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The *Staphylococcus aureus* Exotoxin Recognition Using a *Sensor* Designed by Nanosilica and *SEA* genotyping by Multiplex PCR

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Abstract

Considering the ever increasing population and industrialization of the developmental trend of human life, we are no longer able to detect the toxins produced in food products using the traditional techniques. This is due to the fact that the isolation time for food products is not cost-effective, and even in most of the cases, the precision of practical techniques like bacterial cultivation and other techniques suffers from operator errors, or the errors of the mixtures used. Hence, with the advent of nanotechnology, the design of selective and smart sensors has turned into one of the greatest industrial revelations of the quality control of food products that, in few minutes time and with a very high precision, can identify the volume and toxicity of the bacteria. In this research, based on the bacterial antibody's connection to nanoparticles, a sensor was used. In this part of the research, as the basis for absorption for the recognition of bacterial toxin, medium sized silica nanoparticles of 10 nm in the form of solid powder were utilized with Notrino brand. Then the suspension produced from the agent-linked nanosilica, which was connected to the bacterial antibody, was positioned near the samples of distilled water, which were contaminated with *Staphylococcus aureus* bacterial toxin with the density of 10^{-3} molar, so that in case any toxin exists in the sample, a connection between the toxin antigen and the antibody would be formed. Finally, the light absorption related to the connection of antigen to the particle-attached antibody was measured using spectrophotometry. The 23S rRNA gene that is conserved in all *Staphylococcus spp.* was used as the control. The accuracy of the test was monitored by using the serial dilution ($l0^{-6}$) of overnight cell culture of *Staphylococcus spp.* bacteria (OD_{600}: 0.02 = 107 cell). It showed that the sensitivity of PCR is 10 bacteria per ml of cells within few hours. The results indicated that the sensor detects up to 10-^{4}molar density. Additionally, the sensitivity of the sensor was examined after 60 days; by the 56 day, it had confirmatory results, which started to decrease after this time. Comparison of practical nanobiosensory method with the conventional methods including culture and bio-technology methods (such as polymerase chain reaction) confirmed its accuracy, sensitiveness and uniqueness. It also reduces the time from hours to 30 minutes.

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1. Introduction

One of the most critical issues in food industry is producing healthy foods. Nowadays, consumers demand the foods with least processing and no micro-organisms, additives and conservatives along with a longer life [1]. Waste and expiry of foods and edibles, and their removal from the food industry cycle cost billions of dollars annually.

Different causes might be counted as the factors involved in this phenomenon; among which are the long distance, air travel and transfer, delayed results report of quality control tests by the corporate and reference laboratories as a result of applying common standard methods, and also false negative results received in many cases as the microbe mass vanishes and *toxin Bacteria* remains [1, 2]. This latter creates awful results in case of natural disasters and large-scale import and export of meat and dairy products. Also millions of innocent people are afflicted by such evil traps, and a large amount of inflected food imported to a country are used before testing, especially in unnatural cases like civil wars and bioterrorism, for lack of testing possibilities or time limitations and poison that may kill many people pre-planned. Therefore, with the developments in food technology and the introduction of nanotechnology in this industry, in general, and quality control of food products in particular, it is exactly the right time for the large-scale production of these findings [3]. Firstly, because of time factor efficiency, which is a very important element in transportation of food product cargos in the ports and customs (in some unexpected cases like flood and earthquake, there is no time for quality control and tests), and secondly from the financial point of view, it is much better compared to other similar products available in the international markets, and the last but not the least, it is very sensitive and exact as the two important measures in terms of security and confidence coefficient [4].

Regarding the time needed to observe the microbe culture, resulting in food products' quality control, that is of least 48 hours or even one week for the observation of primary results in the case of Salmonella and some of the strains that need pre-enrichment and enrichment, applying biosensors and nanobiosensors are of great importance. The reason for that is the time needed for maintenance and declaring the results for approval or disapproval of the product before presenting it to the customer is very long, which decreases the lifelong, imposing an indirect loss to the producer in the Research and Development Department (R&D) and also the Quality Control Department of some food factories [2, 5].

One of the most important parts of industries in all countries, which is totally interrelated with their food industry, is their food safety. Using advanced technologies in this part is a new approach met with great popularity. The convergence of nanotechnology and food science results in great potentials and that is why around 200 big companies around the world have made huge investments in this field and even introduced some products to the market. Besides the presented products, regarding the great potentials of applying the nanotechnologies in food industry, a massive revolution in food and agriculture industry is expected in the next two decades so extensive that its effects would be much bigger than the mechanized agriculture and the Green Revolution [6].

The present study has tried to design a sensor with nanotechnology functionality in order to diagnose *Staphylococcus (S.) areus*, as one of the most prevalent factors creating food poisoning with great sensitivity and discrimination [7].

2. Materials and Methods

2.1 *Potentiometer based on a polymer molecule frame*

To build physically and chemically resistant patterns for exotoxin staff, we initially should make a proper solution of this sensor from its pure solution. In so doing, a pure solution with 1.10^{-5} was made from the pure solution presented by the Sigma Company properly diluted by distilled water. At all levels of working with toxin, everything was done at standard temperature, the solution were prepared on a daily basis, and the experiment was done at 25°C. First, a concentration of 10^{-5} molar of exotoxin *S. aureus* type A from the Sigma Company was taken by double distilled water (the toxin soluble used was prepared daily).

2.2 *Providing diluted version from pure toxin bacteria to define the sensor sensitivity*

Based on the exotoxin bacteria from *S. aureus* brochure received from the Sigma Company, there exists 2 mg of toxin, which in 200 µl vial volume equals the 400 mg of toxin weight per each micro liter. To calculate the commercial exotoxin molecular mass based on this label, in each 1 mg of exotoxin that has a mass of 202 mg in the previous vial weight of 400 mg, 2 mM of that is found. Based on molar definition (mole by liter or mM in ml), we solved the resulting 2 mM in 1 ml to get 1 molar toxin. Then1 molar exotoxin was used for serial dilution, which defines the sensitivity, and the least dilution was recognized by the sensor.

Using the double distilled water, 1 ml of 1 molar toxin was mixed with 9 ml of double distilled water, and after sampling, the exotoxin dilution equaled 0.1. Again, 1 ml from the previous dilution was mixed with 9 ml of double distilled water, and the dilution equaled 0.01. This continued in the same way until the dilution equal

to 0.000001 was obtained. It is noteworthy, however, that to obtain the above mentioned dilutions, we used Elisa plate to simplify applying the sensor and entering the electrode into the dilution containers [8].

In this study, silica nanoparticles (size 10 nm on average) as solid powder from neutrino brand were utilized as the basis of absorption to recognize the exotoxin bacteria (to recognize exotoxin, we used the linking reaction between nanoparticles and antibody as the base recognition factor). Nanosilica, which was used as the surface of these particles, has hydroxyl factors making the surface modifiable. Moreover, it transforms into carboxyl after modifying the hydroxyl factor, and the Nanosilica solid powder is transformed into liquid after the modification. It was obtained as explained below, and then dried and used to connect to the antibody.

To connect the nanoparticles to the antibody, the method of surface modification of silica nanoparticles was applied. The antibody was directly stabilized on the silica nanoparticles, which received the linkage agent from carboxylic acid and; in fact, the linkage of modified nanoparticles to the antibody was presented. This biosensor provides advantages like short time analysis and sensitivity in toxin recognition. Stöber process was used to obtain silica nanoparticles. For this purpose, silica nanoparticles carrying amino were used as pre-material, and the given nanoparticles were created with the carboxylic acid agent group in the context of DMF solution. The studies indicate that nanoparticles have distinctive characteristics in bio-analysis and biotechnology operations [9, 10]. The new modification process on nanoparticles described in this study resulted in the formation of an amide link between the nanoparticles and the antibody proteins by an active ester reaction between the amino group in the antibody chain and the carboxylic acid agent. The related phases are presented in Figure 1.

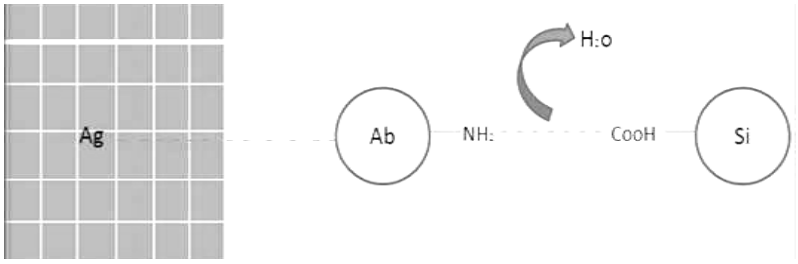

Figure 1. Modified Stöber process (a physico-chemical process)

2.3 *Modified Stöber process*

A physico-chemical process for generation of monodispersed particles of silica)**:** According to the following schematic figure, first 4 ml of Tetraethoxysilane was added to a mix of 33 ml of ammonium hydroxide, and 47 ml of ethanol and reaction atmosphere was put on a magnet stirrer in the lab temperature for 24 hours. Ultimately, colloidal silica was observed. Then 0.3 ml of APTES catalyst was added to the reaction atmosphere, and stirring of the mixture was continued for 24 h at 25°C on a magnet stirrer. This way, the silica nanoparticles changed into amino group. To change from amino group to carboxylic acid, 1g of amino silica nanoparticles was depressed in 50ml DMF solvent in a flask. Then 0.5 g of succinic anhydride and 5ml of triethanolamine were added to the flask. This mix was stirred for 20 h at 70°C, so that the surface of the obtained nanoparticles would be modified by the carboxylic acid agent. The particles were totally washed in DMF solvent by several depression (against sonicitor and using ultrasonic waves) and then by centrifuge (TURKOGLU, E. A., 2012).

2.4 *To check sensor sensitivity*

To check the sensor sensitivity, the amount of nanosilica, antibody and antigen was changed, and any increase or decrease in the sensor sensitivity in the mentioned phases was checked. First, the nanoparticles carrying agents produced in the latter phase, which are susceptible to link to antibody, were put in different antibody dilutions. In this phase, a bond between carboxylic nanoparticles and amine group from amine the ending of the antibody is expected. Thus 5ml of the modified nanoparticles was put in different antibody dilutions, and the mixing operation was continued slowly for 10 minutes. Then 1.5, 1.10, 1.20 and 1.40 molar antibody dilutions were applied. As the bonding operation ended, 10^{-3} molar toxin solution was added to each of the above solutions, and after a 1minute slow mix in the test tube, chromogen soluble was added to them. Then the color change

degree of each of them in 520 nm wave length was checked (according to red and orange colors out of the seven color spectra).

To enhance the sensor's sensitivity, the amount of nanosilica powder used was increased. So that 6, 8, and 10 mg of nanosilica carrying agent were obtained through the Stöber process, and were prepared to connect to the antibody based on the previous steps. Then the suspension resulting from the nanosilica carrying agents connected to the antibody bacteria were put in the distilled water samples infected by toxin bacterium *S. aureus* with 10^{-3} molar concentration in order to form a link between the toxin antigen and the antibody in terms of toxin presence in the sample. Finally, the results of light absorption (due to the presence of chromogen in the sensors containing different amounts of nanosilica) were analyzed and studied using a spectro-photometer. Ultimately, in order to analyze the last possible parameter in designing the sensor the toxin dilutions were examined. In other words, the optimum gram of bonded nanosilica with the ideal amount of antibody obtained in the latter step was put in different dilutions of the antibody to measure the least recognizable amount in this sensor for the optimum nanosilica and obtained antibody.

2.5 *Life time*

In the third step of the second phase of the project, the life span of the designed sensors was targeted to study. More specifically, it was studied that how long the recognition qualities in each of the sensors based on the molecular frame polymer or in the sensors resulting from the bonding between antibody bacteria and nanosilica last, or how long they can recognize the toxin. This issue was tested in the days 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 60 of the sensor design.

2.6 *Multiplex PCR*

Prior to molecular analysis, the specimens were aseptically transferred into the culture medium for microbial analysis. They were streaked onto the brain heart infusion broth (Merck), mixed and incubated overnight at 37°C. After incubation, the growing colonies were picked up and used for bacterial DNA isolation using the method adopted by Gillespie and Oliver [9].

Identification of the isolates was done by cultural, morphological and biochemical characters according to Cremonesi et al. and Da Silva et al. [11, 12]. The results were compared to determine the sensitivity of the multiplex PCR assay in identifying the bacteria with culture.

The sample of growth colonies was transferred into 1.5ml micro-tube, and the bacterial cells were precipitated by centrifugation at 3,000×*g* for 5 min at 4°C, and the aqueous phase was discarded. Some 30µl of 10% SDS was added to the bacterial cells and transferred to a sterile mortar containing enough volume of liquid nitrogen (LN). After freezing, the cells were squeezed by formation of crystals. The bacterial cell wall was smashed with pestle mechanical strikes and disrupted without using any lytic enzymes based on the method described before to extract the fungal DNA. After transferring of the lysed cells into the micro tube, Phenol-chloroform was added and centrifuged in 10,000×g for 10 min. The supernatant (phenol) was removed, and chloroform and alcohol (1:24) were added and centrifuged at 10,000×g for 2 min. Then 20µl RNase was added. The amount of 7.5µl NaCl for precipitation of DNA and 600 µl isopropanol were added and kept in room temperature (RT) for 10 min. After centrifugation at 10,000×g for 10 min at 4°C, the supernatant was removed, and the precipitate, which contained DNA, was washed two times with 70% ethanol, and dissolved in distilled H_2O.

3. Results and Discussion

3.1 *The results of sensor life time*

The results from the second phase of the project concerning the configuration of the sensor's sensitivity life time showed that the sensor's sensitivity towards the antigen bonding decreases in the day 30, while the sensor (with a higher percent of methacrylic acid monomer) stays totally sensitive even in the day 52, and then starts to decrease in sensitivity levels (Figure 2). Therefore, we can conclude that the sensor has a longer life time [13].

3.2 *The sensitivity results of the sensor based on nanosilica parameter*

According to the results obtained, 8g of nanosilica powder has more bonding with the amine antibody concluded from the water molecules formed in ester reaction between amine group and carboxyl. So, based on 8ml water obtained from the reaction, the optimum amount of the powder used is 8g, and any amount more than that should make no difference in the ester reaction.

3.3 The sensitivity results of the sensor based on antibody parameter

The results of this part showed that the degree of light absorption in vertical axis is decreased

from 1.20 dilution level, and is reduced from 0.01, and this decrease in the light absorption levels depicts the lack of bonding in higher dilutions [14].

Figure 3 shows the changes in absorption levels based on different antibody dilutions. As illustrated, the absorption curve is linear up to 1.20 dilution levels, and the maximum potential of modified nanoparticles to bond with antibody happens in 1.20 dilution.

3.4 *The sensitivity results of the sensor based on antigen parameter*

Based on the obtained results in the previous phase, toxin recognition tests in the 1/20 dilution level of the antibody bonding coefficient were performed, and different toxin dilutions, including 1/5, 1/10, 1/25, 1/50, 1/75, and 1/100, were put in the mentioned reaction atmosphere.

In other words, with a fixed 8g optimum amount of modified nanosilica linked to 1/20 antibody dilution, different exotoxin dilutions were analyzed. The sensor recognition system can trace toxin up to a 1.78 dilution with high accuracy, but higher dilutions produce no meaningful absorption response [15].

Table 1. Primers of multiplex PCR

Primer name	Oligonucleotide sequence (30-50)	Amplication size (bp)	Target gene	Reference
23S1200-F	TTT-GG-TCC-TTG-TCC-GGA-TGT-AGC	499	23S rRNA	[11]
23S1698-R	AGA-AT-CTT-CAC-GCT-CTC-TC			
A1	GGT-TAT-CAA-TGT-GCG-GGT-GG	102	SEA	[12]
A2	CGG-CAC-TTT-TTT-CTC-TTC-GG			

The results of DNA extraction obtained in 1.5% agarose gel by using liquid nitrogen are demonstrated in Figure 4 This method is very suitable for Gram-positive bacteria, especially *Staphylococcus spp*, with stiff wall due to the peptidoglycan. The time to extract DNA from the bacteria was roughly estimated in 15 min. By using liquid nitrogen, not only the application of expensive commercial enzymes was omitted, but also the spent time was halved comparing with the commercial enzymes [16].

Traditional methods of working with culture context to recognize toxin bacteria are very time consuming and exhausting. Nowadays, it is very important to employ fast and accurate techniques to recognize pathogens transferred from food products. Since, in most cases, these infections in food products are at low levels, we need fast and sensitive methods to assure the health of food supplies. Current methods of detection and recognition of bacteria and toxins are mostly based on the use of culture context and biochemistry tests, which take 4 to 7 days.

Regarding the time needed to observe the results of microbe culture in the food quality control issues, a 48-hour result score for biosensors and nanobiosensors is definitely of paramount importance, especially in the case of some of the strains that need pre-enrichment and enrichment, and some others like Salmonella even 1 week, to obtain the primary results. Because in many food factories, quality control is a very prolonged process in the Research and Development Department (R&D), and the time needed to approve the product to be presented to the consumer reduces the foods' health life, this would result in an indirect loss to the producer. However, in some branches of food production such as grains, beans, oil, and can, the case is much different from the meat and dairy industries. It can be concluded that the approval timing is a key crucial factor in the quality control systems and in the return of investment to the producer.

It is noteworthy, however, that in isolation operations and even in military and strategic issues, this case is much more functional and considerably highlighted. For instance, in ISO systems, in addition to quality control tests by the producer, the material contractor must also pass checks and tests, because food production is a very interconnected chain; therefore, in import and export of food products and any related material for relevant companies and industries, the release timing as well as the accuracy and exactness of results are very influential in total functionality and profitability of the cycle.

Another remarkable point is that, in most cases, the microbe culture, which is identifiable by the afore-mentioned and traditional methods, is eliminated due to competitive effects of the presence of other pathogens or environmental factors such as temperature, acid atmosphere, etc; hence, the toxin bacteria resistant to temperature remain active and unaffected. This sensor is capable of detection and recognition of such cases by itself, and can greatly assist quality control researchers and experts in food health and safety management systems.

Therefore, inspired by the above-mentioned discussion, speed, accuracy, and detection

conditions are among the most prominent factors, which prove the advantage of sensors to traditional and current methods in food industry quality controls.

Studies show that nanoparticles contain unique characteristics for bioanalysis and biotechnology applications. The new nanoparticles' modification process, explained in this method of antibody and nanoparticle bonding, leads to the formation of amide bonding between the nanoparticles and the exotoxin protein. This bond is formed by an active ester reaction between the amine group of exotoxin chain and the carboxylic acid agent. This biosensor enjoys advantages such as a decreased analysis time and increased sensitivity in toxin detection [11]. Nonetheless, among the disadvantages of this method, we can mention detection sensitivity limitation due to the lost capability of bonding in some antibodies to link to antigen as they are engaged with silica nanoparticles.

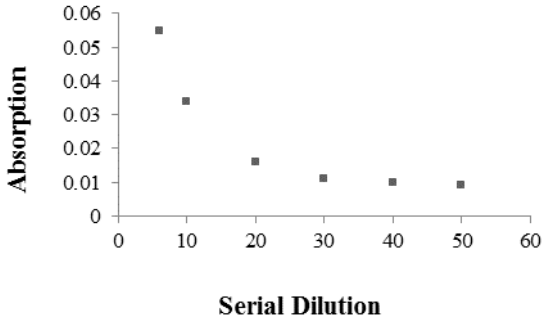

Figure 2. Life time sensor

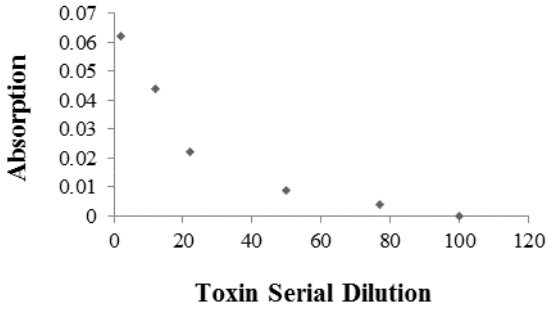

Figure 3. Absorption level changes in toxin recognition system in presence of modified nanoparticles and based on antibody dilution coefficient

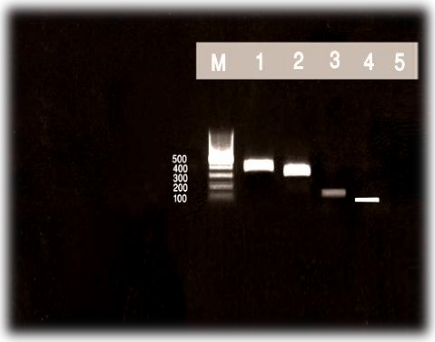

Figure 4. PCR sensitivity test by using 23S rRNA gene (499 bp): lanes 1-6; 106, 105, 104, 103, 102 and 101 standard *S. aureus cells*; M, 100 bp DNA molecular marker.

Hence, the antibody itself is the determining factor of the sensor sensitivity, and not the density of exotoxin. In a situation where the antibody is utilized less than the optimized amount mentioned in the design, it would bond to the modified nanosilica; however, its linking capacity to the antigen or the same toxin would be decreased due to the engagement with the nanosilica. This occurs when it causes the sensor sensitivity to diminish. But most of the times that we do not have any problems about the existence of antibody, the sensitivity level of this kind of sensor, in comparison to the molecular frame polymer sensor, is significantly higher and more precise. In the second part of the comparison, the total cost of the sensor with regard to the microbial characteristics of the sensor can be mentioned. The molecular frame polymer sensor demands a very high cost for the initial design; however, in its simulation and final mass production, this deficiency would be eradicated. Nevertheless, the total cost of the designed sensor based on the antibody-nanoparticle link is many times more ideal and is dependent on the type of the nanoparticle utilized as well as the antigen and antibody used from the bacteria or any pathogen microorganism.

The production of potentiometric sensors based on molecular frame polymers is also perfectly cost-effective and cheap regarding the machine. After the production of the sensor, the intended experiment can be carried out by making use of a simple potentiometer. That is, the materials needed for the sensor are expensive

but the machine needed for the recognition and result indication is very simple and accessible; however, the chemicals used in producing the sensor are often hazardous and pollutant for the environment. Regarding the world natural environment EPA, the materials used in the antibody connected to the nanoparticle sensor, in contrary to the molecular frame polymer sensor, are environment friendly and use lesser amount of chemicals in comparison to the first method sensor. In addition, the materials used in producing the elements comprising the first sensor are very expensive, and raise its production cost. Also other cons of this type of biosensors are their short lives and the decrease in their sensitivity. This is the matter for both of the sensors, but the sensitivity loss in type 1 sensor is much more evident in comparison to the type 2 sensor with the passage of time. Therefore, based on the studies done, and the review of the official scientific literature, the designed sensor enjoys a higher sensitivity based on the antibody nanoparticle connection, has less total cost, lasts longer, and disseminates less harmful materials targeting the environment and human health. However, the amount and the type of antibody purity are of paramount importance in determining the sensitivity and the usage period of the sensor [2, 4].

4. Conclusion

Generally, the production of biosensors, which is based on using the connection of antibodies to nanoparticles, requires technical knowledge and expert for their purposeful connection to antibody. The production of nanoparticles used is also a very technical and professional process, which was, albeit, used in this research as the ready and trade market type, and their purchase demands very high costs depending on their type.

Comparison of our nanobiosensor to conventional methods like culture and bio technology methods (such as polymerase chain reaction) shows many advantages for it including accuracy, sensitiveness and uniqueness. In the other way it reduces the time from hours to less than 30 minutes.

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