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參展科別 生物化學

作品名稱 **DEVELOPMENT OF PAPER-BASED ORIGAMI BIOSENSOR PLATFORMS FOR COLORIMETRIC DETECTION OF BIOCONTAMINANTS**

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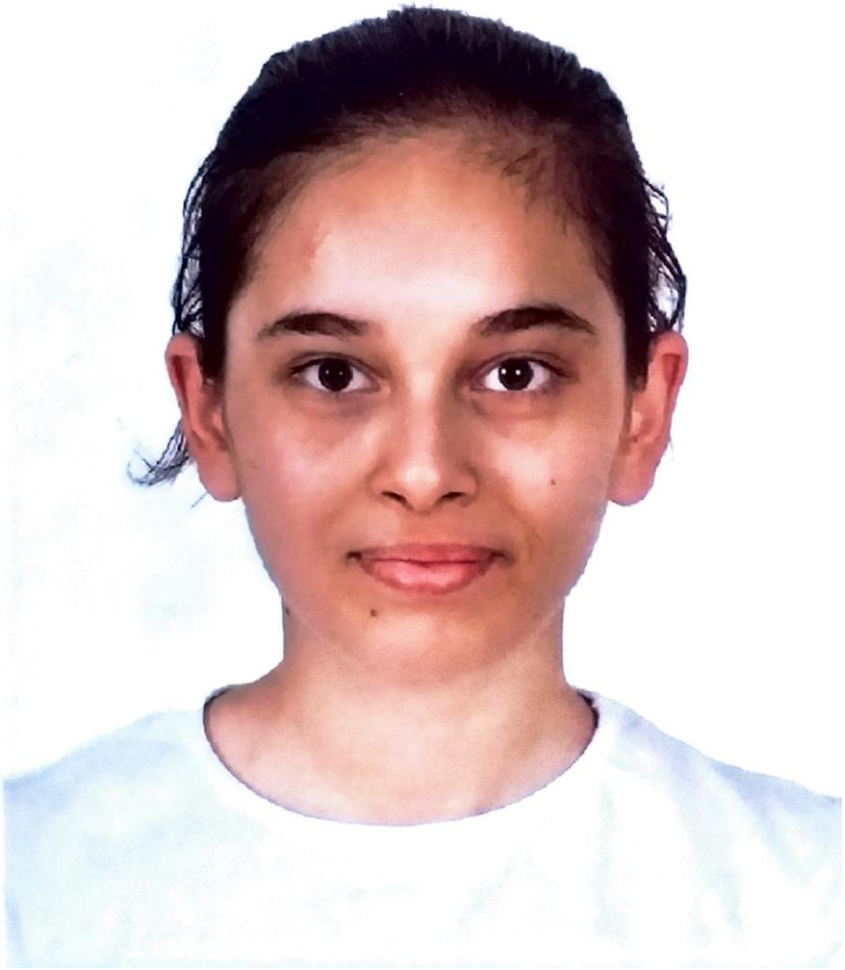
關鍵詞 **Paper-based biosensor、Origami、Escherichia coli**

作者照片



Abstract

Infectious diseases caused by bacteria from biological pollutants pose a great burden in terms of diagnosis and treatment, and millions of people worldwide die from bacterial infections. Detection of bacteria plays a critical role in clinical diagnosis and control of contamination, but is not accessible due to the high cost, complex devices and equipment required.

In the project, an alternative to existing methods, a paper-based biosensor for the detection of model organism E. coli bacteria, which is visible, low cost, easy to use, can be integrated with a smartphone, is based on rapid color change in the exposed environments, drinking and pool water, wastewater, beverage products. platforms were developed. For the specific detection of E.coli bacteria, two different biosensors have been developed that can perform colorimetric detection in a user-friendly origami design, minimizing microchip and processing steps based on antibody-bound PVDF membrane and filter paper-based immunological method. In the presence and absence of target bacteria E.coli, the lowest detection limit of the biosensors obtained by using paper-based platforms that create a distinctive color on them, depending on the concentration, was $0.9x10^3$ bacteria/ml for origami biosensor, $2.7x10^3$ bacteria/ml for microchip biosensor and the widest dynamic linear operating range was calculated as 10^3-10^7 bacteria/ml.

With the biosensor platforms we have developed, the use of only one smartphone for both qualitative and quantitative, visible results and analysis within minutes constitutes the originality of our project. With these promising results, the biosensors we have developed can also be used for the detection of different biological pollutants, do not contain complex devices and can be easily produced in large scales. We believe that the biosensors we have developed for the detection of biological pollutants in water and beverages, especially in regions where test laboratory infrastructure is not available, will contribute to the literature, public health, health economy and sustainable development goals such as clean water and sanitation, health and quality life, and life in water.

Aim

Within the scope of this project, it is aimed to develop paper-based biosensor platforms for the detection of E. coli bacteria, which is used as a model organism, in drinking water, pool water, waste water and various beverage products, which are the environments where humans are exposed to this bacteria. In accordance with this purpose,

- ✓ Design and manufacture of paper-based biosensors
- ✓ Characterization of parameters such as flow parameters, protein adsorption, well volumes of paper-based biosensors,
- ✓ Development of microchip-based and paper-based biosensor systems with two different designs, developed with the user-friendly origami biosensor technique in order to minimize the processing steps and size of the system and using them for qualitative and quantitative E.coli detection in water and beverage are aimed.

1. Introduction

1.1. Detection of Biological Contaminants

Biological pollutants are living entities or products derived from these organisms that invade an environment, producing undesirable effects. Bacteria, viruses, archaea, protists, fungi and various insects can be considered as biological contaminants. These pollutants reach people through media such as soil, water, air, food, and pose a serious threat to public health (McCarthy and Shugart, 2018). Among biological pollutants, infectious diseases, especially caused by bacteria, constitute a great burden in developing countries, and millions of people worldwide die from infections such as pneumonia, bacteremia, gastroenteritis, osteomyelitis, meningitis, endocarditis, and toxic shock syndrome (Mathur et al., 2018). Escherichia coli, which is used as a model organism within the scope of the project, is a bacterium that can also be found in the intestinal environment of living things. Some subtypes such as Enterohaemorrhagic E. Coli and their toxins cause life-threatening diseases. These species can easily be transmitted and cause epidemics (McCarthy and Shugart, 2018).

Detection tests play a critical role in clinical decision making and contamination control, but they tend to be costly and often not accessible (Gous et al., 2018). Current methods used in the detection of biological contaminants are given in Figure 1.

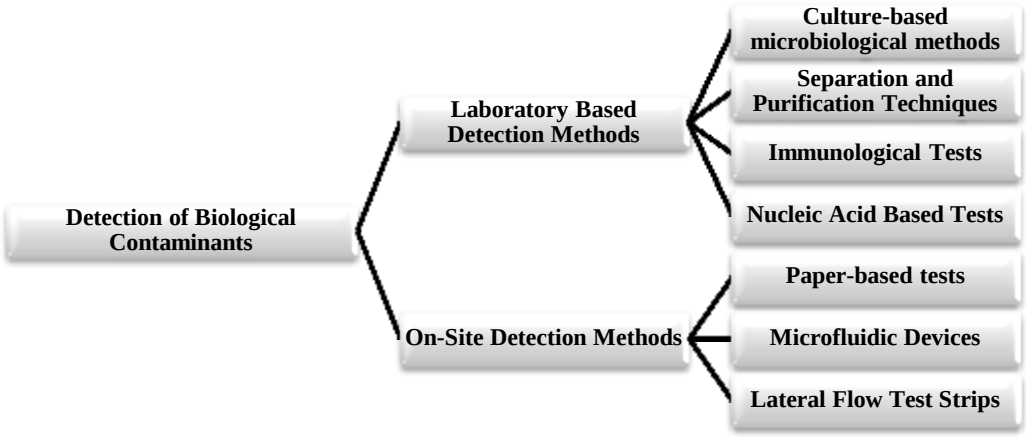


Figure 1. Methods used in the detection of biological contaminants

Ideally, infectious disease detection platforms should be simple to use and interpret, stable under a wide range of operating conditions (such as different temperatures and humidity), portable and disposable. For these reasons, many initiatives have focused on the development of targeted microfluidic immunoassays for limited resources (Chen et al., 2019). Among the recently developed analytical platforms, paper-based lab-on-a-chip technologies are attracting increasing attention due to their low cost and biologically sustainable properties (Quesada-González&Merkoçi, 2018).

1.2.Lab-on-a-chip Technologies

With the rapid developments in fields such as medicine, biotechnology and genomics, the need for chemical/biochemical analyzes has increased and practical applications of lab-on-a-chip technologies have been developed instead of the use of classical methods, laboratories and complex devices. Lab-on-a-chip (LOC) is a new technology that can be used in areas such as food safety, environmental analysis and medical diagnostics, which creates a mini lab on a chip at a small size. These technologies enable one or several analyzes and have important features such as high sensitivity, short diagnostic time, better process control, portability and security. In addition, when compared to existing tests, lab-on-a-chip technologies have many advantages such as low liquid volumes due to its small volume, low production cost, integratability with smartphones and on-site detection (Ghallab et al., 2016;

Yılmaz et al., 2018).

Lab-on-a-chip technologies (LOC) are based on microfluidic systems that allow the processing of small volumes of liquids (100 nL-10 μL sample) in microchannels with at least one size with a length scale of 10-100 micrometers. This definition reveals that performing complex analyzes on a chip is designed to include all phases of a complete laboratory procedure, including sampling, sample pretreatment, chemical reactions, product separation and isolation, detection system and data in a small-scale, automated platform. (Francesko et al., 2019; Park et al., 2020).

1.3.Environmental control and food safety applications of Lab-on-a-Chip Technologies

Based on lab-on-a-chip technologies, microfluidics is a multidisciplinary technology that combines engineering, chemistry, physics, biochemistry, microtechnology, nanotechnology and biotechnology. Microfluidic devices are portable due to their large surface-to-volume ratio, which is useful for in situ testing (Luka et al., 2015). In recent years, efforts have been intensified to combine biosensors with lab-on-a-chip (LOC) technology, which uses microfluidic systems that add a lot of value to biosensor technology (Mark et al., 2010). Biosensors and microfluidic systems are designed to offer an integrated and miniature alternative to traditional repetitive laboratory methods. Working together, they significantly reduce the production of samples, reagents, energy and waste (Dhar et al., 2018). In addition, these microfluidic biosensors can improve analytical performance, low energy and reagent consumption, high specificity and sensitivity, low cost, efficiency, fast reaction rates and portability, real-time sensing compared to traditional approaches, thereby enabling sensing to benefit both developing and developed countries. It allows them to be converted into test procedures (Pol et al., 2017). Such systems are used in the development of devices that provide on-site detection (Point of Care, POC) for drug development, medical imaging, diagnosis of diseases, environmental monitoring and food safety (Francesko et al., 2019).

Portable analytical procedures should be developed to ensure the quality of environmental areas and protect human health from pollutant exposure. Of course, creative solutions must be aligned with the stated sustainable development goals, such as the elimination of plastics and solvents, the use of small samples, and ease of application (Cate et al., 2015). In this context, the use of specific interactions for the long-term development of diagnostic tools and research on paper-based substrates to develop new analytical solutions are showing promising results in environmental applications (Cioffi et al., 2021). Current detection methods of

environmental pollutants are expensive and time consuming, especially advanced environmental monitoring of microorganisms is a major concern. Microfluidic chip-based technologies offer potential alternatives. Environmental pollutants, especially bacterial, viral and parasitic infections and their toxins, pose a serious public health threat. Detecting microbial residues in environmental samples and food products is the first step towards identifying these organisms and protecting one's health and safety from disease (Dhar et al., 2018).

The environmental impact of pollutants as a result of extensive use and subsequent persistence in the environment is a worldwide concern. In this context, efforts to create new methods/technologies for real-time pollution detection are of great interest. Microfluidic laboratory-on-a-chip (LOC) devices are an efficient method for continuous contaminant monitoring in a miniature, integrated manner (Pol et al., 2017).

1.4.Paper-based microfluidic Technologies

Paper-based microfluidic devices are gaining a lot of attention due to their undeniable benefits such as simplicity, cost-effectiveness and no need for laboratory infrastructure and skilled workers. In addition, these devices use small amounts of reagents and samples, provide fast results, are portable and disposable (Cinti et al., 2019). They provide additional advantages such as integratability with various detection systems, high sensitivity, selectivity, simplicity of instrumentation, portability and low total system cost (Du et al., 2012).

1.4.1. Origami-paper-based biosensor Technologies

Origami is the art of folding flat paper to produce three-dimensional sculptures or structures (Kuan et al., 2016; Johnson et al., 2017). This ancient art form has recently come to be used for a new, scientific purpose, as several origami-influenced, paper-based analytical devices have been developed by folding a single plain paper into different designs using a single pattern step. This method provides much easier handling by eliminating the need for complex, sequential, layer-by-layer stacking of individual paper layers using double-sided tape. In addition, these microfluidic origami paper-based sensor systems can be opened to reveal each layer for easy test result analysis (Tian et al., 2017).

The main purpose of utilizing origami in these studies is to simplify the fabrication of three-dimensional (3D) microfluidic channels or multiple working regions within paper microfluidic devices. In these systems, users only need to load their samples into the test zone and apply a single, fixed volume of buffer to initiate the test. In such developed systems, results can be easily obtained by colorimetric reading. In addition, the colorimetric signal can

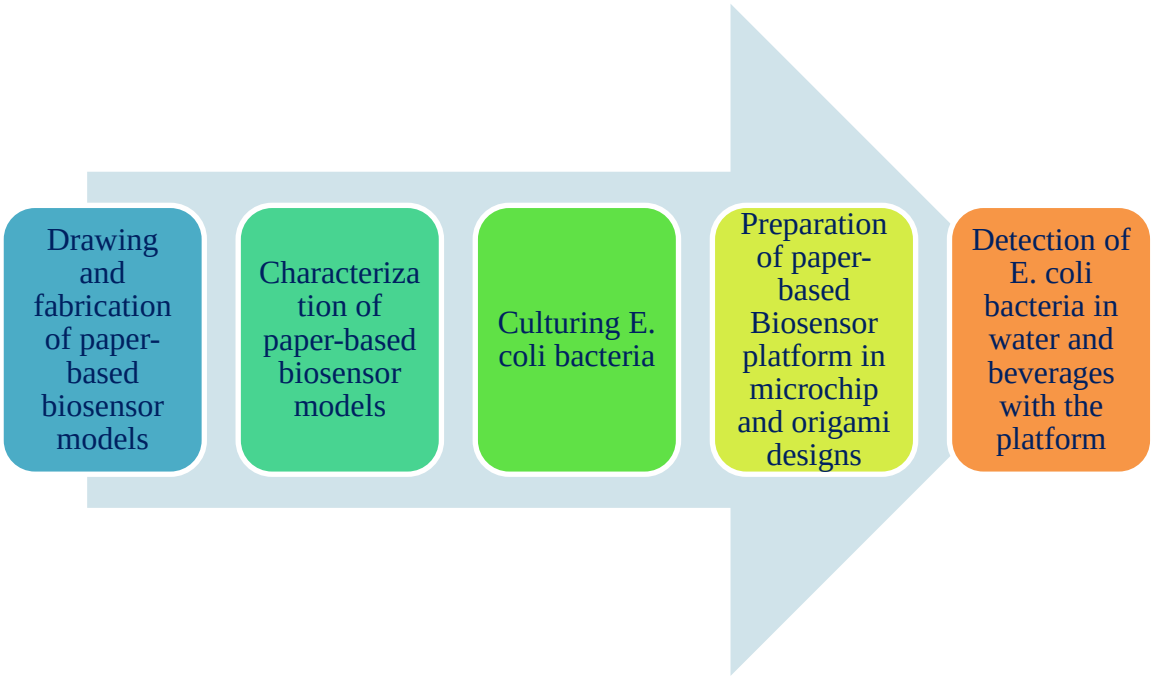
be scanned or photographed using a smartphone and the results can be analyzed and compared with various image processing applications. Several studies continue to be conducted to explore the possibilities of using origami to fabricate PADs to perform biodetections (Ge et al., 2012; Kuo et al., 2019; Chen et al., 2019). It is emphasized that the origami technique, which is an ancient art form, has high future potential for the development of paper-based sensor systems for diagnostics that can bring real benefits to human health.

When the studies in the literature are examined, it is seen that in the study developed by Yamaguchi et al., they developed a microfluidic system for the detection of Legionellapneumophila bacteria in water samples. A fluorescently labeled polyclonal antibody has been used for the detection of L. pneumophila, and a rapid, portable, semi-automatic detection system has been developed for the in situ detection of this bacterial contamination, especially in freshwater. A portable measurement time of 1.5 hours is required for in situ measurement (Yamaguchi et al., 2017). In the study developed by Savaş et al., the gold nanoparticle labeled sensor can detect bacteria with high sensitivity and specificity. This study is more costly due to the use of gold nanoparticles and makes individual use difficult with the use of an electrochemical analyzer at the end of the experiment for detection (Savaş et al., 2018). In the study by Pang et al., a paper-based sensor that detects E.Coli was developed using the ELISA method (Pang et al., 2017). In another study, Değirmenci et al. showed that bacteria can be detected within 8-10 hours with the method they suggested (Değirmenci et al., 2019).

An alternative to existing methods for the detection of E. coli bacteria, which is used as a model organism within the scope of this project, in drinking water, pool water, waste water and various beverage products, which are the environments in which humans are exposed to this bacterium. paper-based biosensor platforms have been developed. In the project, it is recommended to use microchip-based biosensor systems with two different designs, developed with the user-friendly origami biosensor technique in order to minimize the processing steps and size of the system, for the determination of bacteria in liquid media for different purposes.

2. Material and Method

2.2.Project Flow Chart



2.3. Chemicals and Devices

2.3.1. Chemical and Consumables

Chemicals and consumables used within the scope of the project, Ethyl alcohol (Ethanol, EtOH) (70 mL 30mL) (100%), LuriaBertaniBroth, EscherichaColi Bacteria BL 21 Strain, Blue Food Coloring, Ultra Distilled Water, PBS (Phosphate Buffered Saline), BSA (Bovine Serum Albumin), BlockingReagent (1.5g / 50 mL), ELISA Kit Reagent 1, ELISA Kit Reagent 2, Tris - HCl Buffer, Acetate Buffer, Citrate Buffer, Hydrogen Peroxide (H_2O_2), PLL (Polylysine), GNP (Gold Nanoparticles), Triton – X (0.05%, 0.01%), Tween 20 (0.05%, 0.01%), PVA (PolyVinyl Alcohol), Glycerin (0.1%), Paraform Aldehyde, DCM (Dichloromethane (CH_2Cl_2)), TMB (Tetramethylbenzidine), Citric Acid (20 µg/100 µL), Sodium Hydroxide, NaOH (3%), Potassium Hydroxide KOH (5%), Dimethylosulfoxide, DMSO (50%), (2%), (5%), (1%)), Polymethylmethacrylate, PMMA (10% Dichloromethane), Primary Antibody (Anti-E.Coli antibody /GoatPolyclonal) (Ab13627) (4%), Secondary Antibody (Horseraddish proxidase conjugated anti-goat), PVDF (Polyvinylidine Fluoride), 96/Wellplate, Micropipette, Pipette Tip, O Toclave Tape, Parafilm, Filter Paper,

Slides/Lamels, Petri dishes, Pitches, Spatulas, Falcon Tubes, Centrifuge Tubes, Tape, Hole Punch, Beaker, Flask tubes.

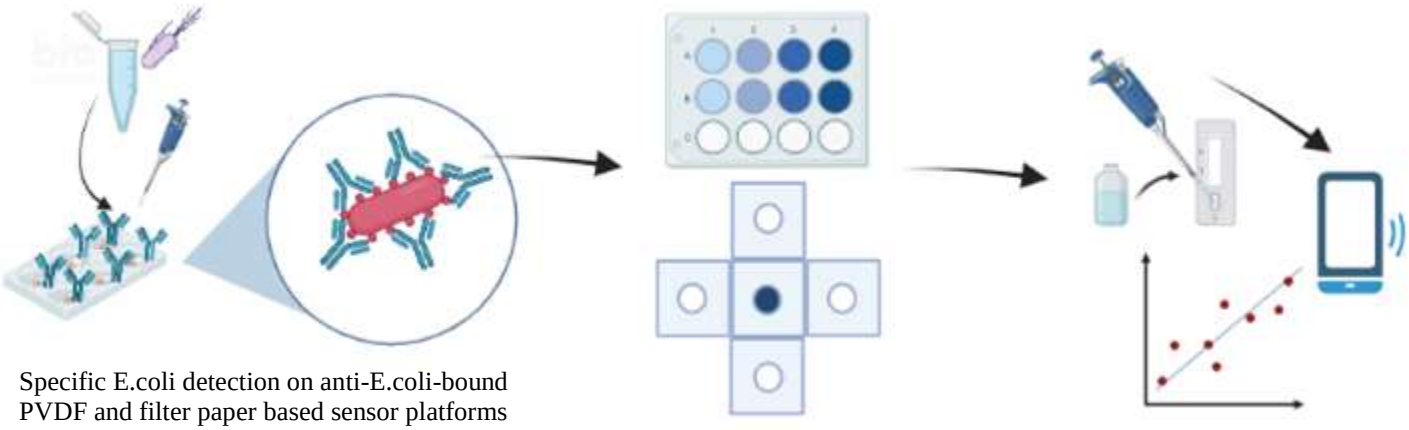
2.3.2. Devices

Devices used in the project: Centrifuge (Nuve NF 400R), Spectrophotometer (MultiscanGO Thermo Scientific), Ultrasonic Water Bath (İSOLAB Laborgerate GMBH), Laser Cutting Device (Paragon Tempero Glass AS2 MS 1/8C DOT 243), Precision Weighing (RADWAG) AS 220/C/2), Vise (AYTUT), Biosafety Cabinet, Vortex (Dragon Lab MX-F), , Fluorescent Microscope (ZEISS), Heated Stirrer, Autoclave, Magnifier

2.3.3. Programs

CorelDRAW X6 was used for the graphical drawing of the paper-based biosensor designs used within the scope of the project, Biorender for the visual drawings of the biosensor system, and MathWorks MATLAB R2018b for the evaluation of the analysis results.

2.4. Paper-based biosensor system for E. coli detection developed in the project



Specific E.coli detection on anti-E.coli-bound PVDF and filter paper based sensor platforms

Concentration dependent color change in biosensors developed in microchip and origami design for E.coli detection

Color analysis of E.coli detection via smartphone

Figure 2. Stages of the paper-based biosensor system for E.Coli detection (The drawing was made in the Biorender program.)

2.4.1. Drawing and fabrication of biosensor models for paper-based biosensor fabrication

For the drawing and production of paper-based sensor models, drawings in different designs (Y, S, +,) were made in the CorelDRAW X7 program. The drawings were placed in a 125 mm diameter circle. Before cutting, the back side of the 125 mm diameter circular filter paper and the PVDF membrane was covered with aluminum tape to make it durable. The drawings were cut by applying a

0.5 mm thick layer of PMMA under the filter papers with a laser cutting device, in which laser production was made using CO_2 in accordance with the visuals in Figure 3. The drawings were tested with the 40% speed scanning method at 6 different powers given in Table 2. and the optimum laser cutting power was determined.

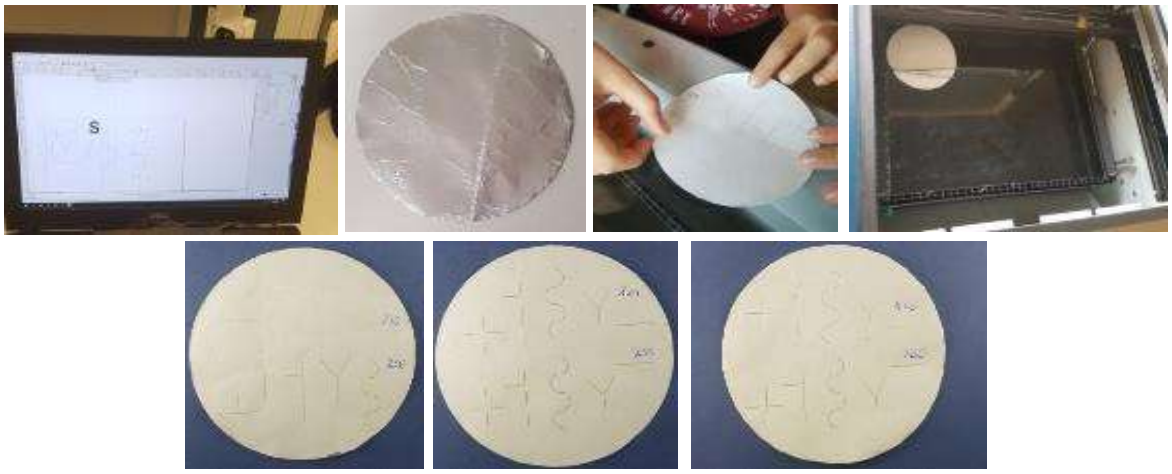


Figure 3. Drawing and fabrication of paper-based sensor models

Table 2. Laser cutting power and speed ratios

	1	**2**	**3**	**4**	**5**	**6**
Power	%10	%30	%25	%35	%20	%40
Speed	%40	%40	%40	%40	%40	%40

2.4.2. Characterization of paper-based sensor models

a. Visual and microscope analysis

The drawings cut at different strengths were first examined under the light with a magnifying glass and then under the light with a microscope in order to reach a more detailed and precise result. In this examination, smoother channel edges, less heat damage and less color change were considered as parameters.

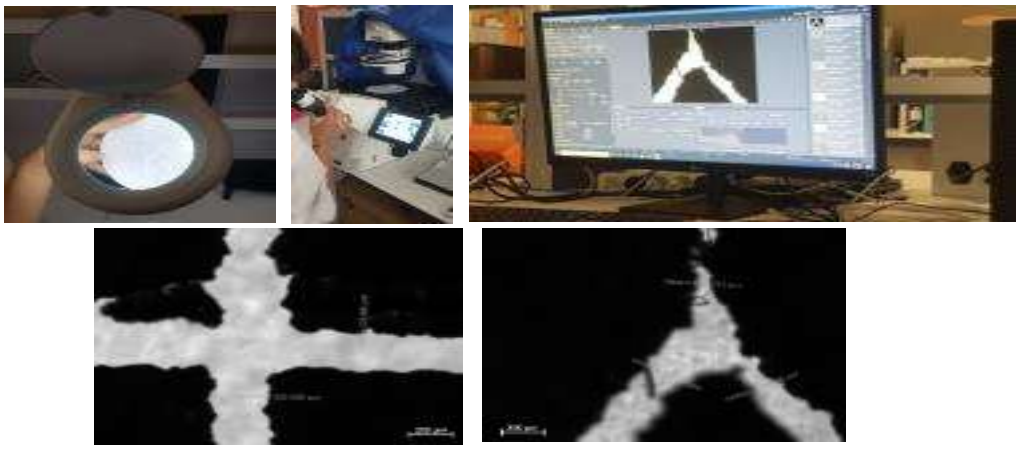


Figure 4. Characterization of paper-based sensor models

b. Optimization of flow parameters with dye

To determine the volume capacity of 15 prepared 10 mm diameter wells and 1.25 mm diameter, dilute blue food dye was added at the rates and in the order shown in the table below.

Table 3. Quantities tried to determine volume capacities

1	**20 µl**	2	**16 µl**	3	**10 µl**	4	**12 µl**	5	**7 µl**
6	**14 µl**	7	**8 µl**	8	**9 µl**	9	**9.5 µl**	10	**9.6 µl**
11	**9.43 µl**	12	**9.45 µl**	13	**9.48 µl**	14	**9.55 µl**	15	**5 µl**

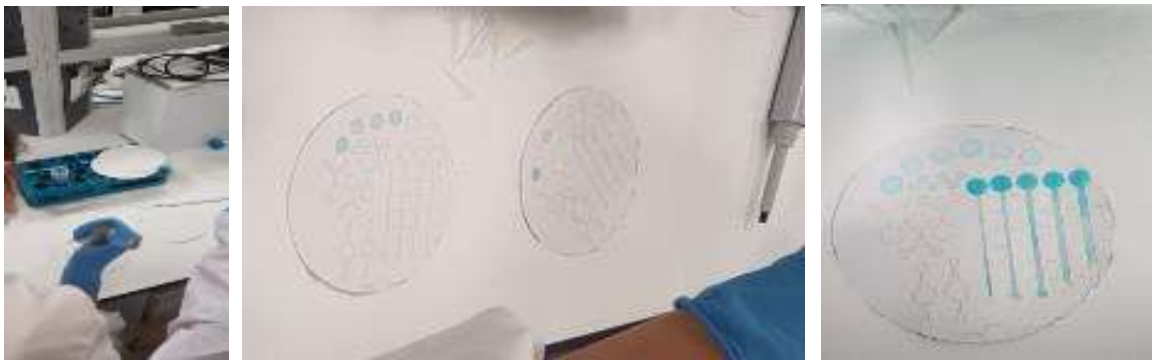


Figure 5. Optimization of flow parameters with dye

c. Determination of the maximum fluid capacity of the wells

During the determination of the maximum liquid capacities of the wells to be used as the sensor analysis medium, the volumes given in Table 4 were tried.

Table 4. Amounts tried to determine the maximum fluid capacity of the wells

10µL	3µL	2µL	2µL	1µL	2.5µL	3µL
2.8µL	2.1µL	2.6µL	1.8µL	1.9µL	2µL	2.2µL
2.3µL	2.2µL	2.1µL	2µL	3µL	2.3µL	2.1µL

d. Protein (BSA) Adsorption Analysis by BCA Method

Bicinchoninic Acid (BCA) method was used for the determination of protein adsorption. 5 mg of BSA was dissolved in 2.5 ml of phosphate buffer with the help of ultrasonic bath. Subsequently, serial dilution from the master stock to 0; 7.81; 15.62; 31.25; 62.5; 125; 250; 500; one thousand; BSA solutions were prepared at a concentration of 2000 µg/mL. On top of the diluted 25µl BSA solutions, 8 ml of Reagent 1 and 0.16 ml of Reagent 2 solutions were mixed and 20 µl was added from the prepared mixture. Mixtures prepared from each concentration in 3 replications were placed in the wellplate.

The initial concentrations were determined by measuring the absorbance of different concentrations of BSA solutions at a wavelength of 562 nm in a spectrophotometer. Filter papers with a diameter of 16 mm and PVDF membranes taken into the wellplate were immersed in BSA solutions of different concentrations in a volume of 20 µl. After the prepared wellplate was covered with aluminum foil, it was covered with aluminum foil for 2 hours at 37.5 ^{o} C and shaken in a heated mixer. At the end of the period, the samples were cooled at room temperature and 25µl of sample was taken and the absorbance was measured in the spectrophotometer at a wavelength of 562 nm by applying the BCA method.

Figure 6. Protein adsorption - BSA analysis

e. Hydrophilization of PVDF Membrane

In order to make the PVDF membranes, which were determined to have hydrophobic structure by BSA Analysis, hydrophilic, 109 PVDF wells were cut, taken into a falcon tube, and ethanol was added to it. It was sonicated in a water bath at 37 ºC for 10 min. At the end of the period, washing was done with distilled water and placed in a petri dish. It was dried in a thermoshaker at 74ºC. Membranes were transferred into the wellplate. 100 µl of different solvents were added to it as given in the table below and incubated for the times specified in the table. After incubation 2.3.2.d. Protein adsorption study was carried out as given under the title. In addition, the wettability tests of the samples were performed. For this purpose, the dry weights of the samples and their weights after soaking with distilled water were measured and compared.

Table 5. Solvents Used to Make PVDF Membrane Hydrophilic

Incubation time	4h	24h
A	Citric Acid 20 µg/100 µL	Polymethylmethacrylate, PMMA (10 µg/ 90 µL dichloromethane, DCM)
B	NaOH 3µg/100 µL	Acetate Buffer
C	Potassium Hydroxide KOH (5µg/100µL)	Ethyl alcohol, EtOH
D	Dimethylosulfoxide, DMSO (50 µL/50 µL)	Glycerine (%0.1)
E	Dimethylosulfoxide, DMSO (20 µL/80 µL)	Triton – X (5 µL/995 µL),(%0.05)
F	Dimethylosulfoxide, DMSO (5 µL/95 µL)	Tween 20 (5 µL/995 µL),(%0.05)
G	Dimethylosulfoxide, DMSO (1 µL/99 µL)	PBS (24h)
H	PBS	

Figure 7. Hydrophilization of PVDF membrane

2.4.3. Culturing E. coli bacteria

In order to culture E.Coli (BL 21 strain) bacteria, first of all, the biosafety cabinet was sterilized with 70% ethanol and all laboratory materials to be used were sterilized by autoclave. In order to reduce the risk of contamination during all processes, the materials were used by passing through the bunsen burner flame. 100 µl of the stock bacterial culture was taken and 900 µl of the LB Broth eppendorf tube containing the necessary medium for the growth of bacteria was placed in a shaker mixer at 250 rpm for 20 hours at 37ºC. At the end of the period, the bacterial culture was centrifuged at 4000 rpm for 8 minutes + 4ºC. 1 ml of phosphate buffer was added to the bacterial culture that precipitated in the centrifuged tube. Absorbance was measured at 600 nm wavelength in the spectrophotometer and the amount of bacterial growth was determined by performing turbidity analysis.

Figure 8. Culturation of E. coli bacteria

2.4.4. Development of the prototype biosensor platform

The biosensor platform was designed in CorelDRAW X7 program. The drawings were placed in a 125mm diameter circle. Before cutting, the back side of the 125 mm diameter circular filter papers/PVDF membranes was covered with aluminum tape to make them durable. A dark PMMA layer with a thickness of 3 mm was placed under the filter papers with a laser cutting device and cut. Afterwards, the platforms were cut with scissors and separated from each other. Then, 500 µl of E.Coli Antibody Ab13627 was transferred to 25 eppondorf tubes with 20 µl in each. Then, 1 µl of Antibody Ab13627 was diluted 250-fold with 249 µl of phosphate buffer (PBS). Then, Antibody Ab13627 was added to 4 biosensor platforms, 2 µl in one of the chambers and the other empty. E.coli bacteria were grown in LB Broth, covered with parafilm and shaken for 20 hours in a thermoshaker at 250 rpm at 37.5 ºC. It was centrifuged at 4000 rpm for 8 minutes. The remaining LB Broth medium was withdrawn and E. Coli bacteria were isolated. 6 mL of PBS was added to the bacteria. 10^7/mL, 10^6/mL, 10^5/mL, 10^4/mL bacteria were prepared in eppendorf tubes by serial dilution of 10 µL of 10^8/mL bacteria (by adding 90 µL of PBS to 10 µL bacteria). Then, the amount of bacteria was determined by absorbance measurement at 600 nm wavelength in a spectrophotometer.

In four 2-well biosensor systems, 2 µL of Primary antibody (diluted in 1 µL/249 µL d. Water) was added to 1 well of each. After waiting for 15 minutes, 2 µl of bacteria for the first biosensor platform, 106 for the second biosensor platform, 105 for the third biosensor platform, 104 for the fourth biosensor platform, 2 µl in each of the 2 chambers on each biosensor platform, were added. Waited 15 minutes. After waiting for 15 minutes, washing was done with PBS. Then, 1 µl of secondary antibody was diluted 250-fold with 249 µl of PBS and 2 µl was added to the chambers of the platforms. Waited for 15 minutes and then the biosensor platforms were washed. As a final step, 2 µl of TMB (Tetra methyl benzidine) was

added to the chambers of the platforms. Waited 15 minutes. Afterwards, a color change was observed. 2 µL of primary antibody diluted 250-fold was added. It was covered with aluminum foil. After waiting for 15 minutes, it was washed with PBS. Waited until it dried. 2 µL of secondary antibody was added. After waiting for 15 minutes, washing was done with PBS. Waited until it dried. 2 µL of TMB was added and it was waited for 15 minutes. Color change was observed.

Figure 9. Development of the prototype biosensor platform

In a 2-well biosensor system, 2 µL of Primary antibody (diluted as 1 µL/249 µL d. Water) was added to biosensor 1 well. In order to be able to measure with cocoa milk (chosen because of its dark color), 1 mL of bacteria with a concentration of 10^7 and 5 mL of cocoa milk were mixed with the help of an automatic pipette. After adding the primary antibody, it was waited for 15 minutes. Then washing was done with PBS. After drying, 2 µL of cocoa milk mixture prepared on them was added to both wells. After waitingwell for 15 minutes, washing was done with PBS.

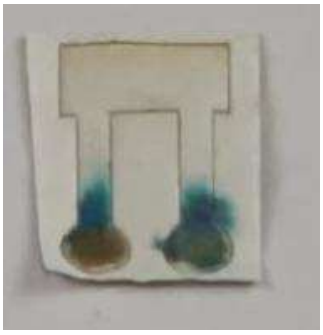


Figure 10. Real sample study on prototype biosensor platform

Waited until it dried. 2 µL of primary antibody diluted 250-fold was added. After waiting for 15 minutes, washing was done with PBS. Waited until it dried. 2 µL of secondary antibody was added. After waiting for 15 minutes, washing was done with PBS. Waited until it dried. 2 µL of TMB was added and it was waited for 15 minutes. Color change was observed.

2.5.Bacteria determination with the help of smart phone with different biosensor platforms

2.5.1. Development of the OrigamiSensor platform

One origamisensor was cut with a laser cutter at 50% speed, 40% power, provided that there is a well of 2.5 mm diameter in each square, in the shape of +, consisting of 2 cm 5 squares on one side of filter paper and PVDF. PVDF to the bottom of the biosensor, through the filter paper; five wells, each of which were punched with a 6 mm diameter hole punch from PVDF, were adhered to the bottom of the filter paper biosensor. The filter paper was placed in the middle of the biosensor after it was kept in the adhered PVDF transfer and capturing buffer medium for 1 night. In the first stage, 5 µL of E.Coli was added to the middle chamber of the origami sensors made of PVDF and Filter paper. Then, 5 µL of Primary Antibody was placed in one of the wells. 5 µL of Secondary Antibody was placed in the other well. After the square on which the Primary Antibody was placed was folded in the middle, 5 µL of PBS was passed over them to ensure the penetration of the Antibody into E. Coli. After waiting for 3 minutes, the same procedure was performed with Secondary Antibody. For this, the system was washed with Washing Buffer, waiting for 3 minutes. 5 µL of TMB was added to the middle chamber of the origami sensor, which was expected to dry. Color change was observed.

Figure 11. Development of the Origami sensor platform

2.5.2. Development of the microchip biosensor platform

2 rectangles with a width of 2.13 cm and a length of 5.7 cm were cut from a 2 mm thick black PMMA disc with a laser cutter at 50% speed and 60% power. 4 wells with a diameter of 6.5 mm were removed from the inside of the rectangles by scanning the edges with a laser cutter at 50% speed and 40% power. 2 more rectangles of the same dimensions were cut from 3 mm thick transparent PMMA. The black and transparent rectangles were attached with double-sided tape, clamped in a vise, and the microchip biosensor infrastructure was obtained. 6 mm diameter PVDF membranes, which were kept in transfer and capturing buffer for 1 night, were placed in 8 wells, 2 of which were in the control group. Then, E. coli bacteria produced by the aforementioned procedure were diluted with 10 and 100-fold PBS and added to a 10-fold diluted biosensor to another 100-fold diluted biosensor. After waiting 3 min, 5 µL of Primary Antibody was added to each well. After a further 3 min, 5 µL of Secondary Antibody was added to each well. Waited 3 minutes. It was washed with a total of 300 µL Washingbuffer. After drying, 5 µL of TMB was added to each well. Color change was observed.

Figure 12. Development of the microchip biosensor platform

2.6. Detection of bacteria in beverages (milk, juice, etc.) with sensor platforms

One of the PMMA biosensors prepared as described in the previous stages was made into a control group and an experimental group. Each group had a total of 4 wells. 6 mm diameter PVDFs were placed in each well, which were kept in transfer and capturing buffer medium overnight. 5 mL of ayran, fruit juice, tap water and lemonade were placed in 4 vials, respectively. 5 µL of consumables were added to the control groups, respectively. E.coli bacteria, which were produced and diluted 10 times, were added to the consumables, provided

that they were 5 mL and 500 µL, respectively, and mixed with a spatula. 5 µL of these mixtures were added to the experimental group, respectively. Then 5 µL of Primary and Secondary was added to both groups. After washing with 50 µL of wash buffer, 5 µL of TMB was added. Each step was waited for 3 minutes. Color change was observed.

Figure 13. Detection of bacteria in various beverages with the microchip biosensor platform

As described in the previous steps, 2 origami biosensors were prepared from the filter paper, one as the experimental group and the other as the control group. After the filter paper was produced in the middle chamber of the biosensor, 10-fold diluted E.Coli was added. Then, 5 µL of Primary Antibody was placed in one of the wells. 5 µL of Secondary Antibody was placed in the other well. After the square on which the Primary Antibody was placed was folded in the middle, 5 µL of PBS was passed over them to ensure the penetration of the Antibody into E. Coli. After waiting for 3 minutes, the same procedure was performed with Secondary Antibody. For this, the system was washed with Washing Buffer, waiting for 3 minutes. 5 µL of TMB was added to the middle chamber of the origami biosensor, which was expected to dry. Color change was observed.

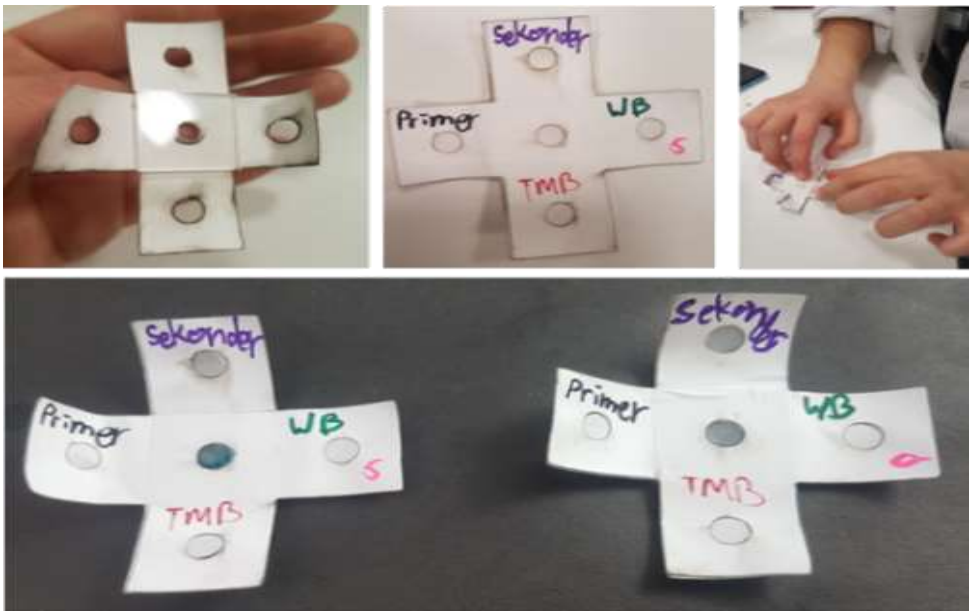


Figure 14. Detection of bacteria in various consumables with the OrigamiSensor platform

2 origamisensors with 3 wells of 5 mm in diameter were prepared as described in the previous steps. After 1 night of capturing and transfer buffer added PVDFs were put into the chambers, 5 µL of E.Coli mixture diluted 10 times with pool water was added to a single well. The biosensor, which was added to both wells with 5µL of Primary Antibody, was left for 3 minutes. It was then washed with 50 µL of PBS. After waiting to dry, 5 µL of Secondary Antibody was added to each well. Waited 3 minutes. Each well was washed with 15 µL of wash buffer. After drying, 10 µL of TMB was added to each well. The same process was carried out with fruit juice. Color change was observed.

Figure 15. Detection of bacteria in pool water and juice with the OrigamiSensor Platform

2.7.Detection of bacteria at different concentrations with the microchip biosensor platform

As described in the previous steps, a black PMMA biosensor platform with 8 wells, two of which is 6.5 mm in diameter and one with a 5 mm diameter, was prepared. 6 mm diameter PVDFs, which were kept in the transfer buffer and capturing buffer for 1 night, were placed in the wells. The first wells of two biosensors with 8 wells were determined as the control group. Additions were made to the remaining 7 wells at the rate of 10^1-10^7 E. Coli, which was prepared by increasing from left to right and diluted with pool water, as in Figure 13. Then 5 µL of Primary Antibody was added to each well. After 3 minutes, it was washed with 50 µL of PBS. After waiting to dry, 5 µL of Secondary Antibody was added to each well. Waited 3 minutes. Each well was washed with 50 µL of wash buffer 2 times. After drying, 10 µL of TMB was added to each well. The same process was applied on the wells formed with filter paper. Color change was observed. Color change was analyzed with Matlab program.

Figure 16. Determination of bacteria at different concentrations with the microchip biosensor platform

3. Results

3.2.1. Characterization of paper-based sensor models

a. Visual and microscope analysis

As the first step of the study, the laser cutting power to be selected for the preparation of the paper membranes to be used was determined. As a result of the microscopic examination made after the power and speed trials given in the method section, the speed value was determined as 40% and the power value as 35%. These values were chosen because the samples created as a result of cutting have sharper lines and are more coherent.

b. Optimization of flow parameters with dye

Due to the potential of the final product to be obtained to have lateral flow, flow rate studies were carried out. Although the results of the study show that the products can be used in this respect, it was seen that liquid transport was not possible due to the hydrophobicity of the PVDF membrane, and it was decided that the use of origami and microchip sensor platforms would be more advantageous instead of lateral flow. Flow study results for the filter membrane can be seen in Table 6.

Table 6. Flow study results for the filter membrane

Flow (Time)	1.	2.	3.	4.	5.
1	06.61	20.85	17.17	18.95	14.81
2	13.7	43.52	20.95	33.21	24
3	45.53	01.20.02	49.07	01.03.99	45
4	01.44.65	01.44.41	01.21.13	01.36.68	01.02.85

c. Determination of the maximum fluid capacity of the wells

During the determination of the maximum liquid capacities of the wells to be used as the sensor analysis medium, the volumes given in Table 4 were tried. The maximum liquid capacity of the wells was determined as 2 µL.

d. Protein (BSA) Adsorption Analysis by BCA Method

Bicinchoninic Acid (BCA) method was used for the determination of protein adsorption. As a result of the study, the protein adsorption amount for the filter paper was determined as 150.965 µg/mm^2 for the BSA protein and 111.391 µg/mm^2 for the PVDF membrane. It was thought that increasing the protein holding capacity and wettability of the PVDF membrane would be beneficial in the continuation of the study and increase the applicability of the final product, and studies were started on the hydrophilization of the PVDF membrane.

e. Hydrophilization of PVDF Membrane

With BSA Analysis, PVDF membranes, whose hydrophobic structure would pose a problem for further use in the study, were conditioned with different chemicals to make them hydrophilic. Then, wettability and protein adsorption studies were carried out. The materials used are given in Table 7 and the results are given in Figure 17 and Figure 18 as the results of wettability and protein adsorption, respectively.

Table 7. Chemicals used for conditioning

Code	Chemical Used for Conditioning	Code	Chemical Used for Conditioning
S1	Citric acid	**L1**	Acetate buffer
S2	Sodium Hydroxide (NaOH)	**L2**	ethanol
S3	Potassium Hydroxide (KOH)	**L3**	Glycerine
S4	Dimethyl sulfoxide (DMSO)	**L4**	Triton X
S5	Dimethyl sulfoxide (DMSO)	**L5**	Tween 20
S6	Dimethyl sulfoxide (DMSO)	**L6**	Phosphate Buffer (PBS)
S7	Sodium dodecyl sulfate (SDS)		
S8	Phosphate Buffer (PBS)		

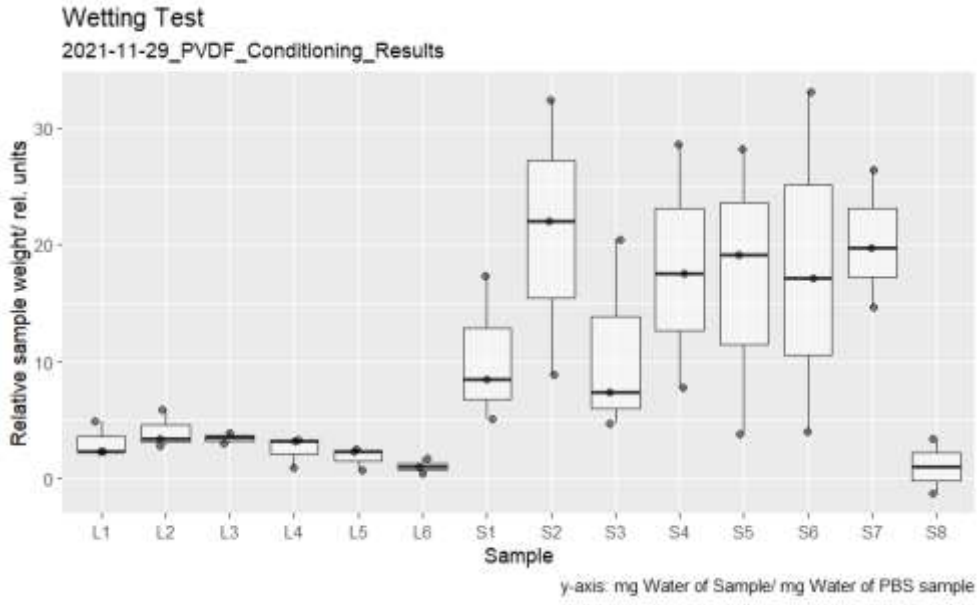


Figure 17. Dry weighing and weight differences of the samples after the wettability study

The wettability study showed that short-term incubation of strong reducing or raising agents and the use of DMSO, a PVDF solvent, were effective to hydrophilicize the PVDF membrane, but the results of the protein adsorption study showed that effective adsorption was achieved for a long time with non-oxidizing/non-reducing and less solvent materials. demonstrated by the treatment.

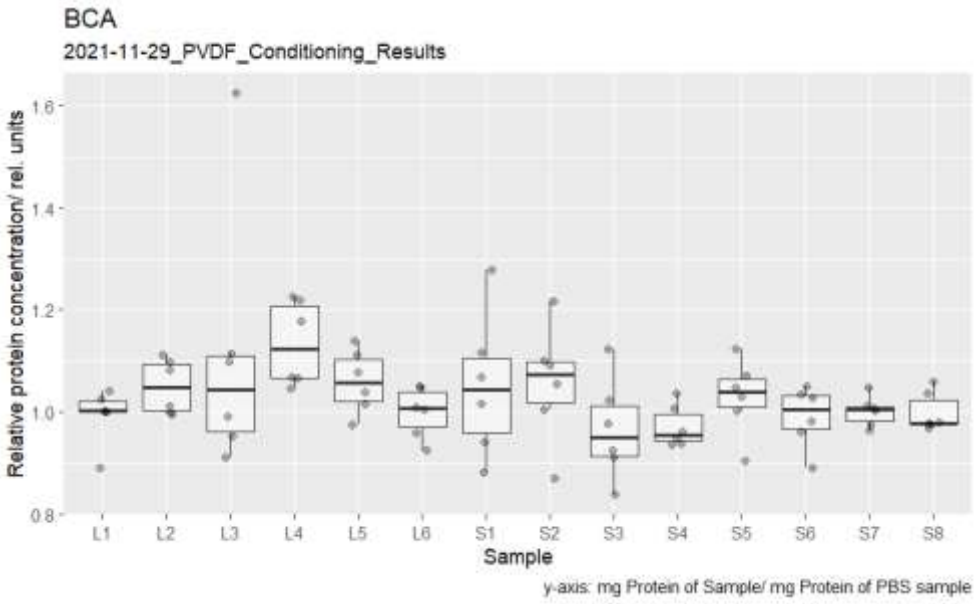


Figure 18. Protein adsorption studies of PVDF membrane samples conditioned with various chemicals

Protein adsorption was accepted as a more important parameter compared to wettability, since the continuation of the study included antibody function on the membranes, and the transfer buffer solution to be used in this direction was chosen as a citrate buffer containing 0.01% Triton X and 96 mM glycine.

3.3.Prototype biosensor platform trials

The prototype was first used for the determination of bacterial samples prepared in PBS buffer solution. Figure 19 shows the results obtained. As can be seen in the figure, there is a visible color difference between the control groups and samples, as well as between samples with different concentrations.

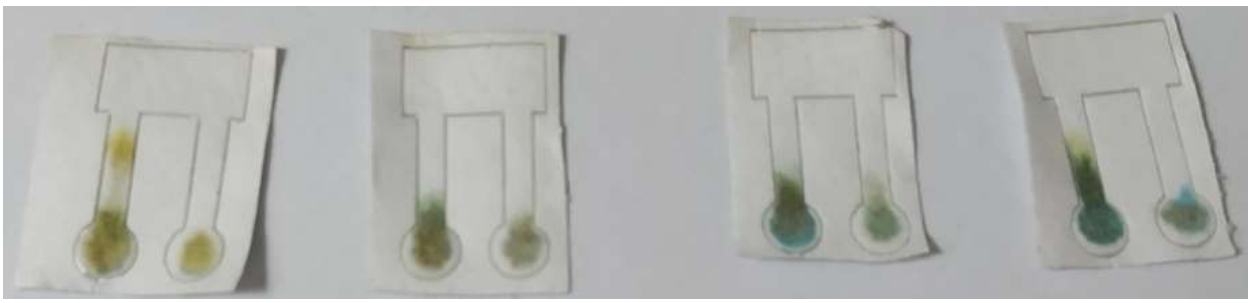


Figure 19. Trial results applied at increasing concentrations from left to right. The well on the left of each platform was used as the sample and the right one as the control

In order for the visible difference to have a quantitative significance, the colors of the images obtained were converted into numerical data. In this process, a code was written to convert these colors into numerical data using MATLAB software. The code gives both an average color difference, how bright the darkest part of the image is, and the average luminance values. Figure 20 and Figure 21 show images that MATLAB has converted to black-and-white and LabColor format for use.

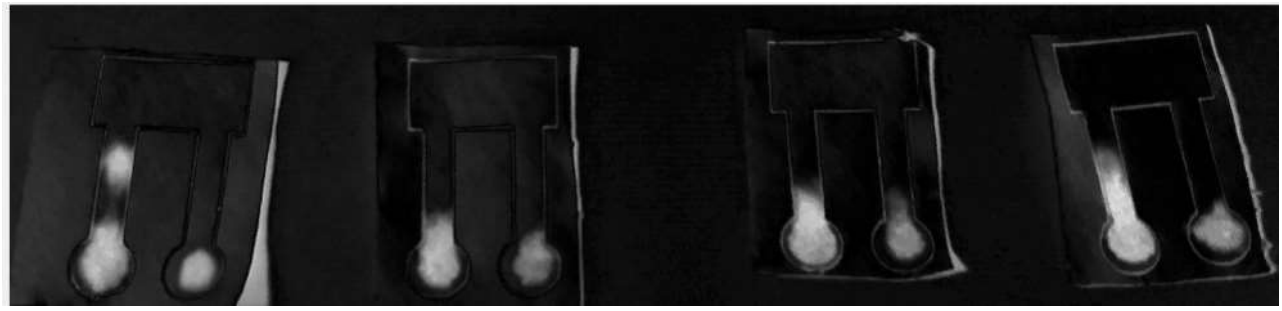


Figure 20. Black and white image used by the Matlab program

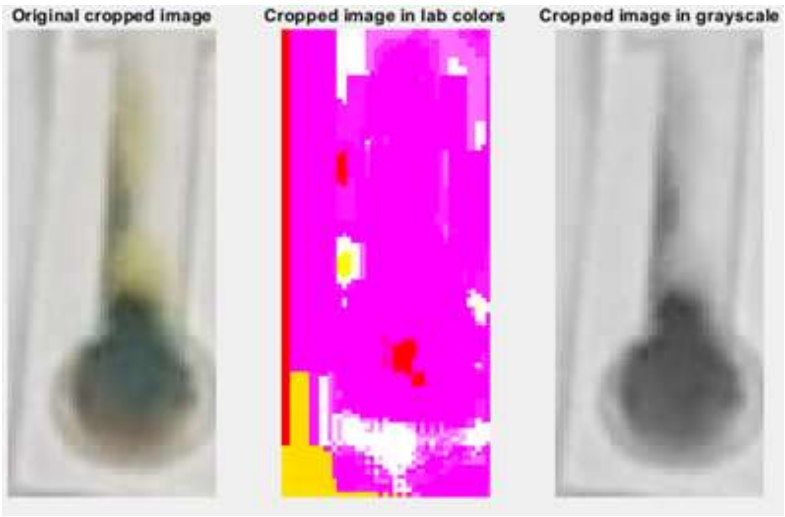


Figure 21. Images used by the Matlab program

The numerical data obtained after the aforementioned process is applied to all samples are shown in Table 8.

Table 8. Color values of the results obtained from samples with different bacterial concentrations.

Concentration	**10^7 cell / ml**	**10^6 cell / ml**	**10^5 cell / ml**	**10^4 cell / ml**	**10^7 cell / ml - control**
Average color difference	18.403	15.860	15.663	15.043	18.450
Darkest spot exposure (black and white)	46.000	60.000	77.000	83.000	98.000
Average exposure (black and white)	88.121	97.401	105.846	116.038	123.847

As seen in Table 8, no dramatic change is observed in the average color difference, but it has been observed that both the darkest spot and the average exposure values decrease with increasing concentration, so the resulting color gradually gets darker. The calibration curve drawn with the obtained data can be seen in Figure 22.

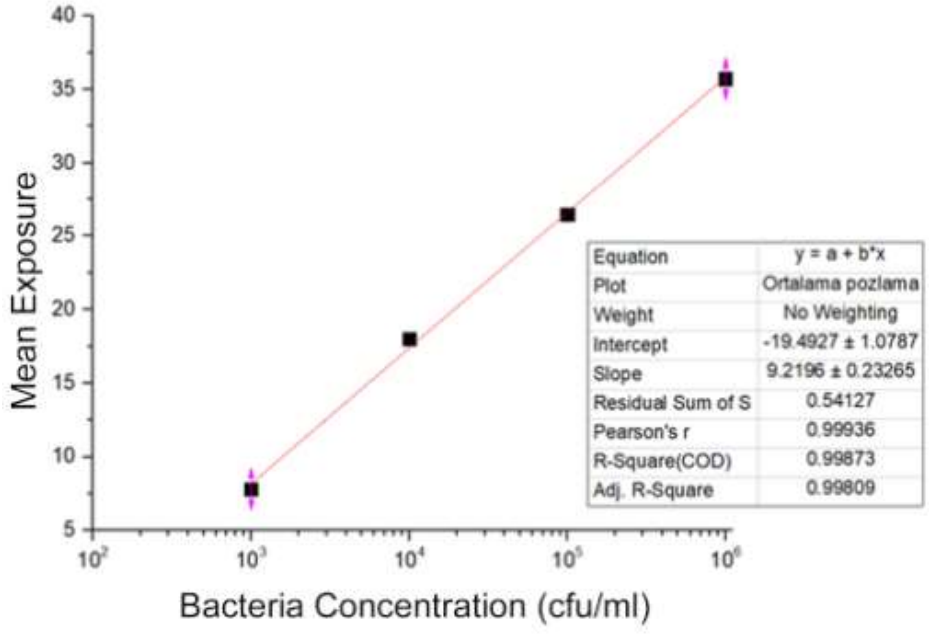


Figure 22. Calibration curve prepared for the existing sensor system

The detection limit calculated using the calibration curve was determined as $0.9x10^3$ bacteria/ml for origami biosensor, $2.7x10^3$ bacteria/ml for microchip biosensor

3.4. Bacterial determination with different biosensor platforms

As mentioned before, two different platforms were used to implement the same method. These are origami and microchip based sensor platforms. As described in the method section, the platforms contain filter paper and PVDF membrane as antibody carriers. In order to avoid problems arising from the natural color difference between the two materials, both materials were tested on the same platform and the color change values were calculated as described in the previous step, using samples at different concentrations.

a. PVDF membrane based sensor system

Figure 23. Color measurement results using PVDF membrane

As seen in Figure 23, determination can be made up to 10^3 bacteria/ml concentration by using the antibody-functional PVDF membranes produced in the study. Figure 24 shows the corresponding calibration curve.

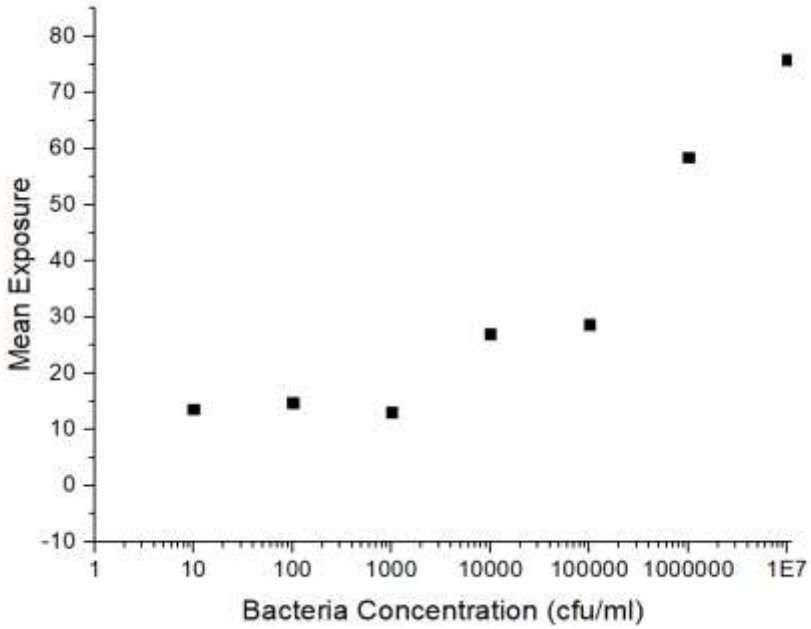


Figure 24. Calibration curve drawn by color measurement using PVDF membrane

As the calibration curve shows, a sensor platform has been developed that provides the chance to measure in a very wide (10^3-10^7 bacteria/ml) range.

b. Filter paper based sensor system

The same process was done for the filter paper after the PVDF membrane. The results and the calibration curve can be seen in Figure 25 and Figure 26.

Figure 25. Color measurement results using filter paper

Although the expected color formation is blue, yellow-green color formation is observed in Figure 23. The reason for this is as follows: TMB is stored in its colorless and reduced form. When HRP (horseraddish peroxidase) enzyme and H_2O_2 are present in the environment, it oxidizes and gives a blue color. This mechanism is based on the fact that the HRP enzyme obtains the two hydrogen atoms necessary for the reduction of H_2O_2 and its conversion to water by TMB (TMB^{1+}) oxidation. As the reaction continues, the amount of oxidized TMB increases and causes precipitation. Failure to stop the reaction and excessive oxidation of TMB (TMB^{2+}) produces a yellow-orange color. Under normal conditions, “stop-solution” is used to prevent the formation of this color. In addition, the addition of anionic surfactant before oxidation or the use of dilute TMB can prevent yellow color formation. There is no need for these interventions or the use of "stop-solution" when there is no problem such as the rapid disappearance of the blue color or the need for long-term storage.

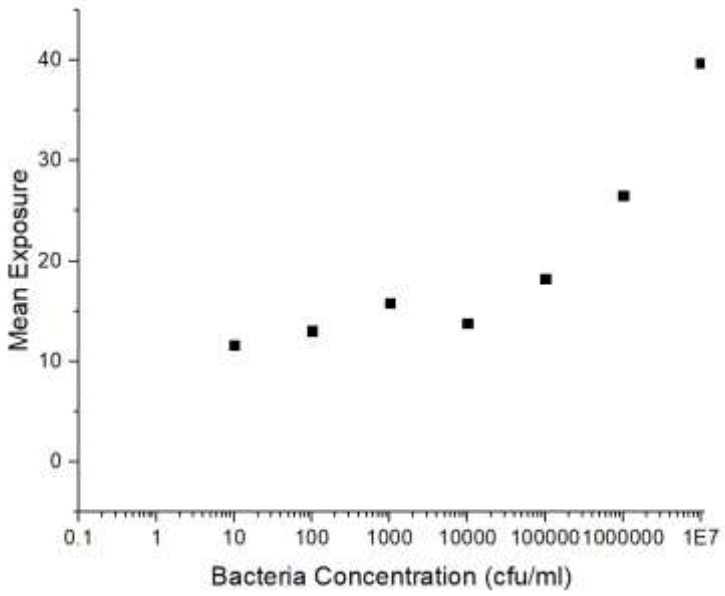


Figure 26. Calibration curve drawn by color measurement using filter paper

It can be seen in Figure 26 that the measurement can be achieved with filter paper in a similar way (to PVDF membrane results), but the lower limit of measurement is higher in comparison.

3.5.Detection of bacteria in various consumables (milk, juice, etc.) with Sensor Platforms

Origami and microchip sensor platforms prepared as described in the previous stages were used in the analysis of different consumables and samples. For this purpose, milk, fruit juice, lemonade and pool water were used as examples. Figure 27 shows the results of the origami-based sensor platform.

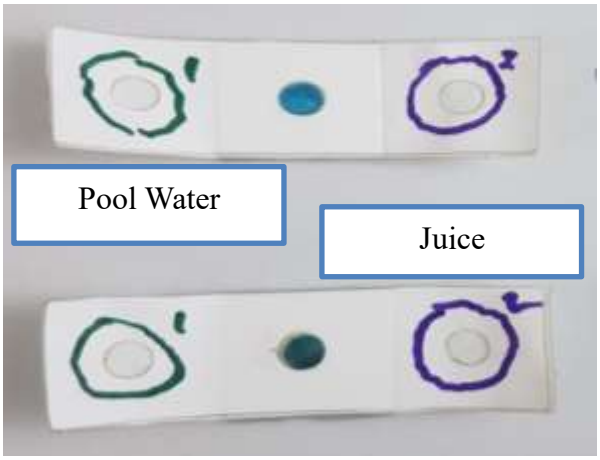


Figure 27. Results of pool water and fruit juice samples obtained with origami sensor platform

As seen in Figure 27, color change was observed in both samples. Samples prepared in a similar way were then tested on the microchip sensor platform. The results obtained can be seen in Figure 28.

Figure 28. Results obtained from ayran, fruit juice, pool water and lemonade samples, respectively. The first row contains the samples, and the second row contains the control groups without added bacteria.

As seen in Figure 28, positive results were obtained from different samples. The color yellowing mentioned before in Section 3.3.b was encountered here as well and was determined to be sufficient for determination.

4. Discussion

Lab-on-a-chip technologies have developed in the last 20 years and constitute an alternative to traditional methods. Due to the unique properties of microstructures, complex analyzes of these systems can be performed more efficiently with lower cost, energy and chemical consumption than traditional methods (Francesko et al., 2019). Within the scope of the project, lab-on-a-chip technology including paper-based biosensor systems that make immunological and colorimetric measurements for E.coli detection has been proposed and the application of the proposed method on two different sensor platforms has been carried out. When the results obtained in the project are evaluated;

- **During the development of the paper-based biosensor system, two different platforms consisting of PVDF membrane and filter paper, microchip and two different biosensors including user-friendly origami designs to minimize the processing steps and size were developed.**
- At the stage of characterizing the paper-based biosensor system;

  o At the stage of determining the laser cutting conditions to be chosen for the sharp lines and coherent structure of the paper membranes to be used as the analysis platform, the speed value was determined as 40% and the power value as 35% as a result of microscopic examination.

  o It was decided that the use of origami and microchip sensor platforms would be more advantageous, instead of lateral flow, in the final design obtained during the optimization of the flow parameters with dye.

  o As the sensor analysis medium, the maximum liquid capacity of the wells was determined as 2 µL.

  o Bicinchoninic Acid (BCA) method was used for the determination of protein adsorption. As a result of the study, the protein adsorption amount for the filter paper was determined as 150.965 µg/mm2 for the BSA protein and 111.391 µg/mm2 for the PVDF membrane. It was thought that increasing the protein holding capacity and wettability of the PVDF membrane would be beneficial in the continuation of the study and increase the applicability of the final product, and studies were started on the hydrophilicization of the PVDF membrane.

  o PVDF membranes were conditioned with different chemicals to make them hydrophilic. Then, wettability and protein adsorption studies were carried out. Protein adsorption was considered as a more important parameter compared to wettability, since

the continuation of the study included antibody function on the membranes, and the transfer buffer solution to be used in this direction was chosen as a citrate buffer containing 0.01% Triton X and 96mM glycine.

• In order to optimize the conditions for bacteria detection, first of all, a paper-based sensor platform prototype was prepared and used for the determination of bacteria samples prepared in PBS buffer solution. In the results obtained, a visible color difference was determined both between the control groups and samples and between samples with different concentrations. In order for the visible difference to have a quantitative significance, the colors of the images obtained were converted into numerical data. **A code was written to convert these colors into numerical data using MATLAB software. The code gives both an average color difference, how bright the darkest part of the image is, and the average luminance values.** In these results, no dramatic change was observed in the average color difference, but it was observed that both the darkest spot and the average exposure values decreased with increasing concentration, so the resulting color gradually became darker. **The detection limit calculated using the calibration curve was determined as $0.9x10^3$ bacteria/ml for origami biosensor, $2.7x10^3$ bacteria/ml for microchip biosensor.**

- The developed paper-based biosensor system contains filter paper and PVDF membrane as antibody carrier. In order to avoid problems arising from the natural color difference between the two materials, both materials were tested on the same platform and the color change values were calculated as described in the previous step, using samples with different concentrations. It is possible to determine up to 10^3 bacteria/ml concentration by using produced PVDF membranes with antibody function. As the calibration curve shows, a sensor platform has been developed that provides the chance to measure in a very wide (10^3-10^7 bacteria/ml) range. The same process has been done for filter paper and measurement can be achieved with filter paper in a similar way, but the lower limit of measurement is comparatively higher. In addition, concentration-dependent blue color change is observed in the biosensor system developed with PVDF membrane, while yellow-green color change is observed in the biosensor system created with filter paper.

- In the final stage, the prepared origami and microchip sensor platforms were used in the analysis of E.coli in real samples such as pool water and different consumables, and color changes were successfully observed when compared to the bacteria-free control groups.

- When the analysis costs of the developed sensor platforms are examined, the E.coli bacteria analysis to be performed with the microchip design prepared using PVDF membrane is only 9.5 Cent; It was calculated that the E.coli bacteria analysis to be performed with the origami design prepared using PVDF membranes is only 14 Cent.

The lowest detection limit obtained using the existing platforms and method was $0.9x10^3$ bacteria/ml for origami biosensor, $2.7x10^3$ bacteria/ml for microchip biosensor, and the widest dynamic linear working range was calculated as approximately 10^3-10^7 bacteria/ml. With the produced sensor platforms, results can be obtained within minutes and only a smartphone is used to measure the result. **Our biosensor systems offer a detection option that has an economic advantage over existing methods,** provides results in a short time and does not require expertise to use. We believe that the biosensors we have developed for the detection of biological pollutants in water and beverages, especially in regions where test laboratory infrastructure is not available, will contribute **to public health, sustainable development goals such as clean water and sanitation, health and quality life, and life in water, and the literature.**

5. Suggestions

Within the scope of our project, we have developed a detection system based on the concentration-dependent detection of the presence and absence of E.coli bacteria in water and various consumables. This system is a **low-cost and easy-to-use detection** kit that is an alternative to the existing systems used in the detection of E.coli bacteria today.

- Since the biosensor system we have developed does not contain complex devices, **it can be easily produced in large scales**. In this way, it will provide a significant benefit to **public health and the country's economy** for contamination screening by providing **routine bacteria detection** in regions where advanced laboratory infrastructure is not available.

- The system we have developed will also pave the way for the determination of bacteria in different mediums **ranging from water and beverage substances to biological fluids such as blood, urine and saliva.**

- Producing the biosensors we have developed in the design of microchip and origami, which can be used in the field, with more advanced technologies and turning them into a

commercial product may be among the suggestions that can be applied in the continuation of the project.

• Our most important suggestion for the future is to make the color produced by the platform permanent and to convert the MATLAB code used into a single integrated smartphone application with visual acquisition.

With these promising results, we believe that our project will contribute to the literature and future studies on the development of new detection systems in order to modify the biosensor system we have developed and use it as an infection marker for the detection of different biological pollutants.

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This project aims to make a paper-based biosensor to detect E. coli at a low cost，easy to use，and integrate with a smartphone for rapid testing. The author optimized several conditions for better devices. The presented results are practical and logical studies for concept proof. However，antibody-based detection is specific，and the biosensor can only detect E. coli may not be practical for application.

Suggestions:

1. Proper space is needed between words in References，for example，2，5，and 6.
2. For their detection，they used E. coli antibody Ab13627 for detecting E. coli，the same strategy of antibody-based detection used in other systems.
3. Using folded paper，it looks like a very simple lab-on-chip.
4. Two bacteria-trapping materials were used，PVDF membrane and filter paper. They thought increasing the protein holding capacity and wettability of the PVDF membrane would be beneficial so the studies were started

on the hydrophilicization of the PVDF membrane. However，two materials showed similar bacteria detection ranges and the lower limit of detection in both cases is still too higher (Figure 25 and 26).

5. Using a smart phone to detect color changes is not novel.

6. Quantatative data need to be reported，such as the E. coli concentrations in different samples，such as pool water，juice etc. they used. Were the bacterial concentrations linear with amount of the samples used ?