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Interdigitated capacitive biosensor based on molecularly imprinted polymer for rapid detection of Hev b1 latex allergen

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ABSTRACT

Allergen protein detection was performed by a surface imprinted layer combined with an interdigitated capacitance (IDC) transducer that allowed label-free measurements. The immobilized imprinted polymers are the probes that bind to rubber allergen proteins extracted from products such as rubber gloves. Copolymers made from methacrylic acid–vinylpyrrolidone–dihydroxyethylene-bisacrylamide (MAA–NVP–DHEBA) are soluble in aqueous solution and eliminate the denaturation of protein. When deposited as a coating onto an IDC microelectrode transduction system, such materials lead to sensors that produce capacitance responses that are clearly dependent on the concentration of the latex protein (10–900 ng ml^{-1}) in pH 7.4 buffer. The biosensor can detect Hev b1 within minutes and with a detection limit of 10 ng ml^{-1}. Different but related hevein allergenic proteins isolated from natural rubber latex from the rubber tree (Hev b1, Hev b2, and Hev b3) were distinguished by the imprinted material, depending on the dimension and conformation of these proteins with a selectivity factor of 4. They recognized *Hevea* latex proteins better than non-Hev b proteins, such as lysozyme, ovalbumin, and bovine serum albumin, by a factor of 2. Moreover, the sensor exhibited good operational stability of up to 180 days when used continuously at room temperature.

The detection of latex allergens is an important and challenging issue that needs to be addressed by the biosciences. The use of natural rubber latex (NRL)[1] has increased greatly since the onset of the HIV/AIDs epidemic that became a major public health problem. It became quickly evident that the use of rubber gloves and condoms would help to control this epidemic and also reduce infections from other infectious diseases such as viral hepatitis and influenza [1]. Latex allergens from the rubber tree (*Hevea brasiliensis*) cause severe and chronic allergies to health care workers and other sensitized persons, and allergies to components in latex rubber is a growing problem. *Hev b1* (rubber elongation factor) is a 14.6-kDa water-insoluble protein that is present on rubber particle surfaces. Hev b1 is one of the most important protein-associated latex allergens, with extremely high involvement in sensitizing patients to latex allergies and child patients with spina bifida [2,3]. However, it is derived from the fragmentation of *Hev* b3 (22–27 kDa), small amounts are found in latex gloves, and it is relevant to spina bifida patients [4]. Another latex allergen, Hev b2, is a 35- to 36-kDa 1,3-β-glucanase protein that contains a number of epitopes that bind to immunoglobulin E (IgE) and has immunological cross-reactivities with similar proteins in other plant-derived foods [5]. Other distinct *Hevea* allergens found in rubber tree latex that are clinically relevant to NRL allergies have also been detected in NRL products with no changes to their allergenicity, including Hev b5 (a 16-kDa acidic NRL protein), Hev b6.02 (mature hevein), and Hev b13 (a 43-kDa *Hevea* latex lipolytic esterase) [6,7]. A conventional method for detecting rubber latex allergens in rubber gloves involves an assay for their total protein content (commonly using the Lowry or biuret method) followed by confirmation with biochemical or immunological assays [8–10]. These methods are sufficiently sensitive to detect NRL proteins in the complex matrix of manufactured latex rubber gloves, but they are time-consuming and not cost-effective. Because of the increasing demand for rubber-based gloves that has led to a decrease in processing time by the manufacturers, this in turn has led to an increase in the retention of latex proteins. Different manufacturers of gloves use different methods, standards, and processing times. There have been differences of up to 3000 times in the latex

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[1] *Abbreviations used:* NRL, natural rubber latex; IgE, immunoglobulin E; IDC, interdigitated capacitance; MIP, molecularly imprinted polymer; DHEBA, *N,N′*-dihydroxyethylene-bisacrylamide; MAA, methacrylic acid; NVP, *N*-vinyl-2-pyrrolidone; SDS, sodium dodecyl sulfate; AIBN, 2,2′-azobisisobutyronitrile; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; MALDI TOF/TOF, matrix-assisted laser desorption/ionization tandem time-of-flight; UV, ultraviolet; NIP, nonimprinted polymer; AFM, atomic force microscopy; SEM, scanning electron microscopy; ELISA, enzyme-linked immunosorbent assay; RSD, relative standard deviation.

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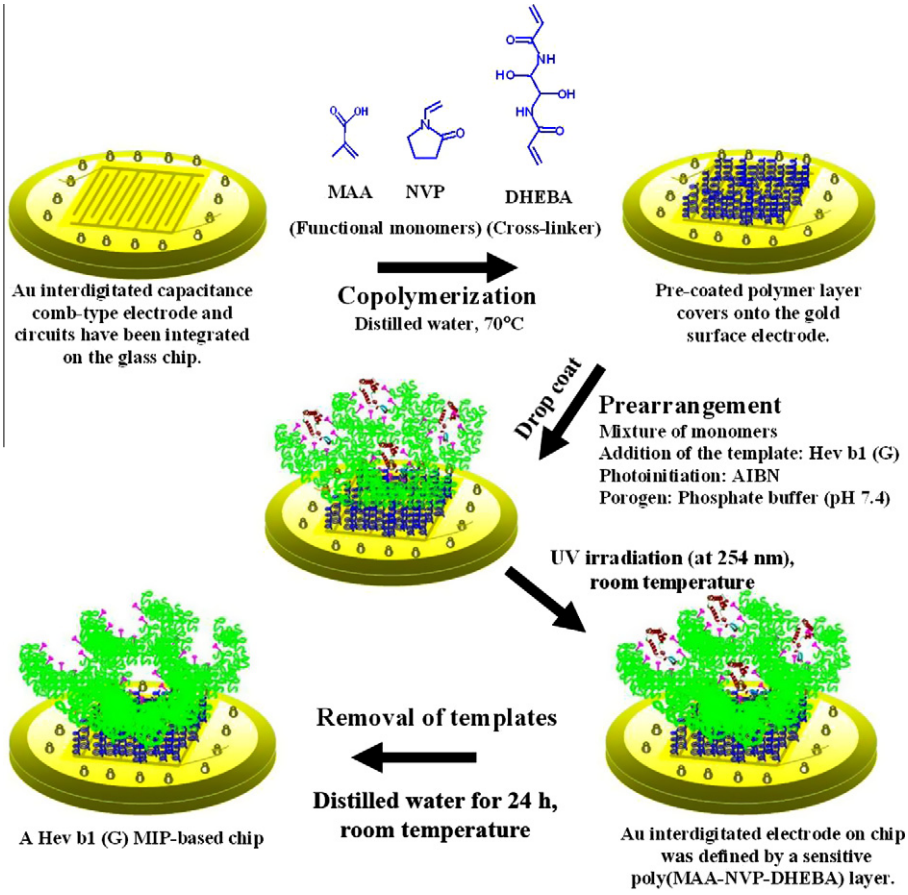
contents of 10 different brands of gloves [11]. Most latex-allergic patients can be sensitized to more than one latex allergen. Thus, substantial efforts must be made to monitor different kinds of rubber allergenic proteins, and this requires a concentration of aqueous extracts to produce material for a reliable and rapid screening system.

During recent years, biosensors have come to play an increasingly important role in the detection of chemicals and biomolecules for clinical analysis and environmental monitoring. The interdigitated capacitance (IDC) biosensor is a label-free, inexpensive, and promising detection method that is performed in the presence of an electrical field [12]. It is regarded as an effective method for sensing the formation of antigen–antibody complexes on electrode surfaces by probing the features of the interfacial capacitance of the electrode and allowing the detection and quantification of target molecules in complex mixtures. There is increasing attention being given to the use of molecularly imprinted polymers, especially in the field of biosensors. This is because molecularly imprinting polymer materials can be said to represent artificial antibodies that offer fully artificial, robust, and highly selective materials. It is even more attractive to integrate IDC electrodes with molecularly imprinted polymer (MIP) layers that can be used to produce sensitive chemical sensor systems for detecting drugs such as fenvalerate [13] and pazufloxacin [14] and small molecules such as glucose [15]. Other studies have also shown that IDC microelectrodes display great promise in the field of immunosensing and biosensing [16], due to their capacity to rapidly monitor the changes of the capacitance properties of their surfaces. Copolymerization of appropriate functional monomers with cross-linking agents and comonomers may be used to generate recognition materials in the form of self-supporting thin films [17–20] that can considerably broaden the applications of conventional polymeric materials for analysis and biosensor purposes.

This article reports the development of an affinity-based interaction/sensing approach using a chemical support (MIP) for application to the detection of Hev b1 allergen in rubber glove samples. A surface imprinting approach that enhances recognition by the imprinted polymer surfaces is used to ensure generation of sufficient receptor binding sites from the bound protein templates during the polymerization process. We demonstrate that the adsorptive cross-link made of a copolymer between methacrylic acid and vinylpyrrolidone with *N*,*N*′-dihydroxyethylene-bisacrylamide (DHEBA) as the cross-linker allows some control of the binding characteristics of the resultant polymeric recognition material. As a result of surface diffusion from the imprinted layer, the specific spatial requirements for achieving significant levels of binding capacity and affinity of the corresponding polymer, compared with those obtained with a conventional polymer surface reference material, are obtained. This is followed by a substantial increase of the dielectric constant and by corresponding changes of the surface of the capacitance components on the IDC transducer system when analyte binds.

In this study, an IDC microelectrode (for detection) and MIP (for specific capture) were integrated to form chemical probes that resulted in changes to the electrical capacitance due to modification of the dielectric constant of the polymer after binding of the rubber protein molecules. The MIP-based capture system was prepared by a solution-based imprinting polymerization with Hev b1 proteins extracted from rubber gloves as the template (Scheme 1). The



Scheme 1. Schematic illustration of the surface imprinting process of a precoated IDC microelectrode on the chip. The monomeric solutions of the MAA and NVP functional monomers and DHEBA as a cross-linker and the Hev b1 (G) template in phosphate buffer (pH 7.4) are dripped onto the coated transducer surface to produce self-organizing receptor sites on the thin film surface.

technique was based on a simple casting of the template-containing polymer solutions on the cross-linking prepolymer layer coating on the surface of an IDC electrode attached to the glass chip. This avoided the unfavorable action of photochemical stress on the templates and prepolymerization complexes. The presence of Hev b1–MIP–IDC biosensors fabricated as a lab-on-chip device is directly incorporated into a miniaturized sampling system that allows online measurement of the allergen proteins. So, with this approach, we can detect the rubber allergens without labeling them (label free), and the approach provides an inexpensive production process and low consumption of sample. The new flow-through conductometric sensor was applied to measurement of the Hev b1 rubber allergen in simple extracts from the complex matrices of manufactured rubber latex gloves. We show that substantial differences occur in the capacitance signals of the Hev b1 imprinted electrode and nonimprinted reference electrode after exposure of the protein analyte to an interdigital capacitor in an aqueous solvent using the IDC-based assay protocol. Parameters that influence the protein-specific selectivity of the imprinted polymer on the IDC sensor are also discussed.

Materials and methods

Chemicals and biologicals

Methacrylic acid (MAA), *N*-vinyl-2-pyrrolidone (NVP), DHEBA, sodium dodecyl sulfate (SDS), Triton X-100, and Tris–HCl were obtained from Aldrich Chemical (Milwaukee, WI, USA). 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Janssen Chimica (Geel, Belgium). Ammonium persulfate was obtained from Amresco (Solon, OH, USA). Lysozyme, ovalbumin, and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rubber latex was obtained from a commercial rubber latex manufacturer in Songkhla, Thailand, and kept in a refrigerator at 2–4 °C. Cellulose dialysis bags with a molecular weight cutoff of 3700 Da were purchased from Sigma. All solvents used were of analytical reagent grade and used without further processing.

Protein extraction and purification

Allergenic proteins isolated from rubber tree sap are identified with an "L" in parentheses, and proteins extracted from rubber latex gloves are identified with a "G" in parentheses. The protein Hev b1 (G) was obtained from latex gloves, and the proteins Hev b1 (L), Hev b2 (L), and Hev b3 (L) were obtained from rubber latex, by protocols similar to those described previously [21] using 1% SDS in 20 mM phosphate buffer solution (pH 7.4) as a detergent. Briefly, fresh rubber latex was centrifuged in an 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 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 at 60,000*g* at 4 °C for 45 min to obtain the supernatant, which contained Hev b1 (14.6 kDa) and Hev b3 (23 kDa). After the first step of centrifugation at 60,000*g*, 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 at 60,000*g* to obtain the supernatant containing Hev b2 (35 kDa). The extracted proteins were dialyzed against distilled water for 48 h at 4 °C and then lyophilized.

Purified Hev b1 (G) was obtained from manufactured rubber latex gloves as described previously [21]. Briefly, powdered rubber latex 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 for 15 min at 10,000*g*, 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 generation of proteins that were used in templating polymerization for rubber latex allergen.

Crude proteins were purified by size exclusion chromatography with Sephacryl S-100 (1.5 × 64 cm, Pharmacia, Uppsala, Sweden) 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 (polyacrylamide gel electrophoresis) using 15% polyacrylamide gels under nonreducing conditions and stained with Coomassie blue. The major band representing the target protein was excised, destained, and extracted from the gel pieces. Extracts were dried in a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectrometric analysis of the NRL proteins was performed using an Ultraflex III matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF) mass spectrometer from Bruker Daltonics (Bremen, Germany) [22]. Database searching (NCBInr) and protein identification were performed with the Mascot computer program (Matrix Sciences, London, UK) according to a recently described procedure [23]. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [24]. Western blot analysis was performed with the serum from latex-sensitive patients to identify whether the Hev b1 protein extracted reacting IgE antibodies recognizing Hev b1 with positive results obtained. Alignment of the amino acid sequences of proteins was carried out with the Geno3D program and visual inspection of the model by Discovery Studio 1.6 (Accelrys, San Diego, CA, USA).

Interdigitated array microelectrodes and fabrication of MIP biosensors

We used a gold interdigitated electrode with dimensions of 4 mm × 4 mm × 1 μm and with a 10-μm gap between the digits, where a planar inductor interdigital capacitor pair is photolithographic. The capacity of the interdigital capacitor in the air was approximately 5–10 pF. The IDC microelectrodes had a conductivity of 62.3 ± 2.1 Ω/cm in 0.01 mM NaCl solution and increased to 108.5 ± 4.0 Ω/cm in 0.2 mM NaCl solution. The sensor coating layers were prepared from an aqueous solution with distilled water as porogen solvent with a functionality ratio of MAA and NVP of 6:2 and a 17 mol% content of DHEBA as cross-linking agent. After adding the radical polymerization initiator (AIBN), polymerization was continued under stirring conditions at 70 °C until the gel point was reached. The polymerizing solutions were diluted with distilled water and used for the coating process and patterning for rubber protein allergen. To prepare the template layers, the polymer coating was as already described except that the pH had been adjusted to 7.4 with 100 mM KOH (to prevent denaturation of the protein template) and ammonium persulfate as catalyst was mixed with Hev b1 (G) in 0.1 mM phosphate buffer (0.2 μl, 12.5 mg ml^{-1}). The freshly prepared template-containing polymer solution was coated onto the surface of the prepolymer-coated electrode. The curing process of MAA–NVP–DHEBA–polymer layers was by overnight ultraviolet (UV) exposure at 254 nm under ambient conditions. After the immobilization step, the chip was immersed in 5 × 100 ml of distilled water for 24 h at room temperature. This removed the templates, leaving the nanometer pits of the imprinted process on the IDC microelectrodes. The coated layers were responsible for transferring the capacitive signal of the analytes when they were rebound in the MIP cavities. The reference electrode with a nonimprinted polymer (NIP) was used to evaluate

the efficiency of the MIP electrode, which was fabricated in the absence of the template during the polymerization process.

IDC electrode and measurements

In this work, we coated IDC microelectrodes with both the imprinted and nonimprinted reference polymers. Allergen detection was performed using a 4284A LCR precision bridge (Agilent Technologies, Santa Clara, CA, USA) with software developed in-house. In the sampling and capacitive analytical system, a liquid port for delivery of the sample from a sample reservoir, a peristaltic pump, and the flow-through microcell/integrated MIP sensor with a sample volume of 100 μl were connected to the test and counter electrodes on the network analyzer. The tested frequency range was from 0.1 kHz to 1 MHz, with an alternating potential voltage of 100 mV. After washing the electrode several times with a background solution, the difference in capacitance before and after the protein binding was taken as the signal produced by reaction between the MIP biosensors and proteins. Finally, capacitance (C) values were extracted from the measurements at certain frequency (f). The signal response toward the protein of the sensor was reported as ΔC (fF [femtofarad]), where ΔC is the capacitance shift in response to the addition of known amounts of the protein of interest. After washing several times with distilled water, sensors were stored at room temperature until use.

Sample and assays

To validate biosensor performance, we sampled one disposable powder glove and one powder-free glove from a glove manufacturer in Songkla, Thailand. Rubber-based gloves were cut into small pieces and extracted in 0.2 M phosphate buffer (pH 7.4) containing 0.5% SDS. After that, the mixtures were centrifuged at 10,000*g* for 15 min at 4 °C. The liquid was dialyzed against distilled water for 48 h at room temperature. A typical protein sample had a conductivity ranging from 150 to 153 Ω/cm. The crude protein extract from the rubber gloves was analyzed using the developed MIP–IDC biosensor at the optimized sensor conditions. After several washing steps, a further IDC measurement was carried out by following the protocol described above. Every experiment was carried out in triplicate on any particular day of experimentation.

Results and discussion

MIP immobilization

Atomic force microscopy (AFM) was used to confirm the immobilization of the chemical detectors (artificial antibodies) onto the coated biosensor surface. The MIPs were made by photochemical polymerization of the monomeric mixtures after being doped with a free radical initiator in distilled water as a porogen solvent. An Hev b1 (G) imprinted poly(MAA–NVP–DHEBA) was fabricated on the glass chip by following the method mentioned in Materials and methods and as presented in Scheme 1. A section analysis with an AFM image showed that the depth of the imprinted sites was approximately 70 nm. Due to the surface imprinting process using solution-based polymerization with templates, we were able to generate imprinted surfaces at several nanometer thicknesses of film by making changes to the dilution of the prepolymer. The Hev b1 (G) imprinted surface had nanosized pits on the gold IDC microelectrode. An MIP layer, 200 nm thick, was measured by the AFM spectroscopy method using a Nanoscope III (Digital Instruments, Santa Barbara, CA, USA) after the film was scratched with a needle (see Fig. 1A and B). At 200 nm thick, the layer was

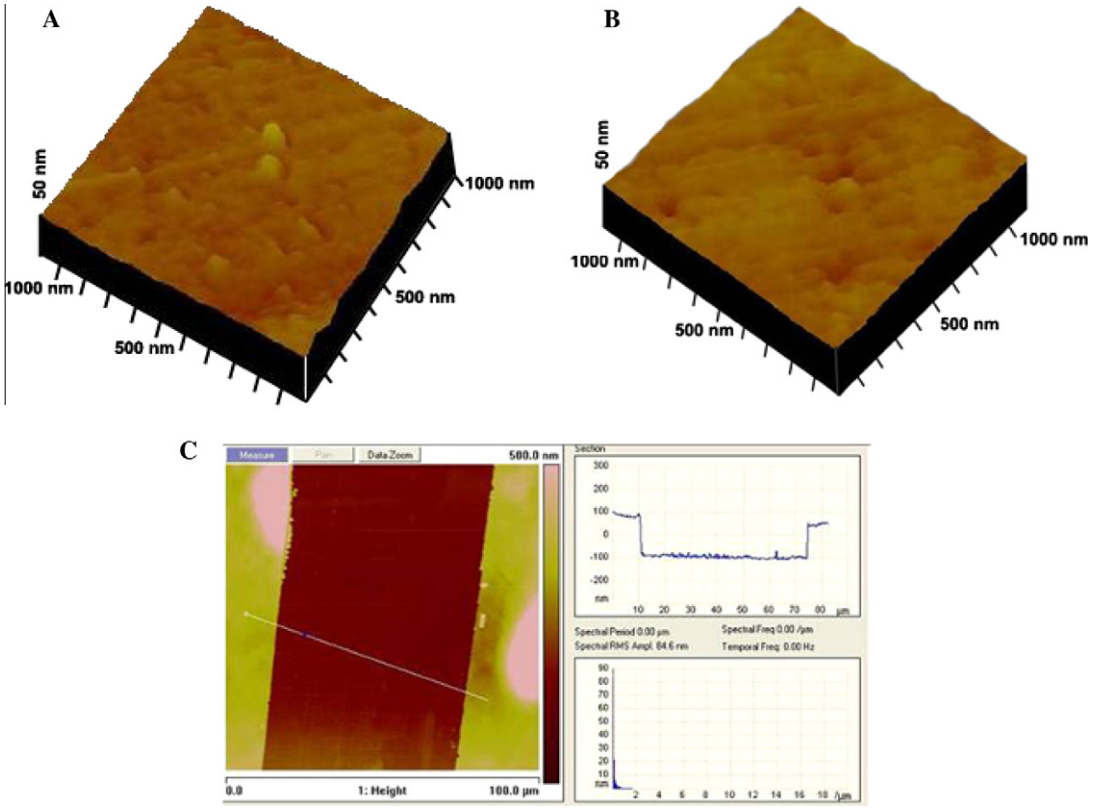


Fig.1. Contact mode AFM images: (A) MIP with partially removed template showing the rubber allergens retained in the polymerized material; (B) imprinted layer showing Hev b1 (G) pits on an MAA–NVP–DHEBA polymer after hardening of the layer and washing with distilled water for 24 h; (C) cross-section analyses of the scratch on the imprinted layer and the measured 200-nm layer.

also suitable for imprinting the polymer and provided the optimal imprinting effect to achieve the optimal capacitance signal (when the measured capacitance was carried out in the open air after the electrode was soaked with sample at ambient temperature). The size distribution of the rubber allergen imprints is reflected through the surface imprinting process, and when the templates are removed by solvent, the hardened material is left with polymeric-coated cavities that have a memory for the contours and charge distributions of the chemical groups in the original biological molecule. Fig. 1A illustrates the regular pattern of the imprinted sites and some remaining rubber allergens included in the imprinted pits on the surface of the polymer layer coated onto an IDC transducer. The surface imprinting procedures allow generation of micropatterns of the rubber allergen protein on the polymer surface, resulting in randomly distributed nano-pitch pits on the coated surfaces (Fig. 1B).

Sensor coating layer

We determined the effect of the washing procedure on the efficiency of extracting the template from the resulting MIP coating layer on the IDC surface. Allergenic rubber proteins are well known for their ability to adhere to the inner and outer surfaces of manufactured rubber gloves. Therefore, we needed to ensure that remaining allergens in the used electrodes could be effectively removed. For this reason, we always first performed the removal of remaining templates from the films by soaking the coated electrodes with distilled water at room temperature (25 ± 1 °C). We could see that the resistance of the surface, when measured in the air, gradually decreased the more times the imprinted electrodes were washed in distilled water up to 24 h. A 24% increase in the capacitance response in the air occurred for the MIP film after consecutive immersions of the MIP-coated electrodes in distilled water. This indicates that the templates diffuse through the imprinted cavities within the MIP matrix. In contrast, there was only a 7.1% increase in capacitance for the reference polymer-coated surface. This capacitance behavior indicates that water penetrates into the bulk of the polymer layer in this type of polymer material. The washing of the MIP electrode with water reduces the contribution of any nonspecific binding and also effectively extracts the specifically bound template Hev b1 (G). Under the washing protocol used, the existence of Hev b1 (G) imprints on the surface of polymer films was shown to be approximately 90%, as determined from the amount of template that was removed from the sensor coating layers.

Capacitance characteristics of immobilization process

Fig. 2A illustrates a scanning electron microscopy (SEM) image obtained with the micro-interdigitated capacitor, showing the interdigitated comb capacitor structure on top of a glass chip, and an equivalent circuit model of the biosensor is depicted in Fig. 2B. The equivalent circuit consists of ohmic resistance of the electrolyte (R_s) between two electrodes and double layer capacitance (C_{dl}), and electron transfer resistance (R_{ct}) and the Warburg impedance (Z_o) around each electrode was proposed for interpretation of the capacitance components of the gold planar microelectrode arrays. The total current through the electrode area is the sum of the Faradaic current and double layer current in the branch circuit and the impedance and electron transfer resistance, with both of them being connected in parallel with double layer capacitance. If there is a change in the dielectric constant, then a counter change occurs in the capacitance value between the two sets of array electrode. Unlike other detection mechanisms used to quantify protein concentration, the capacitance method does not need labeling of protein sample. The protein bound to the surface of

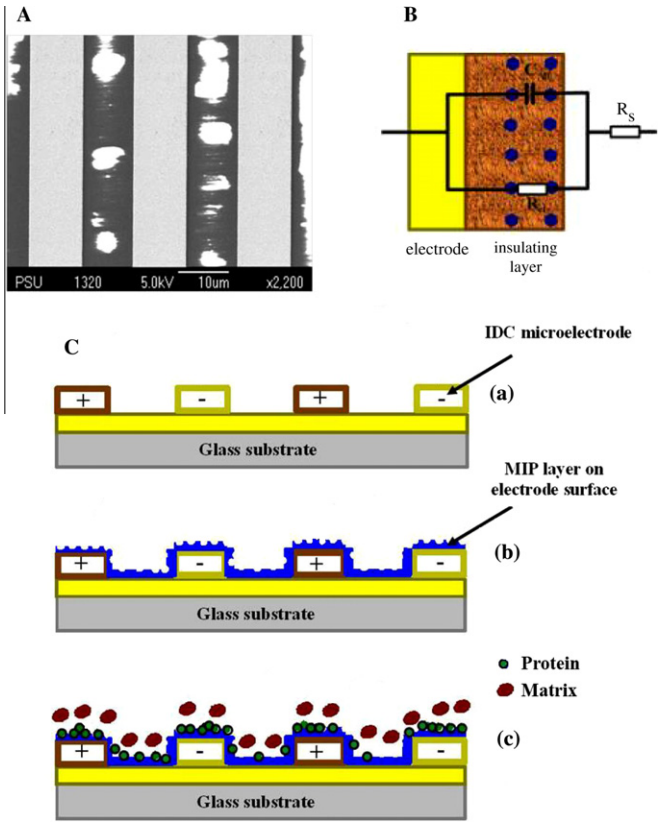


Fig.2. (A) SEM image of interdigital capacitor. (B) Equivalent circuits used to describe impedance spectra of coated electrodes. R_s, electrolyte resistance; R_{ct}, charge transfer resistance; C_{dl}, double layer capacitance. (C) Hev b1 (G)–MIP capacitive biosensor fabrication and process: (a) bare electrode; (b) with MIP layer; (c) with Hev b1 (G) binding. Circles: Hev b1 (G); ovals: matrix.

the immobilized capture molecularly imprinted polymers yields dielectric properties between the IDC electrode changes, and measurement can be taken immediately after the protein binding (Fig. 2C). Taking into account that the capacitance of a planar capacitor is determined by its geometry (area and thickness) and dielectric constant, any modification of its capacitance with no change of geometry indicates a definite modification of the dielectric constant [25]. Fig. 3 shows the Bode plots and phase degree of bare electrodes and electrodes coated by Hev b1 (G) MIP thin film. As expected, the impedance spectra of the bare microelectrodes

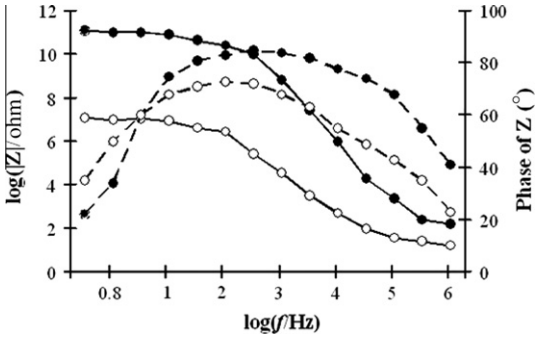


Fig.3. Bode plots referring to representation of the impedance magnitude (or the real or imaginary components of the impedance) and phase angle as a function of frequency for IDC electrode (open symbols) and IDC electrodes coated with Hev b1 (G)–MIP (filled symbols) exposed to 0.1 mM phosphate buffer solution (pH 7.4) containing Hev b1 (G) at a concentration of 250 ng ml^{-1} at room temperature. Impedance: solid lines; phase degree: dotted lines.

can be described by the interfacial interactions, the Randles equivalent circuit with an essential contribution of Warburg's impedance. Modification of the molecularly imprinted polymer of the IDC electrode led to major alterations to the impedance spectra. At a frequency of more than 100 Hz, the coated electrodes had mainly capacitive properties (the phase angle became close to 90°). The log $|Z|$ versus log f function was a straight line with a slope of −1 and remained nearly unchanged at low frequencies (where the resistive components dominated) and was decreased at high frequencies. The strong capacitance properties of the MIP-coated electrode at 1 kHz allow us to relate the changes in the capacitive current at this frequency to changes in the electrode capacitance; hence, this frequency is used for further experiments. We observed an inductive behavior after 10 MHz; therefore, capacitance values for frequencies higher than 10 MHz were not considered. According to the equivalent circuit model obtained by impedance measurement, we can calculate the value for the double layer capacitance and reaction resistance on the interfacial electrode of IDC biosensor at 1560 μF/cm^2 and 3975 MΩ/cm^2, respectively.

Optimal content of cross-linker

The presence of imprint structures in the polymerized MIP coating obtained by the solution-based imprinting polymerization was demonstrated by AFM. Regular patterns of bioimprinting sites in a thin film of sensor coating are preferred over a large number of densely imprinted sites. This can allow pronounced revisits of protein templates onto the surface of the polymer layer. Prepolymer formulations generally contain the functional monomer and protein template at high concentration levels such as comonomers, cross-linking agents, and porogenic solvents. Under polymerization conditions, functional monomers supposedly change to form stable dimers or oligomers. Sometimes, disposition of pendent groups of a functional monomer can be hidden within polymer domains and so limit some conformational changes and impair the pendent's ability to establish the subtle network of noncovalent contacts essential for efficient and selective protein binding.

In this work, we determined the optimal amount of DHEBA for cross-linking the MAA–NVP–DHEBA–polymer layer to allow the formation of a complementary shape with a high affinity for the template in the imprint. This also produced the most effective sensor response from the Hev b1 (G) MIP when immobilized onto the IDC electrode. As can be seen in Fig. 4, this degree of cross-linking, 10 mol%, produced a lower imprinting effect than at the other cross-linker contents, indicating a low stability of the binding sites on the MIP thin layer. In contrast, a high cross-linked density of the polymer beyond 15% caused a reduced diffusion of the protein template onto the binding sites of the MIP, resulting in just surface imprinting on the polymer, and tended to interact more by binding nonspecifically bound protein to the residues of the functional groups of the cross-linker. We successfully generated a sensitive and highly selective MIP for recognizing Hev b1 (G) by using mixtures of MAA and NVP as the functional monomers (mole ratio 6:2) with 15 mol% DHEBA and a solubilized Hev b1 (G) as template. The fact that the affinity and selectivity for the Hev b1 (G) are superior to those for other components (an imprinting factor of 4) indicates that there should be a significant population of template selection sites on this polymer. At this degree of cross-linking, the fraction of the monomer being nonfunctional due to steric restriction was low. The high binding affinity and the excellent molecular level, complementary to this template–monomer combination, were anticipated to give rise to a high concentration of monomer–template complexes in the polymerization mixture. The decreased capacitance on the binding events of the protein analyte on the electrode surface can be accounted for by the template interacting with the binding sites within the imprints, which are associated with the change in the dielectric property of the selective imprint material due to the orientation polarization changes on binding.

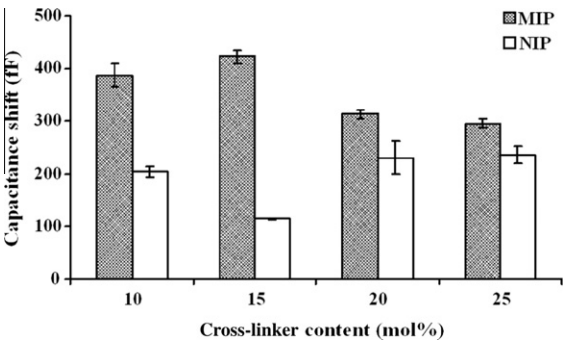


Fig.4. Effect of the amount of cross-linking monomer (DHEBA) on the capacitive responses of the MIP and NIP layers on the IDC microelectrode biosensor exposed to Hev b1 (G) in phosphate buffer solution (pH 7.4) at a concentration of 400 ng ml^{-1}. The MAA and NVP acted as functional monomers at a mole ratio of 6:2.

Hence, the imprinting approach has proven to be successful in reproducing the structural features of the latex allergy protein within the imprinted matrix, allowing the artificial recognition surfaces to interact with the accessible surface residues of Hev b1 (G) in solution. We propose to exploit the new selective Hev b1 (G) imprinted MAA–NVP–DHEBA–polymer by measuring the change to its capacitive property after binding of the specific molecule (targets) into the imprint cavity and to investigate any analytical applications for detecting rubber allergen proteins in products such as rubber gloves.

Chemical parameters

We varied the medium pH values of the reaction conditions by using phosphate buffer solutions. The highest sensor effect to Hev b1 (G) was approximately pH 7.4 (see Fig. 5A). At pH 5.0, both the MIP and NIP thin films gave the lowest capacitance shift response for Hev b1 (G) (Fig. 5A). The allergen undergoes a renaturation process in acidic conditions; therefore, all measurements at pH 5.0 were performed with freshly prepared samples [26]. The results can be explained if the Hev b1 (G) (pI = 5) does not bind to the binding sites on the polymer film surface at pH 5.0. As the amount of Hev b (G) increases in the MIP layer due to the increase in pH and the MIP polymer material returns to its original size when at pH 7.4, which mimics the conditions under which the polymer were created, a similar response to the original interaction is produced. Therefore, we chose pH 7.4 to gain the highest allergen sensitivities because we were mainly interested in detecting Hev b1 (G) from manufactured rubber gloves. Using the same layer compositions, we tested the effectiveness of our sensor coating immersed in a phosphate buffer solution containing Hev b1 (G) (250 ng ml^{-1}), an NaCl concentration (0.01–0.2 mM), and a small amplitude ac potential (100 mV). Increasing concentrations of NaCl to 0.2 mM resulted in a decrease in the selective sensor responses to Hev b1 (G) (see Fig. 5B). Chloride ions will displace water molecules from the inner coordination polymer layer. A 70% increase of the magnitude of capacitance was observed for the MIP-based electrode, whereas there was a 180% increase in the magnitude of capacitance for the NIP-based electrode. The reduction of the selective signal of the biosensor as the amount of Na^+ increased was due to the polymer cross-link binding to the salt instead of the protein template, causing the network to lose this proportion of its cross-linking and, thereby, allowing the protein to be released from the MIP. Therefore, the concentrations of salts must be kept

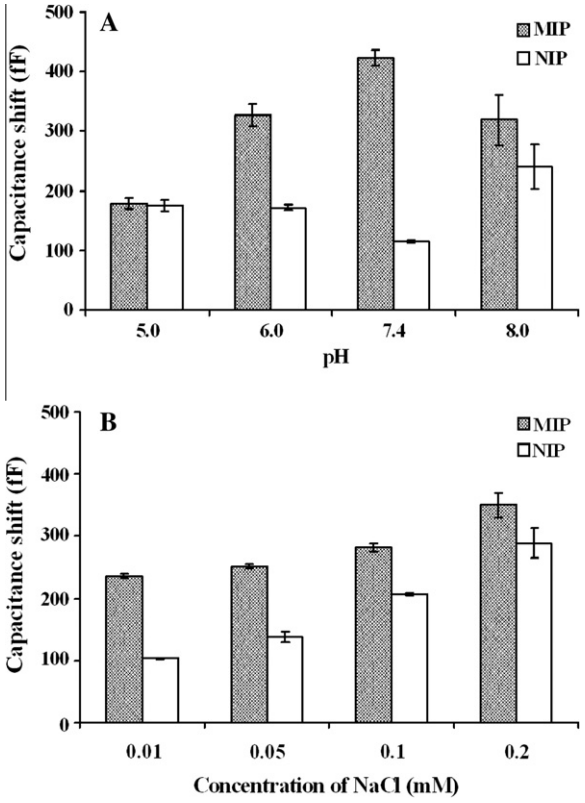


Fig.5. (A) Effect of pH of the medium on the capacitance response on 400 ng ml^{-1} Hev b1 (G)–MIP sensor and reference sensor at 1 kHz and room temperature. (B) Effect of NaCl on the capacitive shift response of the MIP and NIP copolymer of MAA and NVP at a 6:2 mole ratio and 15 mol% DHEBA (pH 7.4). Responses were initiated by the addition of 250 ng ml^{-1} Hev b1 (G).

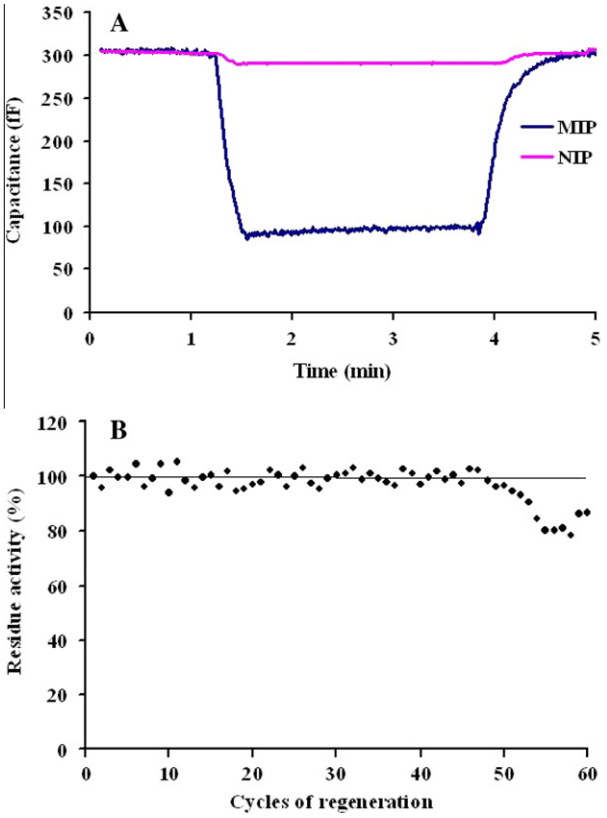


Fig.6. (A) A typical sensor response of both a nonimprinted control polymer and Hev b1 (G)–MIP illustrating the effectiveness of rubber allergen adhesion via surface imprinting using solution-based polymerization with Hev b1 (G) proteins (pH 7.4, room temperature, and a flow rate of 1 ml min^{-1}). (B) Reproducibility of the response from the Hev b1 (G)–MIP modified electrode to injections of a fixed volume of standard solution of Hev b1 (G) (250 ng ml^{-1}) with regeneration and reconditioning steps between each individual assay.

below 0.01 mM for effective molecular recognition and selectivity by the selective MIP layer.

Capacitance measurements and optimization of capacitive biosensor

We were successful in detecting a single rubber protein allergen–MIP interaction using this IDC microelectrode transducer system. It can be applied for rapid and selective detection and provides an increased active area for interdigitated electrode arrays that can be used to increase the sensitivity to binding events. The adhesion of the protein allergen to the IDC surface was measured by an IDC-based assay protocol, and the effect in Fig. 6A could be interpreted as the adhesion of the rubber protein into the selective layer. A capacitance decrease of approximately 63% on the MIP-modified electrode and a decrease of 3.7% for the NIP-modified electrode after binding of a Hev b1 (G) (250 ng ml^{-1}) allergen were observed. The efficiency of the imprinting process is greatly enhanced and redissolution of the template can occur without the process being modified, and it achieved a rapid signal response within 5 min, enabling multiple measurements without time-consuming surface regeneration. This complete availability of the surface imprinting protocol is a reflection of the thin film of the cross-linking polymer, allowing fast and efficient mass transport. In the current study, the optimization of capacitance for the Hev b1 (G)–MIP–IDC biosensor was carried out to assess whether regeneration of the selective electrode removes any noncovalently bound Hev b1 (G) analyte without disrupting the activity of the biomimetic recognition materials on the electrode. The residual activity of the imprinted electrode was obtained from the capacitance change as a result of the binding between Hev b1 (G) (i.e., 250 ng ml^{-1} Hev b1 (G) standard) and the Hev b1 (G) imprinted layer on the IDC transducer system before and after regeneration. Successful regeneration allows the surface to be reused by the capacitive biosensor many times. The criterion for successful regeneration of the electrode surface is whether postregeneration binding still retains more than 90% binding efficiency compared with that before regeneration [27]. We found residual binding values above 95% after up to 50 cycles of regeneration steps (Fig. 6B).

Concentration dependence of the biosensors

As can be seen from Fig. 7, the MIP sensor gives a signal response that depends on the concentration of Hev b1 (G) in the range of 10–1000 ng ml^{-1}. In contrast, the reference sensor shows only a slight capacitance response toward added Hev b1 (G) in phosphate buffer solution (pH 7.4). The sensitivity of these sensor layers, therefore, is in the ng ml^{-1} range of template concentrations. The sensor response was selective with a selectivity factor of approximately 6 at low rubber protein concentrations (10–400 ng ml^{-1}), but it was highly selective when challenged with protein concentrations of between 400 and 900 ng ml^{-1} with a factor of 10 for Hev b1 (G). The template polymerization procedures used produced a pronounced titration point at approximately 400 ng ml^{-1} Hev b1 (G), with a capacitance change of approximately 450 fF that corresponds to the formation of a Hev b1 (G) imprint cavity. This observation indicates that for the IDC sensor, there may be an optimal thickness for the MIP layer for achieving

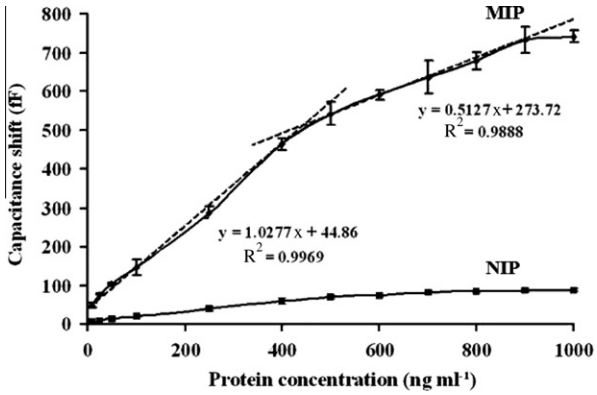


Fig.7. Concentration dependence of the capacitance response to Hev b1 (G) for the imprinted poly(MAA–NVP–DHEBA)-coated interdigital capacitor and the control. Each point represents the average of three independent measurements. A separate regression line was fitted for the data in each stratum. Those regression lines are the dashed lines in the plot.

a favorable compromise among the response characteristics, sensitivities, and selectivity. This observed concentration dependence could depend on the surface diffusion because we assume that at very low concentrations the template will rapidly bind to the highly specific affinity sites. Because of the multiple-point interactions experienced in this environment, template dissociation from these sites, and thus surface diffusion, should be slow. In the presence of higher template concentrations, however, the sparsely populated high-affinity sites are rapidly saturated; further template binding will then occur at randomly distributed nonselective sites, the dissociation kinetics of which is presumed to be much faster because of the lack of specific interactions. Further increases in the quantity of allergen on the nonimprinted electrode produced a very slight increase in the capacitance responses due to the electrostatic repulsions of the charges on the additional proteins that result in hydrophobic protein–protein aggregations on the electrode surface. Therefore, the increase in capacitance sensitivity (i.e., ~1000 times) is related to the specific protein–MIP interactions and the dynamic structural changes that occur on binding. Incorporation of this protein–monomer complex into the surface imprinting procedures may successfully compete with their structurally identical, conformationally unrestricted, and readily accessible surface cavities. In addition to these, the corresponding control materials also generated signals in the absence of Hev b1 (G) in a phosphate buffer.

Fitting the isotherm data to the Langmuir adsorption models enabled quantification of the association energies and densities of the individual binding sites of the MIP onto the capacitance electrode, with the values of the amounts bound being plotted against the concentrations of Hev b1 (G). The binding constants were determined from the following equation:

$$\frac{B}{[Hev\ b1]} = \frac{(B_{max} - B)}{K_d},$$

where K_d is the equilibrium dissociation constant, B is the amount of Hev b1 bound to the polymer, B_{max} is the apparent maximum number of binding sites, and [*Hev b*1] represents the equilibrium concentration of Hev b1. The binding profile of our thin films indicated simple two-phase binding interactions. This finding demonstrates that the polymerizable component provides the significant imprinted sites with heterogeneous binding characteristics. Thus, the Hev b1 (G) MIP had pronounced affinity (K_a = 1.25 μM) that was approximately 62.5 times that of the corresponding NIP (K_a = 0.02 μM). The capacity (B_{max}) was 0.021 μmol/mm^2 for Hev b1 (G), which is more than 10 times higher than the polymer layer's capacity.

Selectivity and sensitivity of Hev 1 (G) MIP layer on interdigital capacitor

Incorporation of protein–monomer complexes into the cavity formed by the Hev b1 (G) MIP may shape binding sites around complexes with the potential to enhance selectivity and substrate specificity. It is justified to expect that the surface imprinting procedures used may also form pits imprinted with the ability to memorize specific structural features, in particular the size and shape of the template. Thus, the Hev b1 (G) surface imprinted polymer should show an enhanced level of substrate specificity that will preferentially recognize the original template by providing a geometrical fit and a conformation memory for the rubber protein allergen. The results indicate that the NRL latex proteins are capable of inducing changes in the capacitance of MIP due to being located in the Hev b1 (G) imprinted cavity, but at different degrees of fitting for each rubber allergenic protein (see Fig. 8). The cross-reactivity results are illustrated in Fig. 9. The experiment was carried out with an NIP-based sensor to confirm that the test proteins did not bind specifically to the sensor with a similarly embedded Hev b1 (G) MIP. High sensitivities are obtained only when the analyte is identical to the template. As shown in Fig. 8, solutions of Hev b1 (G) of 250 ng ml^{-1} in buffer are readily detectable, whereas the Hev b1 isolated from rubber tree-derived latex produced a 1.8 times lower sensitivity. Although they have exactly the same size, they differ slightly in their exact conformation around the receptor site. Compared with Hev b1 (G), the different allergenic proteins Hev b2 and Hev b3 isolated from natural latex proteins produced a smaller capacitance signal response on a Hev b1 (G) MIP-based electrode by a sensitivity factor of 2.25. The Hev b1 (G) MIP has a cross-reactivity to Hev b2 (L) of 47% and to Hev b3 (L) of 43%. These two latex allergens are different in size; the molecular weight of Hev b2 is 35 kDa and that of Hev b3 is 23 kDa, so they do not fit comfortably into the smaller Hev b1 (G) imprints. Although Hev b3 has structurally similar epitopes to the template and Hev b3 is related to Hev b1 by a sequence identity of 47% [28], the Hev b1 (G) MIP yielded similar bindings to Hev b2 (p*I* = 9.3) and Hev b3 (p*I* = 4.8) [29]. Part of the structure of Hev b2 of the allergenic NRL proteins may be similar to part of the structure of Hev b3. However, this interaction area between this part of the larger latex allergens and individual cavities of the MIP will be restricted to the upper edge of the recognition site, and this will

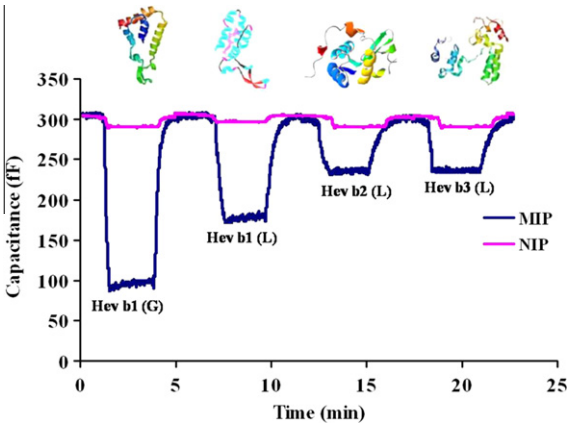


Fig.8. Hev b1 (G) imprinted MAA–NVP–DHEBA copolymer on the IDC transducer. The sensor response shows different degrees of enrichment for hevein latex allergenic proteins tested at an analyte concentration level of 250 ng ml^{-1} at room temperature and pH 7.4.

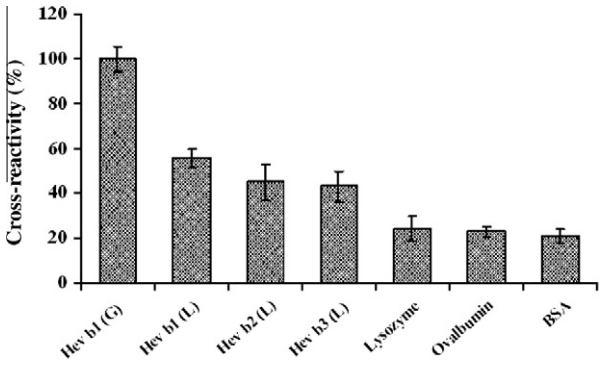


Fig.9. Selectivity pattern of Hev b1 (G) imprinted polymer as obtained by IDC measurements. The proteins Hev b and non-Hev b are detected at a concentration of 250 ng ml^{-1} in phosphate buffer (pH 7.4) at room temperature. Each value represents the average of three independent measurements.

not be sufficient for strong noncovalent binding to the imprinted layer. When comparing Hev b1 (G) and the structurally related protein Hev b1 (L) with a non-Hev b protein (lysozyme) that has similar dimensions (14 kDa), there was a clear difference observed in the binding, thereby demonstrating the sensor effects of the imprinted layer for these proteins. The much lower cross-reactivity value of the lysozyme protein (cross-reactivity value = 25%) can be due to its having a more globular structure and also indicates the difference in surface functionality and protein configuration between the template and lysozyme that renders lysozyme as unable to fit well into the cavities. The selectivity value of the distinct larger nonrelated proteins (ovalbumin and BSA) is as low as approximately 20%.

The specificity shown with the MIP sensor could be explained on the basis of electrostatic interactions combining with size and shape memories on the template. The Hev b1 (G) imprinted polymer can distinguish between Hev b-related proteins and non-Hev b proteins such as lysozyme, ovalbumin, and BSA; the Hev b1 (G) MIP favors binding to Hev b. The imprinted polymer has the defined oriented protein assembly found on the surfaces of the latex allergens. Hev b1 (G) selective polymers can also distinguish between extractable Hev b1 allergens from latex mattresses made from rubber tree latex and rubber latex gloves.

Analytical characteristics

The calibration curves constructed from the capacitance shift parameter dependency provided reasonable results. The capacitance shifts of Hev b1 (G) measured on the MIP sensor had good linear relations with its concentration in the two ranges of 10–400 ng ml^{-1}, with a correlation coefficient (R^2) of 0.9969, and 400–900 ng ml^{-1}, with a correlation coefficient (R^2) of 0.9888. When the capacitance shift response of the Hev b1 (G) microelectrode was taken as the threshold of the signal, the detection limit of this biosensor was 10 ng ml^{-1} and corresponded to a 3:1 signal/noise ratio, which is comparable to the sandwich enzyme-linked immunosorbent assay (ELISA) for assessment of latex allergen proteins that had a detection range of 125–4000 μg L^{-1} with a detection limit of 1.25 μg of Hev b1/g rubber [30,31]. The MIP can undergo at least 50 generation steps without losing its recognition ability, which is limited to only four to six cycles for the natural compounds. 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. A calibration curve was prepared by dissolving Hev b1 (G) in 0.1 mM phosphate buffer (pH 7.4) to attain solutions having Hev b1 (G) between 10 and 1000 ng ml^{-1} and then comparing them with those measured for Hev b1 (G) in spiked samples. The recovery data were in the range of 95–100% with a relative standard deviation (RSD) of 4%.

Analysis of rubber latex gloves

To examine the selectivity and sensitivity of the Hev b1 (G) imprinted polymer in the presence of a complex matrix, rubber gloves supplied by a local manufacturer were chosen to test the MIP-based capture system. The sensor responses of Hev b1 (G) imprinted and nonimprinted electrodes to powder-free gloves and powdered latex surgical glove samples are shown in Fig. 10. The effects on the sensor of a spiked sample with standard Hev b1 (G) solutions of 50, 100, and 200 ng ml^{-1} are also compared. As shown in Fig. 10B, the sensor effect was not influenced by the complex matrix in the manufactured rubber latex gloves and finished products of natural rubber latex. A nonspecific effect was obtained for the imprinted polymer and nonimprinted polymer reference sensor, a capacitive sensor response that is very slight and did not change with increasing Hev b1 (G) in the samples. Moreover, the sums of the absolute capacitance changes of the imprinted and nonimprinted electrodes are similar for all samples tested, indicating that small compounds are present in the powder-free examination gloves and powdered latex gloves after extraction and dialysis and can compete for adhesion to the interaction sites on the polymer surface.

When one powdered latex glove was treated with SDS and centrifuged, the capacitive effects were observed only for the contents of the pellet from three different batches in which the average of three independent measurements was shown. The amount of Hev b1 (G) in a specimen of the rubber latex gloves was found to be 25 ± 6 μg/g rubber material. The IDC measurement for nonpowdered gloves was examined, and the protein level is shown to be

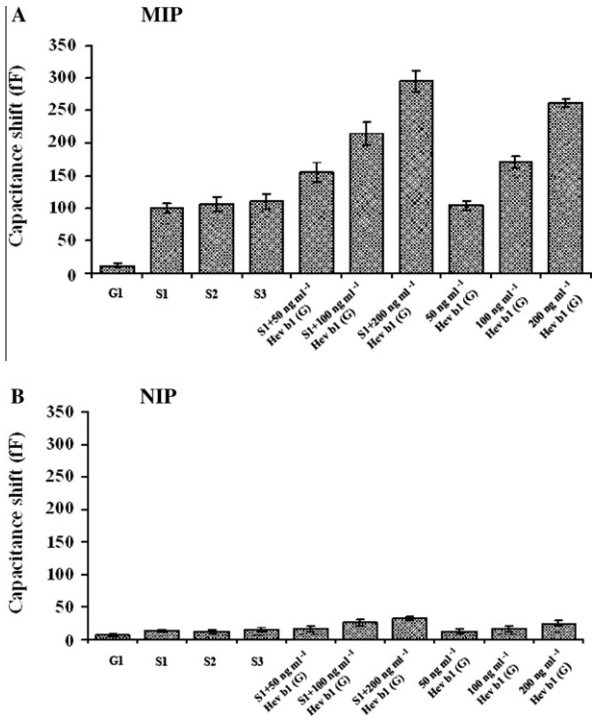


Fig.10. Response of Hev b1 (G) imprinted (A) and nonimprinted (B) reference polymer layer-coated IDC sensors for the rubber latex allergen proteins to Hev b1 (G) from powder-free gloves (G1) and powdered rubber latex gloves (S1, S2, and S3) and powdered gloves (S1) spiked with Hev b1 (G) standards and pure Hev b1 (G) standards. Measurements were carried out in 0.1 mM phosphate buffer solution (pH 7.4) at room temperature and a flow rate of 1 ml min^{-1}. Each value represents the average of three independent measurements.

very low at 3.1 ± 0.5 μg/g. The values of protein content obtained from the MIP–IDC biosensor-based assay correspond well to those of previous reports by other authors [30–32].

Conclusions

This article has described the production and properties of a biosensor for the detection of Hev b1 rubber tree latex allergens using an efficient surface imprinting process with the rubber allergenic proteins combined with an advanced microelectrode technology. This biosensor, based on MIP–protein interactions, can be used for quantification of *Hevea* latex allergens present as contaminants in rubber latex gloves. The MIP–IDC sensor showed a rapid shift in capacitance and a reasonable capacitive response that was clearly dependent on the concentration of the Hev b1 allergen proteins. The ability of the artificial recognition material to recognize the presence of defined organized structures on the protein surface allows interaction and binding that can be recognized by the sensor system. The MIP chemical probes on the IDC sensor could distinguish Hev b1 from analogs such as lysozyme, ovalbumin, and BSA. This is extremely beneficial for the application of this analytical system to quantify hevein latex allergens in real-life samples. Moreover, the IDC microelectrode is an effective approach to miniaturize MIP biosensors that will allow the detection of specific allergens in small sample volumes without reducing sensitivity and selectivity. The IDC microelectrode-based on-chip MIP biosensors, when integrated with microfluidic systems for sample delivery, can further improve the overall stability and reproducibility of the interdigitated electrode-based biosensor. Interdigitated electrodes can be further designed in different array formats and integrated into microdetection devices/systems for biomedical and biotechnological applications. Using an array of capacitors may allow us to quantify more than one marker at the same time and enhance the diagnostic power of the biosensor.

Acknowledgments

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