



Development of a rubber elongation factor, surface-imprinted polymer–quartz crystal microbalance sensor, for quantitative determination of Hev b1 rubber latex allergens present in natural rubber latex products

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ABSTRACT

Molecularly imprinted polymers (MIPs) for screening to detect rubber latex allergens (Hev b1) in natural rubber based products were designed as artificial recognition polymeric materials coated onto a quartz crystal microbalance (QCM). The polymers were prepared using a stamp imprinting procedure after mixing optimum amounts of methacrylic acid–vinylpyrrolidone–dihydroxyethylene bisacrylamide and Hev b1 latex allergen proteins, obtained from rubber gloves. QCM measurements showed that the resulting polymer layers after removal of the proteins used in their preparation could incorporate structures and features down to nanometer scale of protein templates into the imprinted polymer much better than a non-specific control polymer under controlled sensor conditions and an optimized polymerization process. This selective polymer but not the non-selective polymer clearly distinguished between the latex allergen Hev b1 and proteins such as lysozyme, ovalbumin and bovine serum albumin, with a selectivity factor of from 2 to 4, and the response of the rubber elongation factors by an astonishing factor of 12. The imprinted cavities recognized specific binding sites and could distinguish among related hevein latex allergenic proteins isolated from fresh natural rubber latex; Hev b1, Hev b2, and Hev b3 with a selectivity factor of from 4 to 6. The different QCM measurements obtained presumably reflected slightly different conformations and affinities to the MIP binding sites. The sensor layers selectively adsorbed Hev b1 within minutes in amounts ranging from 10 to 1500 $\mu\text{g L}^{-1}$ and with a detection limit of 1 $\mu\text{g L}^{-1}$. This work has demonstrated that this new sensor provides a fast and reliable response to natural rubber latex protein, even after being extracted from the matrix of rubber gloves.

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1. Introduction

Hev b1, rubber elongation factor, is a 14.6 kDa protein found on particles of rubber in natural rubber latex (NRL) obtained from *Hevea brasiliensis* [1]. Hev b1, is a recognised allergen and when present in gloves and other manufactured rubber products, is of great concern to allergic persons. Biomedical chemists are continuously trying to establish new sensitive detection methods for these types of potentially dangerous allergens and have recently detected Hev b1 in NRL gloves using an immunoblotting technique [2,3]. Currently, thirteen NRL proteins (Hev b1 through Hev b13) have been classified as major latex allergens by the International Union of Immunological Societies (IUIS). They can cause IgE-mediated reac-

tions and some NRL protein allergens have the potential to elicit Type I, immediate type hypersensitivity reactions in genetically predisposed individuals [4].

Conventional methods for detecting rubber tree latex allergens in rubber gloves involves the extraction of protein and using the Lowry or Biuret method to estimate the total protein content [5,6]. The minimum amount of NRL protein allergens required for sensitization and elicitation of allergic reactions is not known, but it is known that levels of less than 100 μg per 1 g of rubber product produced a marked decrease in allergenicity [7]. Patients allergic to NRL allergens often give a positive skin prick test reaction when the total concentration of the four latex allergens exceeds 1 $\mu\text{g g}^{-1}$ rubber [8]. The immunoassay and enzyme immunometric assay methods, such as ELISA, RAST and Western blot now used to detect known rubber latex allergen proteins [9,10] have a detection limit of 1.25 μg of Hev b1. More sophisticated methods such as the MALDI-TOF mass spectrometer test [11] used for the specific determination of rubber allergen proteins are probably even

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more sensitive. Although such available methods provide satisfactory results those that require human serum samples are difficult to standardize or require a well-trained operator for the instrumentation. A specific, reliable and sensitive method is still required, that is rapid and easy to handle. Latex glove manufacturers would then be encouraged to use the method for quality control and reduce the variability and amounts of allergen in their processed rubber latex products. A suitable sensor system would be one that allows for the testing of allergenic proteins in a vast number of samples without the need for large numbers of operating personnel. In that case it would be possible to assess the efficiency of the manufacturing process to reduce or eliminate NRL allergens especially Hev b1 in their end products. The main advantage of such systems would be to help to reduce the incidence of latex sensitization or latex allergy.

In order to achieve this goal, we have designed a new approach for sensing rubber protein allergens. Templated polymerization of proteins on polymers, for recognition purposes, provides enormous possibilities for the study of structure, conformation, assembly and physicochemical properties of proteins [12–14]. Molecularly imprinted polymers (MIPs) are biomimetic materials used for sensitive imprinting conditions, such as the selective detection of various chemical compounds and biomolecules [15–17]. A surface imprinting approach was suggested by Mosbach and coworkers that 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 a high interaction with proteins [18]. Other techniques have also evolved to produce recognition sites of proteins for polymers, including epitope imprinting [19] and imprinted polymer arrays [20], protein crystal imprinting [21] and protein imprinting that employs grafting a polymer brush [22]. In the present work, we introduce a surface imprinting approach with the Hev b1 latex allergen as the template. This is a particularly interesting and challenging task for several reasons. First, NRL protein allergens are heat and chemically stable and Hev b1 proteins have been detected on NRL gloves by immunoblotting using latex particles. This may imply that the NRL allergens are one of the last proteins to be washed off the manufactured products. This stability could provide many specific protein recognition sites in the polymer layers. Any NRL allergens that might be eluted from the surgeons' gloves or other manufactured rubber products could be detected by rebinding to the selective polymers. Also this method could be adapted for use to detect remaining NRL allergens in other NRL finished products by ensuring that the imprinted polymer was capable of protein recognition in an aqueous medium.

The aim of the current study was to seek a specific and reliable method for detecting the Hev b1 rubber latex allergen in natural rubber based products by means of a protein surface-imprinted polydihydroxyethylene bisacrylamide (DHEBA-MIP) sensing element incorporated onto a quartz crystal microbalance (QCM) transducing analysis system. In this work, a surface imprinting technique, which involved simple fabrication steps, was used to create an imprint of the rubber protein employing a layer of the cross-linked poly(DHEBA) that rapidly cured under UV-light at room temperature. These allergens had been extracted from fresh natural rubber latex articles and from latex gloves and were used as the template on a glass substrate. The optimum conditions for preparing the mixed polymer sensor and the stamping process must be carefully controlled as the polymer coating on the QCM electrode is performed by spin-coating when the pre-polymer is not yet totally polymerized on the electrode. This includes the measuring conditions (background solution, pH and temperature for final polymerization). Moreover, the sensor composed of an optimized Hev b1-imprinted polymer on the QCM electrode was also checked for its calibration characteristics (sensitivity, operation and linear concentration range and detection determination limits). The con-

structed sensor was used to detect the allergenic proteins in spiked samples and the amounts of Hev b1 present in real-life samples were assessed using the newly developed MIP-QCM sensor method.

2. Experimental

2.1. 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 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 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 3700 Da were from Sigma (St. Louis, MO, USA). All solvents used were of analytical-reagent grade.

2.2. Protein extraction and purification

All allergenic proteins extracted from latex 'mattresses' made from rubber tree latex are denoted by (L). The extractable Hev b1 protein from medical gloves is denoted by (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 [23]. 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 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 (L) and Hev b3 (L). Crude protein was dialyzed through a cellulose dialysis bag against several changes of distilled water. The protein extract was purified by gel permeation chromatography on a Sephacryl™ S-100 column (1.5 cm × 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⁻¹. The collected fractions were concentrated and analyzed by SDS-PAGE on 15% gels and protein bands detected with Coomassie staining. The major bands representing the target proteins (Hev b1 (L) and Hev b3 (L)) were excised, destained, digested with trypsin and extracted from the gel pieces. Extracts were dried in a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA), and the sequence of the allergenic proteins was confirmed using an Ultraflex III MALDI TOF/TOF (Bruker Daltonik, Bremen, Germany) mass spectrometer [11].

In addition, the collected bottom fraction (BF) of latex (after initial centrifugation at 60,000 rpm) was washed three times with isotonic buffer and recovered by centrifugation. The rubber particles were then 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). The supernatant was dialyzed against distilled water at room temperature for two days. The sample was lyophilized and purified as described above for Hev b1 (L) and Hev b3 (L).

Hev b1 (G) was obtained from rubber latex gloves, as described previously [23]. 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

Table 1

Polymer compositions for preparing of Hev b1 rubber allergens surface-imprinted polymer.

Polymer	Functional monomer (mmol)			Cross-linker (mmol)		Porogen (mL)
	MAA	NVP	ACM	DHEBA	MBA	
MIP1	0.7	0	0	0.15	0	0.8
MIP2	0	0.7	0	0.15	0	0.8
MIP3	0	0	0.7	0.15	0	0.8
MIP4	0.6	0	0.2	0.15	0	0.8
MIP5	0	0.2	0.6	0.15	0	0.8
MIP6	0.6	0.2	0	0.15	0	0.8
MIP7	0.6	0.2	0	0	0.15	0.8

natural rubber latex. This protein was lyophilized for use as purified Hev b1 (G) for patterning process.

2.3. QCM

The imprinting was performed on quartz crystal microbalances with two-electrode geometry. One electrode was incorporated with the imprinted polymer layer and the other one was a reference electrode coated with the non-imprinted polymer (NIP) control polymer, prepared in the same way but 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 [24].

2.4. Protein stamp preparation

The preparation of the protein stamps could be achieved by direct molecular self assembling of the proteins onto very flat glass slides (roughness approximately 2 nm; Schott, Mainz, Germany). The glass slides were cleaned with detergent then with distilled water three times, followed by drying in the oven at 120 °C for 2 h before it was coated. A 5 μ L sample of protein in 0.1 mM phosphate buffer (pH 7.4) was applied to the glass surface and then allow to assemble at 25 °C for 2 h. During incubation, the Hev b1 adsorb on the glass through polar interactions and remain adhered to it at the dry state. The observed dependence of the morphology of the film on the concentration of the Hev b1 protein was examined on the glass surface, where the micrometer-sized aggregation patterns on the stamp could be observed at high concentrations of 10 and 100 mg mL⁻¹. However, when a Hev b1 protein solution at a concentration of 0.1 mg mL⁻¹ was used, the desired nanostructure pattern of the adsorbed Hev b1 protein was generated.

2.5. Sensor layers

Molecularly imprinted and corresponding non-imprinted polymer film layers coated on the QCM electrodes were created according to a previously described procedure [25]. The chemical structures of functional monomers and cross-linkers used in this study are presented in Fig. 1. The compositions of this preparation of surface imprinted films are shown in Table 1. The polymerizing mixture was dissolved in distilled water followed by adding AIBN (1%, w/w) and purged with N₂ (for 30 s) and pre-polymerizing until reaching the gel point. The viscous pre-polymer was then spin-coated onto a QCM electrode at 3000 rpm to yield layer heights in the range of 80–400 nm. The final stamp was pressed into the

pre-polymerized coating localized on the electrodes of a QCM. The pre-polymer mixtures were then exposed at 25 °C to a UV-source to initiate photochemical polymerization. The template removal was carried out by incubating the resulting coated electrodes with distilled water at 40 °C for 30 min and followed by thoroughly rinsing with water in the measuring cell at pump velocity of 2 mL min⁻¹ until the return of signals to the baseline. The nonMIP-based electrodes were also fabricated with a pre-polymerized mixture including Hev b1 (L) of the rubber latex as the template during the polymerization process.

2.6. Atomic force microscopy (AFM)

Tapping mode AFM (Nanoscope III AFM, Veeco, USA) was used to test (the assembly, morphology and tunability of the nanostructure) the adsorbed Hev b1 on the stamps and stamping procedures. A silicone cantilever with a spring constant of 40 N m⁻¹ was employed. Topographic and phase images were collected at a scan rate of 1–2 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.

2.7. QCM assays with imprinted polymers

The coated quartz electrode was mounted in a self-made measuring cell of 250 μ L volume and thermostatted at 25 °C. Home-built oscillator circuits and a self-programmed frequency readout and processing software were used. The 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 in a stopped-flow system and a 2 mL min⁻¹ flow rate for solution delivery. 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 $\pm$ 1 °C). The sensors were rinsed with distilled water to remove bound proteins and allergens followed by a background solution at the end of analysis. The frequency shift responses of QCM with the prepared MIP and NIP control polymers were measured when exposed to Hev b1 (G) solution at its initial concentration. In order to define the optimum sensor technological parameters of the prepared sensor the frequency of the sensor (Hev b1 (G)-MIP film) was measured and presented in this work. For the sample measurement using the sensor, the signal response toward the analyte sensor was reported as a frequency shift response ($-\Delta F$). Moreover, the selectivity of the MIP sensor was affirmed by comparing the signal responses of the MIP sensor and reference sensor having a NIP control polymer, as a different pair mode. The selectivity of the sensor was examined by comparing the frequency shift response of the 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 carried out in triplicate on any particular day of experimentation.

2.8. 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 described in Section 2.2. The crude proteins from rubber latex and glove extracts were analyzed by the proposed Hev b1 (G)-MIP sensor. 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 to the proce-

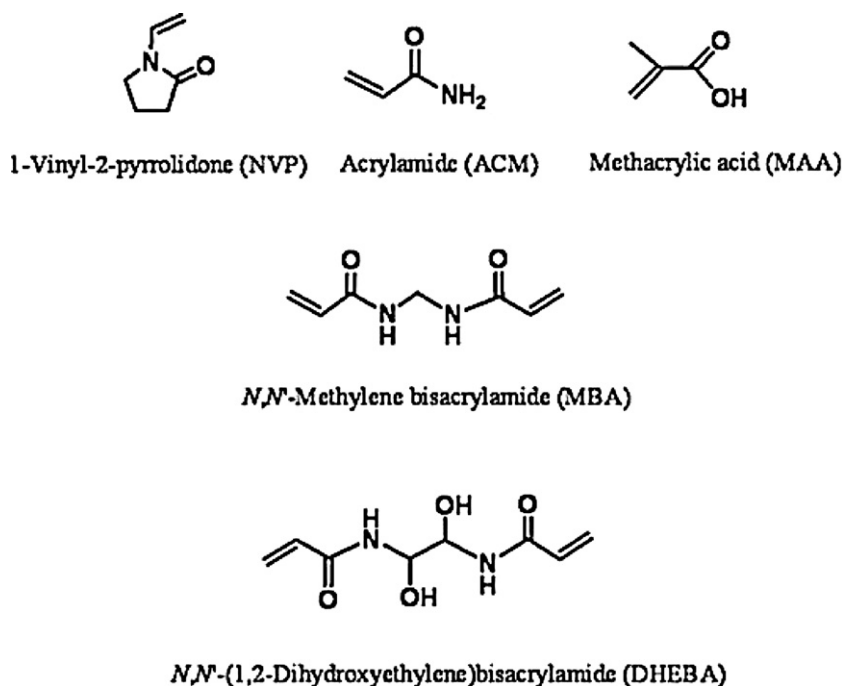


Fig. 1. Chemical structures of functional monomers and cross-linking monomers used in this study.

ture previously described for Hev b1 (G). Spiked samples of allergen protein (250, 500, and 1000 $\mu\text{g L}^{-1}$), 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 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 and 1000 $\mu\text{g L}^{-1}$, and then comparing them to that measured for Hev b1 (G) in the spiked samples. After several washing steps, QCM measurement was carried out by following the protocol in Section 2.7. Every experiment was repeated three times at room temperature ($25 \pm 1^\circ\text{C}$).

3. Results and discussion

3.1. Protein stamp and imprints

An effective approach for miniaturization of biosensors based on-chip MIPs as biorecognition elements requires an appreciable examination of the templating procedures. The initial evaluation was examined with a selective layer fabricated by the prepolymerization of MAA–NVP–DHEBA. Polymers based on the MAA–NVP–DHEBA copolymer can be prepared in aqueous solution and initiated by radicals, and can be devoid of denatured protein. We applied a protein stamp incorporating a surface-imprinted thin layer on a transducer of a QCM sensor and then the template proteins were removed from the templated (MAA–NVP–DHEBA cross-linked) polymer structures, while hopefully retaining a copy of the geometrical features of the latex allergen (Fig. 2). Fig. 3(a and b) shows the topographic images of the rubber allergen protein adsorbed onto the glass surface. Protein stamps of a protein concentration of 0.1 mg mL^{-1} resulted self-organization of the proteins Hev b1 into ordered array of nanostructures by a process that is similar to the molecular driving forces that allow formation of ordered self-assembly on solid substrate. Previous study suggests that physical and chemical properties of the substrates can profoundly affect adsorption, wettability and adhesion [26]. The interfacial behavior of Hev b1 proteins on the glass surfaces is dictated by the balance of enthalpic and entropic effects

from protein/surface-protein segment interactions. During drying, capillary forces can induce rearrangement of natural protein conformation. The adsorbed protein nanostructures can be seen with a superior nanometer three-dimensional spatial resolution for Hev b1 (G) compared with that of Hev b1 (L) in the dry state. Moreover, the physicochemical properties such as protein concentration,

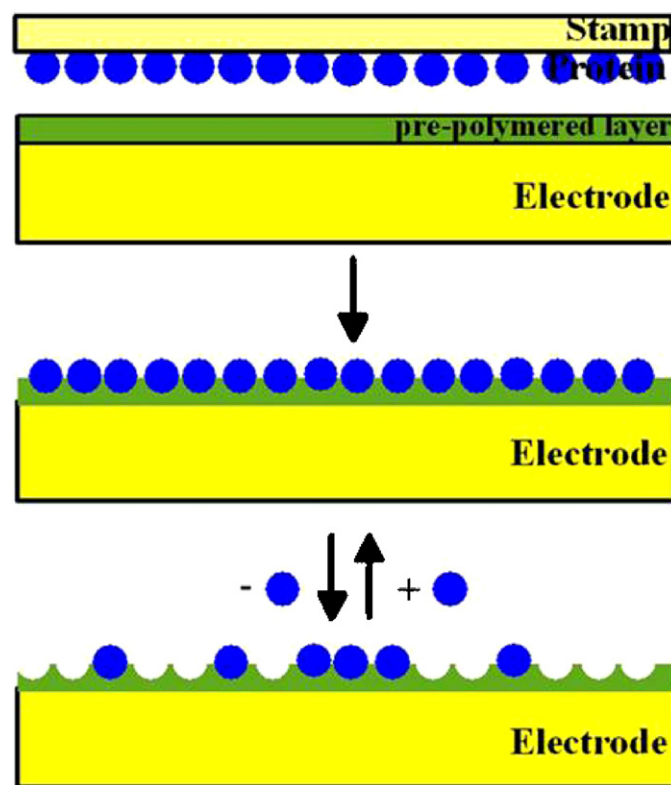


Fig. 2. Schematic for the preparation of the protein surface-imprinted polymer coating on a QCM electrode. A stamp with densely packed rubber proteins is pressed into the pre-polymerized coating, templates are removed after polymerization.

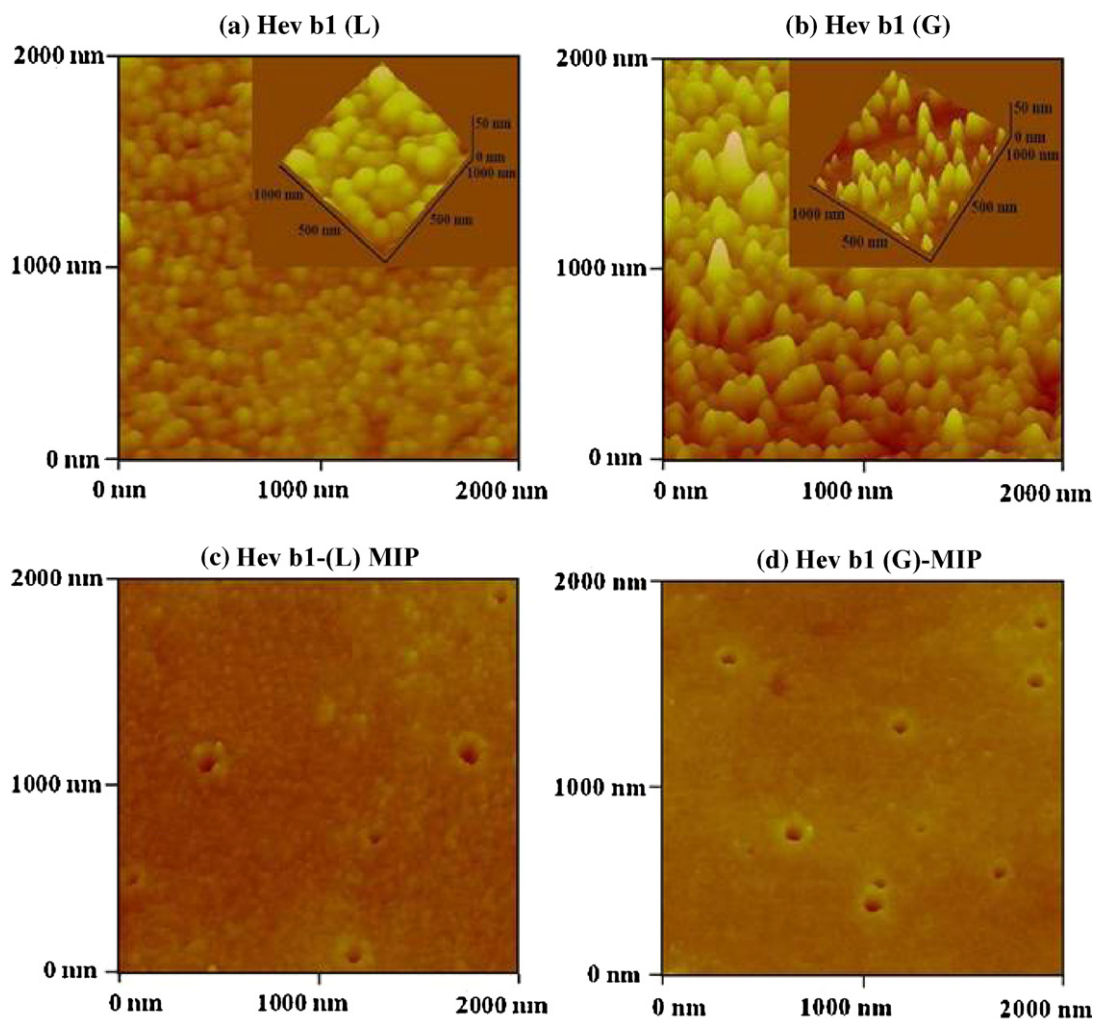


Fig. 3. Topographic AFM images of poly (MAA-NVP-DHEBA-polymer) layer: (a) Hev b1 (L) and (b) Hev b1 (G) self-assembled on the glass surface, (c) Hev b1 (L)-MIP, and (d) Hev b1 (G)-MIP coated on a QCM electrode showing nanopatterned surfaces with nanoscale of lithographic imprints. Layer heights are 200 nm (spin coated, layer height tested with AFM).

pH, surface charge distribution, and ionic strength of the protein solution can play crucial roles in the oriented protein assembly on surfaces.

The imprint process maximizes the surface for interaction between the embedded protein and the molded layer material. A simple spin-coating procedure for surface patterning was found to provide easy access to nanoscale patterns on the polymer surface on the Au coated transducer. Partial curing of a MAA/NVP/DHEBA based UV cross-linkable resin by spin-coating at 3000 rpm (for 10 s) prior to imprinting resulted in a sufficient buildup of the resin molecular weight to prevent its absorption into the stamp protein yet maintaining a low enough viscosity to allow for rapid molding of nanoscale features of the template during the subsequent imprinting process. Film thickness had to be optimized; if it was too high Hevein proteins would not penetrate as effectively into the tighter meshed polymer chains, while a very thin film would not lead to good reproduction of the specific template species patterns in the polymer due to the small interaction area. The AFM test results revealed that optimal imprinting of the allergenic proteins is when the layers have a thickness of 200 nm. An AFM of the imprinted surface, as depicted in Fig. 3c, confirms a pattern height of less than 50 nm. Whereas with the 70 nm-pit in Fig. 3d, the polymer layer imprinted with the Hev b1 (G) mold, shows better contrast

and sharp edges. Our results indicate that the proposed stamp imprinting process is a feasible method for replicating the pattern of the allergenic proteins in a polymer imprint layer and its corresponding surface feature with high resolution at the nanometer scale that may enable high selectivity for the fabricated MIP-QCM sensor.

Various layers of thickness of a Hev b1 (G) imprinted polymer coated on the transducer surface in a range of 80–240 nm were examined for their frequency shift responses of the sensor exposed to 2 mg L^{-1} Hev b1 (G) solution. The frequency shifts increase with an increase of the polymer thickness, with the greatest shift occurring between 80 and 120 nm thickness, and the shift-increase being relatively stable between 160 and 240 nm (Fig. 4). In contrast, with the corresponding NIP layer, the frequency shift appearance was only slight and associated with binding of proteins to non-specific binding sites. The occurrence of this saturation condition with respect to the layer thickness has been associated in the MIP with a minor role played by nonspecific binding. This phenomenon may be attributed to the effects of a gradient in water evaporation being established in the monomeric mixture near the surface of the film layer during the imprinting process. Such a gradient would be expected to lead to an alteration in the density of the created poly-

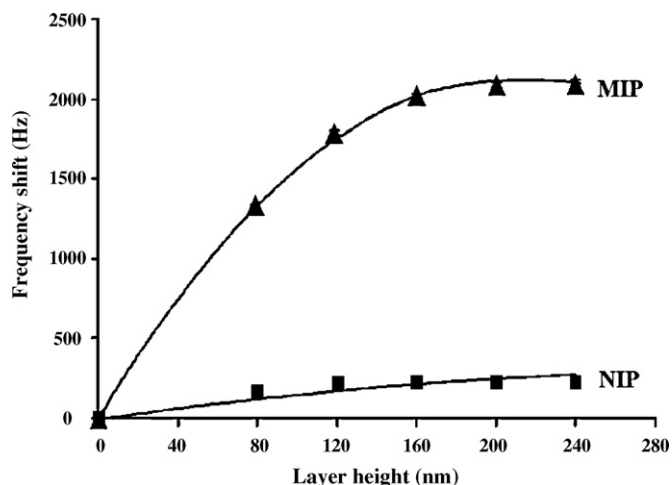


Fig. 4. Effect of imprinted layer height on the frequency shift of the NIP and Hev b1 (G)-MIP (MAA-NVP-DHEBA-polymer) coated QCM exposed to Hev b1 (G) at a concentration level of 2 mg L^{-1} . Measurements were carried out in 0.1 mM phosphate buffer solution (pH 7.4) at room temperature, using an electrode polymerized at 25°C .

mer network and be dependent upon the depth of the MIP layer.

3.2. Optimization of polymer compositions

We investigated the effect of covalent cross-linked polymer layer on non-covalent connection on the surface of nanostructures of the Hev b1 (G) stamps to the growing polymer for the preparation of Hev b1 (G)-MIP-QCM sensor. As shown in Fig. 5, a MAA polymer gives a frequency shift response to Hev b1 (G) higher than the NVP polymer or even higher than the ACM polymer, prepared using DHEBA as a cross-linker. For the monofunctional polymers with a molar ratio of 7:1, an excess of the functional monomer with respect to the template yielded a higher non-specific affinity. 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. The QCM with MIP prepared with MAA-NVP monomers gives a high frequency response for Hev b1 (G) with an astonishing minor non-specific effect with respect to other MIPs (Fig. 5). The polymer functionality of the MAA-ACM copolymer can interact with protein by hydrogen bonding which leads to a high effect similar to that of an MAA polymer but with selectivity factors of

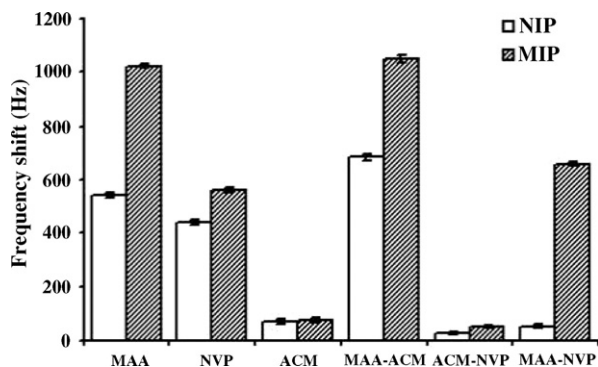


Fig. 5. Effect of the polymer components for an MIP- or NIP-modified QCM sensor on the frequency shift responses to $500 \mu\text{g L}^{-1}$ Hev b1 (G) in phosphate buffer (pH 7.4) at room temperature.

less than 2 toward Hev b1 (G). When the ACM was combined with the 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. For the polymer functionalities of ACM-NVP they are neutral in an aqueous solution and provide a much lower frequency response and a very low selectivity whereas with MAA-NVP, their polymer functionalities occupy more hydrogen bonding from MAA and a good wetting or adhesive properties from the NVP. Moreover, NVP stabilizes the inclusion of protein in the MIP and also stabilizes the protein conformation after attachment [27].

We examined the effect of different cross-linking agents on the stability of hydrogen-bonding interactions between the nanoscale template Hev b1 (G) and the functionality of MAA and NVP functional monomers. 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 imprint polymer layer, in which DHEBA provides an -OH surface on the polymer [28], can be attributed to the greater polarity and higher wettability of poly(methacrylic acid-co-vinylpyrrolidone) cross-linked with DHEBA generated interactions with proteins at a near native condition. The imprinted polymer from cross-linked poly(MAA-NVP-DHEBA) displays a high efficacy of recognition for the target analyte; therefore this polymer has been used for investigations in the following experiments.

3.3. The investigation of the technological parameters

3.3.1. Influence of the background solutions on the sensor response

Fig. 6a shows the effect of the solvent on the frequency shift response of Hev b1 (G) ($500 \mu\text{g L}^{-1}$) to Hev b1 (G)-MIP-modified and NIP-modified QCM electrodes when exposed in either 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 the pH 7.4 buffer was as high as 660 Hz, and was only half the value with distilled water. The frequency response of Hev b1 (G) for the imprinted polymer was low for both NaCl and the Tris-HCl buffer solutions compared to that in distilled water. With water as the solvent, the protein surface is not charged properly to bind to the active sites of the cavities of the MIP, and hence produces a lower selective signal. The selective response of MIP decreased when exposed in NaCl solutions such that it is possible that the electrolytes cause a salting out of the proteins, thus altering the protein structure so that it can no longer be recognized by the imprint. While in the Tris-HCl buffer, the binding interaction of MIP and protein might be inhibited by conjugation of amine-based cross-linkers [29]. The phosphate buffer, which was used in 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.

3.3.2. Influence of the medium pH on the sensor response

The pH of the background solution 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) (pI value = 5.0) on the sensors than when the sensors were exposed to protein at a higher pH (pH 6–8). At acidic pH, renaturation was achieved and this affects the binding motifs on the latex proteins [30]. This effect causes a decrease of adsorption interactions between protein and polymer, and results in a 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. This could be due

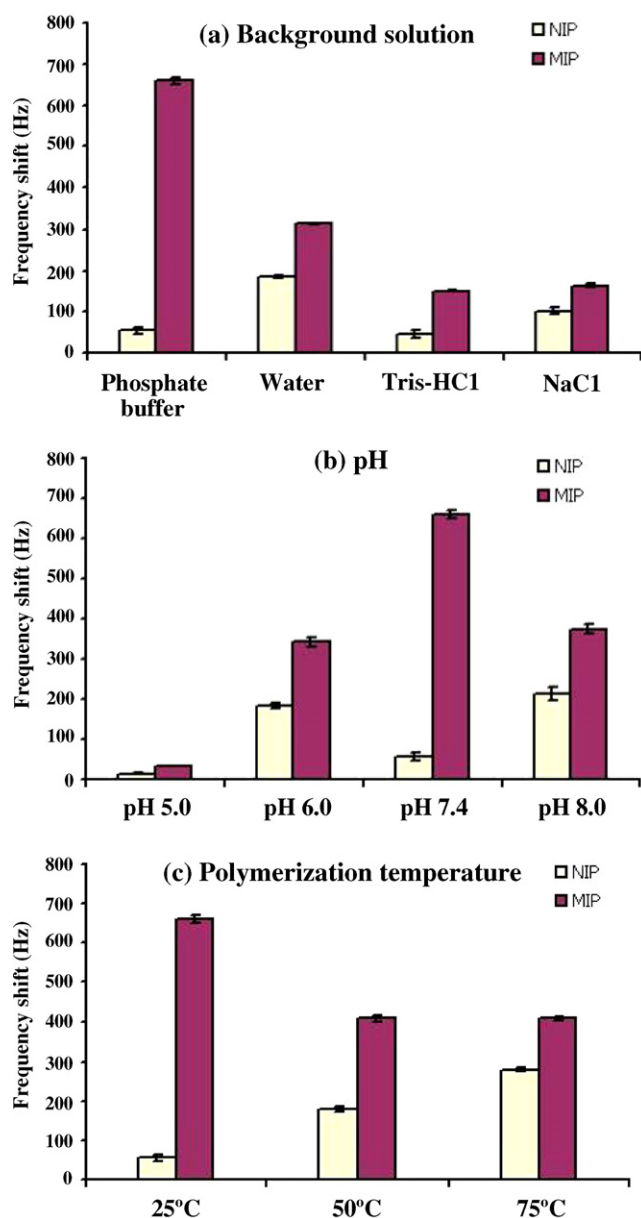


Fig. 6. (a) Effect of the background solution on the frequency responses of the NIP and Hev b1 (G)-MIP (MAA-NVP-DHEBA-polymer) layer coated 10 MHz-QCM using an electrode polymerized at 25 °C. Measurements were carried out in 0.1 mM phosphate buffer solution pH 7.4 and at room temperature. Each value represents the average of three independent measurements. (b) Effect of medium pH on the sensor response using 500 $\mu\text{g L}^{-1}$ of Hev b1 (G) for NIP sensor and MIP sensor, using an electrode polymerized at 25 °C. (c) Effect of polymerization temperature on the frequency shift response of the NIP sensor and MIP sensor. Responses were initiated by the addition of 500 $\mu\text{g L}^{-1}$ Hev b1 (G).

to the structural rearrangement of the MAA-NVP-DHEBA-polymer in the pH 7.4 buffer, which was used in the preparation phase for the sensor layer to provide a successful rearranging of the functional groups of the template and monomers in the imprint cavity with a defined surface structure optimized for recognition of the template. Furthermore, the Hev b1 (G) allergen proteins retained their native structure on adsorption to the MIP layer. Consequently, while strong polymer/surface-protein interactions are desirable for surface coverage and charge compensation, this depends not only upon the binding affinity of MIP, but also on the relative stability of the structure under the exposed conditions.

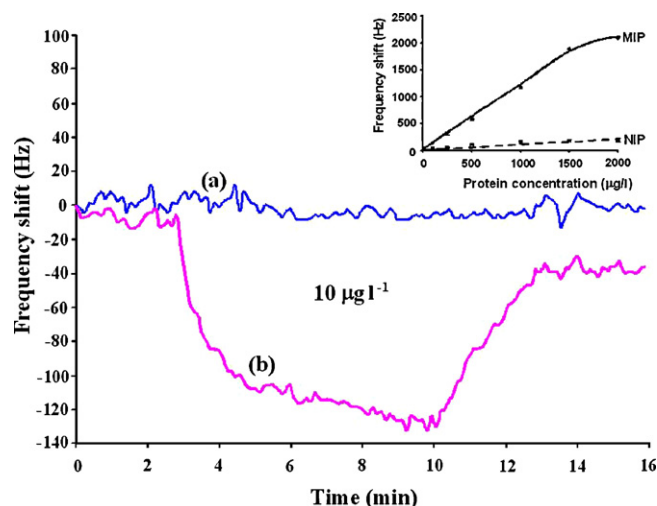


Fig. 7. QCM sensor responses for (a) a non-imprinted reference and (b) a Hev b1 (G)-MIP coated electrodes exposed to 10 $\mu\text{g L}^{-1}$ Hev b1 (G) solution. At the top right are the sensor characteristics of NIP, and Hev b1 (G)-MIP coated 10 MHz QCM. Measurements were carried out in 0.1 mM phosphate buffer solution (pH 7.4) at room temperature.

3.3.3. Influence of the polymerization temperature on the sensor response

The effect of the polymerization temperature on the frequency shift response of the sensor exposed to Hev b1 (G) (500 $\mu\text{g L}^{-1}$) at 25 °C was examined 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). 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 are 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 the template being present in the binding groups of the resulting materials [31]. The selective sensor response of the optimized imprinted polymer for the present study was, after polymerization, the highest at room temperature (25 °C). The incorporation at the optimum condition of the QCM sensor exposed to the protein template may occur by hydrogen bonding between a proton donor and acceptor, typically involving oxygens and nitrogens of protein and MIP. Moreover, a geometrical fit can occur by a part incorporation of the protein template to MIP.

3.4. Binding of template to Hev b1 (G) MIP

Fig. 7 shows the response of the Hev b1 (G) MIP layer and the adsorption by the NIP control electrodes. It appears that the normal baseline of an MIP-based sensor changes to 110 Hz upon the addition of 10 $\mu\text{g L}^{-1}$ Hev b1 (G) in phosphate buffer, pH 7.4. The present study demonstrates that the Hev b1 (G)-MIP sensor has a high sensitivity. This can be explained by the protein readily interacting with the 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. Adsorption leads to a stable plateau value being attained within 10 min, which 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 due to Hev b1 (G) for the non-printed sensor turns

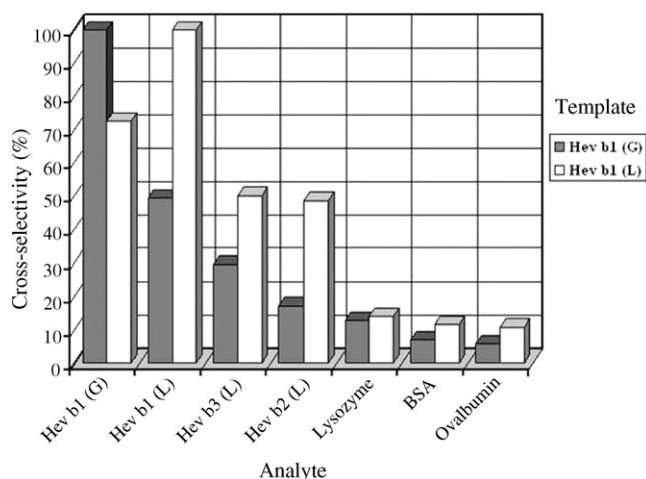


Fig. 8. Selectivity profiles of the surface-imprinted polymers for Hev b1 (G), and Hev b1 (L) templates from stopped flow QCM experiments at an analyte concentration level of $500 \mu\text{g L}^{-1}$ in phosphate buffer (pH 7.4).

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 the imprints. The sensitivity of the QCM was 1.04 Hz ng^{-1} . At the highest concentration measured, the overall mass effect is 2.1 kHz, that corresponds to 2.52×10^{17} Hev b1 (G) proteins bound to the sensitive layer. The mass response generated by the selective layers is correlated to the allergen concentration in the buffer, as shown in Fig. 7, top right. We have found that allergen concentration versus frequency change is linear over $10\text{--}1500 \mu\text{g L}^{-1}$. 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.

3.5. 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. A molecularly imprinted polymer sensor layer selective for non-native and native Hev b1 was synthesized using the same protocol for synthesis. 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 particular MIP on the sensor for the analogue relative to that for the respective template, is shown in Fig. 8. Clearly, in both cases the imprinted sensors results 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.

Both the Hev b1 (G)-MIP and Hev b1 (L)-MIP based receptors provide a high selective recognition for 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 allergen shares a 47% sequence identity with Hev b1 at the amino acid level, and the two molecules have been shown to share IgE epitopes [32]. However, there appears to be cross-selectivity between Hev b1 (G) and Hev b1 (L) because of their similar functional groups and dimension of protein. Hence, it can be concluded that the Hev b1 (G) surface-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” in a non-native conformation to the imprint, 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 the 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, and is specifically able to bind species of naturally derived Hev b1. This may be because the non-native Hev b1 protein refolds to a stable state in the Hev b1 (L)-imprinted cavity to its native conformation. Although the sensor readily detected proteins from other partially purified sources because they had the same epitopes, this sensor would be advantageous to detect the proteins extracted from NRL products other than gloves or were similar epitopes on the proteins recognized on crude latex samples.

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

Using the Hev b1 (G) surface-imprinted polymer as a selective layer in QCM is important for determination of the analyte in the extracts of NRL articles and finished products of gloves. We developed a MIP-QCM sensor-based assay for measuring the analyte, 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 $10\text{--}1000 \mu\text{g L}^{-1}$ ($R^2 > 0.996$). The analytical detection limit, calculated using a signal-to-noise ratio of 3, was $1.0 \mu\text{g L}^{-1}$. 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 the matrix sample from a latex glove spiked with a standard solution of Hev b1 (G) at concentration levels of 250, 500 and 1000 $\mu\text{g L}^{-1}$. The method showed high selectivity and good precision and compatibility with the biological complex matrix. Recoveries were in the range of 97–105%, 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 $22 \pm 4 \mu\text{g g}^{-1}$ of rubber material, respectively, which correspond well to the previous report by other authors using different methods [10,33,34].

4. Conclusions

This paper presents the development and optimization of a surface imprinted polymer for detection of the latex allergen proteins in natural rubber latex products. Surface imprinting allows for the selective recognition of proteins based on shape selection and the positioning of functional groups that can memorize Hev b1 protein in a molecular recognition process. Polymers as sensor coatings enable for a variety of modifications of composition and functionality, ensuring optimum modification of the sensitive surface to the analyte. The results of the present study proved that surface imprinting of thin films does produce molecularly imprinted synthetic receptors that allow accessibility for target analytes and can be a fast and cost-effective method for detection and determination of Hev b1 rubber latex allergens. The fast frequency shifts, as well as the fully reversible responses, also indicates the presence of non-covalent interactions to the bioanalyte in the cavity of the MIP. Hev b1 (G) imprinted methacrylic acid/vinylpyrrolidone/dihydroxyethylene bisacrylamide copolymer as the recognition system on a transducer of QCM sensor showed a high sensitivity and selectivity for the response toward Hev b1 (G). It can readily distinguish between the template and others (i.e. lysozyme, ovalbumin, and bovine serum albumin), and demonstrates 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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