



A new optical interferometric-based *in vitro* detection system for the specific IgE detection in serum of the main peach allergen

R.L. Espinosa^{a,c}, M. Garrido-Arandia^b, A. Romero-Sahagun^b, P. Herreros^{a,c}, L. Tramarin^{a,c}, M. F. Laguna^{a,c}, A. Díaz-Perales^{b,**}, M. Holgado^{a,c,*}

^a Center for Biomedical Technology (CTB), Universidad Politécnica de Madrid, Parque Científico y Tecnológico de la UPM, Campus de Montegancedo, 28223, Pozuelo de Alarcón, Madrid, Spain

^b Center for Plant Biotechnology and Genomics (CBGP), Universidad Politécnica de Madrid, Parque Científico y Tecnológico de la UPM, Campus de Montegancedo, 28223, Pozuelo de Alarcón, Madrid, Spain

^c Department of Applied Physics and Materials Engineering, Escuela Técnica Superior de Ingenieros Industriales, Universidad Politécnica de Madrid, C/José Gutiérrez Abascal, 2, 28006, Madrid, Spain

ARTICLE INFO

Keywords:

Food allergy
IgE detection
Optical biosensors
Interferometry
SiO₂ nanoparticles
Molecular allergen
Component resolved diagnosis

ABSTRACT

Food allergens cause worldwide chronic diseases with a great impact on public health. Immunoglobulins E (IgEs) trigger allergic reactions by specifically binding the allergens to which the allergic patients are sensitized. In this scientific work we report for the first time a new optical interferometric *in vitro* system for the detection of specific IgEs (sIgEs) to the principal peach allergen (Pru p 3) in real serum samples. Interferometric Optical Detection Method (IODM) was employed for reading out the signal of Fabry-Perot based interferometers acting as biotransducers. Pru p 3 was immobilized as bioreceptor onto the sensing surface for detecting the target biomolecules, sIgEs to Pru p 3. Moreover, the demanding low concentration of IgE, compared to other analytes in real serum samples, made it necessary to use nanoparticles (NPs) for two reasons: to collect only the IgEs from the serum sample and to enhance the optical interferometric read-out signal. The methodology was validated in advance by scanning electron microscopy (SEM). Consequently, we report in this article a novel high-performance *in vitro* detection method to recognize sIgE to molecular allergens by means of silicon dioxide (SiO₂) NPs. Finally, this scientific work provides the basis for the *in vitro* component resolved diagnosis (CRD) of sIgEs to molecular allergens.

1. Introduction

Food allergy is a worldwide chronic disease with a great impact on public health. Different patterns of food allergy have been reported depending on geographical regions, ethnic groups and meal cultures (Hu et al., 2010; Taylor, 2006; Ward, 2014). The globalization and complexity of modern food are well known reasons of the increase in prevalence of this disease (Sicherer and Sampson, 2014). In particular, in the Mediterranean area, lipid transfer proteins (LTP) are the principal proteins responsible for food allergic symptoms. Among these allergens, the main peach allergen (Pru p 3) has been described as a primary sensitizer in LTP mediated food allergies (Salcedo et al., 2007).

According to the National Institute of Allergy and Infectious Disease

(NIAID) guidelines, the adverse reaction mediated by food can be considered food allergy when it is a reproducible effect after the exposure to it (NIAID-Sponsored Expert Panel et al., 2010). The immunological reaction can be triggered either by the intake of very tiny amounts of food allergens or compounds from additives, or even by the contact with a given food (Ward, 2014). The severity of conditions is patient depending, and varies unpredictably from mild symptoms (e.g. itching and rashes), even to severe and life-threatening reactions (e.g. vomiting, diarrhea, anaphylaxis and death) (Alves et al., 2016; Johansson et al., 2004; Muñoz-Furlong and Weiss, 2009).

The weakness of usual clinical procedure for food allergy diagnosis (lack of reliability of the clinical history, the low skin prick test [SPT] sensitivity and the oral provocation test [OPT] patient-risks) have

* Corresponding author. Center for Biomedical Technology (CTB), Universidad Politécnica de Madrid. Parque Científico y Tecnológico de la UPM, Campus de Montegancedo, 28223, Pozuelo de Alarcón, Madrid, Spain.

** Corresponding author.

E-mail addresses: araceli.diaz@upm.es (A. Díaz-Perales), m.holgado@upm.es (M. Holgado).

<https://doi.org/10.1016/j.bios.2020.112641>

Received 12 June 2020; Received in revised form 19 August 2020; Accepted 17 September 2020

Available online 19 September 2020

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prompted the increasing interest in finding effective and safer tools to be implemented in clinical practice. The need for these sensitive tests has led to the improvement of the methodologies of serologic immunoglobulin E (IgE) tests (Maloney et al., 2008). Therefore, the *in vitro* component resolved diagnosis (CRD) from molecular allergens is pointing out as the potential methodology for an accurate allergy diagnosis (van Hage et al., 2017a).

The IgE antibody-mediated allergic reaction (type 1 hypersensitivity or immediate hypersensitivity) is one of the categories described to classify the immune-mediated adverse food reactions (NIAID-Sponsored Expert Panel et al., 2010). When the allergic process triggers, the level of IgE in human serum increases (Hamilton and Adkinson, 2003; Kreuzer et al., 2001), but remaining in a low concentration (Papamichael et al., 2007) compared to other serum components. Serum is a highly complex matrix consisting of a mixture of water and several lipids and proteins. Among these, immunoglobulin G (IgG) antibodies are especially remarkable due to their relative abundance in the sample and their ability to recognize the same protein epitopes than IgE. Therefore, the low IgE concentrations along with the matrix effect are the main significant problems to establish the specific IgE (sIgE) detection methodology as the best option for *in vitro* food allergy diagnosis.

In the past decade, many IgE detection methods have been performed in large and expensive automated laboratory platforms such as radio allerge sorbent test [RAST], ELISAs, fluorescence enzyme assays (CAP-FEIA), etc. Notwithstanding their drawbacks in quantifying specific IgE concentration in serum samples (Goikoetxea et al., 2013; Wilmons et al., 2000), these immunochemical techniques are widely but controversially established in daily routines. Different working formats, and the absence of specific IgE for calibration hinder the settlement of a standard quantification methodology, leading to inconsistencies in qualitative results and to a controversial discussion about features required for specific IgE detection procedures (Hamilton, 2010; Matricardi et al., 2016). Therefore, despite the worldwide acceptance of the CAP as the gold standard for this type of test (Johansson, 2004), there is an urgent need to develop reliable and comparable multiple testing assays to allow the reduction of sample volume, testing time, and costs (Hamilton et al., 2010; Maloney et al., 2008).

In this context, new IgE assays such as microarrays and microbeads are addressing clinician requirements for multiplex testing with low volume of sample (Chinnasamy et al., 2014; Gadisseur et al., 2011; Gantelius et al., 2011; King et al., 2007; Lupinek et al., 2014; Pomponi et al., 2012; Ramachandran et al., 2013; van Hage et al., 2017b). In addition, new approaches are combining micro and nanotechnology to manufacture and characterize nanomaterials in order to enhance the sensitivity and accuracy of IgE biosensors (Erathodiyil and Ying, 2011; Jain et al., 2007). Specifically, nanoparticles (NPs) exhibit particular physicochemical properties which can be engineered to make them suitable in the field of biotechnology (Chinen et al., 2015; Daems et al., 2017; Johansson et al., 2004; Kairdolf et al., 2017; Wu et al., 2011; Zhu et al., 2010). For example, the increased binding surface, the NP ability to couple either with the capture or the detecting molecules and their potential to amplify the signal of the biosensor make them suitable for the development of an inexpensive and highly sensitive sensor solution (Loyez et al., 2020; Sapsford et al., 2013; Wang et al., 2010, 2009; Wang and Alcocija, 2015; Zhang et al., 2010; Zhou et al., 2015) and specifically in the food allergy diagnosis field (Kim et al., 2010; Liu et al., 2010, 2012; Teste et al., 2011).

Other challenges to be solved on the IgE testing to achieve a reliable and selective method are the efficient separation of IgE from the matrix (Ashraf et al., 2012; Platt et al., 2014), the need of both secondary antibodies and fluorophores (King et al., 2007; Platt et al., 2014), the time to result (Ashraf et al., 2012), and performing the bioassays in serum samples (Teste et al., 2011; Wang et al., 2010, 2009). In addition to the abovementioned challenges, another crucial requirement is the need of developing a cheap user-friendly device for real-time detection of food allergens in an end-user environment. Hence, the use of both, the

already reported Biophotonic Sensing Cells (BICELLS) (Holgado et al., 2014; Lavín et al., 2013; Sanza et al., 2011) and the described point-of-care device (Maigler et al., 2018), are a potential alternative to other methodologies for direct label-free and reproducible IgE detection. Recently, we have demonstrated the ability of BICELLS to be adapted and easily adjusted into more complex systems for multiplex point-of-care. We have already reported the system capability of detecting molecules such as C-reactive protein (Maigler et al., 2018), metalloproteinase 9 (MMP9) (Santamaría et al., 2017) and dry eye biomarkers (e.g. ANXA1, ANXA11 and PRDX5) (Laguna et al., 2015). And more specifically, in food allergy field we validated the system by detecting IgE in a similar range than the ImmunoCAP (Espinosa et al., 2018).

On the other hand, to the best of our knowledge, there are no reports for the use of silicon dioxide (SiO₂) NPs for both (i) to amplify the interferometric signal of photonic biotransducers read-out in an optical point-of-care device, and (ii) to separate the IgE from IgG in real serum to diagnose food allergy. Accordingly, we set a high-performance *in vitro* detection system for multiplex serologic IgE tests by means of SiO₂ NPs to improve the specificity and selectivity of our biosensors, and to reduce the cross-reactivity and the matrix-effect in real serum assays. Furthermore, the refractive index amplification as a result of the use of SiO₂ NPs also improved the sensitivity of the detection system.

Hence, in this work we developed a CRD test based on a multiplex biosensor coated with Pru p 3 together with SiO₂ NPs capturing specifically the IgE from serum sample and enhancing the optical interferometric signal. Once the effectivity of the methodology was demonstrated, for the very first time, for one of the main sensitizer in LTP mediated food allergies, Pru p 3, we could extend it to other molecular allergens due to the versatility of our biosensor to be adapted up to 63 different biomolecules.

2. Materials and methods

2.1. Biosensing system

2.1.1. Read-out platform, measurement methodology and optical simulation

We carried out all the immunoassay measurements by using the registered interferometric optical detection method (IODM) (Holgado et al., 2016, 2014) and the reported optical read-out platform, but adapted to read the multiplexed 63 optical interferometric biosensing wells, which we call BIWELLS sensors and are described below in this manuscript. The biosensing read-out system scanned two optical references from the biosensor and then, by triangulation it located the coordinates of the 63 BIWELLS. The read-out system can modify the positioning system to read each of the BIWELLS but unchanging its optical interrogation methodology.

The read-out signal used to evaluate the biosensing is based on the Increased Relative Optical Power (IROP) signal also reported in previous studies (Holgado Bolaños et al., 2012; Holgado et al., 2016). In brief, IROP is the measurement unit to quantify the changes in the optical read-out for two interferometric signals when a biofilm coats the biosensor sensing surface. Hence, the Δ IROP methodology is a method to detect the concentration of the objective molecule in an analyzed sample after its bindings to that biofilm. As it was well reported, IROP signal is inversely related to the biofilm thickness, thus, according to the formula of Δ IROP (%) (see Equation (1)), an increase on the biofilm thickness (X nm after bindings) renders a decrease in IROP signal and therefore, a negative Δ IROP (%) signal

$$\Delta\text{IROP}(\%) = \frac{\text{IROP}_{\text{thickness } 1 (X \text{ nm})} - \text{IROP}_{\text{thickness } 0 (0 \text{ nm})}}{\text{IROP}_{\text{thickness } 0 (0 \text{ nm})}} \times 100 \quad 1$$

Eq. (1). Equation for Δ IROP (%) methodology. Thickness 0 is the thickness of the biosensing surface prior to binding (0 nm). Thickness 1 is the thickness of the biofilm when it is attached to the biosensing

surface. IROP signal is lower after bindings according to IODM methodology.

As pointed out above, the photonic biosensor used is the BIWELL, which is a multilayer interferometric Fabry-Perot photonic transducer. In order to amplify its interferometric read-out signal, we proposed the use of NPs at the recognition step. The NPs onto the sensing surface of BIWELLS increased its optical thickness leading to a change in its interferometric profile, being this modification highly NP size and material depending.

Specifically, the size of the NPs modulates the read-out signal, thus, to estimate the good coupling between NP size and the optical read-out platform, we performed a theoretical approach with different NP diameters. To this end, we calculated a simulation of the hypothetical interferometric signal for a Fabry-Perot sensor and NPs of different sizes (50, 100 and 200 nm of diameter respectively) when the percentage of coverage with NPs increased following the IODM methodology. Results

indicated the duplicity of the detection signal values when using NPs of 200 nm, while the optical behavior changed when we simulated a reduction in NP sizes, yielding a lineally decreasing response as the coverage of the surface increased. Thus, the tuned NP behavior and the improvement of the sensor response were achieved by reducing the NP sizes below 100 nm. Therefore, as the theoretical study pointed out an optimum NP size under 100 nm, we selected an 80 nm diameter in order to obtain the best unambiguous and tuned response, also bearing in mind the biological biofilm thickness covering the NPs.

2.1.2. Multiplexed biokit with 63 interferometric biosensing wells

The multiplexed biokit used has 63 optical interferometric BIWELLS. Fig. 1 shows a depiction of the multiplexed 63 BIWELLS integrated in a single biokit. Each BIWELL is an interferometric vertically interrogated Fabry-Perot transducer consisting on a silicon (Si) substrate with a SU8 layer and a thin layer of nitrocellulose (NC) over them (Fig. 1A). The

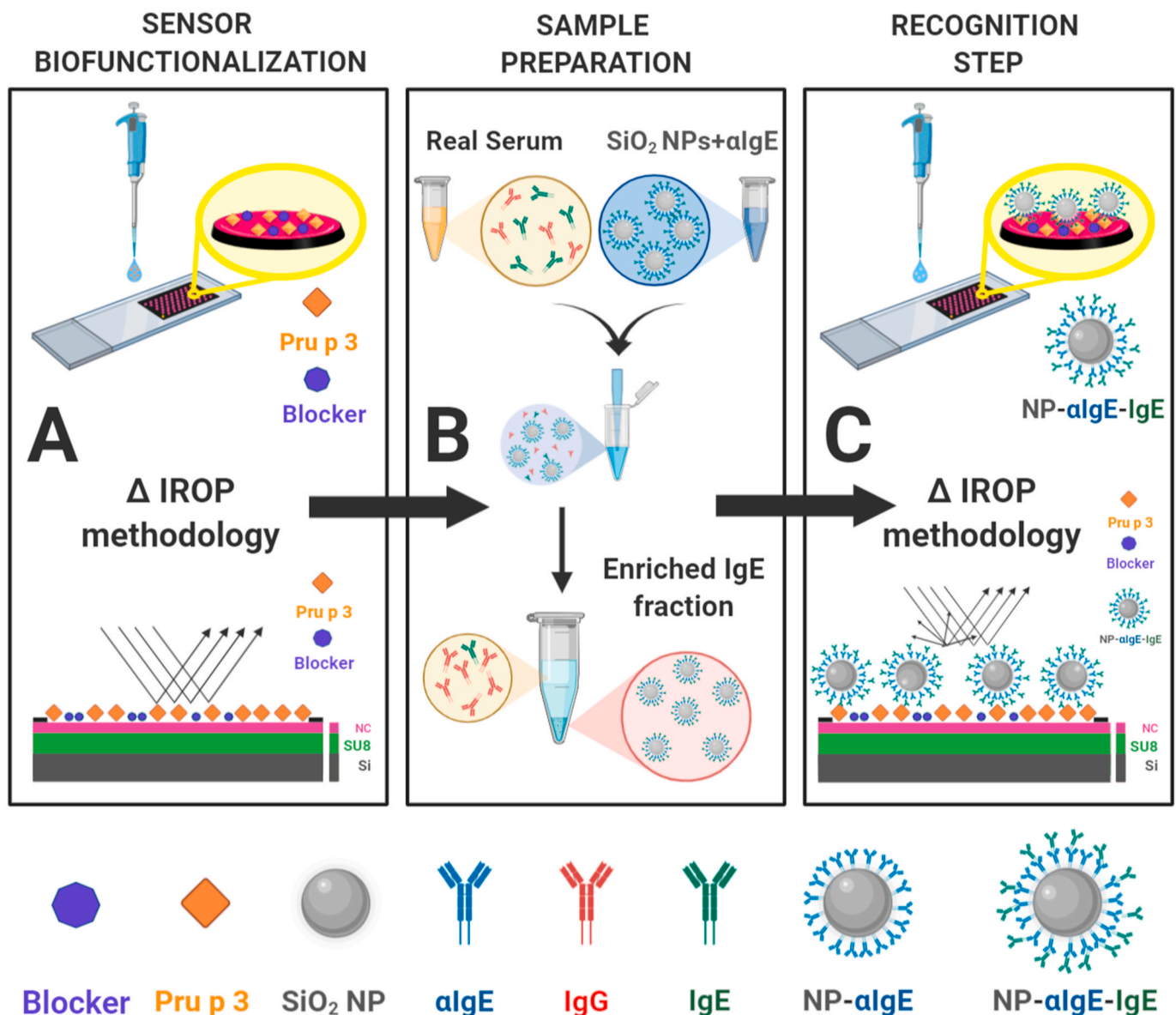


Fig. 1. Depiction of the methodology developed. (A) Biofunctionalization step of a multiplexed 63 BIWELLS biokit (up) and schematic structure of a BIWELL coated with a biofilm of Pru p 3, blocked with casein, and Δ IROP methodology for detecting these molecules (down); (B) Sample preparation protocol to obtain the IgE enriched fraction by filtering serum with biofunctionalized SiO_2 NPs; (C) Recognition step of the multiplexed 63 BIWELLS biokit (up) and schematic structure of the BIWELL when biofunctionalized NPs capture specific IgE (sIgE) from real serum and attach to the Pru p 3 biofilm (down). Captions of the pictures used (created with BioRender.com).

fabrication procedure and the sensor working principle have been well described previously (Espinosa et al., 2018; Holgado Bolaños et al., 2010; Santamaría et al., 2017). Briefly, it is established by the modification of the interference signal when a bioreceptor is attached onto the sensing surface forming a biofilm. Later on, in the recognition stage, the specific binding of the bioreceptor to the objective molecule changes the interference signal again.

Particularly, in this work, we carried out a proof of concept with a single bioreceptor (Pru p 3) to ensure the robustness of the methodology (see Fig. 1A). Moreover, the interference signal at the recognition step shifted as the biofilm covered the sensing surface, but also, the optical dispersion of the complexes of NPs attached to the allergens may lead a decrease in the light intensity (see Fig. 1C). Both effects allowed us to detect target molecules through the Δ IROP (%) methodology. Therefore, when biofunctionalized NPs (coated with IgEs from serum) specifically bound to the allergens immobilized onto the sensing surface we obtained the highest Δ IROP (%) signal.

2.1.3. Biokit functionalization

The whole batch of immunoassays was performed with recombinant Pru p 3 (rPru p 3) produced in *Pichia pastoris* and purified using the methods previously described (Díaz-Perales et al., 2002). We automatically dropped rPru p 3 over each BIWELL (1.37 μ L, at 50 μ g/mL in PBS [pH 7.5]) and incubated them for 120 min in humid environment at 37 °C. After incubation time, we washed biokits directing a stream of mQ water onto the surface of each BIWELL and dried them with filtered air. After that, we measured the Δ IROP (%) signal of rPru p 3 and confirmed this value to be within the admissible values for a thin biofilm of a molecule of such size, according to the IODM methodology (i.e. Δ IROP (%) within range of -75 to -100% of signal). The surface was then incubated with a 10-fold diluted blocking buffer (from a 10x Casein blocking buffer, Sigma-Aldrich) for 1 h, at room temperature (RT) in gentle agitation to reduce the non-specific binding. Finally, we confirmed the effectiveness of this step by measurement of Δ IROP (%) signal again (Fig. 1A).

2.1.4. SiO₂ nanoparticles preparation

In order to perform an efficient separation of IgE from the serum matrix, we established the use of biofunctionalized NPs with anti-IgE (α IgE) over their surface as filtering methodology for capturing selectively total IgE from serum. This strategy allowed discarding IgG and its ability of recognizing the same protein epitopes than IgE, and therefore established the methodology for carrying out a diagnostic test based on specific IgE detection.

Immunological and optical behavior of NPs had to be optimum and compatible for both, the sample treatment and read-out-platform analysis. Hence, pursuing the choice of the ideal NPs, we tested a wide variety of NPs according to the abovementioned IODM methodology regarding size, material and chemical nature of their surface. The experimental results pointed that 80 nm SiO₂ NPs amino terminated were the best option to work with (as the theoretical study had predicted).

It is worthy to mention here that the use of a given size of mono-disperse SiO₂ NPs, not only to separate IgE from IgG in real serum samples, but also to enhance and amplify the interferometric optical signal of biophotonic sensors, is a novelty. To date, to the best of our knowledge, there is not any other procedure which considers both factors at once. Moreover, a whole standardized biofunctionalization protocol needed to be established in order to become a monodisperse stable colloidal solution, to avoid non-specific binding and to assure the sIgE capture from the patient serum.

2.1.4.1. Experimental determination of the optical behavior of NPs over BIWELLS. We performed preliminary tests to determine the functionality of SiO₂ NPs for the biosensing system developed. To find this out,

we covered BIWELLS with SiO₂ NPs. Fig. 2 summarizes the validation of the methodology by means of scanning electronic microscopy (SEM) images (JEOL, JSM-5800 [JEOL Ltd. Tokyo, Japan]). BIWELLS with different percentage of coverage of SiO₂ NPs were obtained and their read-out platform signals were measured. Results revealed a good correlation between the Δ IROP (%) signals and the percentage of SiO₂ NPs attached to the biosensing surface, visualized by SEM images. Fig. 2A shows the Δ IROP (%) signals for three BIWELLS with low, medium, and high percentages of SiO₂ NPs attached to their surface (10, 40 and 90%, respectively). These signals link with SEM images shown in Fig. 2B. Images with scale bar of 50 μ m (Fig. 2B-down) gave an overview of the sensing surface, while lower scales, 5 and 1 μ m (Fig. 2B-up and 2. C), corroborated the correlation between Δ IROP (%) signals and its proportion to the number of NPs attached to the sensing surface. Specifically, two SEM details of the sensing surface almost empty and nearly covered with SiO₂ NPs are seen in Fig. 2C. According to the results obtained we could validate our methodology by means of an external system.

2.1.4.2. Biofunctionalization of SiO₂ NPs with goat anti-human IgE. Biofunctionalization protocol was accomplished after having established the size and material of NPs, and the routinely confirmation procedure of NP biofunctionalization was settled by a 1-step Ultra TMB-ELISA. SiO₂ NPs amine terminated (80 nm) were purchased from Superior Silica (Chandler, AZ, USA). Goat anti-human IgE antibody (α IgE) was obtained from Bethyl laboratories (Montgomery, TX, USA). Phosphate buffered saline (PBS), phosphate buffered saline-tween (PBS-Tween) and anti-Goat IgG horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (st. Louis, MO, USA). NHS-Phosphine, NHS-PEG4-Azide and Ultra TMB-ELISA were obtained from Thermofisher Scientific (Waltham, MA USA).

Amine terminated NPs stored in 20% of ethanol were washed three times with PBS (10 mM, pH 7.5) through centrifugations (8000 rpm, 10 min) and resuspended by sonication (20 min) in PBS. After that, we biofunctionalized NPs with α IgE (Fig. 3A) following the protocol described in previous report (Yuste-Calvo et al., 2019). Finally, the suspension was centrifuged, and the particles washed with PBS three times and resuspended in PBS to obtain the biofunctionalized NPs (i.e. complexes NP- α IgE). We established plate-based ELISA as routinely protocol to confirm the NP biofunctionalization by determining the amount of free α IgE in the supernatant of step A from Fig. 3A. That is, after incubating supernatant with HRP labelled anti-goat IgG antibody (1 h, RT) and washing with PBS-Tween, the presence of free α IgE was detected using Ultra-TMB ELISA subtract and measured in the plate reader platform at 450 nm.

2.2. Specific IgE recognition step in serum sample

As it was pointed above, the batch of immunoassays was performed following the general procedure described in Fig. 1. We collected serum samples of patients sensitized to Pru p 3 from the clinical partners of the European Project AllerScreening: “Point-of-care device based on KETs for diagnosis of food allergies” with grant agreement ID: 768641.

2.2.1. Sample preparation

The sample incubation protocol test comprised all the previous features needed to assure the binding between the complexes NP- α IgE and the IgE from the patient to form complexes NP- α IgE-IgE. We also established a routine procedure of measuring the amount of free IgE in the supernatant to ensure a high binding ratio. Some factors tested in advance via simulation and experimentally were the concentration of NPs on volume and the incubation time.

Finally, we obtained the IgE-enriched fraction (Fig. 1B and 3. B) on a volume concentration of $6.2 \cdot 10^8$ NP/ μ L by mixing 5 mL of serum with $3.1 \cdot 10^{12}$ biofunctionalized NPs, incubating them for 1 h at RT and

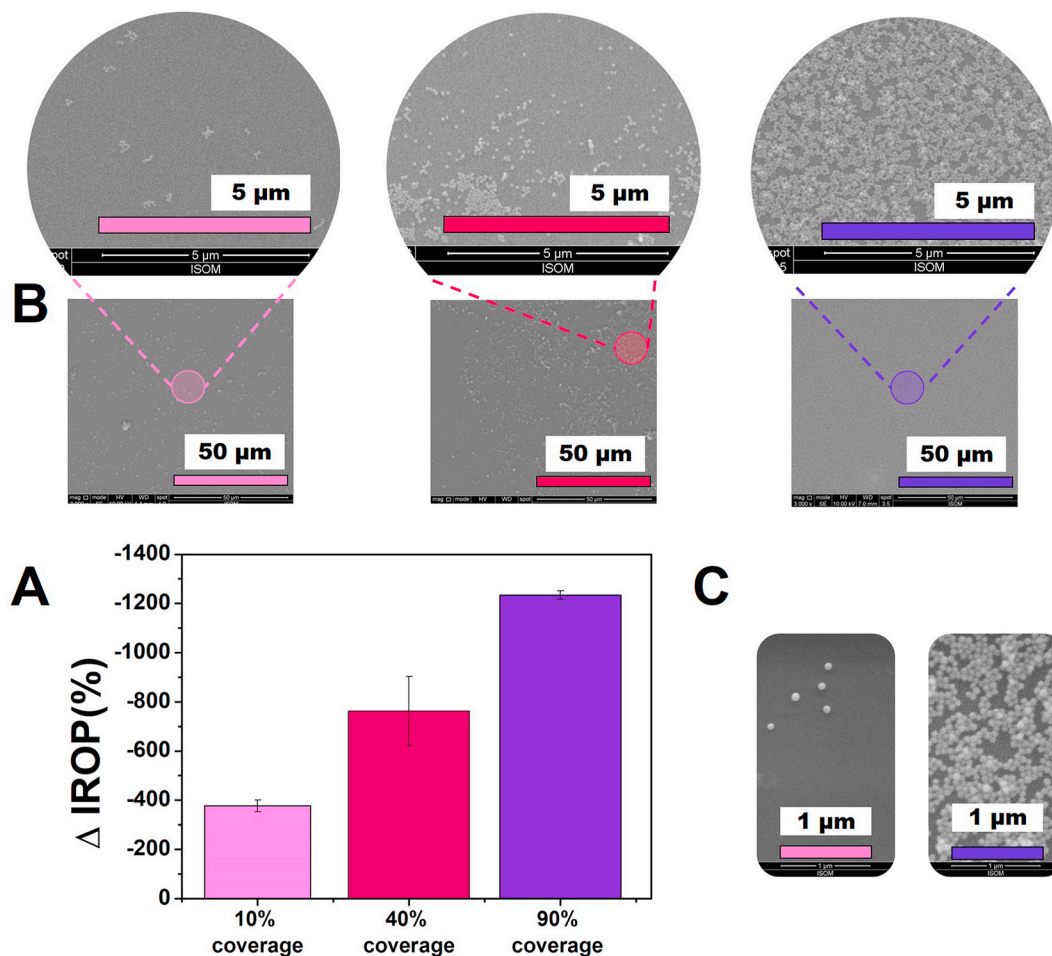


Fig. 2. $\Delta IROP$ (%) signal to evaluate the optical behavior of SiO₂ NPs and SEM images. (A) $\Delta IROP$ (%) signal of three BIOWELLs covered by SiO₂ NPs in different percentages of coverage over the surface (from left to right: low [10%], medium [40%], and high [90%] coverage of NPs attached to the sensing surface); (B) Corresponding SEM images to each BIOWELL represented in A (low-left, medium-center and high-right) with two different scale bars. 50 μm (down) and 5 μm (up); (C) SEM images of small area (scale 1 μm) of BIOWELLs with lower and higher $\Delta IROP$ (%) signal. The NP shape and size are clearly observed, as well as the sensing surface almost covered by SiO₂ NPs (in C-right).

establishing three washing steps with PBS-Tween and centrifugations (20 min, 5000 rpm), prior final reconstitution in PBS (pH 7.5). IgE from the serum of the patient bound to NP- α IgE and formed the NP- α IgE-IgE complexes, thus we had complexes NP- α IgE-IgE in PBS ready for incubation over biokits. The amount of free IgE was determined by 1-step Ultra TMB-ELISA used as HRP substrate in the fraction not bound to the NPs at the end of step B (see Fig. 3B), following the same protocol than for step A, briefly explained in section 2.1.4.2.

2.2.2. NP- α IgE-IgE binding to allergen procedure

At this point, we determined the procedure to assure the specific binding of complexes NP- α IgE-IgE to the allergen coating the BIOWELLs. We tested different incubation times at 37 °C in humid environment, and the preferred association time was 1 h with $\Delta IROP$ (%) signals over -500 $\Delta IROP$ (%). Less incubation times led to a substantial signal reduction with comparable values to non-specific binding (e.g., -200 $\Delta IROP$ (%) for 30 min of incubation time).

Once diffusion, dissemination and kinetics reactions occurred, the complexes NP- α IgE-IgE specifically bound to the allergen. We settled a quick washing protocol including two washing steps with PBS in gentle agitation and a last washing step with Milli-Q water to remove non-specific bindings from the sensing area.

2.2.3. Recognition step protocol

After establishment of protocols (i.e. BIOWELL biofunctionalization,

NP concentration, sample preparation, incubation time and washing steps for complexes), we performed the multiplexed immunoassay using biosensors immobilized and blocked with rPru p 3 and casein, followed by a recognition immunoassay with our in-house-biofunctionalized SiO₂ NPs (NP- α IgE-IgE) (general recognition step can be seen in Fig. 1C). A pool of LTP allergic patient serum was employed to form the complexes NP- α IgE-IgE. Biofunctionalized SiO₂ NPs without human IgE (NP- α IgE) were used as a negative control. Binding was assessed by means of $\Delta IROP$ (%) signal (read-out platform).

As described before, 63 BIOWELLs coated with rPru p 3 and blocked with casein were directly incubated with NP complexes with and without IgE from the serum. We dropped 2 μL of complex over each BIOWELL (1 h at 37 °C in humid environment), washed with PBS and mQ water and dried. Fig. 4A shows the schematic distribution of the NP complexes: NP- α IgE-IgE complex covered the right side of the biokit, and the NP- α IgE complexes covered the left side of the multiplexed biokit. The central part of the biokit remained uncovered in order to have the background signals after a new washing and drying step.

2.3. Statistical analyses

For descriptive purposes, we present medians and percentiles (P) 25 and 75 of $\Delta IROP$ signal in those BIOWELLs non-incubated with NPs, NP- α IgE-IgE and NP- α IgE. Due to the sample size and non-normal distribution of the data in the final assay, a non-parametric test (Mann-

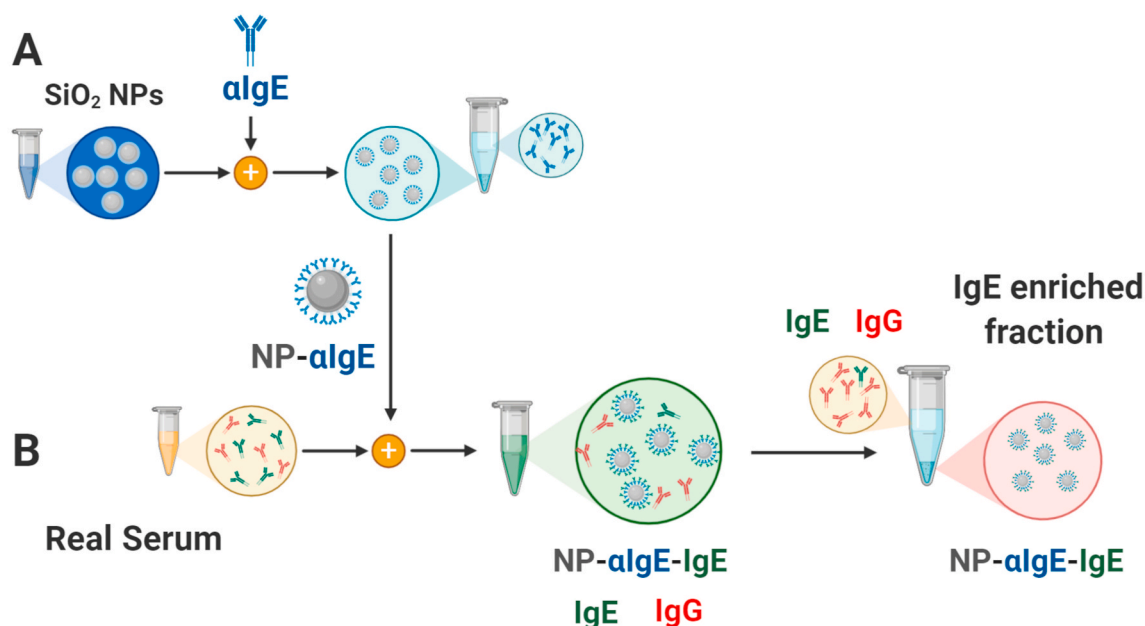


Fig. 3. Biofunctionalization of SiO_2 NPs and IgE-enriched fraction isolation. (A) Schematic process. SiO_2 NPs were biofunctionalized with specific polyclonal anti-human IgE antibodies (αIgE) to form the NP- αIgE complexes; (B) Schematic process to obtain the IgE-enriched fraction. Complexes NP- αIgE were incubated with serum to capture IgE from the patient and to form the complexes NP- $\alpha\text{IgE-IgE}$. The amount of free IgE and IgG in the supernatant are discarded to isolate the IgE-enriched fraction (created with [BioRender.com](https://www.biorender.com)).

Whitney) to compare recognition signal of both NP- $\alpha\text{IgE-IgE}$ and NP- αIgE was conducted. Statistical analysis was performed using OriginPro 8 SR0 (v8.0724) software, where associations are referred to statistically significant associations, this implies a p-value <0.05 .

3. Results and discussion

Here, we report a novel assay framework inspired by the chemical and optical properties of SiO_2 NPs and by the flexibility of the multiplexed biosensor. We demonstrated the SiO_2 NP ability to capture target analytes after NP biofunctionalization and their capability to amplify the optical signal of the biosensor. We applied the developed methodology to the multiplexed biosensors, previously immobilized with molecular rPru p 3 allergen in 63 different sensing points and blocked, and later on, determined the selectivity of the surface and the recognition event according to the above-mentioned ΔIROP methodology.

We obtained good preliminary results after having settled the whole protocol for detecting our target analyte, sIgE. Fig. 4A summarizes the final above-described assay in section 2.2.3. A spatial distribution of the rPru p 3 + blocking ΔIROP (%) signal over the surface of one BIWELL is represented in Fig. 4B. The median (P 25 and 75) ΔIROP (%) signal for rPru p 3 + blocking (n:48) was -183.9 (-195.6 , -168.7). Fig. 4C shows the control values (left) and the specific recognition of NP- $\alpha\text{IgE-IgE}$ conjugates (right). Each graph represents the ΔIROP (%) signal for the immobilization of rPru p 3 blocked with casein (orange group of columns in both graphs) and the ΔIROP (%) signal for the recognition step for the negative control in blue (Fig. 4C, left) and for complexes NP- $\alpha\text{IgE-IgE}$ captured by the bioreceptor in green (Fig. 4C right). The whole data for the complexes NP- $\alpha\text{IgE-IgE}$ is over -500% of ΔIROP (%) signal with reasonable repeatability of the methodology to detect specific binding.

Fig. 5. A represents the ΔIROP (%) mean and its standard deviation increase for each type of recognition. As shown, when incubating samples from patients allergic to LTP over biokits coated with rPru p 3, statistically significant differences ($p < 0.01$, data not shown) were found between negative control (NP- αIgE , in blue) and the complexes (NP- $\alpha\text{IgE-IgE}$, in green). The median (P 25 and 75) for complexes NP- $\alpha\text{IgE-IgE}$ (n:24, right group of columns) and NP- αIgE (n:19, left group of

columns) were -764.4 (-934.7 , -607.2) and -304.5 (-318.2 , -293.6) ΔIROP (%) (data not shown). These results revealed the performance of the methodology for detecting specific IgEs from serum of patients when IgE are captured by SiO_2 NPs well biofunctionalized and the NP- $\alpha\text{IgE-IgE}$ complexes bind to a specific sensing surface.

The specificity of the system is also demonstrated in the three-dimensional depictions shown in Fig. 5B. Each one represents the spatial distribution of the ΔIROP (%) signal measured in 49 measurement points within the center of one BIWELL of each type. We can appreciate a generalized low ΔIROP (%) signal along the control BIWELL (Fig. 5B-left). Some higher values may correspond to isolated NP- αIgE complexes non-specifically bound to the sensing surface. Meanwhile, the high contrast in ΔIROP (%) signal due to specific binding of IgE from patients to their bioreceptor, with signals between -1800 and -600 ΔIROP (%), is depicted in Fig. 5B-right.

Notwithstanding substantial differences between complexes with and without serum, few unexpected interactions between NP- αIgE and allergen-coated BIWELLS still appeared. Even though this limitation seems not to be an important concern, as the ΔIROP (%) signal values registered in controls were lower than the background signal and differences in the ΔIROP (%) signal from recognition to blocking were under an established threshold, we are already exploring further optimization measures towards reducing non-specific signal values. This reduction would lead to an increase in sensitivity and specificity of the system (as lower levels of sIgE could be detected and distinguished from non-specific binding signal). Apart from that, our approach represents a new methodology dealing with standard techniques. Thus, we are developing new assays to evaluate both the specificity and the sensitivity of the system, by correlating results with ImmunoCAP and ELISA, with expected accuracy of the preliminary results.

4. Conclusions

For the very first time, we reported the use of a 63 multiplexed interferometric sensor coated with rPru p 3 to detect the specific IgE from serum samples of allergic patients. The novelty of the methodology lies on the use of a given size of SiO_2 NPs as filtering and signal

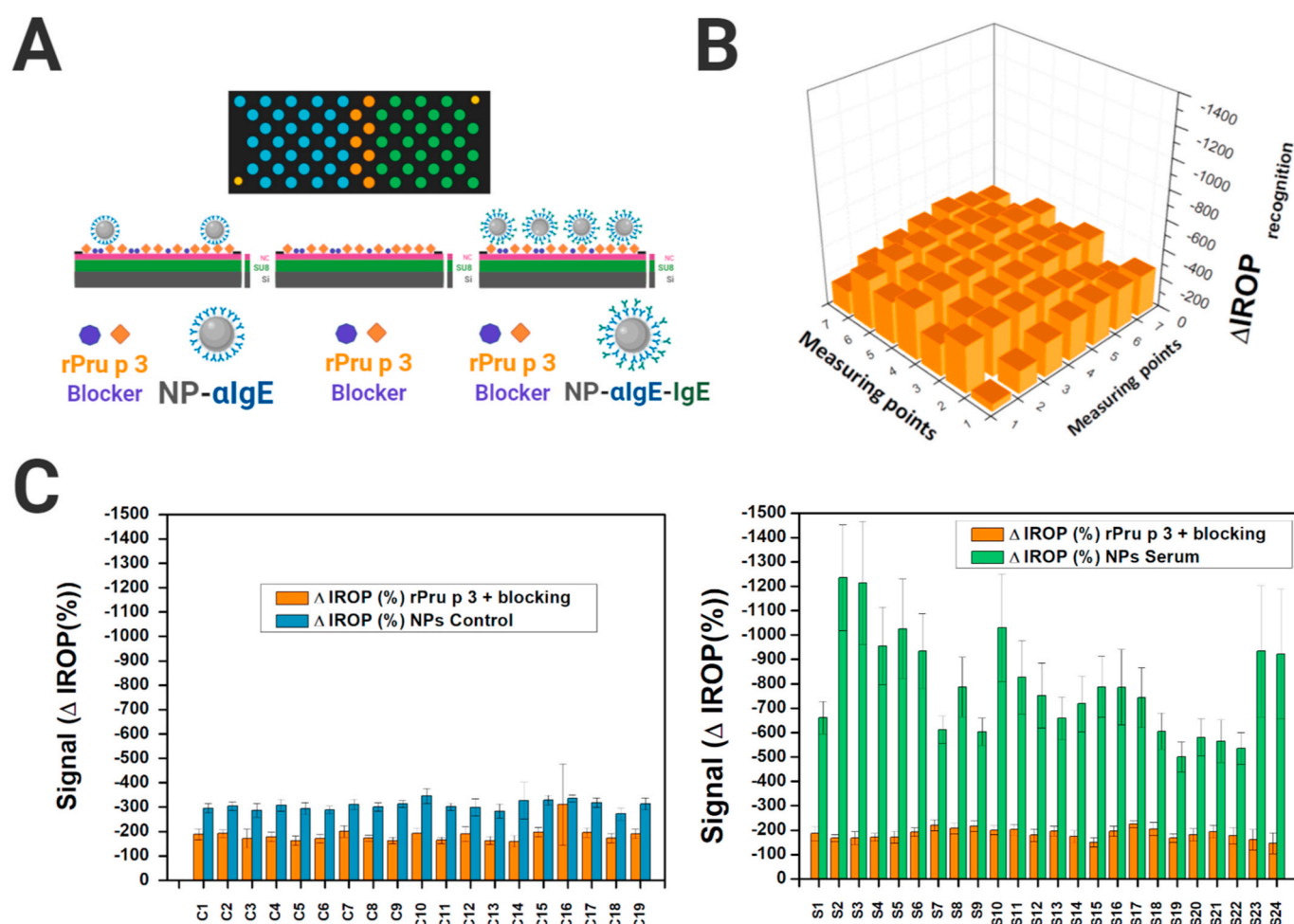


Fig. 4. Recognition assay and results. (A) Depiction of the distribution of NPs complexes over the 63 multiplexed biokit at the recognition step and detail of NP- α IgE-IgE, NP- α IgE and only rPru p 3 + blocking covering the interferometric sensing surface; (B) Spatial distribution of the rPru p 3 + blocking Δ IROP (%) signal over the surface of BIWELL number 30 (each of the 1–7 values corresponds to a Δ IROP% signal at the corresponding measuring point over the BIWELL); (C) Δ IROP (%) signal for 19 and 24 BIWELLS immobilized with rPru p 3 and blocked with casein (orange columns), and Δ IROP (%) signal at the recognition step for NP- α IgE complexes (blue columns, left) and for NP- α IgE-IgE complexes (green columns, right). BIWELLS are named as C for control complexes (C1–C19) and S for complexes with serum (S1–S24). (Created with [BioRender.com](https://www.biorender.com)). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

amplification system to separate IgE from IgG and to improve the interferometric optical signal, respectively. Hence, the methodology settled in this work addressed a novel technical solution for the matrix effect, especially when extremely accurate low-level measurements are required. We solved the non-specific adsorption problem in diagnostic test based on specific IgE detection by separating the objective molecule, IgE, from the rest of the serum out of the sensor. Moreover, we improved the system sensitivity and limit of detection since we amplified the optical signal by selecting the right size, material, and concentration of NPs. Finally, the demonstrated effectivity of the methodology for one allergen makes it a promising alternative to CRD diagnostic tests based on molecular allergens and specific IgE detection due to the versatility of our biosensor to be adapted to customized layouts. The following step will be to demonstrate its accuracy in a multiple assay with molecular allergens and comparing the relevant results with other standard accepted techniques.

Funding

This work was supported by the European Commission, European Union [grant number H2020-NMBP-X-KET-2017. 768641-Allerscreening Project]; the Spanish Ministry of Economy and Competitiveness,

Spain (currently, the Ministry of Science, Innovation and Universities) [grant numbers BIO2017-84548-R and TEC2017-84846-R]; the Community of Madrid, Spain [grant number FOODAL-CM_P2018/BAAA-4574 and BIOPIELTEC-CM 2018/BAA-4480].

Credit authors statement

R.L. Espinosa: Writing - original draft, Investigation, Methodology, of the measurement system, biokits and immunoassay procedure, Formal analysis, Data curation, Visualization. **M. Garrido-Arandia:** Investigation, Methodology, of nanoparticles biofunctionalization and immunoassay procedure, Formal analysis, data curation, Data curation. **A. Romero-Sahagun:** Investigation, Methodology, of nanoparticles biofunctionalization and immunoassay procedure. **P. Herreros:** Fabrication, Methodology, packaging of biokits. **L. Tramarin:** Fabrication, Methodology, packaging of biokits. **M.F. Laguna:** Conceptualization, Supervision, Methodology, Writing - review & editing, Funding acquisition. **A. Díaz-Perales:** Conceptualization, Supervision, Methodology, Writing - review & editing, Project administration, Funding acquisition. **M. Holgado:** Conceptualization, Supervision, Methodology, Writing - review & editing, Project administration, Funding acquisition.

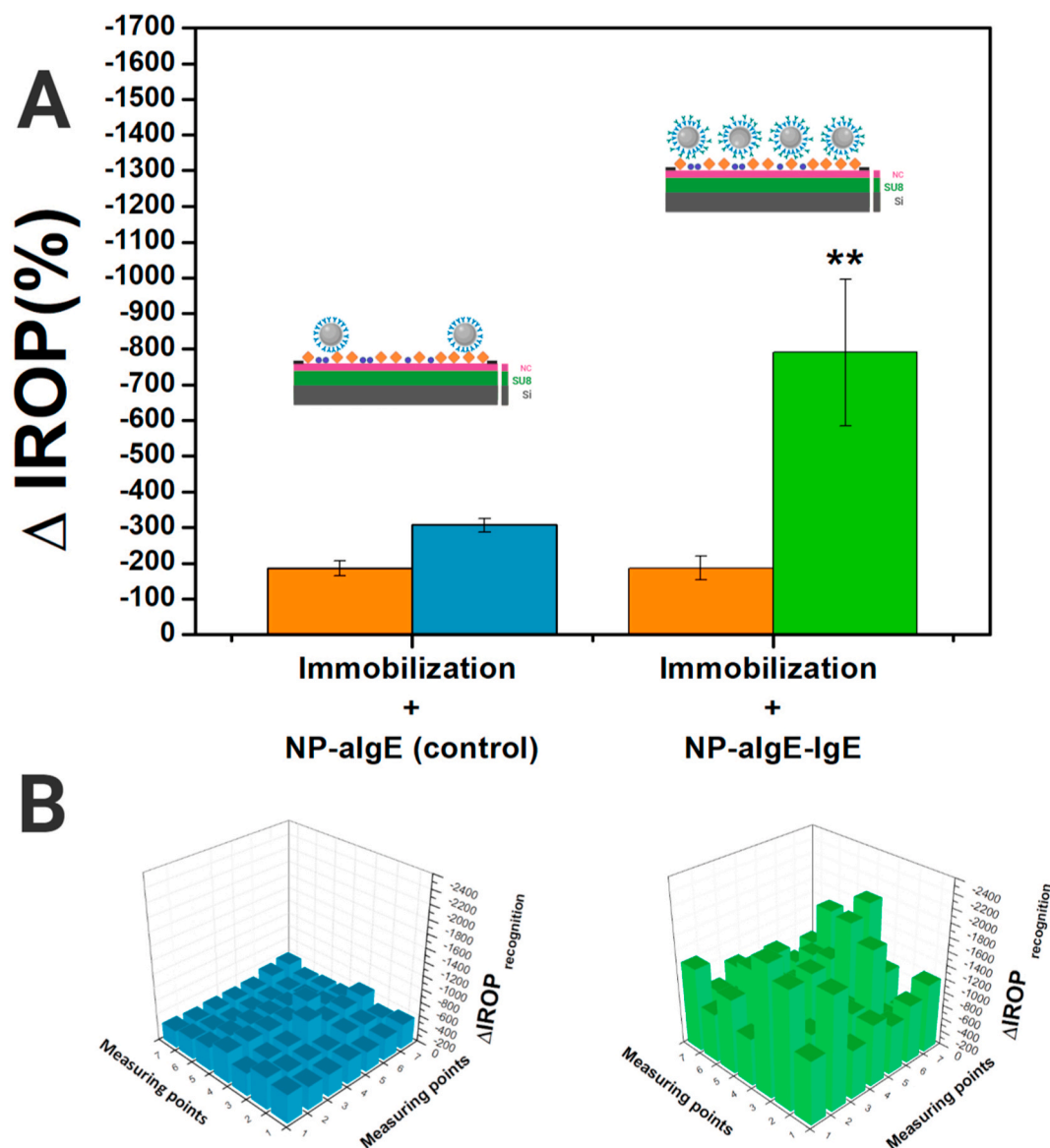


Fig. 5. Recognition assay results. Mean values and 3D depictions. (A) Mean Δ IROP (%) signal for NPs without serum incubation (n:24 NP- α IgE BIWELLS) (left) and NPs with serum incubation (n:19 NPs- α IgE-IgE BIWELLS) (right); (B) Three-dimensional depiction of three random BIWELLS for NP- α IgE (left) and NP- α IgE-IgE (right) (each of the 1–7 values corresponds to a Δ IROP% signal at the corresponding measuring point over the BIWELL). (Created with [BioRender.com](#)).

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

Authors acknowledge to BiOD for the support on the read-out platform and to Gustavo Bregante Torralba for his support on the biokits fabrication and packaging.

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