

Pollen-imprinted polyurethanes for QCM allergen sensors

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Abstract Molecularly imprinted polymers for detecting plant pollen were designed as artificial recognition materials for quartz crystal microbalances in the gaseous phase. Imprints of birch (diameter, 25 μm) and nettle (diameter, 15 μm) pollen can be generated by polydimethylsiloxane stamping technique as proven by atomic force microscopy. If pollen grains are able to access the cavities and thus are incorporated, the resulting sensors display Sauerbrey-like negative frequency shifts. Non-Sauerbrey behaviour can be observed as soon as pollen is prevented from entering the selective hollows: this results in grain mobility on the electrode surface leading to frequency increases. Access to the cavities is determined by the diameter ratio between pollen grains and imprints as can be revealed during cross-selectivity measurements of nettle and birch pollen imprinted layers. When the amount of pollen grains on the electrode surface exceeds the number of available imprints, the excess particles move freely, resulting in positive, non-Sauerbrey frequency shifts.

Keywords Molecular imprinting · Plant pollen · QCM · Non-Sauerbrey behaviour

Introduction

Combining rationally structured polymer layers with mass sensitive transducers such as quartz crystal microbalances (QCM) [1, 2] has proven to be a highly promising approach for designing chemical sensor systems in a variety of analytical applications [3, 4]. The background for QCM sensitivity is that any mass rigidly attached to the device surface leads to a frequency decrease according to Sauerbrey's equation [5, 6]. The concept is especially interesting when sensing biological analytes, because binding of a single species already contributes substantial frequency effects. The necessary selectivity is usually achieved by a suitable recognition material selectively incorporating the analyte of interest [7, 8]. Molecular imprinting [9], where self-organisation between a forming polymer matrix and a suitable template leads to tailored interaction sites, is a comparably straightforward way for actually synthesizing such artificial recognition materials. Originating from chemistry, the concept has been successfully extended to biospecies [10–13] leading to sensors for measurements in aqueous phase. A question yet unanswered concerns the limits of applicability of the imprinting process in terms of template dimensions: the lower limit is in the range of small molecules, whereas the upper limit is yet unknown. In fact, such limitation is for two reasons: first, polymer layers becoming too thick are less suitable as sensor materials due to possible swelling and transduction limitations. Second, recognition in molecularly imprinted polymers (MIP) is based on directed, non-covalent interaction between layer and analyte. Hence, a larger interaction surface should lead to stronger binding and consequently to decreased reversibility of the interaction phenomenon. In the present work, we introduce a surface imprinting

Dedicated to Prof. Dr. Ernst Kenndler on the occasion of his 65th birthday.

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approach with plant pollen as the template. This is a particularly interesting and challenging task for two reasons: first, pollen grains—usually being 20–50 μm in diameter—are exceptionally large bioanalytes and hence are highly suitable for testing a possible upper limit in imprinting, and second, the measurements have to be carried out in the gas phase.

Pollen is the sexual germ cell in seeding plants and possesses a haploid genome, which is transported to the ovaries of other plants for pollination either by wind (anemophilous plants) or insects (entomophilous plants). The grains have an inner—less stable—wall made of cellulose, the so-called intine, and the outer, chemically and mechanically very robust, exine consisting of sporopollenin that can only be destroyed by very strong oxidants, such as ozone or hydrogen peroxide. It is a nitrogen-free biopolymer [14, 15] containing mainly long-chain fatty acids, phenylpropanoids, phenolics and traces of carotenoids. Due to its insolubility in acids and organic solvents, chemical analysis of sporopollenin is limited to solid-state techniques such as ^1H NMR [16, 17], ^{13}C NMR [18, 19] and Fourier transform infrared [20].

Pollen transferred by wind represents a substantial problem for humans suffering from allergies. Proteins, lipids and enzymes are released by pollen when exposed to aqueous systems. They can act as antigens, i.e. allergens, hence causing a misguided immune reaction in sensitised individuals. Therefore, substantial effort has to be undertaken in monitoring different kinds of pollen and their concentration in ambient air so as to obtain a reliable, rapid alerting system. This is not possible at the moment: currently, pollen load in air is analyzed or even only estimated by observing anthesis (i.e. the full flowering period) of the plants of interest, which allows conclusions on the current pollen load. Another strategy includes traps accumulating pollen from ambient air for subsequent counting under the light microscope. However, such a trap needs a certain sampling time to collect sufficient amount of pollen meaning that analytical results can be obtained only after at least 2 h of collecting. In both cases, the methods are thus rather slow, as detection in the range of minutes would be appreciable. Therefore, a sensor system would guarantee rapid analysis without the necessity of larger numbers of operating personnel. Possible applications would especially cover indoor monitoring, e.g. for assessing the efficiency of pollen filters included in air conditioning for instance in office buildings, hospitals or passenger cars but also distributed sensing for allowing early warning systems for environmental surveillance. The latter would also be helpful for modelling incidence pathways of pollen exposition depending, e.g., on weather conditions. The main advantage of such systems would of course be their in-time operability.

Hence, in this work, we present a novel approach for a pollen sensing system based on surface imprinted layers as recognition materials on QCM. We carried out the experiments with two different analytes, namely nettle (15 μm diameter) and birch (25 μm diameter) pollen, the latter being one of the major allergens, as can be seen by the fact that the major birch allergen Bet v1 acts as primary sensitizer causing IgE to show cross-reactivity to homologous allergens in fruits [21].

Experimental

For fabricating the QCM, we screen-printed gold electrodes onto 10 MHz AT-cut quartz blanks of 15.5 mm diameter purchased from ZQC (China) as described in previous works [22]. For stamping, we assembled pollen on polydimethylsiloxane (PDMS) attached to glass plates guaranteeing flat surfaces. Figure 1 shows the procedure applied: first, we covered the PDMS completely with pollen and then blew off the excess material with compressed air. Due to the attractive interaction between pollen and PDMS, a monolayer remains on the surface after this procedure.

All chemicals were purchased from Fluka and Merck, respectively, in analytical or the highest available synthetical grade and used without further processing. For imprinting, we used a polyurethane with a composition of 65.0 mg bisphenol A and 40.2 mg 4,4'-diisocyanatodiphenylmethane acting as monomers and 12.0 mg phloroglucinol as the cross-linker. The mixture was dissolved in 200 μL tetrahydrofuran (THF) at 70 $^{\circ}\text{C}$ followed by adding 10 μL pyridine serving as a catalyst and pre-polymerizing until reaching the gel point. Then we added 800 μL of THF and spin-coated the resulting solution onto the gold electrodes. Film thickness had to be optimised: if it was too high, the pollen grains were buried deeply enough in the layer so that they were irreversibly bound. On the other hand, very thin films did not lead to correct reproduction of the specific pollen patterns in the polymer due to the small interaction area. It turned out that a

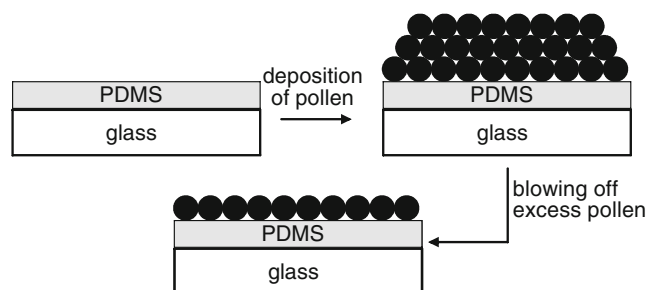


Fig. 1 Schematic of pollen stamp preparation: The pollen grains adhere to PDMS due to adhesive forces. Excess pollen is blown off with pressurised air resulting in a monolayer stamp

polymer layer height of 500 nm proved most advantageous. After pressing the stamps into the spin-coated pre-polymer films, we kept the resulting sandwiches at ambient conditions overnight followed by removing the pollen by sonicating in an ultrasonic bath for not more than 10 s.

Then we integrated the sensors into a measuring cell containing a piezo actuator and an air-stream for ensuring gas phase measurements of pollen. For actual sensing, the pollen was deposited on the piezo actuator before closing the measuring cell. Preliminary experiments showed that the optimal operating frequency of the actuator for generating airborne pollen in the cell is 1,000 Hz combined with a slight air stream of some 10 ml/min at ambient conditions.

The resulting frequency shifts were assessed both by passive measurements with a network analyzer (Agilent E5062A) as well as by operating the QCM in a custom-made oscillator circuit and reading out the resonant frequency with an Agilent 53131A frequency counter transferring the data to the computer via an Agilent GPIB USB interface. Atomic force microscopy (AFM) measurements were carried out by means of a VEECO Nanoscope IVa atomic force/scanning tunnelling microscope in contact-mode AFM.

Results and discussion

During the preliminary polymer screening, it turned out that optimal imprinting results could be achieved with layers having a thickness of 500 nm: the first main challenge in pollen imprinting is removing the template species from the polymeric matrix because the large interaction area between each individual pollen grain and the forming polymer of course increases binding strength. This also makes removing the template species a tedious task. As can be seen in the AFM image depicted in Fig. 2, applying ultrasound indeed leads to a polymer surface comprising the necessary moulds. Repeated AFM tests as well as light microscopy images revealed cavities uniformly distributed over the polymer surface. Besides sonication, layer height plays the second important role: exceeding 500 nm for the spun-on pre-polymer, it no longer releases the template at all. This limit cannot be avoided because the pollen cannot be removed by chemical etching, as it requires conditions harsh enough to affect the polymer matrix. Compared to the template dimensions, this seems rather small but corresponds very well to previous results obtained with erythrocytes, where similar layer heights turned out to be useful [23]. We trace this fact back to the binding mechanism between the polymer and the template and analyte, respectively: the main driving force here is geometrically aligned non-covalent interaction between

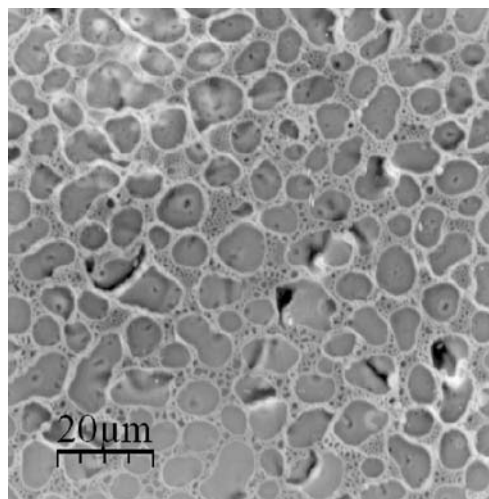


Fig. 2 Contact mode AFM image of a polyurethane imprinted with pollen

the recognition site in the final MIP and its respective target. From the main interaction types—H-bond, electrostatic, π - π stacking and Van-der-Waals forces—in the case of biospecies MIP, especially the first two have to be taken into consideration. Especially for hydrogen bonds, it is well known that if their number exceeds a certain threshold, the bond between the two binding partners becomes irreversible. The most well-known example for this behaviour is the DNA double strand being entirely held together by these non-covalent interactions between the nucleobases. Obviously, the same holds true for biological species: if the layer height exceeds the optimal value, binding of the template species becomes so strong that they can no longer be removed from the material. However, this also implies that there is an upper limit in analyte size for being suitable for surface imprinting procedures.

Hence, in the first step, the imprinting approach has indeed proven successful in reproducing the geometrical features of the pollen grains within the polymer. For additionally testing the actual sensing properties, we coated a QCM with such a material and applied it for analyzing air samples containing pollen. Figure 3 depicts the frequency behaviours of both an imprinted and a non-imprinted material when exposed to different such environments. Upon starting the air stream in the measuring cell, the frequency on both channels increases by several hundred Hertz. When then switching on the piezo, the frequencies increase further by about one third of the first frequency shift. Turning off the buzzer finally leads to frequency decrease, although the initial resonance level is not reached due to adhesion of pollen particles on the sensor surface. From the imprinting point of view, the most favourable feature of the sensor response characteristic is that the difference between the MIP and the reference is negative, thus clearly indicating mass uptake into the sensor layer.

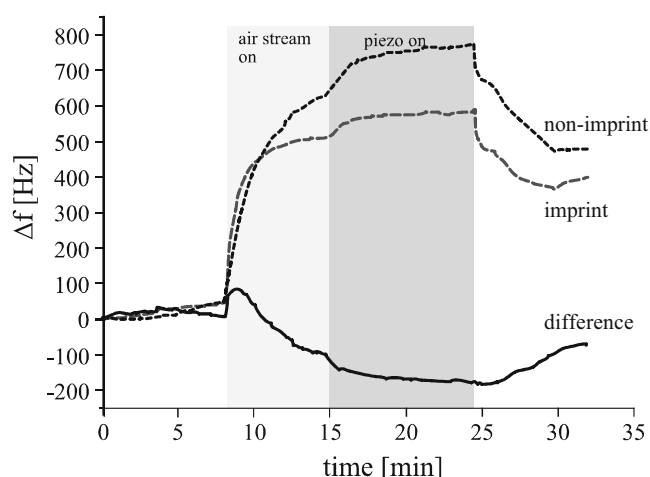


Fig. 3 Sensor characteristic of a QCM coated with nettle pollen MIP and a non-imprint, respectively

However, the net positive frequency shifts still require further elucidation. From previous measurements with yeast cells, we know that increasing frequency usually is a result of weakly bound species rolling over (flat) sensor surfaces [24], where the amount of mobile species determines the scale of the frequency increase: when the amount of yeast cells on a flat surface is high enough for aggregation, the positive frequency shifts turn into the expected negative ones. The reason for this behaviour is that, for the Sauerbrey equation being valid, it is necessary that the layer on the resonator is ideally rigid and hence shows no viscous interactions. However, mobile particles lead to slip between the device surface and the adhering layer causing the frequency to increase rather than to decrease [25]. Hence, for further investigating the positive pollen effects, we removed the sensor from the measuring cell and recorded its responses when rationally exposing it to varying amounts of pollen. The result of such a procedure can be seen in Fig. 4: when a small amount of pollen is carefully dispersed over the quartz (section I), the MIP gives rise to a negative effect of about 200 Hz on the measuring electrode and a positive effect of similar dimensions on the reference electrode. When further increasing the amount of pollen grains on the QCM surface (section II), the frequencies both on the imprinted and on the non-imprinted channels increase. However, on the latter, the effects are substantially larger, reaching 400 Hz. Slightly agitating the QCM manually (section III) leads to a frequency decrease of about 100 Hz on the measuring electrode, whereas the frequency on the reference substantially increases (by almost 1.5 kHz). Finally, subsequently adding pollen (section IV) results in a significant positive frequency response on both electrodes.

The frequency decrease on the MIP electrode on exposing to the small amount of pollen during the first part of the experiment is the result of the selective layer

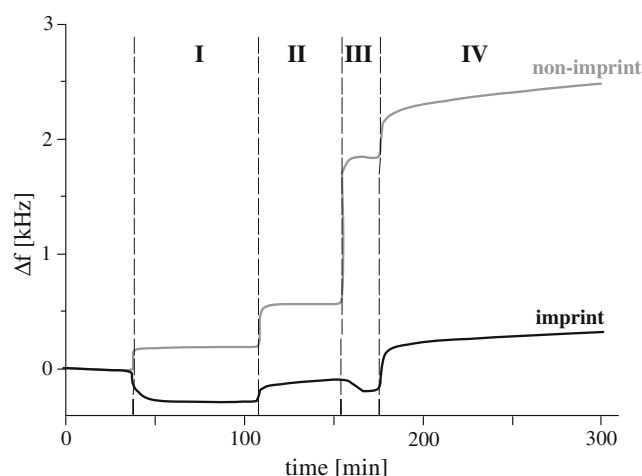


Fig. 4 QCM sensor response of MIP and non-imprint, respectively, towards nettle pollen-imprinted layer under ambient conditions. *I* exposure to nettle pollen, *II* increasing the amount of pollen, *III* QCM slightly shaken, *IV* further addition of nettle pollen

binding the individual grains via the cavities thus yielding Sauerbrey effects. However, in section II, the amount of pollen on the surface exceeds the amount of available binding sites. Hence, as soon as all of them are occupied, additional pollen on the layer surface can move freely on the surface. This then results in non-Sauerbrey behaviour. On the other hand, the reference electrode shows only positive—i.e. non-Sauerbrey—frequency responses indicating free mobility of the pollen on the surface. The increase in sensor response from section I to section II results from the increase in freely mobile particles on the surface due to adding further pollen. Thus, generally speaking, it exhibits a very flat surface and therefore generally shows non-Sauerbrey behaviour in this experimental setup. The frequency decrease on the measuring electrode after mechanically agitating can be explained by additional pollen grains having been incorporated from the second adlayer by recognition sites due to the external motion. This is a well-known effect also in macroscopic systems, where shaking large assemblies of globular structures results in more dense packing. The same shaking leads to substantial frequency increase on the reference electrode. However, the overall amount of pollen present on the device surface is the same as in the previous step, so this has to have a different reason. The non-Sauerbrey effect indicates that the number of freely moving particles has increased, although the overall amount of pollen remained constant on the device surface. Therefore, a part of the pollen has to have formed aggregates on the surface that are too large to move on the oscillating surface. Agitating the quartz mechanically partly destroys such aggregates (as can also be seen on the imprinted channel) and thus increases the number of mobile species on the surface. Further increasing the quantity of pollen on the sensor (section IV) again leads to higher

amounts of mobile particles on the surface and hence yields larger positive frequency shifts. Damping spectra obtained with a network analyzer corroborate this finding: as Fig. 5 shows, depositing pollen on a QCM channel leads to the same increase in resonance frequency as well as to somewhat improved damping. In contrast to the previous measurements, no oscillator circuit has been used in this case. Therefore, any electronic artefacts can be excluded and the non-Sauerbrey behaviour indeed be traced back to the grain mobility on the device surface.

In fact, the different positive and negative frequency shifts thus allow for directly distinguishing between selective and non-selective interactions between the analyte and the respective sensor. However, the question about the selectivity of the sensor materials still remains to be answered. For doing so, we carried out cross-selectivity measurements with nettle and birch pollen, respectively, by exposing the corresponding two MIPs to these analytes. Most of all, the two pollen species differ in size: whereas individual nettle pollen has a diameter of around 15 μm , birch pollen reaches 25 μm . Figure 6 summarises the results of these cross-selectivity measurements carried out in gas phase. On the birch pollen MIP with its larger diameter, both species lead to negative frequency shifts upon exposition. Concerning the own template, this is not surprising, as it is obviously strongly bound by the molecularly imprinted recognition material. However, the nettle pollen also gives a mass effect, as it is obviously able to enter the larger birch pollen cavities. In spite of the difference, pollen and cavity dimensions are in a similar range in this case, which prevents free mobility of the individual grains on the surface. Hence, they oscillate together with the sensor layer on the device, leading to the observable mass response, i.e. negative frequency shift. A reason for this may be the fact that all measurements take place in the gas phase: in contrast to aqueous solutions, air does not offer any “solvation” of the respective biospecies

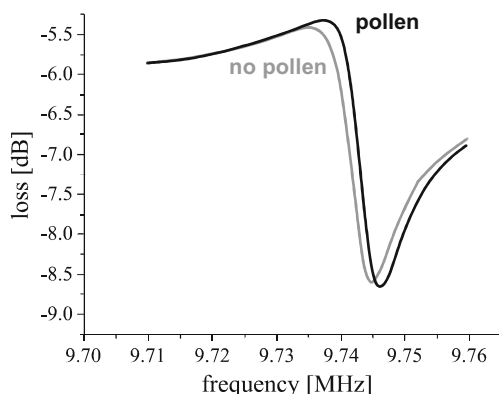


Fig. 5 Damping spectra of non-selective QCM before and after exposition to pollen

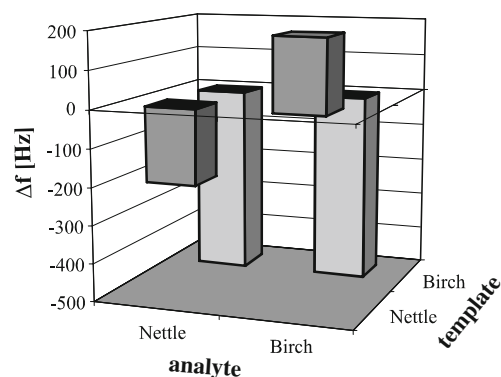


Fig. 6 Selectivity histogram for birch and nettle pollen MIP, respectively, on a QCM

and hence pushes the recognition equilibrium further towards the sensor layer. Hence, also more weakly bound species may lead to stronger non-specific adsorption, which in this case translates to a Sauerbrey-type sensor response due to the very restricted mobility of a particle within the imprinted cavity. Another reason for this fact is that the cavities are rather shallow compared to the size of the pollen particles (500 nm layer height compared to 15–25 μm in diameter). In combination with the multilayer deposition on the surface during recognition (which can be seen from all frequency shifts on the individual channels being positive, i.e. anti-Sauerbrey), this explains the comparably small mobility of the pollen within the MIP material.

However, for the nettle pollen MIP, the picture is completely different: in this case, one can observe negative frequency shifts on the QCM when being exposed to nettle pollen and to positive ones when exposed to birch. Furthermore, the amount of frequency increase for birch is in the same range as for a non-imprinted reference material. Therefore, the positive effect has to be explained by size of the birch pollen grains: physically, they do not fit into the smaller nettle pollen imprints. Hence, the interaction area between the pollen and an individual cavity is restricted to the upper edge of the recognition site, which is not sufficient for strong, non-covalent binding leading to a simultaneous oscillation of the sensitive layer and the incorporated analyte, as being necessary for meeting the prerequisites of the Sauerbrey equation. In consequence, this leads to mobility of the pollen grains on the surface and the frequency increases related to it. Both the broadband selectivity of the birch MIP and the high recognition ability of the nettle MIP strongly support that interaction between the outer shell of a bioanalyte and the pore walls in the polymer is the main driving force for binding between an individual cavity and an analyte particle: it has to be strong enough to prevent the pollen grains from moving freely over the surface and, on the other hand, weak enough for

releasing them again. Though selectivity improvements on the individual sensors still require fundamental effort, the sensor system as such is already feasible with the present results: combining the two imprints and a reference material together yields a three-electrode array that is highly suitable not only for detecting the presence of pollen but also for determining its origin via chemometric approaches. Assessing and optimizing selectivity of individual layers will require further effort both in the field of templating and in generating gas streams of defined pollen content.

Conclusion

Pollen monolayers can be generated very straightforwardly on PDMS due to the strong adhesion between them. They can be applied as stamps in an imprinting process in forming polyurethanes leading to tailored interaction materials on QCM. Such materials show mass effects as expected until all interaction sites are occupied. Then, they reveal non-Sauerbrey behaviour in the same way as non-imprinted QCM. The reason for this is pollen mobility on the surface, which gives rise to frequency increase rather than decrease. The effect depends on the amount of pollen deposited and can be verified by network analyzer measurements. Cross-selectivity assessment revealed birch MIP (pollen grain diameter 25 μm) give rise to negative effects both for birch and nettle pollen (diameter 15 μm). In contrast to this, nettle MIP exhibit Sauerbrey-type mass signals for nettle pollen and non-Sauerbrey behaviour for birch pollen. This is the result of the respective interaction areas, which is obviously too small in the case of birch pollen on nettle MIP. Here, the contact area is restricted to the ring-shaped region at the upper edge of the individual cavities, which is not sufficient for hindering mobility.

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