

Ultrafast Multiplexed-Allergen Detection through Advanced Fluidic Design and Monolithic Interferometric Silicon Chips

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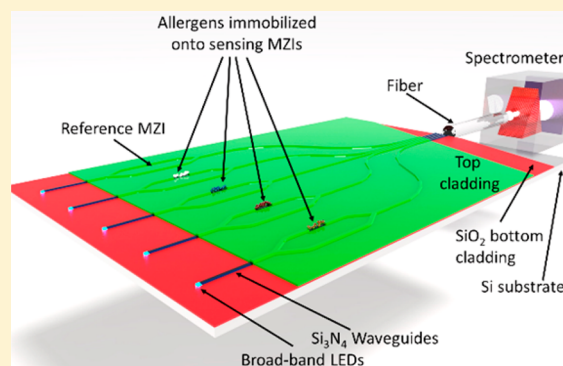
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Supporting Information

ABSTRACT: A silicon-based miniaturized sensor chip combined with an advanced microfluidic module for the simultaneous, label-free immunochemical determination of four allergens, bovine milk protein, peanut protein, soy protein, and gliadin, is presented. The sensor chip consists of an array of 10 broad-band Mach–Zehnder interferometers (BB-MZIs) monolithically integrated on silicon, along with their respective broad-band light sources. The BB-MZIs were biofunctionalized with the targeted allergens and their responses during immunoreaction were monitored by multiplexing their transmission spectra through an external miniaturized spectrometer. The assay is performed by running mixtures of calibrators or samples with the antibodies against the four allergens followed by an antisppecies specific antibodies solution. Employing a fluidic module of nearly one-dimensional geometry, that provided for uniform delivery of the reagents, CV values <6% were achieved for the responses of the 10 BB-MZIs, allowing for reliable multianalyte determinations. The analysis is completed in 6.5 min, and the detection limits were 0.04 $\mu\text{g/mL}$ for bovine k-casein, 1.0 $\mu\text{g/mL}$ for peanut protein, 0.80 $\mu\text{g/mL}$ for soy protein, and 0.10 $\mu\text{g/mL}$ for gliadin. The assays were accurate (recoveries 88–118%) and repeatable (intra- and interassay CVs <7% for all four allergens). Finally, the sensor was evaluated by analyzing samples from a cleaning in place system (CIP) of a dairy industry and the results obtained were in good agreement with those received by the respective ELISAs. The analytical characteristics of the sensor combined with the short analysis time and the small chip size make the proposed system an ideal tool for on-site multianalyte determinations.



Food allergies are considered a global health issue, since approximately 1–2% of adults and up to 8% of children are affected worldwide.¹ The problem is intensified by the fact that, in most cases, even traces of food allergens can trigger an allergic reaction with symptoms ranging from mild ones to severe anaphylactic reactions that may be proven fatal.^{2,3} Unfortunately, the only way to prevent an allergic reaction is to completely avoid the consumption of allergen-containing food. U.S.A. and EU authorities in order to protect consumers from the accidental consumption of allergy-inducing foods, have made mandatory the labeling of packaged foods indicating the presence of allergenic ingredients.^{4–7} The list of allergenic ingredients include milk, egg, soybean, peanut, fish, crustacean

shellfish, wheat and tree nuts, celery, mustard, sesame seeds, lupine, and mollusks.^{4–7} Gluten is another food ingredient for which labeling is mandatory, since gluten intolerance is the cause of celiac disease, a serious genetic autoimmune disorder with a prevalence of 0.5 to 1% worldwide.⁸ According to U.S.A. and EU regulations, food products labeled as “gluten-free” should contain less than 20 mg of gluten per kg, whereas the indication “low gluten” accounts for less than 100 mg of gluten per kg.⁹

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To comply with these regulations, the food industry should test both unprocessed and processed food against the presence of the major allergenic substances.^{2,10,11} In addition, in order to avoid unintentional contamination of food due to the processing of different types of products using the same facilities, food manufacturers employ cleaning in place systems (CIP) for automated cleaning of the production lines. To ensure the effectiveness of CIP procedure, regular monitoring of the process by analyzing rinsewater samples regarding the presence of allergens is required.¹²

Several methods have been developed for the determination of allergenic ingredients in food matrices and rinsewater samples. These include LC/MS-MS, DNA-based and immunological methods as well as immunosensors. LC-MS/MS methods are considered as highly reliable techniques for the simultaneous detection of multiple allergens.^{13,14} DNA-based methods are characterized by high sensitivity but suffer from a relatively large number of false positive results due to the fact that the presence of DNA does not guaranty the presence of the protein.^{11,15} On the other hand, immunological methods, mainly in the form of commercially available enzyme-linked immunosorbent assay kits (ELISA),^{16–21} have been widely adopted for allergen determination due to their high detection sensitivity, specificity and ease of use. Nonetheless, these methods lack the ability for multianalyte determinations in a single run. Flow-cytometric bead-based immunoassays detecting simultaneously 3–14 allergens have also been successfully developed.^{22,23} However, all these methods are not suitable for on-site analysis and should be performed in a laboratory environment by highly skilled personnel. Currently, on-site determinations are performed employing lateral flow immunochromatographic strips or dipsticks that, however, can provide only semiquantitative determinations of a single allergen.²⁴

Immunosensors have emerged in the past years as an attractive alternative solution for quantitative on-site determinations of multiple allergens in samples from food industry facilities. Still, most of the sensors developed are targeting to single-analyte detection,^{25–40} whereas the few examples of simultaneous allergen determination in food products involve surface plasmon resonance (SPR) techniques also confined to laboratory settings and are dependent upon bulky equipment that has to be operated by highly skilled personnel.^{41,42} The only exception, to the best of our knowledge, is a recently published portable electrochemical sensor capable of determining five allergens in different food matrices employing, however, immunomagnetic separation of the analytes from the sample and enzyme-labeled antibody for detection.⁴³

Our group has developed an optoelectronic platform built around silicon chips which accommodate an array of ten Mach–Zehnder Interferometers (MZIs) along with their respective silicon avalanche diodes (LEDs) monolithically integrated on the same silicon substrate. Continuous MZI transmission spectra collection is accomplished through an external miniaturized spectrophotometer.^{44,45} The broad-band nature of the LED along with the particular design of the planar waveguides implemented a new detection principle, that of broad-band Mach–Zehnder interferometry (BB-MZI). This detection principle has been explored, so far, for the real-time label-free immunoreaction monitoring^{44,45} as well as the determination of single analytes in certain food matrices.^{35,46} The aim of the present work was to investigate the multianalyte capabilities of the BB-MZI chip and to improve

its analytical performance. Special emphasis was given to the design of the fluidic module, an aspect often overlooked during immunosensors development, so as to achieve uniform delivery of the reagents to the 10 transducers of the same chip and improve the repeatability of the responses among the different interferometers of the chip. To this end, different microchannel geometries were investigated, ranging from the simple single volume unit to more elaborate channel configurations that interrogate the individual interferometers either along or across the waveguides. In addition, we investigated the assay configuration and the capability of regeneration and reuse of the chip as a means to suppress the analysis cost. The study was performed through the development of an immunosensor for the multiplexed determination of four allergens, namely, soy protein, peanut protein, bovine milk protein, and gliadin. The optimized chips were evaluated with respect to the determination of the four allergens in rinsewater samples from the CIP system of a food industry and the results were compared with those obtained for the same samples using the respective in-house developed ELISAs.

■ EXPERIMENTAL SECTION

Reagents. k-Casein from bovine milk (MW approximately 19000 Da), gliadin from wheat, bovine serum albumin (BSA), rabbit polyclonal antigliadin antibody (G9144), goat anti-mouse IgG antibody, goat antirabbit IgG antibody, goat antimouse IgG antibody Horse Radish peroxidase (HRP) labeled, goat antirabbit IgG antibody HRP labeled, and 3-(aminopropyl)triethoxysilane (APTES) were obtained from Sigma-Aldrich (Munich, Germany). Monoclonal antibodies against bovine k-casein (clones 33–4G10 and 33–6A10), soy protein (clones 26–6C9 and 26–5G4), peanut protein (clones 51–5G9 and 44–4G2) prepared according to a published procedure,⁴⁷ as well as soy and peanut protein extracts prepared as reported previously,⁴⁸ were provided by RIKILT Wageningen UR (Wageningen, The Netherlands). Microtiter plates were from Greiner Diagnostic GmbH (Bahlingen, Germany). Double-distilled water was used throughout the study.

Instrumentation. Silicon chips ($8.0 \times 4.2 \text{ mm}^2$) accommodating an array of 10 integrated BB-MZIs along with their respective LEDs (Figure S1) were fabricated as described in previous publications.^{35,44,45} The measurement setup included a custom designed docking station that supported both the fluidic and electrical connections. An optical fiber aligned to the docking station collected the transmission spectra from the edge of the chip and transferred them to a miniaturized spectrometer (QE65000, Ocean Optics; Dunedin, FL; Figure S2). The operation of the spectrometer was synchronized by a dedicated electrical multiplexer that sequentially turned on/off the 10 LEDs of the BB-MZI array every 1 s. Thus, one spectrum from each one BB-MZI of the chip was recorded every 10 s by the PC, which controlled the measuring apparatus through a dedicated Labview application. In order to deliver the solutions necessary for the analysis, a microfluidic module was attached onto the biofunctionalized chip. This module was made of a $800 \mu\text{m}$ thick poly(methyl methacrylate) (PMMA) foil with drilled fluid inlet and outlet holes. The fluidic cell was created by photolithography of a $75 \mu\text{m}$ thick dry film photoresist (Pyrallux PC1015, DuPont) laminated on the PMMA that also provided the seal to the chips by acting as a pressure sensitive adhesive layer.^{35,45} The chips, after biofunctionalization, were

assembled with the fluidic module, placed on a handling frame and inserted into the docking station of the measuring apparatus (Figure S2). All reagents were delivered to the chip using a peristaltic pump. An injector (Rheodyne 772Si) equipped with a 100 μL loop was placed between the pump and the docking station for the introduction of reagents in flow.

Chips Biofunctionalization. The BB-MZI chips were chemically activated through hydrophilization by oxygen plasma treatment (30 s, 10 mTorr) in a reactive ion etcher followed by immersion in a 0.5% (v/v) APTES aqueous solution for 2 min. The silanization procedure was completed by gentle washing of the chips with distilled water and curing at 120 $^{\circ}\text{C}$ for 20 min. For the biofunctionalization, multiple chips were placed on a custom designed holder with milled recessions (Figure S3). Allergen solutions (100 $\mu\text{g}/\text{mL}$ in 50 mM carbonate buffer, pH 9.2) were then deposited on the sensing windows areas of the 10 BB-MZIs using the BioOdyssey Calligrapher Mini Arrayer (Bio-Rad Laboratories Inc.; Hercules, CA). The 10 BB-MZIs were divided into four groups of interferometer pairs and each pair was spotted with one of the four allergen solutions, according to the layout of Figure 1. The remaining two BB-MZIs were spotted with 100

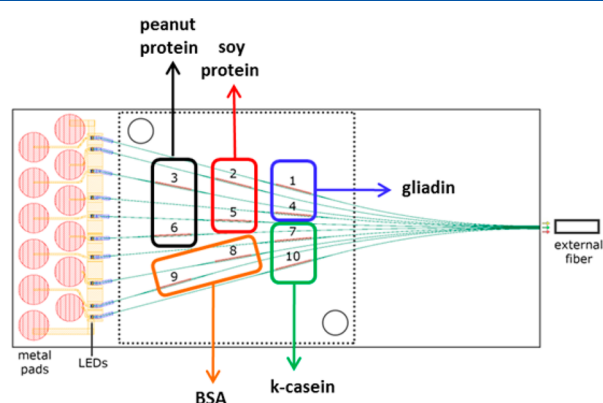


Figure 1. Schematic of the chip depicting the BB-MZIs pairs spotted with the different biomolecules.

$\mu\text{g}/\text{mL}$ BSA solution to monitor nonspecific binding. Appropriate spotting protocols were developed based on deposition of multiple overlapping spots in order to uniformly cover the BB-MZI sensing areas. The spotted chips were incubated overnight at 4 $^{\circ}\text{C}$ (75% humidity). Then, they were washed with 10 mM PBS, pH 7.4 (washing buffer), blocked through a 1 h incubation with 10 g/L BSA in 0.1 M NaHCO_3 , pH 8.5, washed again, dried under a nitrogen stream, and kept in a desiccator at 4 $^{\circ}\text{C}$ until use.

Calibrators and Samples. Bovine k-casein, whole peanut, and soy protein solutions were prepared at a final concentration of 1 mg/mL in 10 mM PBS, pH 7.4, containing 0.5 g/L NaN_3 . Gliadin stock solution was also prepared in the same buffer through overnight stirring at 4 $^{\circ}\text{C}$. Then, the solution was centrifuged for 20 min at 10000 g, and the gliadin concentration at the supernatant was determined by measuring the absorbance at 280 nm using an extinction coefficient value of 0.9551 L/gr $\cdot\text{cm}$.⁴⁹ Calibrators either for single allergen or mixtures thereof were prepared in assay buffer (10 mM PBS, pH 7.4, containing 5 g/L BSA and 0.5 g/L NaN_3) or in tap water. Rinsing water samples were collected from different product lines of Delta Foods S.A. after the final step of the CIP

procedure (cold wash cycle). To each of these samples, sodium azide was added to a final concentration of 0.5 g/L and the samples were aliquoted and stored at -20°C for up to 2 months. Additionally, 23 rinsing water samples spiked with different quantities of the four allergens have been provided by Delta Foods S.A. for the correlation study with the respective in-house-developed ELISAs.

Allergens Detection with the Multianalyte BB-MZI Immunosensor. Prior to the assay, equal volumes of calibrators or samples and a solution containing antibodies against the four allergens in assay buffer (for bovine k-casein: Mab 33–4G10 and 33–6A10 at 0.5 $\mu\text{g}/\text{mL}$ each; for soy protein: Mab 26–5G4 and 26–6C9 at 3 $\mu\text{g}/\text{mL}$ each; for peanut protein: Mab 51–5G9 and 44–4G2 at 2 $\mu\text{g}/\text{mL}$ each; and for gliadin Pab 1:80 dilution) were mixed and preincubated for 10 min at room temperature (RT). For the assay, 100 μL of the preincubated mixtures of calibrators/samples with the antibodies were introduced to the flow, followed by 100 μL of a mixture containing 10 $\mu\text{g}/\text{mL}$ of antimouse IgG and 10 $\mu\text{g}/\text{mL}$ of antirabbit IgG antibody in assay buffer. Then, 100 μL of a 50 mM HCl solution was injected, followed by equilibration with assay buffer prior to the next run.

Signal Analysis. The transmission spectra collected during the assay were processed with Discrete Fourier Transform and presented as phase shifts in TE polarization for each one of the 10 BB-MZIs of the chip. The phase shift corresponding to each calibrator or sample represents the sensor signal (S). Thus, for the preparation of the calibration curves, the percent ratios of the calibrators signal (S_X) to the zero calibrator signal (S_0) were plotted against the respective allergen concentration in log-log scale.

RESULTS AND DISCUSSION

Selection of Assay Configuration. A competitive immunoassay format based on immobilization of the respective analyte onto the sensor was followed. The selected assay format can be realized in a single step (immunoreaction of allergen-specific antibodies with the immobilized allergen) or in two steps where, after the reaction with the allergen-specific antibodies, a secondary antibody is run over the chip. In Figure 2a, the signal evolution for the single step assay format is presented. As shown, the polyclonal anti-gliadin antibody presented a slower kinetic with respect to monoclonal antibodies used for the other three allergens. Nonetheless, signals ≥ 1 rad (signal at least 3 orders of magnitude higher than the baseline noise) were obtained for all allergens after 15 min of reaction. On the other hand, when the two-step assay was implemented, total signals higher than 1 rad (Figure 2b) were obtained after approximately 5 min of reaction with the primary and the secondary antibody, that is, in a total assay time of 10 min. It should be emphasized that no response was obtained from a BB-MZI functionalized with BSA (nonspecific binding). Therefore, the two-step assay configuration was selected, since it considerably reduces the total assay duration and the allergen-specific antibody consumption.

Microfluidic Module Design. An important parameter investigated in the present study was the microfluidic design, since it defines the reagents distribution over the chip surface and could, therefore, have a direct impact on the repeatability of the responses among the sensors of the same chip. The fluidic used in the preliminary experiments, referred as “plain”, was the one depicted in Figure 3a1. It was observed that using

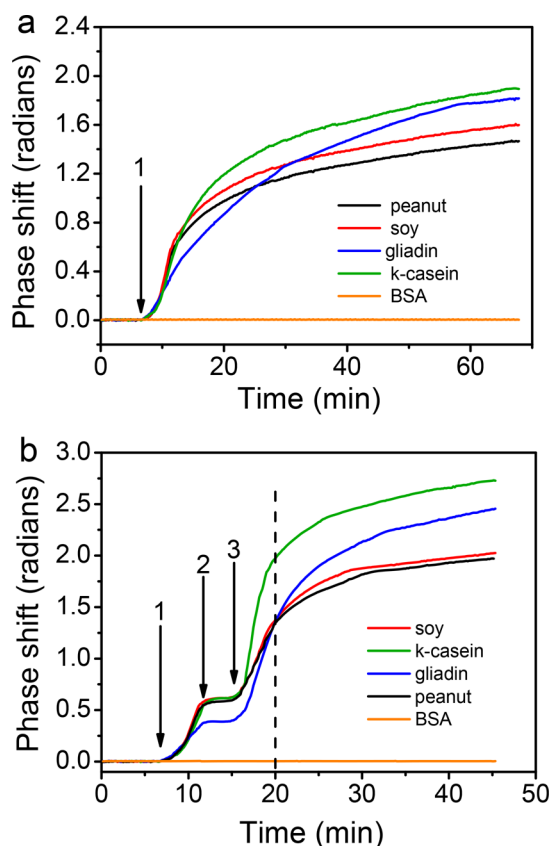


Figure 2. Sensor responses obtained upon running over the chip (a) allergen-specific antibodies (arrow 1) for 60 min or (b) allergen-specific antibodies for 5 min (arrow 1 to 2) followed by assay buffer for 3 min (arrow 2 to 3) and secondary antibodies (arrow 3 to end) for 30 min. Green line: bovine k-casein; blue line: gliadin; red line: soy protein; black line: peanut protein; and orange line: BSA (blank). The dashed black line indicates the signals obtained after reaction for 5 min with the secondary antibodies. Flow rate 50 $\mu\text{L}/\text{min}$.

this fluidic, the CVs of the responses of the 10 BB-MZIs from the same chip ranged between 12 and 15%. These values were not well-suited for the multianalyte determinations targeted in the present study, according to which only two sensors per chip will be dedicated to each analyte. Therefore, the repeatability of the responses of the different BB-MZIs of the same chip is critical for the reliability of the determinations. To improve the intrachip responses repeatability, two more fluidic modules were designed and fabricated, the first referred to as “S-shaped” (Figure 3a2) and the second as “serpentine” (Figure 3a3). The rationale was to compare the single volume plain fluidic with a channel that directs the reagents flow along (serpentine fluidic) or across the waveguides (S-shaped fluidic).

The height of the flow cell for all three designs was 75 μm , while the width was about 3, to 0.8, and 0.3 mm for the plain, the S-shaped and the serpentine fluidic, respectively. The positioning of the three fluidic cells toward the 10 BB-MZIs of the chip is depicted in Figure 3a. The three fluidics were evaluated with respect to the magnitude and repeatability of the zero calibrator signals obtained from the 10 BB-MZIs of the same chip using a flow rate of 50 $\mu\text{L}/\text{min}$. In Figure 3b, zero calibrator signals for the four allergens obtained using the three different fluidics are presented. As shown, the mean signal values obtained for each allergen were similar

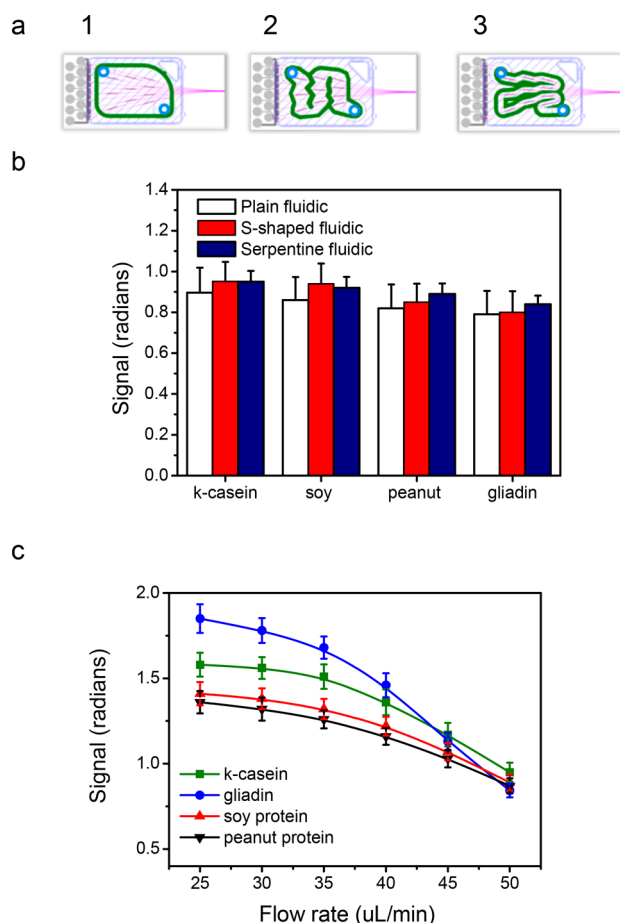


Figure 3. (a) Schematic depicting the layout of plain (a1), S-shaped (a2), and serpentine fluidic (a3) and their positioning with respect to the topology of the BB-MZIs of the chip. Fluid inlet and outlet are visualized by the blue circles. (b) Sensor responses (mean of 10 BB-MZIs $\pm$ SD) obtained for the zero calibrators of the four allergens using the three different fluidics. Plain fluidic: white bars; S-shaped fluidic: red bars; and serpentine fluidic: blue bars. The flow rate was, in all cases, 50 $\mu\text{L}/\text{min}$. (c) Sensor responses obtained for the zero calibrators of the four allergens using the serpentine fluidic vs the reagents flow rate. Green line: bovine k-casein; blue line: gliadin; red line: soy protein; and black line: peanut protein. Each point is the mean value of 10 BB-MZIs. Error bars correspond to $\pm$ SD.

independently of the fluidic used. However, the repeatability of the signals obtained from the 10 BB-MZIs of the same chip was significantly improved when the serpentine fluidic was used (intrachip CVs < 6%) as compared to the plain and S-shaped ones (CVs in the range of 12–15%). The improved performance of the serpentine fluidic could be attributed to the nearly one-dimensional geometry and the associated laminar flow as well as the higher speed of the fluid in the narrower serpentine channel compared to the other two channel types. Thus, the serpentine fluidic was selected for further experimentation.

Flow Rate Optimization. The flow rate of the reagents was optimized with respect to the signal magnitude and repeatability obtained from the 10 BB-MZIs of a chip. In order to run a fixed reagents volume over the chip, an injector facilitated with a 100 μL loop was employed. The flow rate applied ranged from 25 to 50 $\mu\text{L}/\text{min}$. Thus, the total assay duration (reaction with primary and secondary antibody) varied from approximately 480 to 240 s, depending on the flow

rate. In Figure 3c, the responses obtained for the zero calibrators of the four analytes as a function of the flow rate are provided. As shown, when the flow rate was decreased from 50 to 35 $\mu\text{L}/\text{min}$, the signals were increased by approximately 50% for k-casein, soy, and peanut protein and almost 100% for gliadin. The signal increase observed with the decrease of the flow rate could be ascribed to the extension of the assay time. This effect is more pronounced in case of gliadin due to the slower anti-gliadin antibody kinetics compared to the other three allergens (as discussed in section "Selection of Assay Configuration"; Figure 2a). Further decrease of the flow rate to 25 $\mu\text{L}/\text{min}$ provided a moderate signal increase of about 10–15%; however, the total assay time was increased from 390 to 480 s. Concerning the repeatability of the responses of the 10 BB-MZIs of a chip, the CVs were lower than 6% for the whole range of flow rates tested. Thus, in order to achieve the highest possible response in the shortest assay time, a flow rate of 35 $\mu\text{L}/\text{min}$ was selected for the final protocol, providing a total assay time of 6.5 min (3.25 min for each step).

Immunoassay Optimization. All the assay parameters, including the concentration of protein used for coating, the concentration of the antibodies, the composition of the blocking solution and the assay buffer have been optimized and those selected are mentioned in the Experimental section. In addition, in an attempt to improve the assay sensitivity, preincubation of the calibrators with the allergen-specific antibodies was tested. In Figure 4, the percent inhibition values

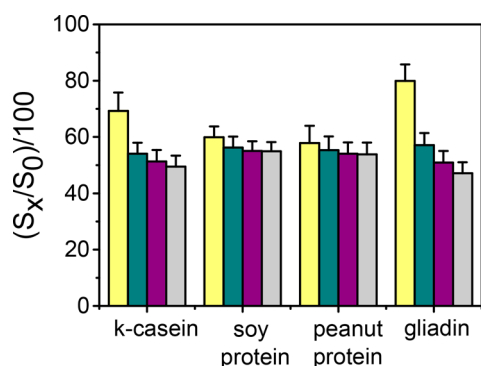


Figure 4. Percent inhibition values with respect to zero calibrator signals obtained using calibrators containing 0.5 $\mu\text{g}/\text{mL}$ of bovine k-casein, 6.0 $\mu\text{g}/\text{mL}$ of peanut protein, 4.0 $\mu\text{g}/\text{mL}$ of soy protein or 0.5 $\mu\text{g}/\text{mL}$ of gliadin when preincubation with the allergen-specific antibody solution for 0 (yellow bars), 10 (green bars), 30 (purple bars), or 60 min (gray bars) was performed prior to the introduction into fluidic cell. Each point is the mean of 3 chips $\pm$ SD (10 replicates per allergen per chip).

with respect to zero calibrators signals achieved using calibrators containing 0.5 $\mu\text{g}/\text{mL}$ of bovine k-casein, 6.0 $\mu\text{g}/\text{mL}$ of peanut protein, 4.0 $\mu\text{g}/\text{mL}$ of soy protein or 0.5 $\mu\text{g}/\text{mL}$ of gliadin for preincubation times of 0, 10, 30, and 60 min are presented. As shown, for the bovine k-casein and gliadin assays, a 10 min preincubation considerably improved the percent inhibition obtained for the respective calibrators tested, while only marginal improvement was observed for longer preincubation times (30 and 60 min). On the other hand, preincubation did not improve the percent inhibition values for soy and peanut protein assays. In order to harmonize the four allergen assay conditions, a 10 min preincubation of the calibrators or the samples with the antibodies solution was adopted in the final protocol.

Immunosensor Regeneration and Stability. Regeneration of the allergen-modified biochips so as to be used for repetitive determinations is of great importance, since it can considerably reduce the analysis cost. Therefore, the regeneration of biofunctionalized chips through removal of the bound antibodies (primary and secondary) after the assay by running an appropriate solution was investigated. A schematic of the assay/regeneration cycle followed is depicted in Figure 5a, along with the expected signal response for two sequential assay/regeneration cycles. The regeneration solutions evaluated were the following: a commercially available IgG elution buffer for affinity chromatography, 0.1 M glycine-HCl buffer, pH 2.5, 50 mM NaOH, and 50 mM HCl. To test these solutions 100 μL were introduced via the injector, after completion of the assay, with a flow rate of 35 $\mu\text{L}/\text{min}$, followed by equilibration with assay buffer. Then, the secondary antibody solution was introduced in order to determine the percent of the allergen-specific antibody remaining onto the surface with respect to the initial zero calibrator signal. As shown in Figure 5b, where the percent ratios of the signals obtained after regeneration with the different solutions are depicted for the four assays, almost complete removal (less than 3% of the zero calibrator signal) of the bound antibodies is achieved for bovine k-casein, soy protein, and gliadin assay with all the regeneration solutions tested. In case of peanut protein assay, however, the use of the commercial IgG elution buffer, glycine/HCl buffer, and 50 mM NaOH failed to remove efficiently the bound antibodies leading to residual signals of 29.9, 18.1, and 8.5%, respectively. On the other hand, when 50 mM HCl was employed as the regeneration solution, a residual signal corresponding to 2.5% of the zero calibrator signal was achieved. Thus, the 50 mM HCl solution was selected as the regeneration medium.

The stability of the immobilized onto the chip allergens versus repetitive assay/regeneration cycles with the selected solution was assessed. The mean values of the zero calibrator signals obtained from single-analyte chips after 20 assay/regeneration cycles performed within the same day are provided for all allergens in Figure 5c.

As shown, the signals obtained from chips functionalized with bovine k-casein or gliadin were stable for at least 20 regenerations. On the other hand, for chips spotted with soy or peanut protein the zero calibrator signal starts to deteriorate after 12 assay/regeneration cycles. Therefore, the biofunctionalized chips could be regenerated for at least 10 times. In addition, it is worth mentioning that the regeneration step is simply performed by injection of the necessary solution in the same manner and at the same flow rate as the assay buffers and samples.

Multianalyte Determination. Once the single analyte assays had been optimized, the potential to perform multianalyte determinations on the same chip was evaluated. For this purpose, the ten BB-MZIs of a single chip were spotted in pairs with peanut protein, soy protein, gliadin, bovine k-casein, or BSA (as shown in Figure 1), so as to determine both the specific and the nonspecific binding signal in a single run. In order to test the specificity of the response of the functionalized sensors, the antibodies against each one of the allergens were sequentially run over the chip, each one followed by reaction with secondary antibody and regeneration. In Figure 6, the responses obtained from the 10 BB-MZIs of a chip functionalized and assayed as described above are demonstrated. As shown, response was obtained from the allergen-

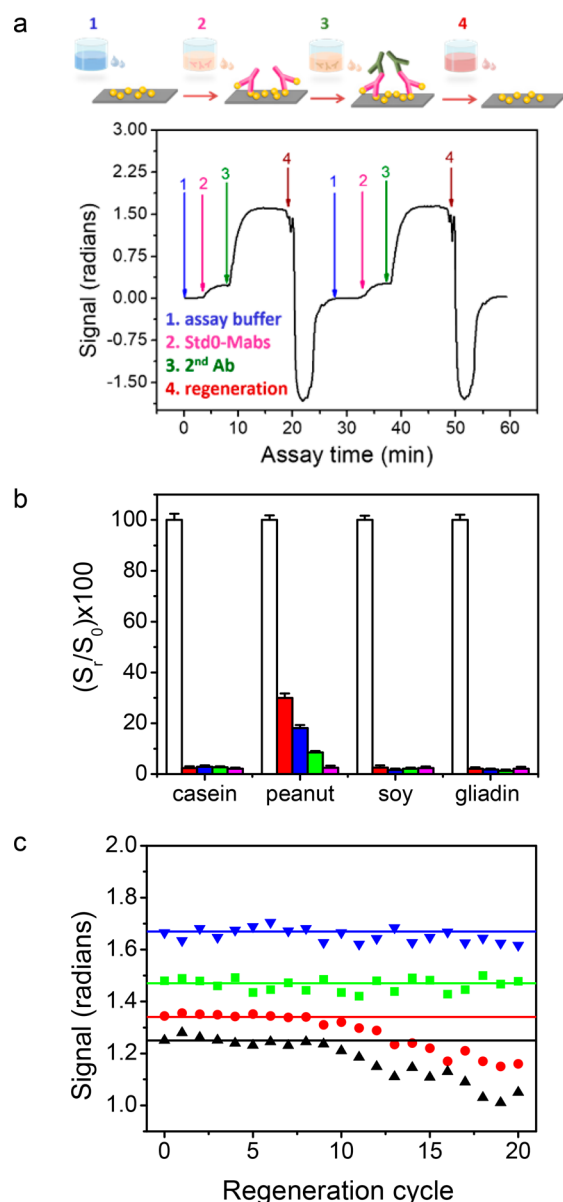


Figure 5. (a) Schematic of the assay/regeneration cycle (upper part) along with an indicative response of a BB-MZI upon two consecutive assay/regeneration cycles (down part). (b) Percentage of residual signal (S_r) determined by flowing the secondary antibody solution after regeneration with IgG elution buffer (red columns), 0.1 M glycine-HCl buffer, pH 2.5 (blue columns), 50 mM NaOH (green columns), or 50 mM HCl (magenta columns) with respect to the initial zero calibrator signal (white columns) for the four allergens. Each point is the mean of 3 chips (10 replicates per allergen per chip, hence, 30 replicates per allergen) $\pm$ SD. (c) Zero calibrator signals obtained after repetitive assay/regeneration cycles using chips functionalized with bovine k-casein (squares); soy protein (circles); peanut protein (up triangle); gliadin (down triangle). Lines correspond to mean values of the first 10 measurements. Each point is the mean of the 10 MZIs of a chip (10 replicates per allergen).

functionalized BB-MZIs only when the respective antibody was run over the chip, indicating the high specificity of the selected antiallergen specific antibodies against the coanalyzed allergens. In addition, the magnitude of the signals obtained for each one analyte from the BB-MZIs functionalized with the same molecule was quite repeatable (CV less than 5%) and

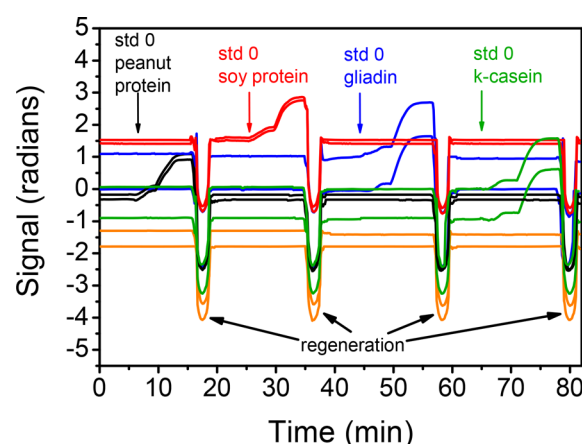


Figure 6. Real-time responses obtained from a chip where 2 out of 10 sensors have been modified with k-casein (green lines), 2 with soy protein (red lines), 2 with peanut protein (black lines), 2 with gliadin (blue lines) and the rest 2 with BSA (orange lines) when antibodies against peanut protein (arrow 1), soy protein (arrow 2), gluten (arrow 3), and k-casein (arrow 4) were sequentially run over the chip, each one followed by reaction with secondary antibody and regeneration.

similar to those obtained from single analyte chips. Moreover, the two BB-MZIs modified with BSA, did not provide any readable signal throughout the experiment, demonstrating the absence of nonspecific binding of (allergen-specific and secondary) antibodies to the chip surface. Thus, the proposed biosensor can be employed for the simultaneous determination of the four allergens in a single-run.

Analytical Characteristics of the Multiplexed-Allergen Immunosensor. Typical calibration curves obtained from the multiallergen functionalized chips for each one of the analytes are presented in Figure S4. The limit of detection (LOD) was determined as the allergen concentration corresponding to a signal equal to $-3SD$ of the mean zero calibrator signals obtained by running 10 chips (2 BB-MZIs per allergen per chip). In Table 1, the LOD, the IC_{50} value, and the linear working range for each one of the assays are provided.

Table 1. Analytical Characteristics of the Four Allergen Assays

analyte	bovine k-casein	gliadin	soy protein	peanut protein
LOD ($\mu\text{g/mL}$)	0.04	0.10	0.80	1.0
IC_{50} ($\mu\text{g/mL}$)	0.64	1.1	4.2	6.6
linear range ($\mu\text{g/mL}$)	0.10–2.0	0.20–20	2.0–16	2.0–20

The accuracy of the developed immunosensor was evaluated through recovery experiments using tap water (as an allergen-free sample) and rinsing water samples from the final step of the CIP procedure applied onto different product lines of Delta Foods S.A. The samples were analyzed prior to and after the addition of known amounts of the four allergens. It was found that the tap water and the rinsing water samples provided signals similar to those obtained using the zero calibrator prepared in assay buffer indicating that no detectable quantities of the four allergens existed in these samples. The percent recovery values determined for both the tap and rinsing water samples spiked with the allergens are provided in Table S1. As

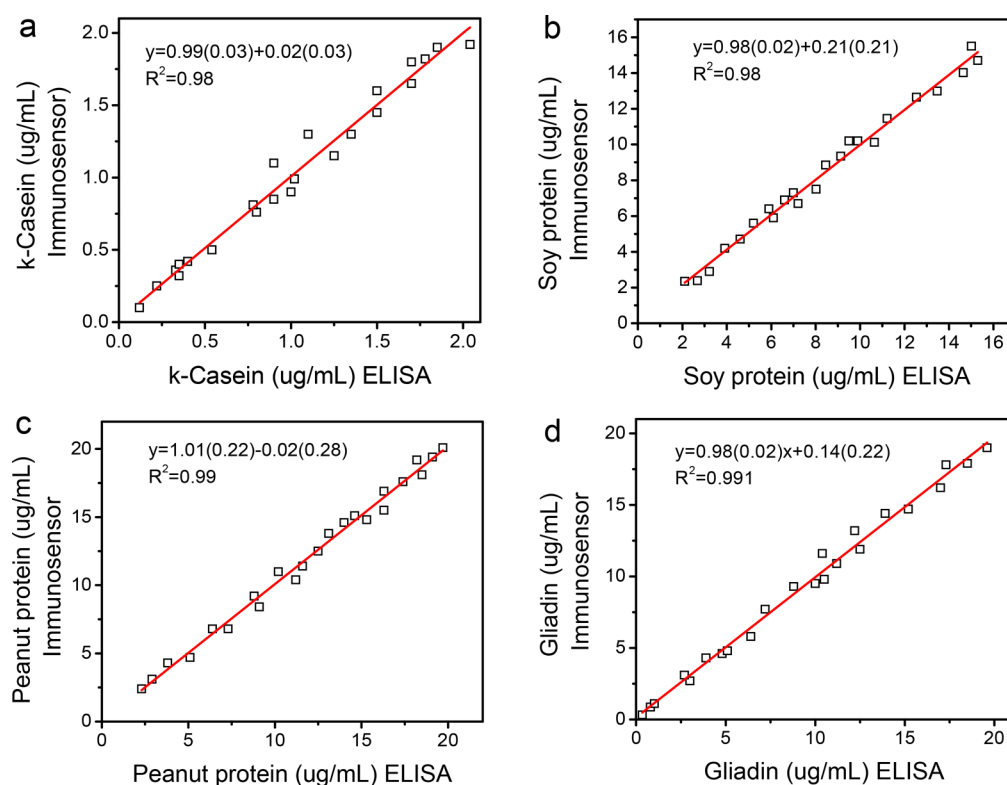


Figure 7. Correlation of the results obtained with the immunosensor developed to those provided by single-analyte ELISAs for (a) k-casein, (b) soy protein, (c) peanut protein, and (d) gliadin for CIP water samples spiked with different concentrations of the four allergens. The linear regression equations are provided as inserts.

shown, the recovery values ranged between 87.5 and 110% for all samples and analytes, verifying the accuracy of the determinations performed in rinsing water samples with the immunosensor developed. Furthermore, 23 rinsing water samples collected from the different product lines of Delta Foods S.A., after the final step of the CIP procedure and spiked with various amounts of the four allergens were analyzed with the immunosensor developed and the in-house developed ELISAs (see [Supporting Information](#) for ELISA protocol and calibration curves). As shown in [Figure 7](#), where the correlation of the values obtained with the two methods is provided, there is an excellent agreement between the results obtained with the developed immunosensor with those provided by the respective ELISAs. These findings further supports the accuracy of the determinations performed with the immunosensor developed.

The precision of the developed four-allergen immunosensor was assessed through determination of intra- and interassay CVs. For this purpose, tap water was spiked with two concentrations of each one of the allergens within the dynamic range of the assays (0.4 and 1 $\mu\text{g/mL}$ bovine k-casein, 0.4 and 2.5 gliadin $\mu\text{g/mL}$, 5 and 10 $\mu\text{g/mL}$ soy protein, and 4 and 16 $\mu\text{g/mL}$ peanut protein) to create two control samples. For the determination of the intra-assay CVs, each control was run three times in the same day, while the interassay CVs were calculated by running the control samples two times per day in 12 days over a period of one month. The intra-assay CVs ranged from 4 to 8%, and the interassay CVs ranged between 12% and 16% for all assays.

The stability of the biochips was also determined by running chips spotted and dried, as described in the [Experimental Section](#), over a 3 month period. The functionalized chips were

found to be extremely stable since the variation of the mean signal values obtained was less than $\pm 7\%$ for all analytes with respect to the signal values obtained from freshly prepared chips.

Comparison with Other Label-Free Immunosensors for Allergen Detection. The majority of the label-free sensors reported in the literature (listed in [Table S2](#)) for the determination of the allergens targeted in the present study are capable of determining a single analyte, whereas only two reports refer to multianalyte sensors.^{50,51} The proposed sensor provides comparable or higher sensitivity in a total analysis time similar or much shorter than most of the single-analyte sensors. There is only one report regarding a piezoelectric sensor³⁰ that determines gliadin with 10 $\times$ lower LOD, which, however, requires 40 min to complete the assay. Regarding the label-free multianalyte sensors, there are two reports, one based on SPR⁴² and the other on imaging SPR (i-SPR).⁴¹ The SPR sensor was employed for the detection of soy and peanut protein in milk powder with LODs of 500 and 40 ppm, respectively, in an assay time of 5 min. The i-SPR sensor, on the other hand, was capable to simultaneously detect 13 allergens in cookies and dark chocolate, including k-casein, peanut and soy protein, and provided LODs of 200, 3200, and 5000 ppm, respectively, in an assay time of 7 min. Thus, the proposed sensor offers considerable higher sensitivity with respect to label-free multiallergen sensors with comparable analysis time.

CONCLUSIONS

A miniaturized immunosensor based on silicon chips accommodating 10 MZIs integrated along with their corresponding light sources for the simultaneous, multiplexed

label-free determination of four allergens is demonstrated for the first time. The chips were combined with a specially designed fluidic that provided uniform delivery of the reagents onto the 10 BB-MZIs of the chip due to its nearly one-dimensional geometry. As a result, the repeatability of the responses of the 10 BB-MZIs of the same chip was considerably improved compared to other fluidic designs allowing for reliable multiplexed determinations. The total assay time was only 6.5 min and the LODs achieved were 0.04 $\mu\text{g/mL}$ for bovine k-casein, 1.0 $\mu\text{g/mL}$ for peanut protein, 0.80 $\mu\text{g/mL}$ for soy protein, and 0.10 $\mu\text{g/mL}$ for gliadin. The sensor was evaluated using rinsing water samples from the CIP system of a dairy industry and the results were in good agreement with those received for the same samples by the respective in-house developed ELISAs. The LODs achieved are lower or at least comparable with the respective commercial single-analyte ELISAs and immunochromatographic strips currently used for allergen determination in rinsing water samples from CIP systems.²⁷ Moreover, the immunosensor developed could be regenerated and reused for at least 10 times decreasing the analysis cost. Thus, taking into account that four allergens are determined per run and that the chip could be reused for at least 10 times, the foreseen cost per analysis and analyte is approximately \$0.16 (based on an associated cost of \$6.5 for chip and fluidic fabrication, chip chemical activation and biofunctionalization, and the immunoassay reagents required per run), calculated for a 8000-chip batch. The analytical characteristics of the proposed sensor, combined with the short analysis time and the small chip size, make it ideal for on-site determination of different panels of analytes, especially targeting point-of-need applications.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02321.

Chip layout; Images of docking station and external optical fiber position; Immunosensor calibration curves; ELISA procedure for the detection of four allergens in rinsing water; Recovery experiment results; Comparison of the immunosensor developed with other literature immunosensors for detection of allergens (PDF).

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The manuscript was written through contribution of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Ultra-fast multiplexed-allergen detection through advanced fluidic design and monolithic interferometric silicon chips

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Chip layout

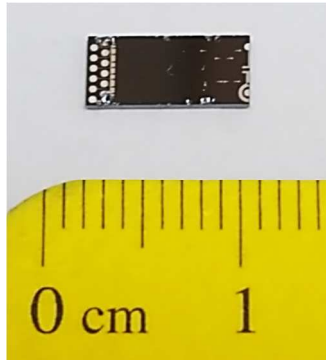
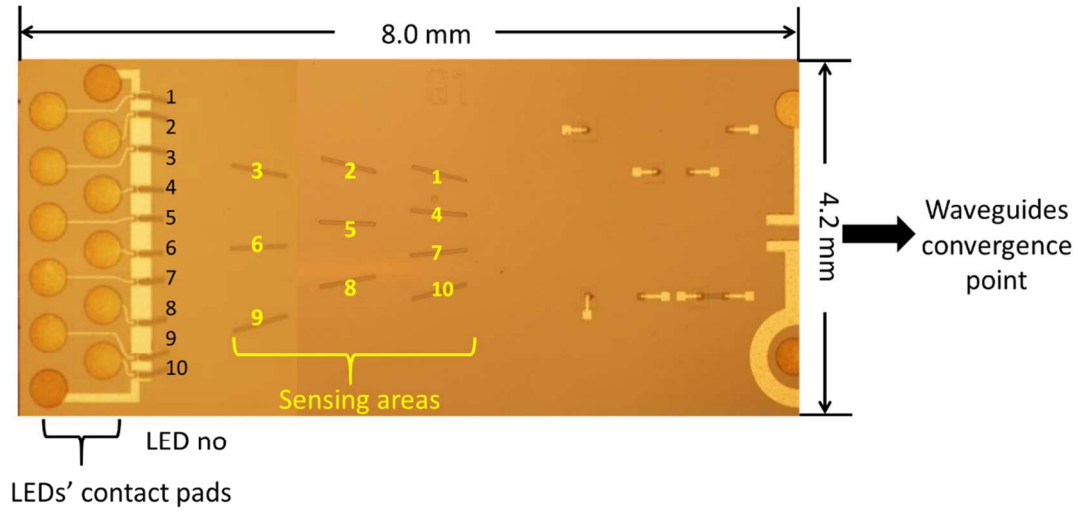


Figure S1. (Up) Optical microscope image of the chip. Shown are the 10 LEDs' contact pads, the position of the 10 LEDs, the position of the 10 BB-MZIs sensing areas, and the point where the 10 waveguides converge. The rib waveguides are not visible under the optical microscope. The dimensions of the chip are also indicated. (Down) Image of the chip along with a ruler.

Docking station and external optical fiber position

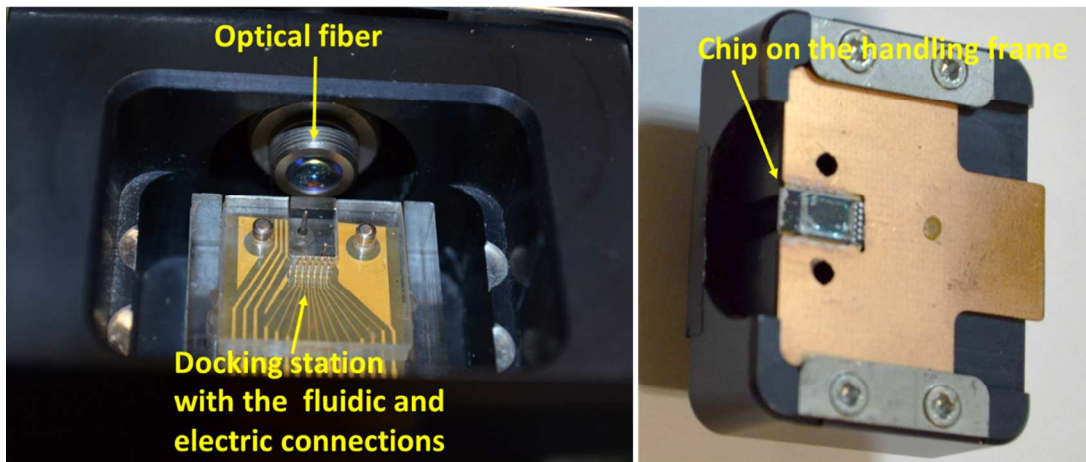


Figure S2. (Left) Image of the docking station along with the external optical fiber that transfers the transmission spectra of the 10 BB-MZIs of the chip to the spectrophotometer. (Right) Image of the chip with fluidic on top placed on the handling frame ready for placement onto the docking station.

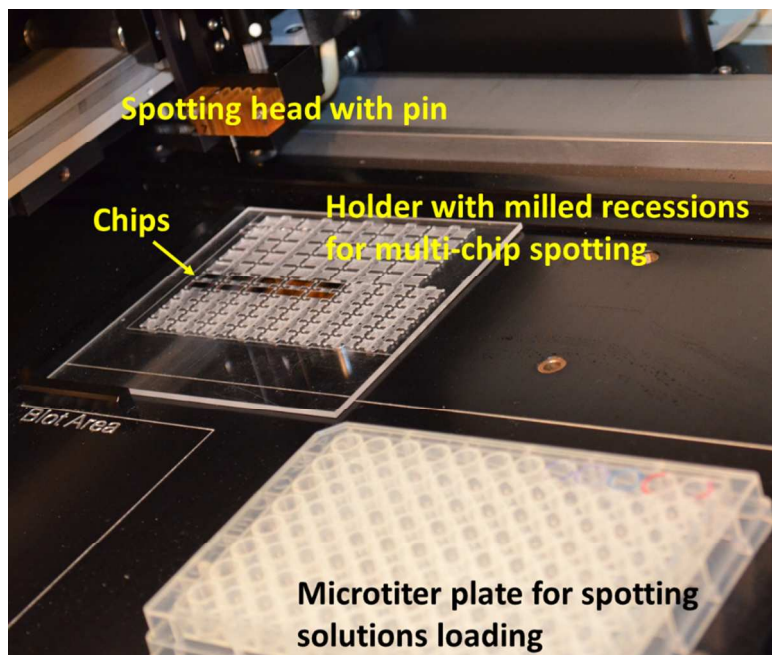


Figure S3. Image of the spotter interior showing the positioning of the holder with milled recessions for multi-chip spotting.

Immunosensor calibration curves

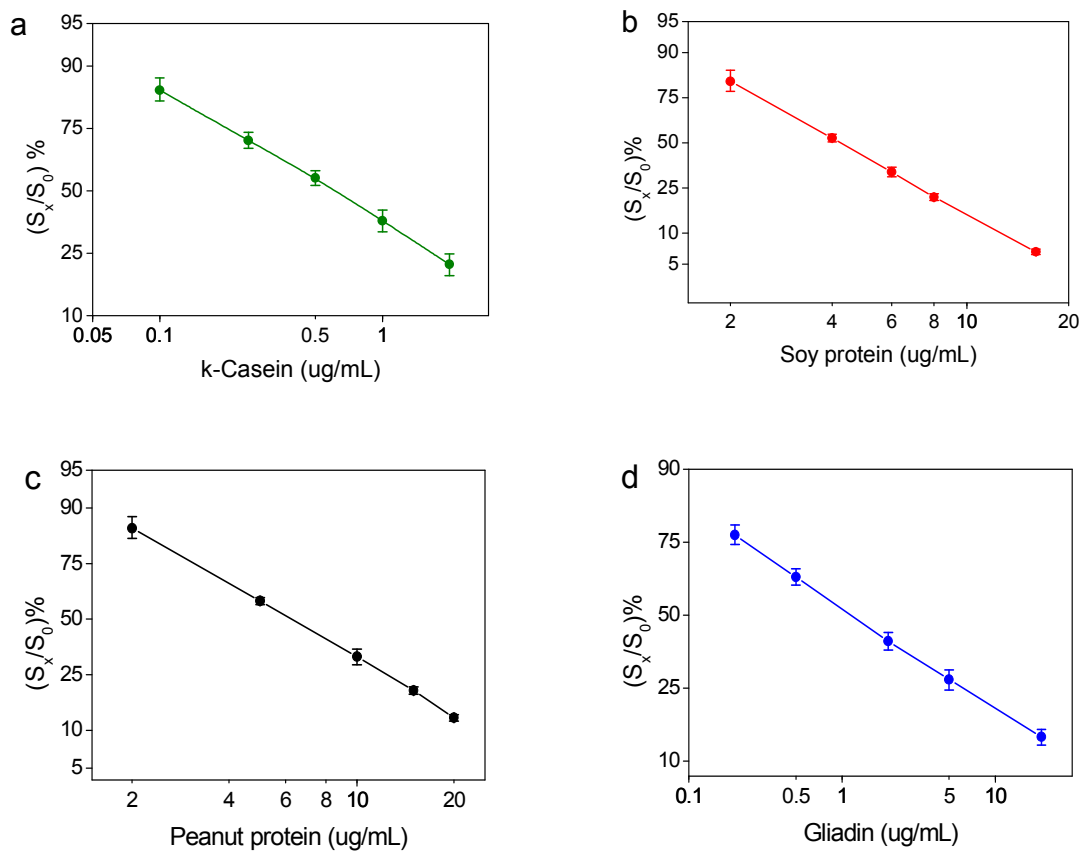


Figure S4. Typical calibration curves of (a) k-casein, (b) soy protein, (c) peanut protein, and (d) gliadin obtained for the four-allergens with the multi-analyte immunosensor. Each point is the mean value of 5 chips (10 MZIs) $\pm$ SD.

ELISA procedure for the detection of four allergens in rinsing water

Microtitration wells were coated with 100 μL /well of allergen solution (1 $\mu\text{g/mL}$ bovine k-casein, 2 $\mu\text{g/mL}$ soy protein, 5 $\mu\text{g/mL}$ peanut protein and 0.5 $\mu\text{g/mL}$ gliadin) in coating buffer overnight at room temperature (RT). The wells were then washed twice with 300 μL of washing buffer and 300 μL of blocking solution (1 % (w/v) BSA in 0.1 M NaHCO_3 solution) were added per well and incubated for 1 h at RT. Prior to the assay, equal volumes of calibrators or samples and a solution containing antibodies against each one of the allergens in assay buffer (for bovine k-casein: Mab 33-4G10 and 33-6A10 at 40 ng/mL each; for soy protein: Mab 26-5G4 and 26-6C9 at 25 ng/mL each; for peanut protein: Mab 51-5G9 and 44-4G2 at 25 $\mu\text{g/mL}$ each; and for gliadin Pab 1:1000 dilution) were mixed and pre-incubated for 10 min at RT. For the assay, 100 μL of the mixtures were added per well and incubated under shaking for 1 h. Then, the wells were washed four times with washing solution and 100 μL of a 5 $\mu\text{g/mL}$ anti-mouse IgG or anti-rabbit IgG HRP conjugate in 0.1 M Tris-HCl buffer, pH 8.25, containing 0.5 % (w/v) BSA, and 0.9 % (w/v) NaCl were added per well and incubated for 30 min at RT. After washing the wells four times with washing solution, 100 μL of H_2O_2 /ABTS chromogenic substrate solution were added to each well and the optical density was measured at 405 nm after 30 min of incubation. ELISA calibration curves for each one of the allergens are constructed by plotting the $(S_X/S_0) \times 100$ values versus the allergen concentration; where S_X is the absorbance at 405 nm corresponding to the various calibrators and S_0 is the respective value of zero calibrator.

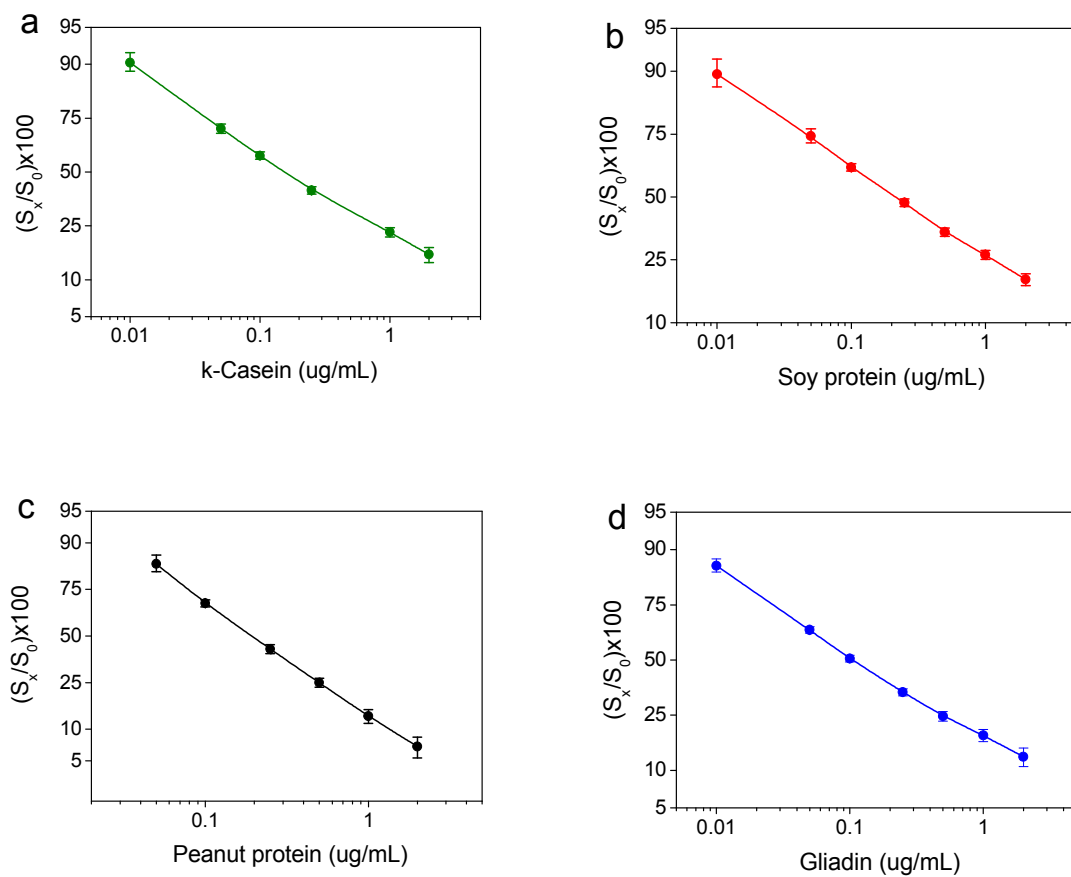


Figure S5. Typical ELISA calibration curves of (a) k-casein, (b) soy protein, (c) peanut protein, and (d) gliadin. Each point is the mean value of 4 replicates $\pm$ SD.

Table S1. Recovery experiment results.

Sample	k-CASEIN		% Recovery
	Amount added (µg/mL)	Amount determined (µg/mL)	
Tap water	0.40	0.37±0.01	92.5
	1.2	1.1±0.1	91.7
	3.6	3.9±0.2	108
Machine SIN-A	0.40	0.39±0.02	97.5
	1.2	1.1±0.1	91.7
	3.6	3.2±0.2	88.9
Machine SIN-B	0.40	0.41±0.02	102
	1.2	1.1±0.1	91.7
	3.6	3.7±0.2	103
Machine PLA-1	0.40	0.36±0.02	90
	1.2	1.3±0.1	108
	3.6	3.4±0.2	94.4
PEANUT PROTEIN			
Tap water	4.0	4.2±0.2	105
	8.0	7.0±0.2	87.5
	16	16±0.6	100
Machine SIN-A	4.0	3.8±0.2	95.0
	8.0	7.4±0.3	92.5
	16	14±0.5	87.5
Machine SIN-B	4.0	3.6±0.1	90.0
	8.0	8.8±0.4	110
	16	17±0.7	106
Machine PLA-1	4.0	3.6±0.1	90.0
	8.0	8.4±0.3	105
	16	14±0.6	87.5
SOY PROTEIN			
Tap water	2.5	2.4±0.1	96.0
	5.0	4.9±0.2	98.0
	10	8.8±0.3	88.0
Machine SIN-A	2.5	2.6±0.1	104
	5.0	4.5±0.2	90.0
	10	9.2±0.3	92.0
Machine SIN-B	2.5	2.3±0.1	92.0
	5.0	4.8±0.2	96.0
	10	11±0.4	110
Machine PLA-1	2.5	2.2±0.1	88.0
	5.0	5.2±0.2	104
	10	9.3±0.4	93.0
GLIADIN			
Tap water	2.5	2.4±0.1	96.0
	5.0	5.1±0.2	102
	10	8.7±0.4	87.0
Machine SIN-A	2.5	2.6±0.1	104
	5.0	4.6±0.2	92.0
	10	9.2±0.3	92.0
Machine SIN-B	2.5	2.4±0.1	96.0
	5.0	5.3±0.2	106
	10	9.4±0.4	94.0

Machine PLA-1	2.5	2.2±0.1	88.0
	5.0	5.1±0.2	102
	10	11±0.4	110

Table S2. Comparison of the immunosensor developed with other literature immunosensors for detection of allergens

Sensing principle	Label	Allergen	Analysis Time (min)	LOD (ppm)	Ref. #
Mach-Zehnder	no	k-casein	10	0.06	35
SPR	no	k-casein	10	0.45	37
SPR	no	a-casein	10	0.127	27
SPR	no	a-S1-casein	6	0.058	12
SPR	no	b-casein	10	0.085	38
Electrochemical	no	casein	30	0.05	28
Electrochemical	streptavidin-PbS Qdots	casein	20	0.04	31
LSPR	no	casein	30	0.01	39
Fiber optic SPR	nanobead particles	Ara h1	20	0.09	34
Electrochemical	alkaline phosphatase	Ara h1	230	0.0038	26
Electrochemical	graphene oxide/Qdots-aptamer	Ara h1	10	0.056	36
Electrochemical	alkaline phosphatase	Ara h6	60	0.00027	25
Piezoelectric	no	gliadin	40	0.008	30
Piezoelectric	no	gliadin	53	4.0	40
Impedimetric	no	gliadin	60	0.2	29
Magneto-electrochemical sensor	HRP	gliadin	30	0.057	32
Electrochemical	HRP	Ara h1	120	0.018	50
		Ara h2		0.00007	
Giant magnetoresistive	magnetic particles-streptavidin	Ara h1	15	0.007	51
		Ara h2		0.0002	
		gliadin		0.0015	
i-SPR	no	k-casein	7	200	41
		peanut protein		3200	
		soy protein		5000	
SPR	no	soy protein	5	500	42
		peanut protein		40	
Magneto-electrochemical sensor	HRP	casein	10	0.170	43
		Ara h1		0.007	
		gliadin		0.075	
Developed sensor	no	bovine k-casein	6.5	0.04	
		soy protein		0.80	
		peanut protein		1.0	
		gliadin		0.10	