Songkla, Thailand, and kept in a refrigerator at 2-4 °C. Cellulose dialysis bags with a MWCO of 3,700

Da were purchased from Sigma (St. Louis, MO, USA). All solvents used were of analytical-reagent grade and used without further processing.

2.2 Protein extraction

Allergenic proteins isolated from natural rubber latex are identified with an (L) and the proteis extracted from latex gloves are identified with a (G). Hev b1 (G), Hev b1 (L), Hev b2 (L) and Hev b3 (L) were obtained from latex gloves and rubber latex by a protocol similar to that described previously [15] using the detergent, 1% sodium dodecylsulfate (SDS) in 20 mM phosphate buffer solution (pH 7.4). The extractable protein was dialyzed through a cellulose bag against distilled water for 48 h at 4°C, and lyophilized. Crude proteins were purified by size exclusion chromatography with Sephacryl^{TM} S-100 (1.5 x 64 cm, Pharmacia) using 50 mM phosphate buffer pH 7.4 containing 0.1% SDS and 0.15 M NaCl as the eluent. Protein fractions (2.0 ml) were collected and examined by SDS-PAGE. The active fractions were pooled and concentrated. The sequence of the target protein was confirmed using an Ultraflex III MALDI-TOF (Bruker Daltonik GmbH, Breman, Germany) mass spectrometer [16].

2.3 Fabrication of the sensor device

The interdigitated capacitance electrode of comb-type with ten fingers was used in this study, after being sintered at 550 °C for 3 h. The electrodes were modified as depicted in Fig. 1. The thickness of the film after sintering was about 1 μm as measured by an atomic force microscopy (AFM) method. These electrodes were modified as depicted in Fig. 1. For generation of an IDC coating, a monomeric mixture consisting of 60% MAA (50 mg) and 20% NVP (20 mg) that acted as the functional monomers and 15% DHEBA (30 mg) as the cross-linker and 1% AIBN (1.5 mg) in 800 μL distilled water was the pre-coated polymer

layer. After rinsing with nitrogen gas the monomer was pre-polymerized up to the gel point at 70°C until it reached a suitable viscosity. Then 2 μL of the pre-coated polymer was drop coated onto the IDC electrode as a very thin layer to increase the binding of the film to the transducer and avoid flaking of the resulting thin films. For the generation of Hev b1 (G) MIP film layer, the experiment was carried out with an aqueous solution of ammonium periosulfate mixed into a 5 μL sample of 10 mg ml^{-1} protein in 0.1 mM phosphate buffer solution (pH 7.4) in a vial, 2 μl of the mixed solution after being adjusted to pH 7.4 by 100 mM KOH in order to prevent denaturation of protein template was dropped on electrodes. The whole content was purged with nitrogen gas for 5 min, and then cured under UV irradiation overnight at room temperature (25 ± 1°C). The template proteins were removed from the polymeric film by immersing the modified electrode into 3 x 100 ml of distilled water at room temperature (25 ± 1°C). Similar IDC electrodes but modified with a non-imprinted polymer (NIP) layer were also fabricated but without the template.

Furthermore, in order to study the effect of the crosslinker content on the MIP's selectivity behaviour, various MIP-modified IDC electrodes were also prepared (using different mole ratios of crosslinker: 10, 15, 20 and 25 mol% with a fixed amount of the functional monomers). Morphological images of modified IDC electrode surfaces were recorded using an atomic force microscope (AFM) (Vecco Instruments Inc., Santa Barbara, USA) with a Nanoscope III STM controller (Digital Instruments Inc., Santa Barbara, USA).

2.4 Sensor fabrication

We prepared the IDC electrode that constituted the integrated sensor array and wire connections with electrode-contacts. The sampling and capacitive analysis system of the sensor consists of a liquid port for delivery of sample from a sample-reservoir (100 μL), a peristaltic pump, a thermo set and the flow-through microcell/integrated MIP sensor

incorporated with a capacitive apparatus controlled by a computer program developed in-house. We operated the stand-alone sensor at a flow rate of 3 ml min^{-1}. The sensor array output signals were monitored using a network analyser (HP 4254A Precision LCR, Hewlett Packard, Germany), that read the capacitance signals from the sensor array with a subsequent display on the Laptop screen. The capacitance measurement of the sensor was performed by applying an alternating potential (100 mV) to the electrodes with a frequency of between 0.1 KHz and 1 MHz. The sensitivity of the MIP-based sensor was measured as a function of the changes in the capacitance of the polymer upon exposure to Hev b1 (G) with concentrations that ranged from 1 to 800 ng ml^{-1}. A control experiment was carried out with the corresponding NIP-based sensor. For the sample measurement using the sensor, the signal response towards the analyte of the sensor was reported as ΔC, where ΔC is the capacitance shift response to the addition of known amounts of the analyte of interest. The influence of parameters (operating frequency, pH and electrolyte) on the capacitance change of the IDC sensor for Hev b1 (G) was studied using the sensor layer that gave optimal capacitance change. The imprinting factor that represented the effect of the imprinting process was the ratio of ΔC of the MIP-base electrode to ΔC of the NIP-base electrode for the analyte under the IDC sensor conditions. All experiments were carried out in triplicate at room temperature ($25 \pm 1°C$).

2.5 Sample analysis

The rubber based gloves used were cut into small pieces and extracted in 0.2 M phosphate buffer pH 7.4 containing 0.5% SDS. The rubber pieces were then removed by centrifugation. The supernatant was dialyzed through a cellulose bag (molecular weight cutoff of 3,700 Da) against distilled water for 48 h at 4°C. The glove extract was analysed for the target protein by the developed IDC at the optimized sensor conditions. The quantitative analysis of Hev b1 (G) in natural rubber latex samples were performed by the standard

addition method and considering the response, i.e., the capacitance shift that was related to the concentration of the analyte.

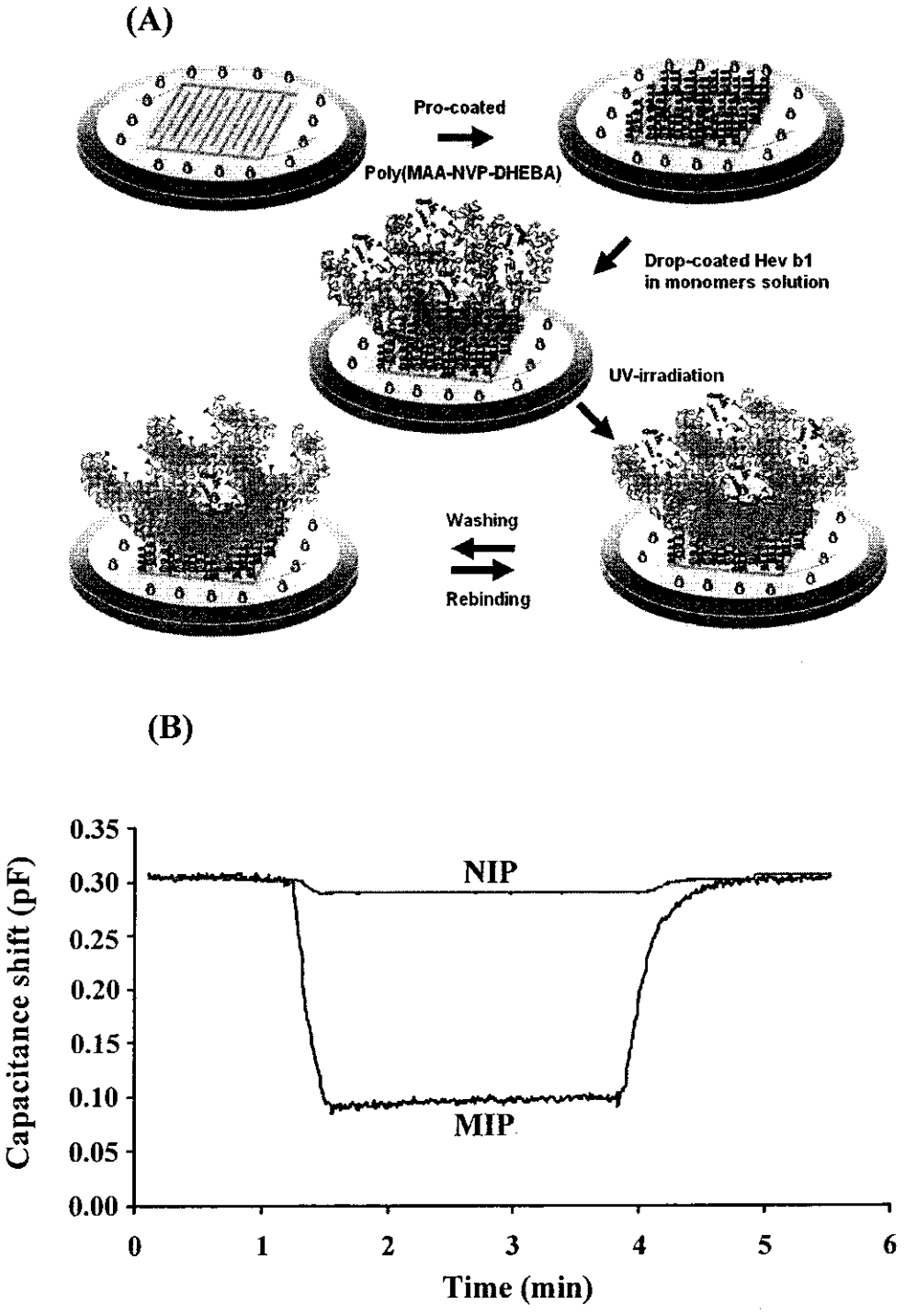

Fig. 1. (A) Schematic illustration of self-assembling organization and crosslinking of monomers to create imprinted sites on the gold electrode and (B) signal response of non-imprinted and Hev b1 (G) imprinted polymer to Hev b1 (G).

3 Results and discussion

3.1. The MIP based IDC sensor

The MIP film of poly(MAA-NVP-DHEBA) was prepared on the gold electrode under UV irradiation using Hev b1 (G) as the template. DHEBA was chosen as the cross-linking monomer for imprinting of the Hev b1 (G) proteins on the polymer, since it can minimize non-specific interactions between protein and the polymer and give reasonable adhesive properties for the polymer [17]. The MAA functional monomer was used for the imprinting of Hev b1 (G), because its acid group is capable of interacting with the amine group of the template via hydrogen bond interactions, while the NVP monomer provided good wetting and adhesive properties. During the preliminary polymer screening, it was found that when a 5:2 mixture of MAA and NVP monomers with respect to the template was used, the resulting MIP yielded the greatest sensor responses. The deposited thin-film of both the MIP and the corresponding NIP was soaked in 500 ml of distilled water, and capacitance measurements were performed in the open air after every hour of electrode soaking at ambient temperature. After soaking in distilled water for 5 h, the ΔC of MIP (4.9×10^{-13} F) was almost 2.5 times higher than that of the corresponding NIP (2.1×10^{-13} F). The capacitance of the blank electrode without the polymer being insulated by protein imprinting was 1.39×10^{-10} F. The change in capacitance of the MIP films corresponds to the insulating polymer properties when the protein template was bound to the MIP polymer. The capacitance of the IDC sensor increased when the protein templates were dissociated from the imprint sites of the MIP by water. Accordingly in the case of the NIP-based sensor, no Hev b1 (G) molecules were involved in the polymerization process but the capacitance shift may be an effect from the residue of non completely bound polymer. During the preliminary imprinting study, it turned out that the optimal imprinting results were achieved with layers having a thickness of 300 nm. This allows the imprinted layer to provide a large interaction area between each individual allergenic protein and the forming polymer increases the binding strength. As can

be seen in the AFM image depicted in Fig. 2, the occupation of the tailor-made cavities by Hev b1 (G) occurs during the imprinting process. The correlation between the occupancy of surface imprinting and the capacitance effect was studied by comparing AFM measurements with results from the interdigitated capacitive responses. All unbound Hev b1 (G) was removed and the occupancy of imprinted pits was estimated to be 90%. Only a minimal amount of allergy protein was seen to be bound non specifically and its contribution to the capacitive sensor effect on the imprinted layer might be negligible. Increasing these layer heights (>300 nm) for the selective layer on IDC produces a relatively stable shift increase. Since recognition in the MIP is based on non-covalent interactions between the layer and protein, hence a larger surface for interaction leads to a stronger binding and consequently to decreased reversibility of the interaction. When both the Hev b1 (G) and NIP-IDC electrodes were exposed to Hev b1 (G), the capacitance on both channels decreased within a few minutes, as shown in Fig. 1(B). This rapid response indicates that only the surface, but not the bulk interactions are involved because for bulk interactions the response would be much slower due to it being strongly hindered by diffusion. The decrease in the capacitance signal of the MIP and NIP film when exposed to the latex allergen in 0.1 mM phosphate buffer (pH 7.4) can be explained in terms of a change in the dielectric value due to the interaction between the allergen and the cavity of the MIP. When the protein formed a complex with the binding site on the MIP, the dipole moment and distinct charge distribution of protein increased because of the radio frequency electromagnetic field leading to an increment of the dielectric value in the medium. Moreover, the conformation and ion surface charge of the protein complex can be changed and lead to a space on the surface layer where ions can migrate to and therefore, the capacitance in the sensor system is decreased. The NIP allows protein to bind to the polymer by non-specific binding and also decreases the capacitance on the IDC sensor system.

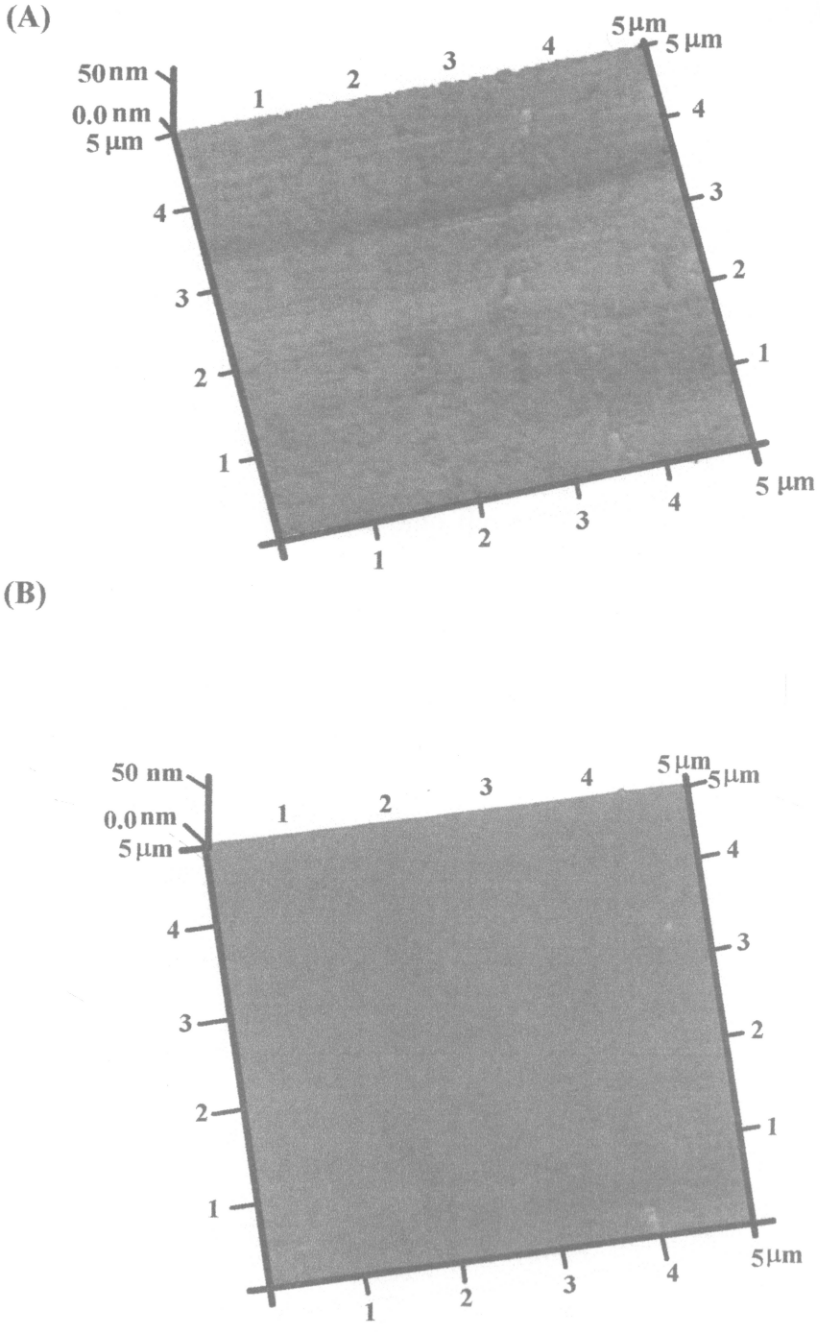

Fig. 2. Contact mode AFM images of: (a) imprinted layer showing Hev b1 (G) pits on poly(MAA-NVP-DHEBA) (as described in Fig. 1) and (b) non-imprinted layer after hardening of the layer and washing with distilled water for 5 h.

3.2. Effect of crosslinking on imprinting effect

In this study, the optimized amount of the cross-linking monomer was examined to find the most effective sensor response of Hev b1 (G)-MIP onto the IDC electrode.The imprint

capacity, reflecting the efficiency of recognition of MIPs was interpreted by comparing the capacitance variation of MIP with the corresponding NIP. As seen in Fig. 3, the degree of the ΔC is a function of the cross-linker content for both MIP and NIP. The selective layer with 15 mol% of DHEBA yielded the highest capacitance shift and the optimal selectivity was achieved with an imprinting factor of 4 for the template protein Hev b1 (G). At 10 mol%, the MIP shows a lower capacitance change. This degree of cross-linking gives a lower imprinting effect than at the higher cross-linker content of 15 mol%. This may be caused by the low stability of the binding sites onto the MIP thin-layer. A high cross-linking of the polymer produces a decrease of the capacitance shift of the MIP layer to the template and a high non-specific adsorption on the non-imprinted layer. The MIP film-layer with a cross-linker beyond 15 mol% exhibited a significant decrease in the recognition efficiency. This result can be explained because a high cross-linked density of polymer causes reduced diffusion of the protein template into the binding sites of MIP and tends to interact more with the non-specifically bound protein binding with residues of the functional groups of the cross-linker.

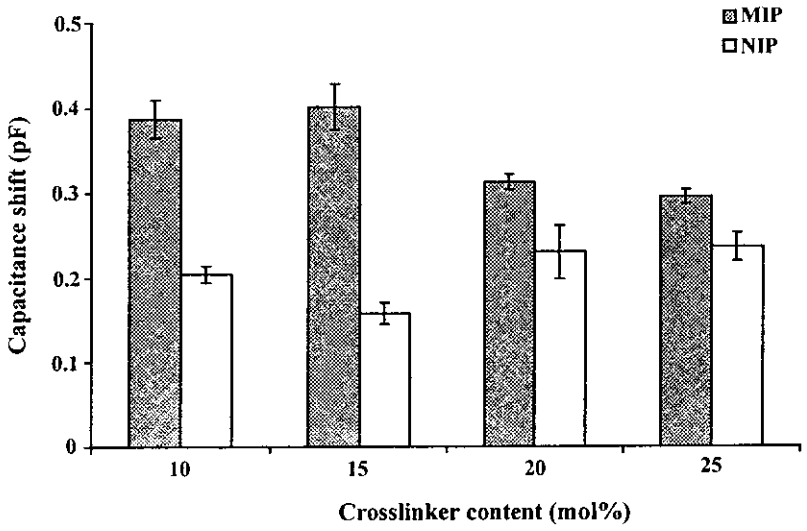

Fig. 3. Effect of the amount of cross-linking agent on the capacitive shift of non-imprinted and Hev b1-(G) imprinted coated IDC exposed to Hev b1 (G) at a concentration lavel of 250 ng ml^{-1}.

3.3. Effect of technological parameters on the sensor response

3.3.1 Effect of applied frequency

In the present work, the effect of the operating frequency on the sensor response was studied to identify the optimum operating frequency. For this purpose, the capacitance of the sensor was measured over the frequency range of 0.1 kHz-1 MHz using a Hev b1 (G) concentration of 100 ng ml^{-1}. Fig. 4 shows the effect of the applied frequency on the capacitance shift sensor. It can be seen that the capacitance shift of the MIP sensor was achieved and decreased when the applied frequency was increased from 100 kHz to 1 MHz. The highest capacitance shift occurred at 1 kHz. The results also demonstrate that the capacitance shift is the response to the binding of protein onto the MIP. The capacitance shift increased when the protein formed a complex onto the polymer thin film.

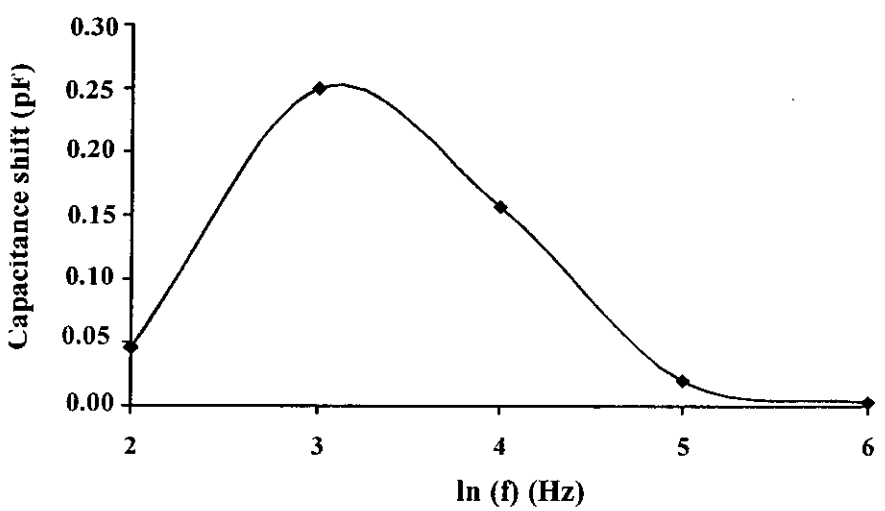

Fig. 4. Effect of the ac frequency on the capacitance shift responses of the MIP-IDC sensor at concentration of 100 ng ml^{-1} Hev b1 (G), measurements were carried out in 0.1 mM phosphate buffer solution pH 7.4 at room temperature.

When applied at a frequency below 1 kHz, the sensor produced a low capacitance shift, as the partial diffusion of protein occurred and reorientation of the protein conformation to fit with the binding site of MIP also probably occurred. When an applied frequency of over 3 kHz was applied to the sensor system, a decrease of the capacitance shift was shown. This may be

because of a high diffusion of protein that is not suitable for binding to the specific cavities of MIP on the polymer thin film. A frequency of 1 kHz was therefore chosen for operating the sensor in all remaining experiments.

3.4.2 Effect of the medium pH

The effect of the pH of the medium on the capacitance shift response of the Hev b1 (G)-MIP sensors was investigated (Fig. 5A). At pH 5.0 both the MIP and NIP thin-films gave the lowest capacitance shift response for Hev b1 (G). These results can be explained if the Hev b1 (G) does not bind with the binding sites on the polymer film surface at a pH of 5. At this low pH value (pH 5) the Hev b1 (G) (pI = 5.0) may have lost the functional groups necessary to interact with the binding sites of the cavities or have a changed structural conformation that is not recognized by the MIP. The optimum capacitance shift of the sensitive layer was at a pH of 7.4, together with giving the lowest value for the non-specific binding in this medium.

3.3.3 Effect of electrolytes

The effect of the electrolyte on the capacitance shift was investigated (Fig. 5B). An increasing concentration of NaCl in the sample solution containing 100 ng ml^{-1} Hev b1 (G) slightly increased the magnitude of the sensor response for both MIP and NIP based sensors. The molecular recognition of MIP decreased as a function of the increased ionic concentration in the solution. The capacitance observed with both MIP and NIP sensors when exposed to Hev b1 (G) and electrolytes (NaCl) can be explained by the enrichment of the protein and counter ions in the polymer, that gives a high local concentration of charged species on the thin film polymer close to the gold electrode surface in the sensing device. Therefore, ions can move through the layer, leading to a high capacitance shift on the interdigital capacitor sensor

system. Therefore, the concentrations of salts have to be kept below 0.01 mM for effective molecular recognition and selectivity of the selective layer.

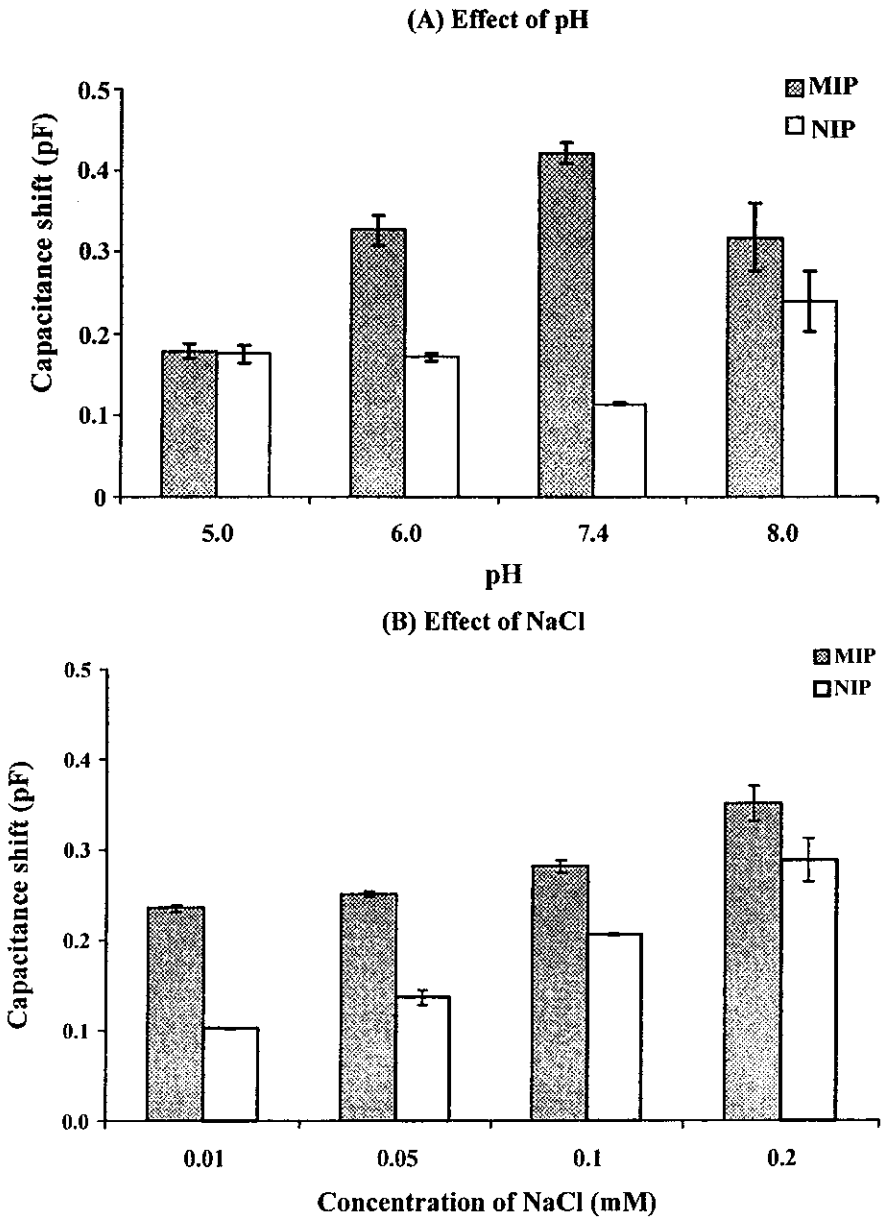

Fig. 5. (A) Effect of pH of the medium on capacitance shift response of Hev b1 (G)-imprinted polymer coated IDC, with IDC measurement on 250 ng ml^{-1} Hev b1 (G). (B) Effect of NaCl of Hev b1 (G)-imprinted polymer coated IDC on the capacitive shift response to Hev b1 (G) at a concentration level of 100 ng ml^{-1}.

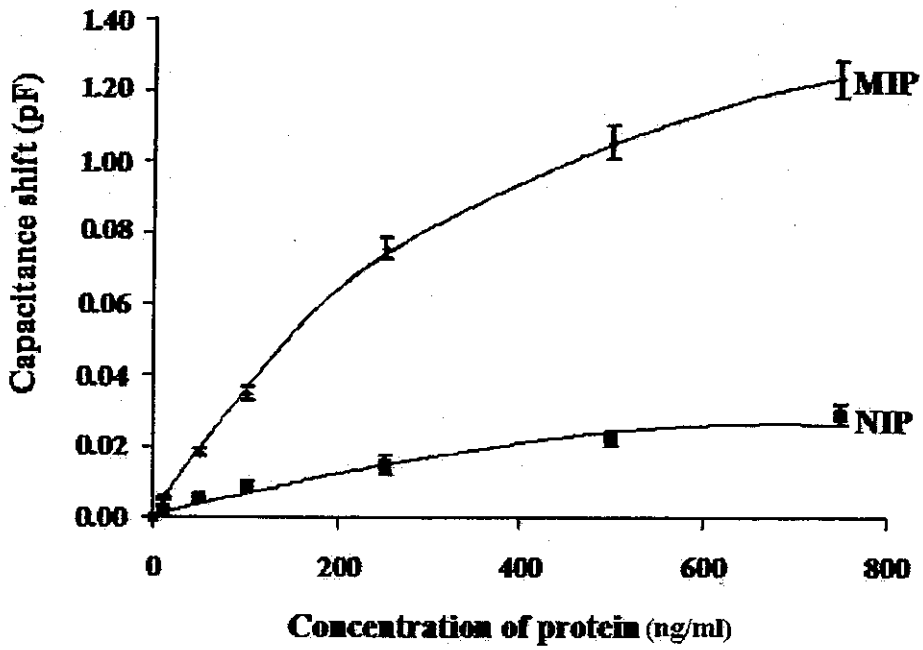

Fig. 6. Sensor characteristics of non-imprinted and Hev b1 (G)-MIP coated IDC electrodes.

3.4 Concentration dependence on the sensor response

Fig. 6A shows the capacitance behavior for a MAA-NVP-DHEBA imprint material when exposed to increasing Hev b1 concentrations ranging from 1-800 ng ml^{-1}. Furthermore, capacitance changes generated by the selective layers depend on analyte concentration. The capacitance decrease on the MIP electrode on being exposed to the small amount of allergen protein is the result of the selective layer binding the individual allergens via the imprinted cavities. At a higher concentration of Hev b1 (G) (> 250 ng ml^{-1}) the response of the MIP sensor seems to be constant. This is probably explained by the saturation of the recognition sites in the selective layer. This indicates that the adsorbed analyte produces an appropriate binding of the analyte molecule at binding sites on the MIP layer. When the amount of allergen on the surface exceeds the amount of available binding sites, some part of the allergen will form aggregates on the surface of the sensor layer. When all the cavities are occupied, additional latex allergens or proteins on the polymer thin layer can be readily removed from the surface by the continuous flow of a peristaltic pump. This causes a

substantial capacitance increase on the reference electrode. Further increasing the quantity of allergen on the sensor again leads to higher amounts of mobile proteins on the surface and hence yields a larger capacitance shift. Consequently, we can trace back to the binding mechanism of the selective materials: Hev b1 allergenic proteins present in latex gloves that maintain their biofunctionality can not only develop new biomedical applications but also improve our understanding of the mechanisms of specific binding and molecular recognition. The mechanism of molecular recognition by the MIP relates to geometrically aligned non-covalent interactions between the protein and the recognition site in the resulting MIP. The interactions created in the cavities can be H-bonds, electrostatic interactions, *pi-pi* stacking and van der Waals forces. In this case the first two types seem to be the most significant.

3.5 Selectivity of the Hev 1 (G) MIP layer on the IDC

To assess the overall selectivity of the MIP electrode, the effect of structurally related Hev b1 (L) and analogs [Hev b2 (L), Hev b3 (L)] and non-related proteins (lysozyme, ovabumin and BSA), on the capacitive sensor were examined in parallel experiments with the corresponding NIP electrodes. The cross-reactivity value of the MIP was obtained by comparing the capacitance shift with analyte to that of the template. When a MAA-NVP-DHEBA layer imprinted with Hevein latex allergenic proteins of Hev b1 (G) was exposed to the Hevein latex allergenic proteins of Hev b1 (L), Hev b2 (L), and Hev b3 (L) the capacitance change response observed for both the MIP and NIP sensor layers is shown in Fig. 7. Fig. 8 summarizes the results of these cross-selectivity measurements carried out in an aqueous medium. The selectivity value for the template was the highest and most significant for Hev b1 (L) (60%), Hev b2 (L) (50%) and Hev b3 (L) (50%). The cross-reactivity shown for the Hev b2 (L) and Hev b3 (L) have to be explained by the size of the Hevein latex allergenic proteins, as they do not fit into the smaller Hev b1 (G) imprints. Hence, the interaction area between the latex allergen and an individual cavity is restricted to the upper edge of the

recognition site, which is not sufficient for a strong, non-covalent binding leading to a capacitance change of the selective layer and the incorporated analyte. Although Hev b1 (G) and lysozyme are similar in dimension (14 kDa), yet the difference in the sensor effects of the selective layer for these proteins is clearly observed. The much lower cross-reactivity value of the lysozyme protein that has a more globular structure, indicates the difference in surface functionality and protein configuration between the template and lysozyme that renders lysozyme unable to fit well into the cavities. The selectivity value of the non-related proteins (ovalbumin, BSA) is as low as ~20%. This result demonstrates that the Hev b1 (G)-MIP-based QCM sensor yields a higher cross-reactivity for the related Hev b protein than the non-Hev b protein.

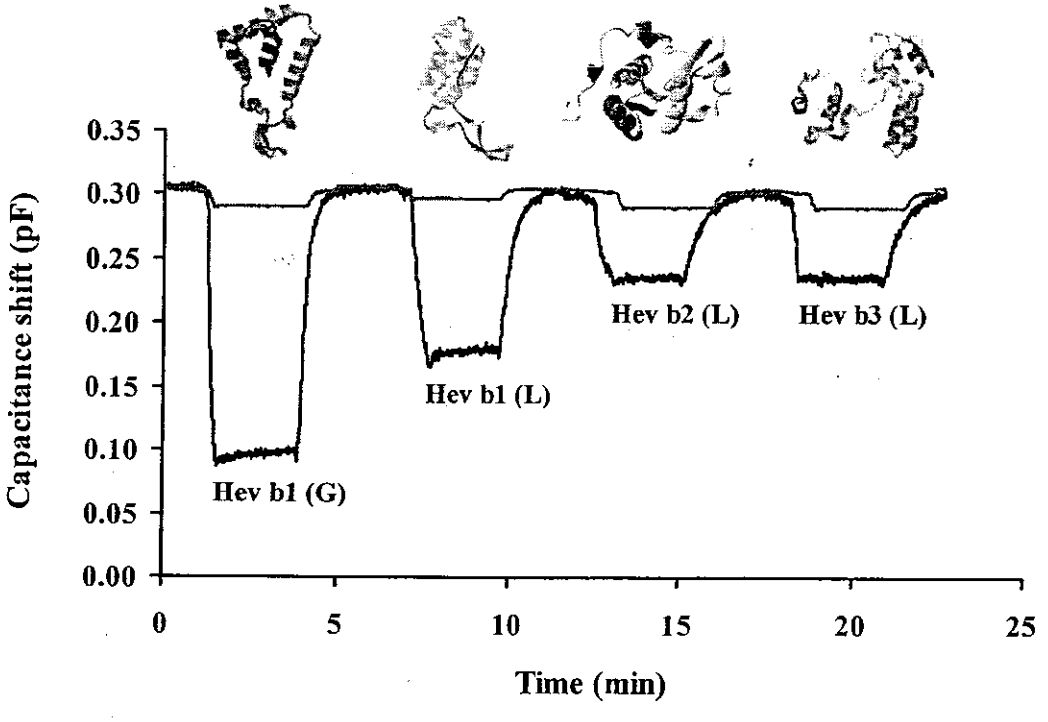

Fig. 7. Capacitive signal responses of the Hev b1 (G)-MIP and NIP-coated IDC sensor, exposed to Hevein latex allergenic proteins of Hev b1 (G), Hev b1 (L), Hev b2 (L), and Hev b3 (L)

Hev b1 (G) MIP can distinguish between the Hev-b related proteins and the non-Hev b proteins like lyzozyme, ovalbumin and bovine serum albumin; the Hev b1 (G) MIP favors

binding to Hev b. In this case, Hev b1 (G) MIP yielded the same uptakes for larger rubber latex allergen Hev b2 (35 kDa), and Hev b3 (22 kDa). Moreover, the Hev b1-selective polymers can distinguish between extractable allergens from latex mattresses made from rubber tree latex and rubber latex gloves, as these have exactly the same geometrical dimension, they just differ by their exact conformation around the receptor site. The antibodies bind to specific regions consisting only small epitopes on latex allergens surface which lead to similar effects for Hev b1 protein allergens. The MIP material interacts with surface accessible residues of the organized protein assembly of an analyte thus also being sensitive to quantitative difference in surface chemistry. The analyte can be recognized on a structural level by the selective layer, that there is a favorable morphology and surface coverage of the bound Hev b1 (G) proteins. Concerning the template, it is obviously strongly bound by the molecularly imprinted recognition material. This indicates that the interfacial behaviour of the Hev b1 (G) protein macromolecules on the molecularly imprinted polymer surfaces, is achieved by the balance of enthalpic and entropic effects from protein/surface-protein segment interactions. Once a protein molecule has been formed, its configuration is fixed, and hence it can take on an infinite number of shapes by rotation about the backbone bonds. The final shape that the latex allergen takes depends upon the intra- and intermolecular forces, which in turn depend upon the state of system: for example proteins in dilute solution, melt phase, or solid phase would each experience different forces. During hardening of the forming polymer in the imprinting process, capillary forces can induce rearrangement of natural protein conformations. Noncovalent forces act on the primary structure to cause a protein to fold into a unique conformational structure and then stabilizes the native structure against a denaturation process. Imprinting polymer yields the defined oriented protein assembly on surfaces of latex allergens, allowing study in surface-protein interaction at a molecular scale in-situ on surface of the cavities. Control of the condition during self-assembly of proteins and the formed polymer material for templating is necessary

for reliable and reproducible preparations of imprinted polymers. Moreover, physicochemical properties such as substrate hydrophilicity, protein concentration, pH, and ionic strength of the protein solution during rebinding play crucial roles in oriented protein assembly on wall surface of cavity of the imprints.

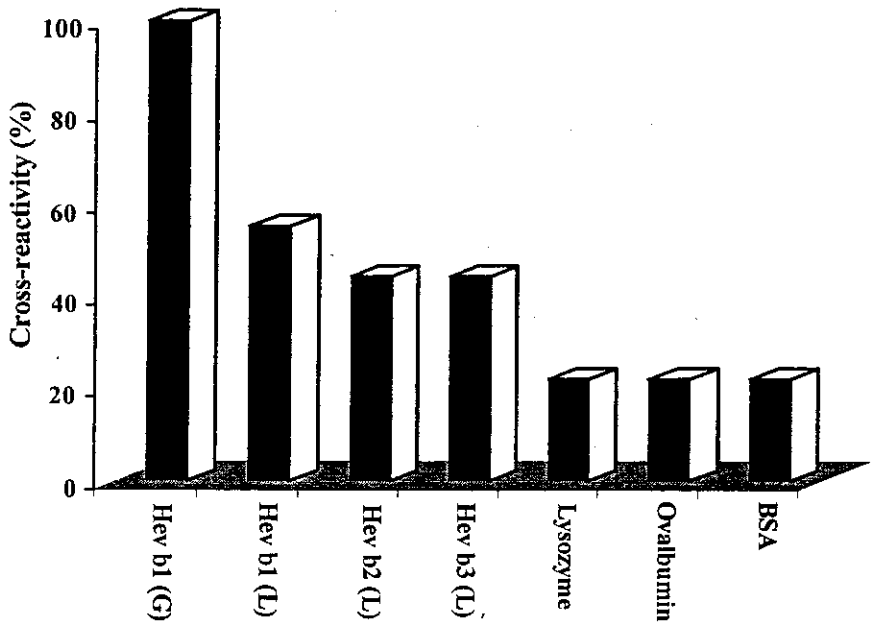

Fig. 8. Selectivity profiles of Hev b1 (G)-imprinted polymers as obtained by IDC measurements.

3.6. Analytical characteristics and real sample analysis

In this study, the analytical characteristic of the IDC sensor modified with Hev b1- imprinted poly(MAA-NVP-DHEBA) was examined and applied to measure the target analyte in the rubber latex glove. The sensitivity (slope) of the sensor was found to be 0.03 and the linearity range was 10-250 ng ml^{-1} with an $R^2 > 0.995$. The limit of detection (LOD) was 10 ng ml^{-1} corresponding to a 3:1 signal to noise ratio. A recovery study was carried out by spiking Hev b1 (G) at 50, 100 and 200 ng ml^{-1} with extracts prepared from latex gloves. The recovery data were in the range 95-100 % with a relative standard deviation (RSD) of 4%. The samples were spiked with standard Hev b1 (G) solution at 50, 100, 250 and 1000 ng ml^{-1} and injected into the IDC sensor at optimum conditions for the IDC measurement that were

performed with a network analyzer. The Hev b1 (G)-MIP IDC sensor was used for Hev b1 (G) assessment in glove samples. The amount of Hev b1 (G) in a specimen of gloves supplied by a local manufacture was found to be 25 ± 6 μg/1g of rubber material.

4. Conclusions

Molecular imprinting techniques were adapted to design a sensor for detecting the Hevea latex allergen protein (Hev b1) in gloves. Selective recognition sites towards the rubber latex allergen proteins Hev b1 remaining in the examination gloves can be generated on poly(methacrylic acid-vinylpyrrolidone-dihydroxyethylene bisacrylamide) layer on the IDC in aqueous medium. The imprinted layer made with the optimum cross-linking monomer level proved to provide a high recognition efficiency and good selectivity towards the template. In addition the imprinting leads to preparation of artificial matrices that are suitable for application as a novel way for a fast and cost-effective detection of latex allergens that have been implicated in life-threatening immunity reactions. The MIP-IDC sensor showed a rapid capacitive shift and reasonable capacitive response that was clearly dependent on the concentration of the rubber elongation factor. The recognition ability of the artificial material that recognizes the presence of defined protein organized structures on a surface by interacting with it. A cross-selectivity assessment revealed that the Hev b1-selective MIP, favored binding to the templating allergen by a factor of 3, compared to a competing one that had a selectivity factor of below 2. The MIP material deposited on the IDC sensor could distinguish Hev b1 from analogues such as lysozyme, ovalbumin and bovine serum albumin. This would be beneficial in the analytical application of quantifying Hevein latex allergen in real-life samples. The results of the present study suggest that the understanding of assembly in 3D on the surface of polymer to forming functional protein assembly of the major latex allergens associated with latex allergy may provide better insight into the structure-function

relationship of the allergens, and may lead to the development of better patient care and management strategies in latex allergy. The newly developed Hev b1-MIP-IDC sensor method has proven to be fast and reliable for detecting Hev b1 in complex matrix obtained by extracting rubber gloves. Moreover, the sensor exhibited the same recognition characteristic after three months of storage in dry conditions.

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References

1. Banerjee, B., Kanitpong, K., Fink, J.N., Zussman, M., Sussman, G.L., Kelly, K.J. and Kurup, V.P. (2000) Mol. Immuno. 37:789-798.
2. Yip, E. and Cacioli, P. (2002) Allergy Clin. Immunol. 110:S3-14.
3. Hayden, O., Podlipna, X., Chen, X. Krassnig, S., Leidl, A. and Dickert, F.L. (2006) Mat. Sci. Engineer. C. 26:924-928.
4. Sussman, G. L., Beezhold, D.H., Kurup, V. P. (2002) J. Allergy Clin. Immunol. 110: S33-39.
5. Salcedo, G., Diaz-Perales, A., Sanchez-Monge, R. (2001) Curr. Opin. Allergy Clin. Immunol. 1:177-83

6. Nieto, A., Mazon. A, Pamies, R,, Lanuza, A,, Munoz, A., Estornell, F., Garcia-Ibarra F. (2002) J. Pediatr. 140:370-372.
7. Saary, M.J., Kanani, A., Alghadeer, H Holness DL, Tarlo SM. (2002) J. Allergy Clin. Immunol. 109:131-135.
8. Tomazic-Jezic, V.J., Lucus, A.D. (2002) J. Allergy Clin. Immunol. 110:S40-S46
9. Beezhold, D.H., Kostyal, D.A., Tomazic-Jezic VJ (2002) Methods 27:46-51
10. Rachkov, A. and Minoura, N. (2001) Biochim. Biophy. Acta. 1544:255-266.
11. Gong, J.L, Gong, F.C., Kuang, Y., Zeng G.M., Shen G.L. and Yu, R.Q. (2004) Anal. Bioanal. Chem. 379:302-307
12. Cheng, Z., Wang, E., and Yang, X. (2001) Biosens. Biolectron.16:179-185.
13. Zhou, L., Ye, G.R., Yuan, R., Chai, Y.Q., and Chen, S.M. (2007) Sci. China Ser B. Chem. 50:547-553
14. Berggren, C., Bjarnason, B., and Johansson, G. (2000) Electroanalysis. 13:173-180.
15. Chen, Z., Cremer, R,, Posch, A,, Raulf-Heimsoth, M,, Rihs, H.P., Baur, X. (1997) J Allergy Clin. Immunol. 100:684-693
16. Marchetti-Deschmann, M., Allmaier. G. (2009) J. Mass Spectrom. 44:61-70
17. Dickert, F.L., Hayden, O., Bindeus, R,, Mann, K-J., Blaas, D., Waigmann, E. (2004) Anal. Bioanal. Chem. 378:1929-1934

SUPPLEMENT MATERIAL

In preliminary screening of polymer, three different types of functional monomers, acrylamide (AAM), methacrylic acid (MAA) and styrene (Sy) were chosen as ligand to dock with the template protein (Hev b1). Geno3D (release 2, France) was used to predict the tertiary structures of target protein. This program is comparative protein structure modelling by spatial restraints (distances and dihedral) satisfaction. The tertiary structures of protein obtained from Geno3D, which amino acid sequences of the proteins were used as a data base to compare with the homology of protein in data blank. A Discovery Studio 1.6 Software (Life Science Modeling and Simulations) was employed to predict the active sites of target protein. AutoDock Tool program was used to estimate the energy of complex, using docking of the most stable conformers by using intermolecular Monte Carlo simulated annealing based conformation search with possibilities of rotating and translating the functional monomer toward the template. In the simulation, the complexes were allowed to rotate randomly at their mass center, and translate into three-dimensional space. For selected functional monomer, the most stable template-monomer complexes were searched and their interaction energy, Δ E, were calculated through the equation as follows:

$$\Delta E = E(\text{template-monomer}) - E(\text{template}) - E(\text{monomer})$$

Table (a) depicts the binding energy of complex. The value of Δ E obtained from the template-styrene complex was lower than that formed with ACM and MAA, respectively.

Table (a): Binding energies ΔE of Hev b1 with ACM, MAA and Sy monomer

Complex	Δ E (KJ mol^{-1})
Hev b1-ACM	-2.64
Hev b1-MAA	-3.46
Hev b1-Sy	-4.07