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Microcantilever resonator arrays for immunodetection of β-lactoglobulin milk allergen

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ABSTRACT

The incidence of allergic disease is globally increasing so much that food allergy is actually considered as one of the main diseases of civilization, as well as among the major costs for healthcare worldwide. In such a scenario, the recent legislations introduced from European Union to protect consumers drastically drive the need for rapid, sensitive, and robust techniques to detect allergens within foodstuffs.

We here report on an innovative immunorecognition method for β-lactoglobulin milk allergen detection, based on microcantilever resonator arrays, a promising class of biosensors. An original sandwich assay that uses the same polyclonal antibody as capture and secondary immunorecognition agent was proposed to overcome the low affinity of the simple direct method. The developed immunoassay showed better Limit Of Detection (LOD) and Limit Of Quantification (LOQ) than commercial ELISA plates, even after an aging of four months. To our knowledge, this work represents the first example in the literature of successfully immunodetection of milk allergens in low concentrations by microcantilever resonator arrays, thus opening new perspectives on alternative diagnostic tools for milk allergens screening tests.

1. Introduction

Food allergy is considered to be one of the diseases of civilization, which occurs as a result of the changing conditions of life together with environmental changes. It is estimated that globally about 220–520 million people may suffer from food allergy and this poses a major burden to healthcare cost worldwide [1]. Occurrence of food allergy depends on many factors, including: individual genetic predisposition, degree of exposure to allergenic food and molecular characteristics of the allergen [2]. Food allergens are proteins that cause an abnormal response by the immune system of the allergy sufferers. Their structure and their biochemical and physicochemical properties determine their allergenic strength [3]. Very small amounts of the allergenic proteins (in the milligram range) can cause a severe or maybe even fatal reaction [4].

The majority of people with allergies reacts to one or a combination of nine common foods: cow's milk, soy, egg, wheat, peanut, tree nuts, sesame, fish and shellfish [5]. Epidemiological studies have reported that cow's milk protein allergy (CMPA) is the most prevalent allergy for infants or young children, with an incidence of about 2% to 7.5% in population-based studies in different countries [6]. In Italy, CMPA is responsible for 42% of food-induced anaphylaxis in the paediatric population [7]. Among milk allergenic proteins, β-lactoglobulin (β-LG) is one of the major allergens in cow's milk, together with α-lactalbumin (α-LA) and caseins [8].

At the end of 2014, new legislation (the EU Food Information for Consumers Regulation 1169/2011) was introduced requiring food businesses (i.e. catering outlets, deli counters, bakeries and sandwich sellers) to provide allergy information on food sold unpackaged. Since mainly children may be extremely sensitive to minute amounts of allergens, severe attention must be paid also to food contaminants. Different methods of protein allergens detection have been proposed. Most of these methods are immunoassay-based, like Enzyme-Linked ImmunoSorbent Assay (ELISA), which are normally used by analytical laboratories, or

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lateral flow that can be used on-site. Such methods are cheap, but poorly accurate, so they are commonly used for qualitative and/or first screening analyses. Other methods have been reported in the literature as second-level analyses such as DNA amplification-based or Mass Spectrometry-based [9]. Such techniques are extremely sensitive and robust, but they are very expensive and required trained personnel.

In this context, biosensors represent a valid alternative to traditional detection methods and attracted considerable interests as powerful label-free, sensitive, portable, cheap, high parallel and fast sensors [10,11], which can be used to detect either a specific allergen or protein, or a specific DNA fragment. Few attempts in food allergens biosensing regarding hazelnut, egg, milk allergens and gluten detection are reported in the literature [12,13].

Cantilever-based biosensing platforms are considered one of the most promising classes of biosensors [14–16], due to their high sensitivity and versatility. They are single-side clamped microbeams opportunely functionalized to capture a specific antigen, commonly exploiting antibody-mediated immunorecognition reaction. When operating in the so-called dynamic mode, the microcantilevers (MCs) act as simple harmonic oscillators: when targets are immobilized on the surface, the mass of the resonator changes and thus the resonance frequency is shifting proportionally [17,18]. Exploiting their impressive mass resolution (down to yoctogram range) [19], MC resonators were applied to the detection and quantification of many interesting targets, such as single cells [20] and microorganisms [21], cancer biomarkers [22,23] and nucleic acids [24,25]. However few works reported the ability to use MC resonator arrays for the identification of biomolecular targets of interest in the food sector [10], and just one paper showed preliminary results on the detection of an allergen (ovalbumin at just one concentration in [26]).

The present work reports on the immunodetection of β-LG milk allergen by MC resonator arrays. After carefully monitoring the coating of the MC arrays with specific antibody, two configurations were tested: a *direct* assay, where the antigen is bound to the antibody through a single immunorecognition event, and a *sandwich* assay, where two immunorecognition events are employed, so that each antigen molecule is sandwiched between two antibodies. The developed sandwich immunoassay showed better Limit Of Detection (LOD) and Limit Of Quantification (LOQ) than commercial ELISA tests with the same antibody. As first example in the literature of immunodetection of milk allergens in low concentrations by MC resonator arrays, the here-presented results could be of importance for all alternative diagnostic tools based on biosensors to identify hidden allergens within foodstuffs.

2. Materials and methods

2.1. Reagents

Sulphuric acid (95–98% w/w), hydrogen peroxide (30% w/w), 3-aminopropyltriethoxysilane (APTES, anhydrous, 99%), glutaraldehyde (GA, 25% v/v water solution) and NaOH were purchased from Sigma-Aldrich. Orthoboric acid and sodium chloride used to prepare borate buffer were ACS reagents (essay ≥99.5%) and were obtained from Sigma-Aldrich. Recombinant Protein G, purified from *E. coli* was from PIERCE. Toluene (anhydrous, 99.8%) was from Carlo Erba Reagents, Tween 20 was from Sigma, Phosphate-Buffered Saline (PBS) was from GIBCO®. Bovine beta-Lactoglobulin rabbit polyclonal antibody was from Bethyl Laboratories.

2.2. Microcantilever array fabrication and measurement

Microcantilever arrays consist of 11 cantilevers with nominal length, width and thickness respectively of 460 μm, 50 μm and 7 μm. They were fabricated using a combination of surface and bulk micromachining techniques, following the process steps detailed in [27].

Cantilever resonance frequencies were monitored before and after each binding event, using a semiautomatic experimental setup based on the optical lever method [28]. Microcantilever arrays were attached on a piezoelectric disk actuator (PI Ceramic) with an adhesive tape. The measurement chamber was then evacuated by a series of a membrane and turbomolecular pumps (MINI-Task System, Varian Inc. Vacuum Technologies) and the temperature was set to 22 °C by using both a temperature controller (by Electron Dynamics Ltd) and a Peltier element (by Supercool), on which the piezodisk is attached. The feedback signal needed to maintain a fixed temperature with a precision of 0.01 °C is obtained interfacing the Proportional Integral Derivative (PID) controller to a thermistor, set in contact with the upper surface of the piezo element. A high frequency lock-in amplifier (HF2LI by Zurich Instruments) is used to generate a sinusoidal signal sent to the piezodisk, which put in vibration the cantilever arrays at the same frequency of the excitation input. A laser diode (by Acal Technology) beam is focused onto the tip of the microcantilever and the reflected light is collimated by means of an optical lens system and focused into the sensitive area of a Position Sensitive Diode (PSD by Edmund Optics). The output signal of the PSD is then amplified, filtered through the lock-in and finally stored in a personal computer. For the fast serial characterization of all microresonators, an automated 2D scanning system composed by two actuators (by Physik Instrumente GmbH & Co) is employed.

2.3. Chemical functionalization and assay protocol

MC arrays were immersed in piranha solution (75% H_2SO_4, 25% H_2O_2) for 15 min and rinsed three times in deionized water in order to remove organic contaminants. Afterwards they were silanized following the procedure reported in detail by Ricciardi et al. [29]. Briefly, the chips were immersed for 10 min at 70 °C in 1% v/v APTES solution in toluene under anhydrous conditions in order to avoid APTES hydrolysis, then rinsed three times in toluene and dried in air. After, the cantilevers were incubated in 0.5% v/v GA solution in borate buffer 0.1 M pH 8.5 for 15 min at 40 rpm and then in 0.01% of sodiumcyanoborohydride solution 5 M in NaOH to reduce amine groups. MCs were shacked for 45 min at 40 rpm, rinsed three-time in deionized water and dried under stream of N_2.

After chemical functionalization, MC arrays were first incubated overnight in a protein G solution (50 μg mL^{-1} in PBS) at 4 °C, and then soaked in antibody solution (at different dilutions, see further) for 90 min at 40 rpm on a shaker at room temperature. After the characterization of cantilever resonances, the arrays were incubated in 100 μL of PBS solution at different allergen concentrations for 90 min at 40 rpm at room temperature. Sandwich assay was performed incubating again the arrays in the antibody solution overnight at 4 °C. After every step of protein binding, MC arrays were washed thrice in PBS-Tween20 solution 0.05% v/v, rinsed three times in deionized water and dried in a nitrogen stream.

2.4. Data analysis

For each of the 11 MCs of an array, the first and the second longitudinal vibrational modes were measured to perform a statistical analysis on the relative frequency shifts induced by analyte adsorption. The resonant frequencies f_i^n (where $i = 1,2$ represents the vibrational mode and $n = 1\text{-}11$ the cantilever position in the array) were characterized before and after the incubation, observing a negative shift Δf_i^n directly proportional to the mass absorbed on the cantilever surface. In order to compare the results from the