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Specific optical-based biosensor to rapid detection of *Salmonella* Typhimurium using FTIR: evaluation in natural orange juice, as an application in food products

Biosensor óptico específico para la rápida detección de *Salmonella* Typhimurium utilizando FTIR: evaluación en jugo de naranja natural, como una aplicación en productos alimenticios

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Abstract

Salmonella is one of the main pathogenic microorganisms present in foods, where natural orange juice is an example, although there are techniques that determine this bacterium, it is necessary to develop a device that allows *Salmonella* detection quickly, easily, and in real time. Therefore, using biosensors is an excellent option to solve this need. In this work, an optical biosensor of amorphous silicon thin films, a little studied material in this area, was applied for the detection of *Salmonella* Typhimurium in natural orange juice, using Fourier transform infrared spectroscopy. The characteristic detection infrared band was identified at 1030 cm^{-1}, related to the functional groups presence of the cell membrane and DNA bacterial, *Salmonella* concentrations in a range from 100 to 1000 CFU/mL were detected and a SEM analysis was carried out. Biosensor did not show cross reactivity with enteropathogenic *Escherichia coli*.

Keywords: biosensor, amorphous silicon, *Salmonella*, FTIR, orange juice.

Resumen

Salmonella es uno de los principales microorganismos patógenos presentes en alimentos, donde el jugo de naranja natural es un ejemplo, si bien existen técnicas que determinan la presencia de esta bacteria en alimentos, es necesario desarrollar un dispositivo que permita su detección de forma rápida, sencilla y en tiempo real. Por lo que, el uso de biosensores es una excelente opción para dar solución a esta necesidad. En el presente trabajo, se utilizó un biosensor óptico elaborado sobre películas delgadas de silicio amorfo, material poco estudiado en esta área, para la detección de *Salmonella* Typhimurium en jugo de naranja natural, mediante espectroscopia infrarroja por transformada de Fourier. Se identificó la banda infrarroja característica de detección a 1030 cm^{-1}, relacionada con la presencia de grupos funcionales de la membrana celular y ADN bacteriano, fueron detectadas concentraciones de *Salmonella* en un rango de 100 a 1000 UFC/mL y se realizó un análisis SEM. El biosensor no mostró reactividad cruzada con *Escherichia coli* enteropatógena.

Palabras clave: biosensor, silicio amorfo, *Salmonella*, FTIR, jugo de naranja.

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1 Introduction

Pathogenic microorganisms are agents that can cause various infectious diseases, becoming a serious health problem worldwide, being *Salmonella* ranked first out of the five major pathogens related to foodborne illnesses resulting in hospitalization and death (Liu *et al*., 2019), for this reason, the development of a device that allows an easy and reliable detection of *Salmonella* is required. Biosensors can be an option to this need, as they are devices that can bind to an analyte of interest by using a biological recognition element (antibodies, enzymes, aptamers) which makes it specific towards this analyte, besides, they have a transducer (optical or electrochemical) which is capable to transform a biochemical signal into a qualitative or quantitative signal that can be interpreted by the user (Ibrahim *et al*., 2020; Huang *et al*., 2018; Maghsoudi *et al*., 2018 & Mendoza-Madrigal *et al*., 2013), where Fourier transform infrared (FTIR) spectroscopy is the optical method that is most often used for bacterial detection and identification (Zarnowiec *et al*., 2015). For the development of this type of device, a wide variety of materials have been used, being amorphous silicon (*a*-Si) thin films one of the least occupied and that have proven to be effective as a platform for the development of biosensors that permit the detection of pathogenic microorganisms (Gómez *et al*., 2020) besides, *a*-Si offers a more flexible fabrication process because it can be deposited at low temperatures (≤300 ºC) and thus allows using glass or plastic materials as substrates, this can be translated into low-cost fabrication and more processing options (Álvarez-Macías *et al*., 2017) also an amorphous material can give more flexibility to the functionalization layer during its formation (Gooding *et al*., 2011); for example, Pitruzzello *et al* (2020) reported a label-free resonant metasurface based on a nanohole array in *a*-Si to detect *E. coli*; Andrei *et al* (2020) studied the integration of CH_3O-PEG750 onto *a*-Si layer in order to develop a surface-enhanced-Raman-scattering-based biochip to detect uropathogenic *E. coli*; while Ma *et al* (2013), developed an *a*-Si thin film transistor array based sensing approach. One of many applications that biosensors have, can be in food, like natural orange juice, that can be available in a range of forms from highly processed to minimally processed. Minimally processed orange juice has a high consumer demand but there is a potential microbiological risk due to acid-tolerant bacteria (Anvarian *et al*., 2016), where *Salmonella* is one of the main microorganisms reported in outbreaks related to consumption of unpasteurized orange juice (Shahbaz *et al*., 2018); for this reason, quick and easy detection of *Salmonella* in natural orange juice samples is important. Therefore, the objective of this work was to apply optical *a*-Si biosensors to detect *Salmonella* Typhimurium at different concentrations, using natural orange juice samples by FTIR spectroscopy, besides observing them by scanning electron microscopy (SEM) and evaluating their specificity.

2 Materials and equipments

The chemical reagents used in this project were the following: trichlorethylene, acetone (Meyer), hydrogen peroxide (Meyer), sulfuric acid (Meyer), (3-amino propyl) trimethoxy silane (3-APTMS, Sigma Aldrich), toluene (Sigma Aldrich), methanol (Meyer), glutaraldehyde (Sigma Aldrich), MilliQ water, phosphate buffered saline (Sigma Aldrich).

Casein peptone, yeast extract, NaCl, and bacteriological agar were used to prepare Luria Bertani agar and broth.

An FTIR Bruker Vertex 70 as well as a scanning electron microscope JSM-6610LV were utilized in this project.

Studied bacteria *Salmonella* Typhimurium ATCC 14028 and enteropathogenic *Escherichia coli* E2348/69 were provided by the Microbial Pathogenicity Laboratory from the Microbiological Sciences Institute of the Benemérita Universidad Autónoma de Puebla (BUAP), Mexico.

3 Methodology

3.1 Self-assembled monolayers (SAMs) on amorphous silicon (a-Si) thin films

a-Si thin films were obtained by plasma-enhanced chemical vapor deposition (PECVD) at Instituto Nacional de Astrofísica, Óptica y Electrónica (INAOE) in Puebla, Mexico. SAMs methodology was carried out considering the process proposed by Gómez *et al* (2020), with the only modification that all washing steps of the thin films were made with manual stirring instead of sonication, in order to avoid

the loss of thin film. The correct functionalization and biofunctionalization of the platforms were confirmed by FTIR spectroscopy, in transmission mode, 120 scans, and 4 cm^{-1} of resolution.

3.2 Detection of Salmonella Typhimurium in natural orange juice samples using a-Si biosensors

One *Salmonella* colony (previously grown in Luria Bertani agar) was inoculated in 5 mL of Luria Bertani broth during 24 h, after this time, optical density was measured at 600 nm (OD_{600}), adjusting this value to 0.3; the cells were washed with phosphate buffered saline four times by centrifugation (10 min/25 ºC/2500 rpm), later, bacteria were resuspended in 4 ml of phosphate buffered saline to maintain an OD_{600}=0.3, from this stock solution, dilutions were prepared in natural orange juice previously sterilized (autoclave, 121ºC/15 min) in order to obtain concentrations: 100, 250, 500, 750, and 1000 CFU/mL, since, according to Dawoud *et al* (2017), *Salmonella* concentrations with values less than 10^3 CFU/mL can develop gastrointestinal diseases in humans; in addition, regulations such as ISO 6579-1 mention that *Salmonella* must be absent from analyzed food sample, therefore, biosensor detections close to 0 would help to comply with international regulations. Biofunctionalized *a*-Si platforms were placed in 500 μL of the bacterial suspensions for 1 h/37 ºC/100 rpm, to continue with the washing step of the platforms (45 min/4 ºC) and their analysis by FTIR (same conditions as mentioned in the previous section), results were compared with obtained results in the spread plate procedure. Scanning electron microscopy (SEM) was used to observe the topography of platforms as well as immobilized bacteria on surface. Detection assays were made three times by triplicate.

3.3 Cross reactivity assays

Cross reactivity analysis was carried out to determine that the developed biosensor does not detect bacteria which it was not elaborated for, such as *E. coli*, a microorganism morphologically similar to *Salmonella*; therefore, methodology and conditions were the same as mentioned in the previous section, using an EPEC inoculum.

4 Results and discussion

4.1 Self-assembled monolayers (SAMs) on amorphous silicon (a-Si) thin films

In Figure 1, the spectra of each stage of SAMs are shown, where presence of characteristic FTIR bands is confirmed; these bonds and their wavenumbers are mentioned in Table 1. Black spectrum (aSi + APTMS) corresponds to the functionalization with 3-APTMS, next SAMs step (functionalization with glutaraldehyde) is showed in the dark grey dashed-line spectrum (aSi + APTMS + GTA), finally the antibody immobilization FTIR analysis can be seen in the upper spectrum (aSi + APTMS + GTA + AB), where bands related to amide I and amide II are shown, these bands are characteristic of proteins, and they are related specifically to carbonyl and amino groups of the peptide bond (Ji *et al*., 2020).With this, the correct elaboration of the biosensor is confirmed.

Table 1. Characteristic FTIR bands related to SAMs process elaborated with: 3-APTMS, glutaraldehyde, and antibodies used as biological recognition element.

Wavenumber (cm^{-1})	Bond	Reference
1470-1540	N-H (1)	(1) Pasternack *et al*. (2008)
1030 and 1100	Si-O-Si (1)	(2) Kennepohl *et al*. (2020)
1490	NH_3^+ (1)	(3) Dragone *et al*. (2013)
1640	C-N (1)	(4) Paluszkiewicz *et al*. (2017)
1700 - 1740	C=O (2)	
1627	C=N (3)	
1650 - 1670	Amide I (4)	
1540 - 1560	Amide II (4)	

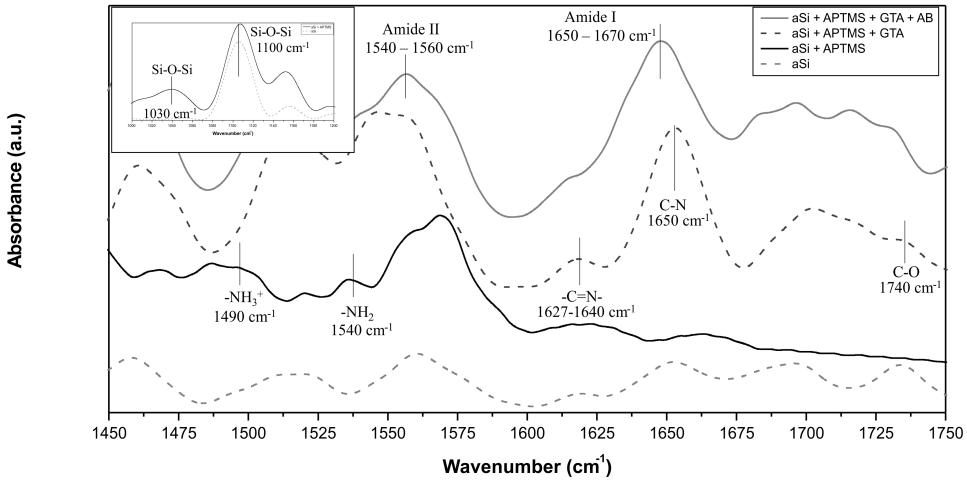

Fig. 1. FTIR spectra from each SAMs stage on *a*-Si thin films, the spectra were obtained in transmission mode.

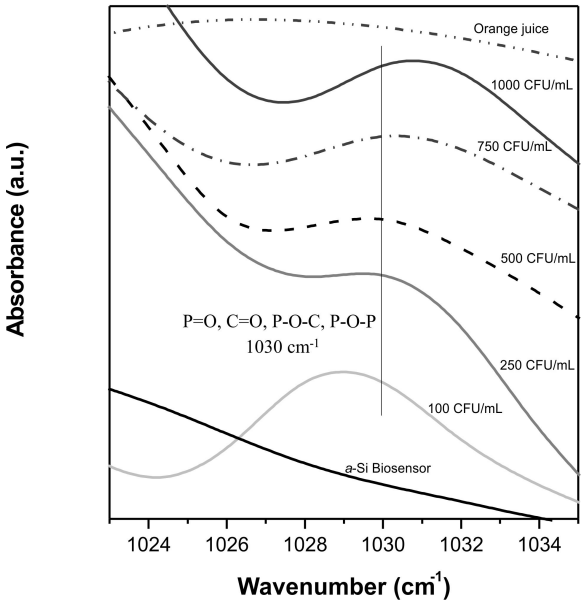

Fig. 2. FTIR detection spectra of *Salmonella* Typhimurium ATCC 14028 on *a*-Si biosensors in natural orange juice samples, spectra were obtained in transmission mode.

4.2 Detection of Salmonella Typhimurium in natural orange juice samples using a-Si biosensors

Salmonella can be present in unsuitable environments because it acquires a state known as "viable but nonculturable", it is considered a state of latency that allows this microorganism to stay alive in the orange juice food (Shahbaz *et al*., 2018). Five different *Salmonella* Typhimurium concentrations could be detected in natural orange juice samples (100, 250, 500, 750, and 1000 CFU/mL). According to Gómez *et al*. (2021) and Muntean *et al*., (2021), after the detection of *Salmonella*, a FTIR band was identified as characteristic at 1030 cm^{-1}, this band is related to bonds (P=O, C=O, P-O-C, and P-O-P) present in the bacterial cell membrane components like phospholipids and glycerophospholipids (Dalebroux *et al*., 2015); as well as the sugar phosphate backbone from DNA (Heaney and Graeff-Armas, 2018), this wavenumber value was considered to confirm the detection of the bacteria on *a*-Si biosensors. In Figure 2, FTIR spectra corresponding to the detection of microorganism in the juice are shown; lower spectrum corresponds to *a*-Si biosensor prior detection, furthermore, orange juice FTIR spectrum was used as control in this study, since this food contains some compounds like vitamin C, carbohydrates (fructose, glucose, sucrose), proteins, and dietary fiber (Chanson-Rolle *et al*., 2016) that could interfere in the identification of the FTIR *Salmonella* band detection, despite this, no similar bands are observed when comparing the orange juice FTIR spectrum with the microorganism detection FTIR spectra. The 5 spectra in the central part of the figure were obtained after bacteria detection process, where the presence of the detection band (1030 cm^{-1}) of interest was confirmed, thus revealing *Salmonella* presence on the biosensors. Besides, it is important to mention that the intensity of this band increases as the bacteria detected concentration increases too, where the minimum concentration (100 CFU/mL) detection band is very notorious.

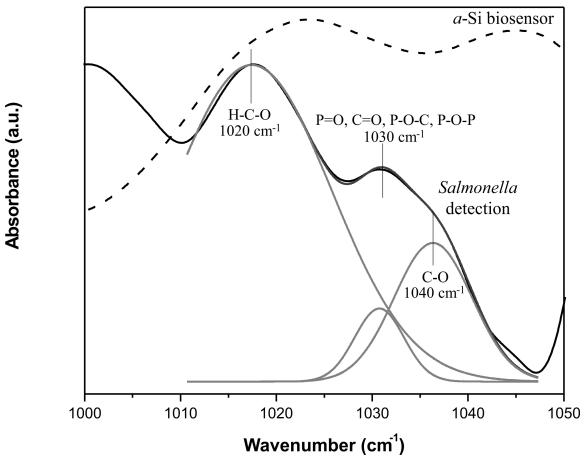

Fig. 3. Deconvolution made in FTIR spectrum related to the detection of 1000 CFU/mL; where dashed line corresponds to the biosensor spectrum and black line to the *Salmonella* detection; gray lines correspond to the bands obtained after deconvolution process.

Deconvolution between 1000 and 1050 cm^{-1} area was made (Figure 3), the generation of three bands is observed, where the FTIR bands at 1020 and 1040 cm^{-1} are related to the biosensor (Figure 3, dashed line), according to Chai *et al*. (2013) and Ramirez *et al*. (2019) band at 1020 cm^{-1} corresponds to H-C-O bond, while the band at 1040 cm^{-1} corresponds to the C-O bond, being both bonds present in the glutaraldehyde molecule; so the band at 1030 cm^{-1} coming from bonds present in the bacterium may not be affected by the bands around it.

In addition to FTIR spectra from Figure 2; in Figure 4, micrographs corresponding to *a*-Si bionsensors are shown. In Figure 4a, topography of *a*-Si biosensor surface is observed, in which changes in the integrity of the platform are seen, these modifications could be generated by the oxidative activity from piranha solution (activation process); where, in the higher areas, amorphous silicon thin film is observed without modifications, while in the deeper areas the platform oxidation is revealed. Meanwhile, in Figures 4b and 4c, glutaraldehyde polymers generated during functionalization step are enclosed with a white circle, and whose structure is similar to the one reported by Guibal *et al* (2014) and Contreras *et al* (2020). Although, the antibodies bound to the surface cannot be observed with the SEM technique, Naja *et al* (2007) reported that after the binding of antibodies to gold nanoparticles and the subsequent analysis by SEM, villi-like structures were observed and these were not previous binding of antibodies to the nanoparticles; in Figure 4, structures can be like the ones reported by them. Finally, in micrograph from Figure 4d, biosensor is shown after the detection of *Salmonella*, where it is observed bacilli-like shaped structures with 2-5 μm length and 0.8-1.5 μm wide, which are characteristic of bacteria under study (Percival and Williams, 2014). Other authors have reported (Hello *et al*., 2018; Haddada *et al*., 2013) that, when biofunctionalization is carried out, uniform surfaces are not obtained, but islands of different sizes are generated, something that we can verify by SEM technique (Figure 4d). Regarding the bacteria observed on the surface, it is proposed that smaller biofunctional islands may be below it, and since the antibody is against complete bacteria, the antigen-antibody interaction would be facilitated.

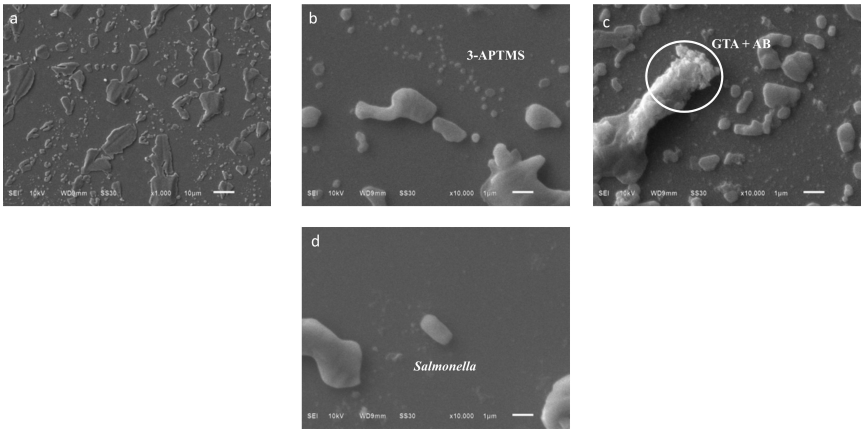

Fig. 4. SEM micrographs of *a*-Si biosensors where: a) biofunctionalized *a*-Si biosensors surface, b) 3-APTMS on *a*-Si biosensors, c) glutaraldehyde and 3-APTMS on *a*-Si biosensors, d) presence of bacteria on *a*-Si biosensors (detection).

Comparing results obtained in this project with other reports, Silva *et al*. (2019) detected *Salmonella* Typhimurium (10^1 – 10^6 CFU/mL) in apple juice samples and phosphate buffer suspensions with a detection time of 1 h using gold nanoparticle polymer inclusion membrane as antibody support and a sensor platform by electrochemical method. Wang *et al*. (2020) detected concentrations between 10^2 – 10^6 CFU/mL with a detection time of 2 h using nickel nanowires with immobilized antibodies, also, there are already commercial biosensors for *Salmonella* detection, for example the Xpress Pathogen Detection System (CDx) from Crystal Diagnostics, however the analysis requires 10 h, another example is the biosensor from Romer Labs which offers validated food pathogen detection solutions for *E. coli*, *Salmonella*, and *Listeria*, having an analysis time of 8 h; none of these biosensors have applications in juices (Di Nardo and Anfossi, 2020).

Considering detecting time established in this project (1 h), the concentrations detected, and the possibility that it might have a quantitative application; the use of this biosensor is a promising development.

It is very important to mention that there are only a few previous studies about the use of *a*-Si thin films for biosensors elaboration and their subsequent application in pathogens detection in real samples, for this reason, the results reported in this study show the efficiency, rapidity, and specificity of these biosensors to be used in the detection of pathogenic microorganisms in this type of samples.

4.3 Cross reactivity assays

Cross-reactivity between antigens occurs when an antibody of a specific antigen binds with a different one, then the two antigens have similar structural regions (epitopes), this allows the antibody to recognize a second antigen as if it were the target antigen (Chadwick, 2008). It is observed in the obtained FTIR spectra (average of all the analyzes) after cross-reactivity process (Figure 5) that there are no differences between each of them by being similar to the FTIR biosensor spectra prior to the detection process, in addition, the spectrum in the upper part shows how the FTIR spectrum should be if the bacteria interaction with the antigen would have existed, in which it is observed the formation of bands related to characteristics functional groups from bacterial cell membrane at 1015 and 1030 cm^{-1}. According to Vining *et al*. (1981), cross-reactivity of antigens

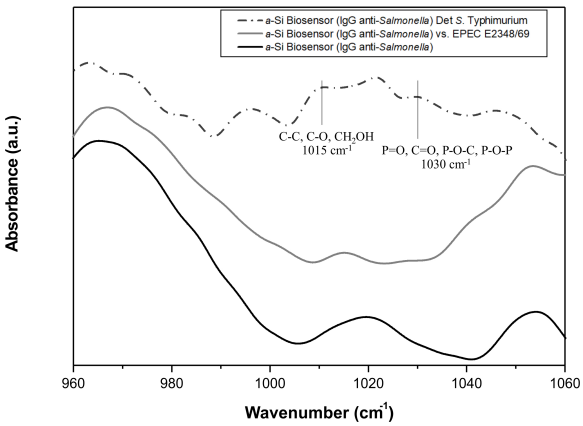

Fig. 5. FTIR spectra related to cross-reactivity assay, where: *a*-Si Biosensor (anti-*Salmonella* IgG) vs. EPEC E2348/69 is an average spectrum of all the spectra obtained in the cross-reactivity analysis. All spectra were obtained in transmission mode.

can be decreased by applying temperatures close to 37 ºC and about 1 hour of antibody-antigen interaction process; values previously mentioned were used during the interaction of microorganisms with the biosensors, additionally, it is reported that cross-reactivity decreases more by using polyclonal antibodies than monoclonal antibodies (Olsen *et al*., 1992); it must also be mentioned that using antibodies purification columns can help to improve the selection of specific antibodies (Levinson & Miller, 2002), in the present project an agarose column with protein A (Thermo-Fischer) was utilized to purify immunoglobulins G against *Salmonella* (whole bacteria). With this analysis, specificity from the biological recognition element obtained and immobilized on biosensors in this investigation was confirmed, this characteristic is very important during the biosensor's development and its application.

Conclusions

Using an optical biosensor developed on *a*-Si thin films, *Salmonella* Typhimurium concentrations between 100 - 1000 CFU/mL were detected, using natural orange juice as analyzed samples, where the band at 1030 cm^{-1} was identified in the FTIR detection spectra, this band corresponds to bonds of characteristic functional groups present in the *Salmonella* bacterial membrane as well as its DNA. In addition, the specificity of the *a*-Si biosensor

with immobilized IgG anti-*Salmonella* antibodies was demonstrated by not detecting enteropathogenic *E. coli*, a similar bacterium to the one studied. Finally, detection of *Salmonella* was confirmed by analyzing the biosensor's topography with SEM technique, where the presence of bacillus-shape structures was observed. The amorphous silicon biosensor in conjunction with FTIR, proved to be useful for the detection of *Salmonella* in an easy and specific way, thus presenting a possible option for the elaboration of this type of device, allowing the detection of pathogenic microorganisms in different real samples.

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