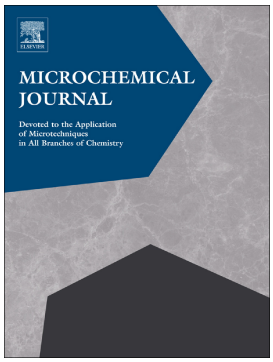

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Paper based analytical device modified with nanoporous material for the fluorescent sensing of gliadin content in different food samples

Evelyn Marín -Barroso[a], *Cristian M. Moreira*[a], *Germán A. Messina*[a], *Franco A. Bertolino*[a], *Mara Alderete*[b], *Galo J.A.A. Soler-Illia*[b], *Julio Raba*[a], *Sirley V. Pereira*[a]*

[a] INQUISAL, Departamento de Química, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco 917, San Luis, Argentina.

[b] Instituto de Nanosistemas, Universidad Nacional de General San Martín, Av. 25 de Mayo 1021, B1650KNA San Martín, Argentina

Author to whom correspondence should be addressed: (e-mail) spereira@unsl.edu.ar. (Tel.) +54-266-4425385; (Fax) +54-266-443-0224.

INQUISAL, Departamento de Química. Universidad Nacional de San Luis, CONICET. Chacabuco 917. D5700BWS. San Luis, Argentina.

Abstract

A novel fluorescent paper based immunosensor for the quantification of gliadin content in different food samples was constructed. The device consists of a paper platform modified with amino functionalized mesoporous material. The nanoporous structure and the aminofunctionality increased the anti-gliadin antibodies immobilization capacity of the sensing surface, conferring high sensitivity to the system. The detection limit reached by the described system allowed us to address the control of gluten free foods, which is extremely important to maintain the food safe consumption by patients with celiac disease, wheat allergy measured by immunoglobulin E, non-celiac gluten intolerance and Dermatitis herpetiformis or Duhring disease. The gliadin determination was performed by applying a noncompetitive immunoassay format, where gliadin present in food samples was recognized by anti-gliadin antibodies immobilized on the mesoporous material and quantified by the addition of anti-gliadin antibody labelled with peroxidase, its substrate: hydrogen peroxide and a mediator: 10-acetyl-3,7-dihydrofenoxacin. This mediator by the action of the enzyme generates resorufin, which was excited by a light emitting diode at 550 nm and emitted a signal at 580nm. The calibration curve obtained for gliadin exhibited a linear range between 0-160 μg Kg^{-1} and a method detection limit of 0.025 mg Kg^{-1}. The obtained values for relative recovery varied between 98.65% and 102.33% for samples enriched with gliadin. Also, the results suggested that the developed fluorescent paper based immunosensor showed good reproducibility and stability, indicating its applicability for high-sensitive gluten free food control analysis.

Keyword: immunosensor; fluorescence; mesoporous material; gliadin; food

1. Introduction

In recent years, paper-based analytical devices have been used with very promising results in terms of public health, food and environmental contaminants. This type of analytical devices has unique advantages over other platforms. Its porous structure and the superficial groups contributed to the immobilization of enzymes, antibodies and DNA, among others. Besides, it is a biocompatible, economical, easy to use and accessible material, features which make it more advantageous than other platform materials such as glass or polymers [1–4].

Paper devices can be coupled to different signal transduction methods, we can list electrochemical, spectroscopic and fluorescent methods [5,6]. Among them, the fluorescence is a methodology that has great relevance in terms of sensors coupling. This is a widely used spectrochemical analytical method for the molecular analysis of samples of different nature. The fluorescence detection system has several advantages such as simplicity, rapid response and high sensitivity [7–9].

The paper platform could be modified with different materials in order to increase the surface / volume ratio and, therefore, the immobilization capacity of the paper surface. The Santa Barbara Amorphous (SBA-15) is one of the mesoporous materials that has aroused great interest among researchers due to its unique properties to improve the surface area. The pore size that can be modified according to its synthesis, the high hydrothermal and mechanical stability as well as the lower susceptibility to shrinkage are outstanding qualities of this material [10–15]. Its silica composition allows to functionalize its surface in a simple way, to enhance its advantage and immobilize different biomolecules [16–18].

Numerous works in recent years have been interested in the development and application of biosensors in the food allergen field. These are biomolecules of different

origins (protein, carbohydrates) which are characterized by generating an activation of the immune system when certain predisposed people are in contact with them [19–21]. Gluten is a protein from the seed of various cereals and is involved in different alterations[22]. The most relevant is celiac disease, but there are other hypersensitivities to gluten that have similar symptoms but cannot be diagnosed within this pathology. Among them, wheat allergy measured by immunoglobulin E (IgE), which is characterized by anaphylaxis after consumption of foods derived from this cereal and non-celiac gluten intolerance, which is not measured by IgE or antibodies [23–25]. The latter, has a prevalence ten times greater than celiac disease and is considered as an emerging disease from the last decades. Another disease related to gluten intolerance is Dermatitis herpetiformis or Duhring disease. This, is an autoimmune pathology characterized by the generation of bullous lesions after consumption of foods containing gluten [26]. All these conditions respond to the same treatment, strict gluten-free diets. This complex protein can be fractionated according to its solubility in alcohol into prolamins and glutenins[27,28].

Gluten free food is identified by a labelling determined by the codex alimentarius, which establishes the allowed gluten values. The maximum allowed value is 20 mg Kg^{-1}, although some very sensitive patients may be intolerable at this amount [29]. The codex alimentarius also suggests the use of a reference spectrophotometric methodology for the measurement of gliadins [30]. Unfortunately, this methodology has drawbacks such as long incubation periods and the need for trained personnel to carry it out. For this reason, it is important to design new sensitive methodologies with low detection limits for the quantification of these proteins to provide safe foods consumption.

In this work, a sensor based on paper platform modified with SBA-15 coupled to fluorescent detection was developed. This system was applied to the determination of

gluten content in food samples through the quantification of gliadin fraction. The SBA-15 surface was amino functionalized to perform the anti-gliadin antibodies immobilization. This antibody specifically recognizes the gliadin protein present in food sample. The bounded gliadin was revealed by the addition of a secondary antibody: anti-gliadin antibody labelled with peroxidase, its substrate: hydrogen peroxide (H_2O_2) and a mediator: 10-acetyl-3,7-dihydrofenoxacin (ADHP). ADHP by the action of the enzyme generates resorufin, which was excited by a light emitting diode (LED) at 550 nm and emitted a signal at 580 nm captured by the fluorescent detector. In this way, this immunosensor was able to determine the concentrations of gliadins in different foods in a simple, sensitive and specific manner.

2. Experimental

2.1. Reagents and solutions

Anti-gliadin antibody (wheat) and anti-gliadin conjugate antibody (wheat) - peroxidase, wheat gliadin, bovine serum albumin (BSA), hydrogen peroxide, 10 (acetyl) 3,7 dihydrofenoxacin, (3-Aminopropyl), Pluronic® P123, tetraethylorthosilicate (TEOS) and glutaraldehyde solution were and purchased from Sigma-Aldrich. Filter paper n# 1 was provided by Whatman. All buffer solutions were prepared with Milli-Q water. All the reagents used were of reactive analytical grade.

The enzyme immunoassay for gliadin quantitative determination: R7001 RIDASCREEN® Gliadin, the patented Mendez cocktail extraction solution: R7006 RIDASCREEN® extraction cocktail and the Set of 3 Gliadin Assay Controls RIDASCREEN® were purchased from R-Biopharm AG-Darmstadt Germany and was used according to the manufacturer's instructions.

2.2. Instrumentation

All pH measurements were made with an Orion Research Inc. (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.).

Scanning electron microscopy LEO 1450VP (SEM). The paper microzones were printed with a Xerox ColorQube 8870 printer.

The system for detection fluorescence signal was constructed using LED operated a 550 nm, being this the source of fluorescent excitation. the sample is placed in such a way that its surface is at 45 degrees from the LED and at a focal length of 30 cm. The fluorescent radiation was detected with the optical axis of the assembly perpendicular to the plane of the device. Light was collected with a microscope objective (10:1, NA 0.30, working distance 6 mm, PZO, Poland) mounted on a microscope body (BIOLAR L, PZO). A fiber-optic collection bundle was mounted on a sealed housing at the end of the lens of the microscope, which was connected to a QE65000-FL scientific-grade spectrometer (Ocean Optics, USA). The entire assembly was covered with a large box to eliminate ambient light [31].

2.3. Sample preparation

The samples used were: common and rice noodles, wheat and stout beer, flour labeled "Sin TACC" and common flour. These were exposed to an extraction procedure after which gliadin content could be determined. In the case of flours 0.3 g were placed in 3 mL of ethanol 60% and exposed to a shaking condition for 30 min, centrifuged at 8000 rpm for 10 min and the supernatant diluted 1:50. Noodles samples were processed with the help of a mortar until obtaining a fine powder while for beer samples 1mL is added in 3 mL of ethanol. After these procedures, in all of samples the extraction process was the same described for flour samples.

The National Administration of Medicines, Food and Medical Technology of our country (ANMAT), also suggest making an extraction with the cocktail of commercial extraction (R-Biopharm, Germany). According to this, 0.25 g of the solids and 0.25 mL for the liquids samples were placed in 2.5 mL of the cocktail solution and incubated in a thermostatic bath at 50 °C for 40 min. Once cold, 7.5 mL of ethanol 80% is added maintaining stirring conditions 1 hour at room temperature. Finally, the sample was centrifuged at 8000 rpm for 10 min and the obtained supernatant was diluted to 1:12.5.

2.4. Synthesis and functionalization of the SBA-15

The synthesis of mesoporous SBA-15 was carried out by dissolving 4 g of P123 in 30 mL of 1.6 M HCl. The mixture was stirred for 45 min, 8.5 g of TEOS was added and the mixture was stirred for 20 h at 35ºC temperature. It was transferred to an autoclave and subjected to 90 °C for 24 h. Then, it was filtered, washed with water and alcohol and let dry at 60 ºC all night. The obtained preparation was air dried and calcined at 500 ° C for 6 h. Finally, the obtained SBA-15 was functionalized by exposing it to reflux treatment for 2 h in 80 mL of toluene and 1 mL of 3-aminopropyltrimethoxylane [32]. The incorporation of amino groups allowed the subsequent immobilization of anti-gliadin antibodies.

2.5. Immunosensor design

For the design and construction of the immunosensor a novel reaction platform was used, Whatman paper # 1 qualitative filter paper, on which a microzone of hydrophobic containment barriers was delimited by wax printing technique. The microzone was designed using Corel Draw and the impression was made with wax. Then, this paper was subjected to a heating plate at 80 °C to achieve a homogenization in the formation of hydrophobic walls by the wax. After, with the purpose of generating more

aldehyde groups for the modification process, the paper surface was subjected to a treatment with oxygen plasma for 2 min.

2.6. Modification of paper surface with SBA-15 and anti-gliadin antibodies immobilization

The synthetized and functionalized SBA-15 was placed on the paper surface, achieving a covalently bonding between aldehyde groups of both materials through an electronic re-arrangement. The amino groups present on the SBA-15 surface reacted with the amino groups of anti-gliadin antibodies by the addition of 5µL an aqueous solution of 5% (w/w) glutaraldehyde in 0.20 M pH 10.00 CO_3^{2-} /HCO_3 buffer (for 2 h at room temperature in a humid chamber). This immobilization was performed by the addition of anti-gliadin antibodies at a concentration of 10 µg mL^{-1} and incubating it 16 h in a humid chamber at 4 °C (Fig.1).

2.7. Determination of gliadin content

To perform the gliadin determination, the modified paper platform was washed with 0.01 M pH 7.2 PBS buffer, blocked with 1% BSA for 30 min and washed with 0.01 M pH 7.2 PBS buffer. The blocking procedure avoid the errors generated by non-specific bonding on the free sites. After that, 5 µL of the different samples already processed were added, incubated in a humid chamber for 10 min and washed with 0.01 M pH 7.2 PBS buffer. In this step gliadin contained in samples was immunologically recognized by the immobilized antibodies and subsequently quantified through the addition of 5 µL of the enzyme conjugate. The incubation and washing conditions were the same described for previous stages.

Finally, the paper platform was immediately placed on the fluorescence detection system. 3 µL of a revealing solution containing H_2O_2 and ADHP were added on the paper modified surface. To avoid the loss of moisture from paper platform, the revealing

procedure should be performed quickly. The fluorescent mediator in the presence of the enzyme generates resorufin, which was excited at a wavelength of 550 nm producing a fluorescent emission signal at a 580 nm. The response fluorescent signal obtained from the enzymatic reaction is directly proportional to the activity of the enzyme and therefore to the gliadin content of the analyzed samples (Supplementary material S1).

The gliadin concentration values in µg Kg^{-1} (ppb) obtained through calibration curve must be further multiplied by the dilution factor of 500. Considering that gliadin fraction protein usually represents 50 % of the proteins present in gluten, this result should be multiplied by 2 to obtain the gluten concentration (Fig.1).

3. Results

3.1. Characterization of the SBA-15

Transmission electron microscopy (TEM), scanning electron microscopy (SEM) images and energy dispersive spectroscopy (EDS) spectra were taken. Figure 2a shows, the unmodified paper surface, fig.2b and 2c illustrates the morphology of the SBA-15 particles, which are elongated; their length being 500 ± 70 nm, and the width 400 ± 50 nm, the images were obtained by SEM. EDS spectra reveals the typical signal corresponding of Si (1.74 KeV), demonstrating proving the satisfactory modification of the paper surface with functionalized SBA-15 (Fig. 2d) [33]. The figure 2e shows TEM image of SBA-15 where it can be seen the pore homogeneity and the hexagonal structure which are typical features of this mesoporous material.

To determine the pore size we used the nitrogen adsorption-desorption isotherms (Fig. 2f). These were obtained at 77 K for SBA-15 and amino functionalized SBA-15 samples. The pore diameter was 5.2 nm and 4.6 nm respectively and the surface area was

473.1 m^2 g^{-1} and 220.8 m^2 g^{-1} respectively. The obtained isotherms were of type IV with hysteresis loops H1 according to reported data for SBA-15 [34].

3.2. Amplification effect of the obtained signal

With the object to study the amplification effect generated by the incorporation of the functionalized SBA-15 in the paper device, the signal intensity obtained using a modified paper surface was compared with a signal corresponding to an unmodified paper surface. The figure 3a shows the signal amplification resultant from de improvement of the surface/volume ratio generated by the modification of paper surface with SBA-15 mesoporous material.

3.3. Optimization of the experimental variables

Different variables involved in the construction and application of the fluorescent paper device were optimized using standards of known concentrations of gliadin.

One of the most important parameters optimized was the concentration of anti-gliadin antibody to be immobilized in the reaction microzone. For this, the saturation test of the peroxidase was carried out (Supplementary material S2). This assay consists of immobilizing a known concentration of HRP enzyme on the reaction microzone and placing different known concentrations of anti-gliadin antibody. As can be seen in the figure 3b, the optimal concentration of antibody immobilization was 10 μg mL^{-1}.

Other parameter evaluated was the optimal concentration of functionalized SBA-15 used in the modification on the paper surface. This optimization procedure was performed using a 40 and 80 μg Kg^{-1} gliadin standard concentration. The optimal functionalized SBA-15 was 1.25 mg mL^{-1} (Fig. 3c).

The total assay time is a significant variable when the real time sensing devices are being developed. For this reason, the incubation time was optimized and significantly reduced when compared to the reference method. This study was performed using three

patterns of known concentrations of gliadin (20, 40 and 80 µg Kg^{-1}), measuring the relative fluorescence for each of them at different incubation times from 5 to 15. The fluorescence intensity increased when the gliadin concentration and reaction time grow. However, the fluorescent intensity did not increase considerably until 10 min, which could be explained by the saturation of the specific antibodies recognition sites. Therefore, the optimum reaction time was 10 min (Fig 4a).

With the aim to avoid non-specific adsorption process on the paper surface, the concentration of blocking agent was also optimized. The best response was obtained using BSA 1%.

3.4. Quantitative test for the detection of gliadin in different food samples

The gliadin content was quantified in the different food samples: free gluten flour, common flour, rice noodles, common noodles, stout beer and wheat bear employing the developed fluorescent paper-based device. Calibration curves were obtained using commercial standards of known concentrations of gliadin from 0 µg Kg^{-1} to 160 µg Kg^{-1}, where 0 µg Kg^{-1} standard was used as a blank for all determinations. In the case of the sensor, a linear relationship was obtained, RFU = 1003.61 + 79.57 x $C_{(mg\ Kg^{-1}\ gliadin)}$ for gliadin concentrations from 0 to 160 µg Kg^{-1}, with a coefficient of variation for the standard of 40 µg Kg^{-1} of gliadin making six measurements was 4.2%. The coefficient of liner relationship was r = 0.999. As for the achieved merit figures, the method detection limit (DL) was calculated by DL= 3.3 of the signal-to-noise ratio and was 0.025 mg Kg^{-1}. The quantification limit (LOQ) 0.076 mg Kg^{-1} was calculated by LOQ= 10 of the signal-to-noise ratio.

The linear regression equation for the case of the commercial standard method, Ridascreen Gliadin ELISA was Abs = 0.166 + 0.026 x $C_{(mg\ Kg^{-1}\ gliadin)}$ with a CV for the

standard of 20 µg Kg^{-1} of 5.3% calculated after six repetitions. The coefficient of linear relationship was r = 0.991 and the corresponding DL was 1.5 mg Kg^{-1}.

We analyzed 14 samples (stout beer, wheat beer, common flour, free gluten flour, rice noodles and common noodles) and 3 control samples. The obtained results were concordant with the composition of the sample, those free gluten foods revealed negative results a those which contains gluten showed positive results. These results were compared with those obtained for the same samples using the reference method Ridascreen Gliadin ELISA (Table 1). A correlation curve showed a slope close to 1, indicating a good correlation between both methods (Supplementary material S3).

The sensitivity obtained by the paper sensor coupled to the fluorescent detection could be evaluated by means of the dilution test. For which serial dilutions of the standard 40 µg Kg^{-1} were made in a 0.01M pH 7.20 PBS buffer. This test allowed us to highlight the excellent accuracy of the immunosensor (Fig 4b). The reliability of the fluorescent device was evaluated by means of the recovery percentages, enriching the samples with a gliadin concentration of 20 mg Kg^{-1} once they were subjected to the extraction process. Relative recoveries values obtained for the enriched samples varied from 98.65% to 102.33% with a CV lower than 5.01%, which showed an adequate precision for the determination of gliadin in food samples (Supplementary material S4).

The selectivity of the system was investigated against 40 µg Kg^{-1} of the following reagents: albumin, casein, glutenin from wheat, gliadin, Ara h1, Ara h2 and β lactoglobulin. The results are shown in the supplementary material (S5) in which we can see that casein is the only one with a cross reaction[35]. The other agents displayed negligible signals. Besides, lyophilized paper device showed the same fluorescent signal response after three months under storage conditions (4°C). This result indicate that the sensor had adequate stability. The reproducibility of the immunosensor was evaluated

with intra and inter-assay approaches. The intra and inter-assay CV obtained from six replicates were 4.82% and 5.43%, respectively, at a concentration of 40 µg Kg^{-1} of gliadin standard and samples (Supplementary material S6, S7).

Finally, the analytical performance of our paper-based device was compared with other systems previously reported in scientific articles for the determination of gliadins in food samples (Table 2). As a result of this comparison, we can affirm that our system is the only one that combines the sensibility of fluorescent detection with a novel paper platform modified with mesoporous material for the quantification of gliadin in food samples. Moreover, our device could reach the lowest detection limit, obtaining results in an ultrasensitive, selective and fast way, with an extremely economical support.

4. Conclusion

In this work we design a paper based device incorporating mesoporous material with fluorescent detection. The new paper based device reached a low limit of detection using a highly sensitive analytical technique such as fluorescence compared with standard spectroscopic methods. The device was developed employing a paper platform modified with SBA-15, a mesoporous material. The nanoporous structure and the aminofunctionality of SBA-15 increased the antibodies immobilization capacity of the sensing surface generating a strong bio-affinity towards gliadin, conferring thus high sensitivity to the system. Moreover, the immobilized anti-gliadin antibodies proved don not have cross reactivity against other agents related with the sample composition, granting the selectivity of the designed system. Apart from these, the fabricated biosensor also exhibits acceptable stability, and good reproducibility. According the described features, the proposed device has demonstrated its suitability for the fast sensing of gliadin content in different food samples.

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Figure captions

Figure 1. Schematic representation of fluorescent paper based immunosensor construction showing the modification of paper surface and the gliadin determination procedure.

Figure 2. Characterization: (a) SEM image of unmodified paper surface, (b) SEM image of paper surface modified with amino functionalized SBA-15, (c) SEM image of paper surface modified with amino functionalized SBA-15, (d) EDS spectra reveals the typical signal corresponding of Si and (e) TEM micrograph of amino functionalized SBA-15 (f) N_2 adsorption-desorption isotherm of SBA-15 (red) and amino functionalized SBA-15 (light blue).

Figure 3. (a) Obtained fluorescent signal intensity using 80 µg $Kg^{-1}$of gliadin concentration standard. This figure compares the signal of the paper surface incorporating SBA-15 (red line) and the unmodified paper surface (black line). For this study, 0.1 M phosphate–citrate buffer, pH 5.0, containing 0.001 M H_2O_2 and ADHP as enzymatic mediator were added and the enzymatic product was measured using excitation at 550 nm and emission at 580 nm. (b) Evaluation of anti-gliadin antibody concentration on the immobilization procedure by the saturation peroxidase method. (c) Effect of the variation of SBA-15 concentration for the paper modification process using 40 µg Kg^{-1} and 80 µg $Kg^{-1}$of gliadin standard concentration.

Figure 4. (a) Study of the reaction time employing 20 µg Kg^{-1}, 40 µg Kg^{-1} and 80 µg $Kg^{-1}$of gliadin standard concentration. (b)Dilution factor test results for 40 µg Kg^{-1} gliadin standard concentration with 0.01 M PBS, pH7.2. Each value of relative fluorescence is based on five determinations.

Tables

Table 1. *Relative* recovery tests for gliadin spiked flour samples.

Samples Nº.	Gliadin content mg Kg^{-1}	Found with spiked gliadin[a]	Relative recovery (%)	CV (%) (n=6)
Rice noodles (2)	Nd	19.92	98.65	3.80
Free gluten flour (3)	3.01	22.87	99.39	5.01
Common wheat flour (3)	59.06	79.56	102.33	3.98
Stout beer (3)	17.83	38.12	100.77	4.69
Common noodles (2)	52.11	73.35	101.72	4.74
Wheat beer (3)	20.03	39.89	99.65	3.75

[a] The data was obtained from six independent experiments (n = 6). The samples were spiked with 20 mg Kg^{-1} of gliadin.

Table 2. Comparison of different techniques with previously reported methods for the gluten determination.

System	*Detection*	*Sample*	*LOD*	*Publication year*	*Ref.*
Immunoassay	Fluorescence	wheat, barley, oat, ric foxtail millet, corn, ry etc.	0.6 ppm	2013	[36]
Magneto immunosensor	Electrochemical	Beer	0.0242 ppm	2011	[37]
Genosensor	Electrochemical	Bread, flour (wheat and rice)	10 ppm	2016	[38]
Aptasensor	Electrochemical	Food	4.9 ppm (gli-1) y 0.5 ppm (gli-4)	2016	[39]
Immunoassay	Fluorescence	Beer, flour and noodles	0.0012 ppm	-	-

Highlights

►A novel device based on the use of a paper platform modified with a mesoporous material: SBA-15 for the fluorescent detection of gliadin was developed.

►The incorporation of SBA-15 combined with fluorescent detection allowed us to achieve determination of very low levels of gliadin

►The proposed analytical device proved to be a suitable strategy to address the rapid analysis of gluten content in food samples intended for celiac patients.

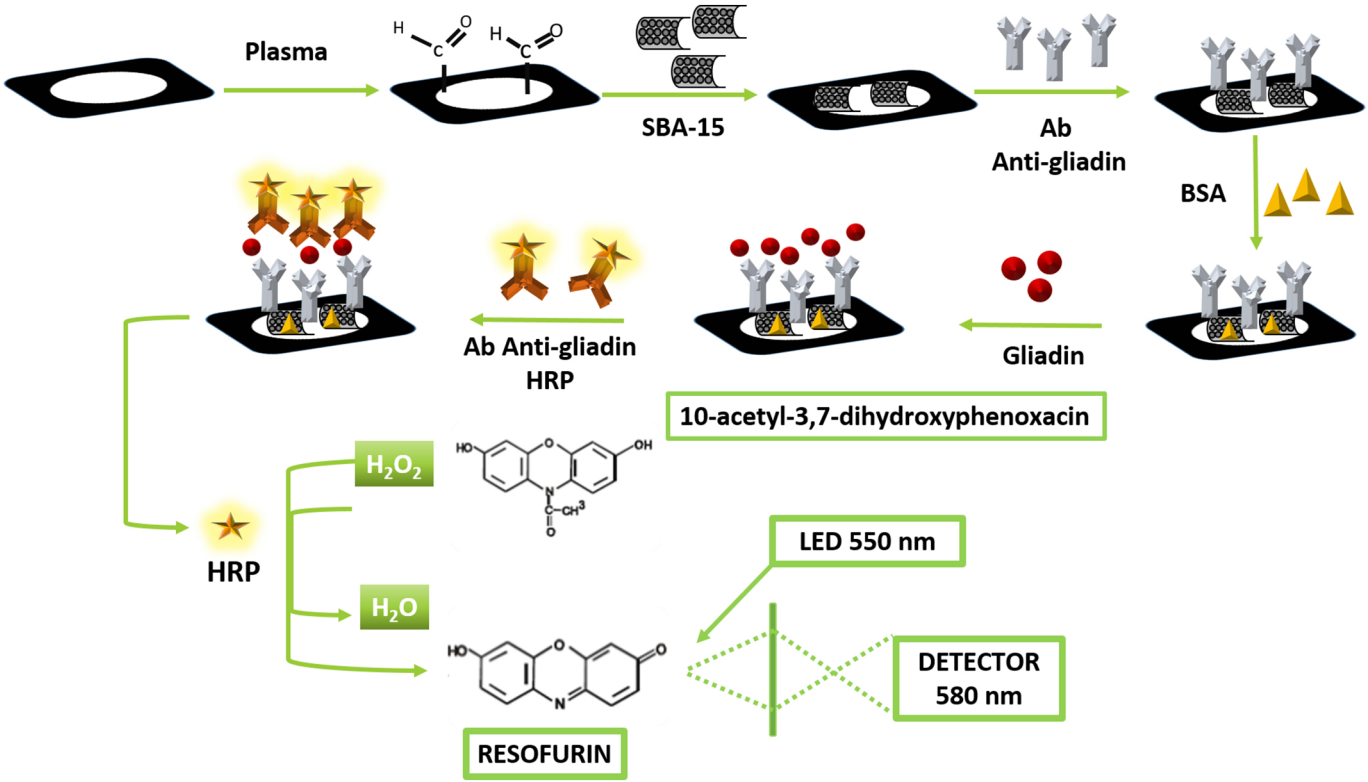
Plasma
SBA-15
Ab
Anti-gliadin
BSA
Gliadin
Ab Anti-gliadin
HRP
10-acetyl-3,7-dihydroxyphenoxacin
H_2O_2
H_2O
HRP
LED 550 nm
DETECTOR
580 nm
RESOFURIN

Figure 1

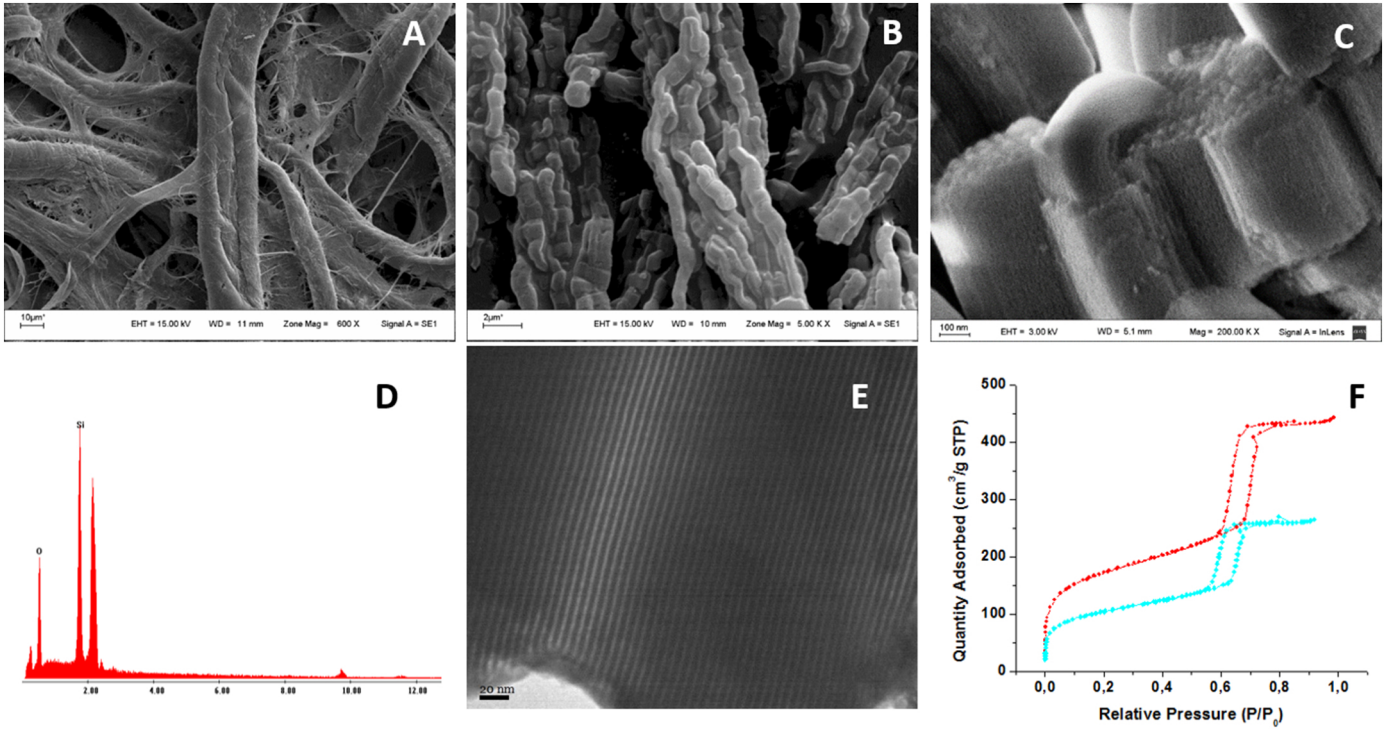
A
10µm
EHT = 15.00 kV
WD = 11 mm
Zone Mag = 600 X
Signal A = SE1
B
2µm
EHT = 15.00 kV
WD = 10 mm
Zone Mag = 5.00 K X
Signal A = SE1
C
100 nm
EHT = 3.00 kV
WD = 5.1 mm
Mag = 200.00 K X
Signal A = InLens
D
O
Si
2.00
4.00
6.00
8.00
10.00
12.00
E
20 nm
F
Quantity Adsorbed (cm3/g STP)
Relative Pressure (P/P0)
0
100
200
300
400
500
0,0
0,2
0,4
0,6
0,8
1,0

Figure 2

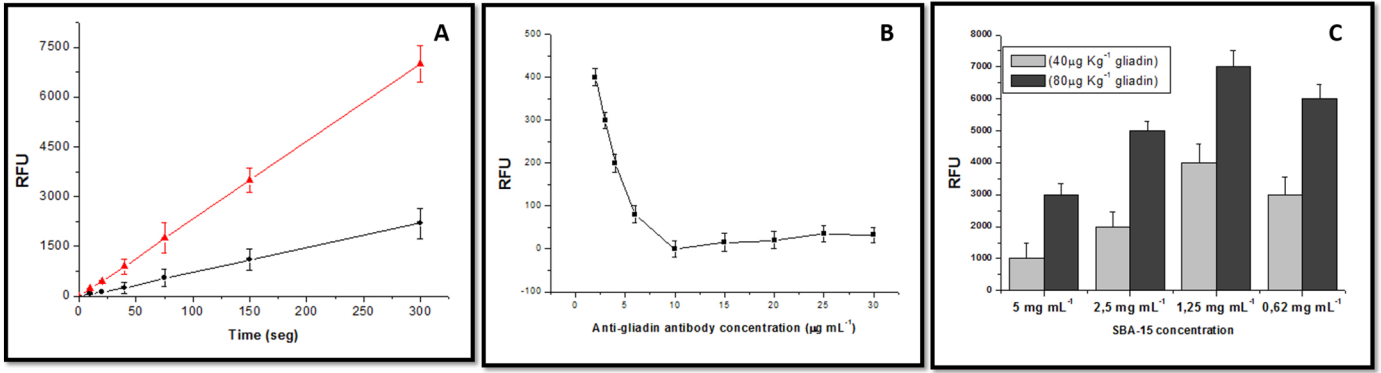
A
RFU
Time (seg)
B
RFU
Anti-gliadin antibody concentration (μg mL-1)
C
(40μg Kg-1 gliadin)
(80μg Kg-1 gliadin)
RFU
5 mg mL-1
2,5 mg mL-1
1,25 mg mL-1
0,62 mg mL-1
SBA-15 concentration

Figure 3

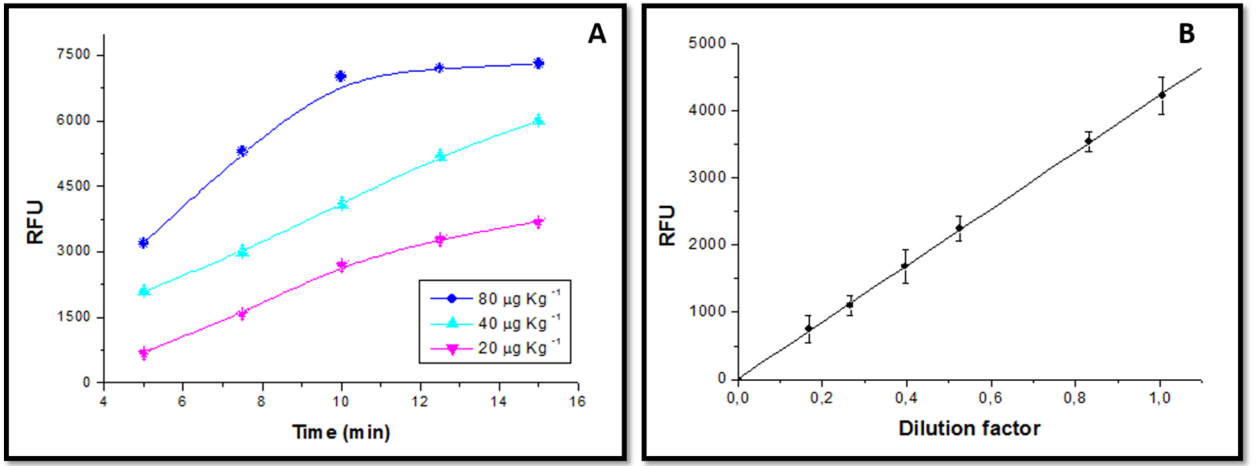
A
RFU
7500
6000
4500
3000
1500
0
4
6
8
10
12
14
16
Time (min)
80 µg Kg -1
40 µg Kg -1
20 µg Kg -1
B
RFU
5000
4000
3000
2000
1000
0
0,0
0,2
0,4
0,6
0,8
1,0
Dilution factor

Figure 4