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Miniaturized flow stacked immunoassay detects *E. coli* in a single step

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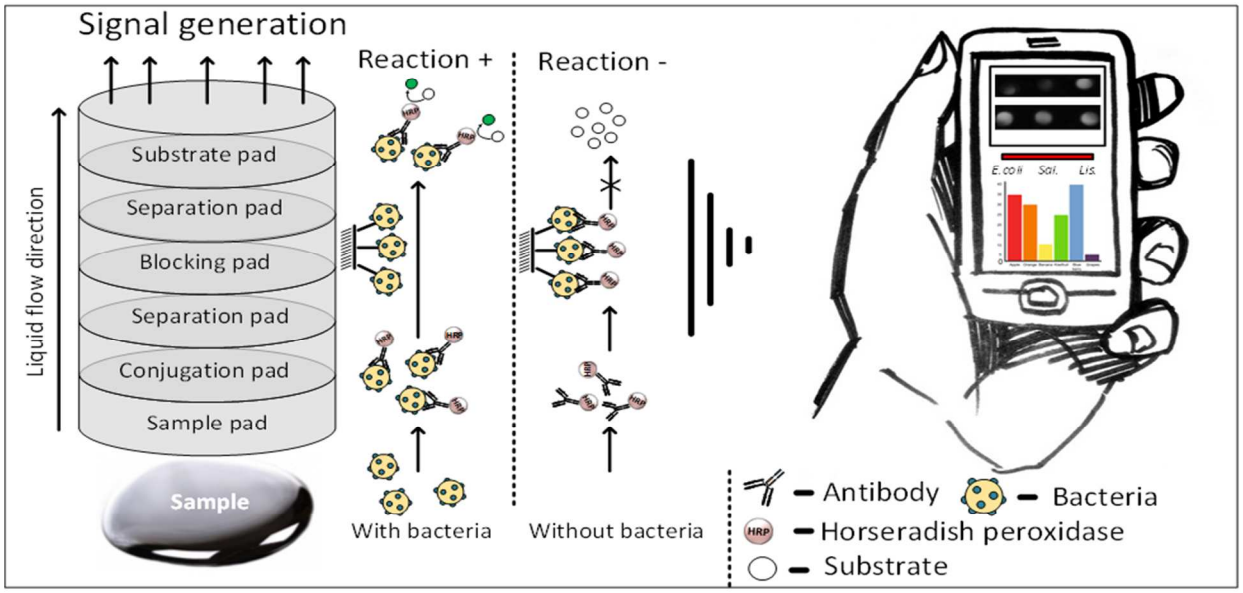
ABSTRACT: Commercially available systems that provide cost effective, fast, simple and portable solution for health and environmental application are few and far between despite huge advancements in biosensor research. We have developed a new system based on stacked membranes, each layer with a specific function. Samples were added onto the bottom-most layer, and as each layer is wetted, the analyte push through to the next layer of membrane. Through migration, the analyte, attached with corresponding antibody conjugated with HRP to produce a measurable signal. To prevent false positive result, blocking layer membranes are added to stop unbounded antibodies from reaching the top membrane. Thus, only analyte/antibody-HRP complex will generate a signal. In order to proof this concept, E.coli was used as the target analyte. After optimization, our sensor sensitivity was adjusted to 100 cells/ml. Different environmental water sources were also tested to demonstrate the sensitivity and specificity of our proposed stacked bioassay. Simplicity, low price, sensitivity and modularity (capability to change to any target analyte) make this idea very promising for future commercialization.



1. Introduction

There is demand for a simple, rapid, reliable, sensitive and affordable solution for a point-of-care test (POCT) device enabling to monitor pathogens (or elicited immunoglobulins thereof, which were induced from their presence in the invaded organism) made for and used outside the conventional microbiological laboratory, requiring minimal skills in a minimal time frame.

A number of classical methods exist to identify pathogens, such as cell plate culture, immunoassays and nucleic acid related tests. However, depending on the method, anyone of them may suffer from one or more disadvantages such as low sensitivity, high price, assay complexity and requirement of a lab environment, and more. In addition, none of these methods can be developed domestic or field consumers in the near to mid term future. Two types of devices have reached the necessary requirements to make them consumer friendly. One is the biosensor-based glucometer and the other the pregnancy test. There are technologies in development seeking to combine these successful technologies [1]. However, presently the gold standard low cost rapid test is the lateral flow immunoassay (LFA) [2]. By 2010, more than 100 companies worldwide had produced a wide range of LFA tests with a total market valued at over 3.36 billion USD [3]. Different parameters (e.g. cost efficiency, portability, simplicity and speed) made LFA more attractive than conventional detection approaches (e.g. ELISA, PCR, cell culture). The format of LFA uses the same rationale as ELISA, where immobilized antibody or antigen is bound onto nitrocellulose membrane instead of a plastic well. The membrane enables a one-step assay, a major advantage when compared to ELISA. Typical LFA is based on four segments, a sample pad, a conjugate pad, a nitrocellulose membrane and an absorbent pad, each serving a given purpose, overlapping one another and combined on a plastic backing support. The sample, added onto the sample pad, migrates through the membrane and is captured by the antibodies immobilized within the nitrocellulose membrane to produce a visible and putatively measurable colorimetric signal. There are various LFA formats, biorecognition molecules, labels, detection systems and applications [4-7]. LFAs have been used to monitor infectious agents [5,8,9], nucleic acids [10,11], proteins [12], cells [13], veterinary drugs [14,15], toxins [16-18] and pesticides [19]. The most popular test to-date is the well-known and well-received pregnancy test. The biggest disadvantage of LFA is that virtually all tests are qualitative, or sometimes semi-quantitative and despite the use of signal amplification (enzyme labels), insufficient sensitivity is obtained [12,20-22]. In the case of the popular pregnancy test, there is sufficient amount of target metabolite and therefore there is no need for quantification, as a positive presence of hormone is sufficient to provide the sought after answer. In other cases (e.g. viral load in HIV treatment or severe Dengue triage), quantification would be needed. Therefore, much effort has been directed to the development of quantitative LFA sensors, to offer accurate concentration information for the target molecule or cell of interest. This can be achieved by combining transducers to the LFA format (e.g. optical [23,24], magnetic [25], or electrochemical [12,26]).

In this research, an alternative flow format is suggested, by re-shuffling the well-known, cheap and available lateral flow materials. Pads created from the same LFA components (sample, conjugate and absorbent pads and nitrocellulose membrane) were arranged in a pile-like structure and samples allowed to migrate from the lowest to the upper-most layer.

In the first step, the analyte is collected and applied so that it is allowed to traverse through a first paper pad until it reaches the confined dried and immobilized HRP conjugated anti-analyte capture antibody laying in wait to conjugate after molecular recognition. Thereafter, the conjugate analyte-antibody complex migrates up to the next 'blocker' layer (figure 1) where a nitrocellulose membrane has been modified with the immobilized analyte of interest, which in the case of the present paper is *E. coli* bacteria. If the sample tested is contaminated with the bacteria in question, then the complex moves on. If the target analyte is abscent then the HRP-labelled antibodies will simply bind in this new layer where the cells have been pre-immobilized as the blocking layer. This means that these labelled markers will not diffuse further. Analyte-positive complexes will migrate to the highest pad, where signal measurement occurs (by chemiluminescence, colorimetry or electrochemistry). To proove the concept, *E.coli* bacteria were chosen as the model target analyte. Thanks to the availability of a huge variety of existing antibodies, and the availability of reasonably priced reaction reagents (enzymes, substrates) [27] this test may have a chance to find its way to commercialization and be used for any analyte of interest as it is indeed a platform technology. We show herein that after optimization, this approach allows a sensitive (100 folds higher than ELISA) and specific determination of *E. coli* cells in the tested samples.

2. Materials and methodsReagents

Phosphate buffered saline (PBS) tablets (Cat. No. P4417) were purchased from Sigma-Aldrich. PBS-0.05%(v/v) Tween (PBST) was prepared by adding 0.5 mL Tween-20 solution (Cat. No. P7949) to 1L PBS buffer. 5% (w/v) Skim milk (SM) solution was prepared by adding 5 g SM powder (70166) to 100 mL PBST solution. Milli-Q ultrafiltered (UF) H_2O (with a resistivity of 18.2 MΩ cm at 25°C) was used in the preparation of all solutions.

2.2. Immunoreagents

Rabbit monoclonal anti-*E.coli* antibody (IgG) with conjugated horseradish peroxidase (HRP) enzyme (ViroStat, Cat. No. VIR-1004) or without (ViroStat, Cat. No. VIR-1001), were purchased from ENCO.

2.3. Bacterial strain growth and maintenance

Five different bacteria strains were used in this study: four *E.coli* strains (DH5α, K12, B and MC1061) and *Salmonella Typhimurium*. Prior to measurements, *all*

strains were cultivated in 10 mL clear LB [28]. Bacteria were grown overnight at 37°C in a rotary thermo shaker (Gerhardt, Konigswinter, Germany) at 120 rpm. Cultures were then diluted to approximately 10^7 cells/mL and re-grown in 25 mL LB at 26°C, without shaking, to an early exponential phase (0.2 OD_{600nm}) as determined by an Ultrospec 2100 pro spectrophotometer (Amersham, Bucks, UK). The cultures were then centrifuged (MiniSpin plus, Eppendorf, Germany) at 12,000 rpm for 5 min. After replacing the supernatant with PBST buffer, bacteria were homogenized by gentle pipetting. This step was repeated thrice. The bacteria were then diluted to the final testing concentrations.

2.4. Device fabrication

2.4.1. Membranes

Sample (Cat. NO. GFB-R4), absorbent (Cat. No. AP-080) and conjugate release matrix (Cat. No. PT-R5) pads were purchased from Advanced Microdevices Pvt. Ltd (India). Amersham protran nitrocellulose membrane 0.45 µM (Cat. No. 10600044) was purchased from GE-Healthcare.

2.4.2. Assay reagent immobilization procedures

Substrate pads were made by cutting 6 mm diameter pads from absorption pads, wetted with 100 µl luminol-H_2O_2 substrate solution (ratio 1:1) (Cat. No.1705040, BioRad) and dried for 3 h at 37°C in the dark. Conjugation pads (D=6mm) were prepared by wetting pads with 80 µl anti-*E.coli*-HRP antibodies (diluted with PBST (PBS (0.203 g l^{-1} NaH_2PO_4, 1.149 g l^{-1} Na_2HPO_4, 8.5 g l^{-1} NaCl) (pH 7.2) with 0.05% (v/v) tween 20) and dried for 2 h at 37°C. The conjugate pads were stored with desiccant gel at room temperature. Blocking layers were made by exposing the nitrocellulose sheets to bacteria (diluted in PBS) for I hr. The sheets were then washed thrice with PBST and incubated at room temperature with blocking solution (PBS, 5% (w/v) SM and 0.05% (v/v) tween 20) for 1 h. The nitrocellulose sheets were then washed thrice before drying at 30°C.

2.4.3. Assembly of the stacked immunoassay system

The stacked immunoassay was assembled, by placing all prepared pads, one on top of each another in the following order (figure 1). The sample, conjugated, blocking and absorbent (substrate) pads were stacked from bottom to top in this order. Each layer is separated with a sample pad to encourage a directed flow towards the centre of the pads and provide space for the conjugation. Finally, in order to provide a flow process with directional integrity, all stacked pads were placed in plastic holders.

2.4.4. Measurement procedure

Each plastic holder (with all pads in the right order) was placed above a 150 µl water sample. Water was observed to seep through the sample pads and migrate, by capillary force, from the lowest to the upper-most layer. Target analytes first diffuse within the layer with the labelled anti-bacterial antibodies, then, move on through the blocking layer, to the absorption pad containing the dried substrate for use by the marker enzyme.

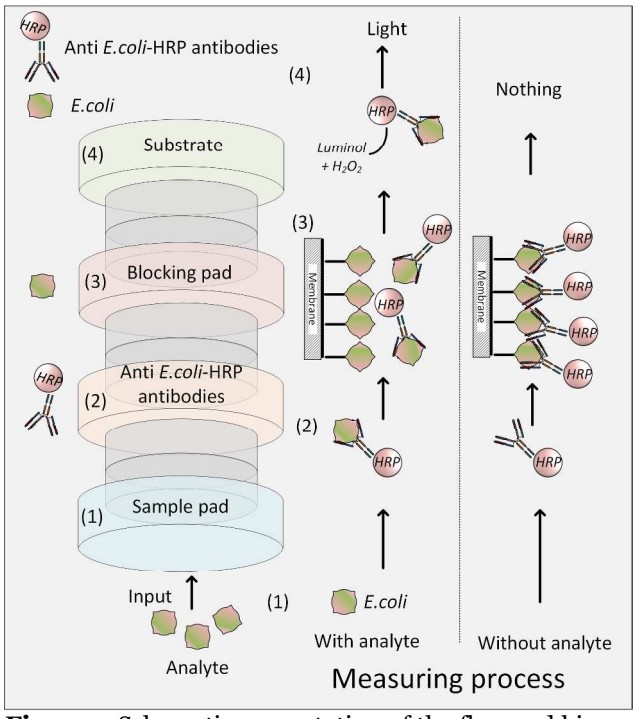


Figure 1:Schematic presentation of the flow pad biosensor. In general it consists of different nitrocellulose pads with various components. (1) sample pad (empty) where the analyte sample will be placed. (2) pad with anti-analyte bioreporter molecule linked to some marker, (3) blocker pad, with immobilized analyte (4) measuring pad (depend on the marker in pad (2) or empty or with specific substrate). There are two main possibilities that can happen during measurements. In the first, sample with target analyte, after deposition on pad (1) will migrate to pad (2) where it will be connected to bioreporter molecules (in this case antibodies). The complex will then migrate through pad (3) and reaches pad (4) to produce a measured signal since the complex is already fromed. Sample without target analyte will migrate from pad (1) to (2) and then will move antibodies (in this case) to the blocking pad (3) where they will be linked to the immobilized analyte and stopped from migrating to the next pad. Thus, no visible signal will be observed

2.4.5. Instrumentation

In samples containing the target bacteria, the light signal produced was captured with CCD camera (Retiga-SRV FAST 1394, InterFocus, United Kingdom). The CCD camera was placed 30 cm above the stacks and serial pictures of 15 sec. exposure time were taken with QCapture pro software. Measurement occured 5 minutes after the stack configuration was exposed to the liquid sample.

2.5. Optimization steps

2.5.1. Immobilization procedure

The effect of the analyte immobilization procedures in enabling the capture of the arriving non-conjugated labeled antibodies on the blocking membrane, was evaluated by exposing 1x2 cm nitrocellulose sheets to anti-*E.coli*-HRP antibodies (1:2,000 dilution) solution diluted in PBST. Three different immobilization approaches were tested: incubation, drop deposition and spreader. In the case of the incubation procedure, nitrocellulose sheets were exposed to 2 mL antibody solutions for different time periods (15, 30 and 60 min), and thereafter rinsed thrice with PBST before incubating with a blocking solution for 1 h. The sheets were again rinsed thrice with PBST and dried at room temperature. In the drop approach, 1 mL of each antibody solution were carefully deposited at the center of a 1x2 cm nitrocellulose sheet and dried at room temperature. For the spray approach, a chromatography dispenser (Z529710, Sigma-Aldrich) was used to dispense 0.1 mL/s antibodies on the 1x2 cm membrane. Three spread durations were tested (10, 20 and 30 s).

2.5.2. Leaching issues

To prevent false positive responses, immobilized bacteria must be strongly attached onto the nitrocellulose membrane and should capture any unbound antibodies during the assay operation. DH5α bacteria (10^6 cells mL^{-1}) were immobilized on the membrane (section 3.4.1) to create such a blocking layer. Its efficiency was evaluated with the following experiment. Modified membranes (with the bacterial target strain immobilized) were cut into circles of 6 mm in diameter and placed on absorption pads (figure 3). 100 µL of an antibody solution (1:4,000 dilution in PBST) was flowed through the blocking layer to the absorption pad. The absorption pad was replaced with a fresh/dry one and another 100 µL of antibodies solution was thus transferred. This step was repeated thrice, and all pads (three adsorption and one blocking) were placed into separate wells of a white opaque 96-well microtiter plate. A 100 µL substrate solution (luminol-H_2O_2 (ratio 1:1)) was added to each well and the light activity was measured with a Luminoskan Ascent luminometer (Thermo Fisher Scientific, United States).

2.5.3. Specificity testing

Two different approaches (ELISA and direct measurements on nitrocellulose pads) were used to determine the specificity of the commercial antibodies to our model *E. coli* DH5α strain of interest. Four different bacterial strains were likewise immobilized on nitrocellulose membranes as previously described in section 3.4.1. Thereafter, each membrane was incubated with anti-*E.coli*-HRP solution (1:2,000) for 1 h. The membranes were then rinsed thrice with PBST and dried at room temperature. Prior to measurement, a 100 µL substrate solution (luminol-H_2O_2 (ratio 1:1)) was pipetted onto each membrane and light activity recorded with a CCD camera.

The ELISA measurements were done using 100 µL of the anti-*E.coli* antibodies solution, which were added into each well of a 96-well microtiter plate (MaxiSorp, Nunc). The plate was sealed to avoid evaporation and the antibodies were allowed to adsorb overnight at 4°C. After incubation, the coating buffer was decanted and the plate was washed with PBS. 150 µL/well of PBST-S.M blocking solution at pH 7.2 was added to reduce the overall background and increase the sensitivity of the assay. The plate was then incubated for 1 h at 37°C and the wells were washed thrice with PBST-S.M. 100 µL of different bacterial strains (10^6 cells mL^{-1}) were added to each well in triplicates before incubation for 1 h at 37°C. Additional empty wells and non-coated wells were used to check the level of background signal. The wells were washed thrice with PBST–S.M. (pH 7.2), 100 µL/well of a solution of anti-*E.coli* peroxidase-labeled antibodies (1:2,000) was added and the plate incubated for 1 h at 37°C. After incubation, the wells were again washed thrice with PBST–SM (pH 7.2) then before each measurement with a Luminoskan Ascent luminometer, 150 µL of oxidizing reagent (H_2O_2) and enhancer luminol reagent solutions were injected. The data were collected and the mean and standard deviation of triplicates were calculated for each point with signals reported in RLU (relative light units).

2.6. Optimization blocking efficiency of the immunoassay

The blocking efficiency of the stack pad biosensor was optimized in testing three configurations (0, 3 and 6 blocking layers), modified with various antibody concentrations (1:1,000, 1:2,000 and 1:5,000 dilution), all immobilized as described in section 3.4.1. A cotton membrane is placed to separate each blocking layer so as to prevent non-directed flow. In a control setup, nitrocellulose membranes were not modified with immobilized bacteria. Prior to setup assembly, these membranes were blocked with the same PBST-SM blocking solution (5%(w/v) SM in PBST) followed by complete drying. Each stack pad configuration was exposed to 150 µL of either pure water or spiked with DH5∝ bacteria (10^6 cell mL^{-1}). The signal generated was captured with a CCD camera, 5 min after sample addition, as previously mentioned (section 3.3.5).

2.7. First whole setup experiments

After optimization, the final setup conformation was established. The order of the pads in the plastic holder were (from lower to upper layers), sample pad, conjugation pad (with anti-*E.coli* antibodies conjugated to HRP), six blocking layers (with 10^6 cells mL^{-1} DH5∝ cells) and absorption pad (with dry substrate). To prevent non-directed liquid flow out of the layers, each active layer was separated with a pad made from cotton. Two different samples were tested, clear water and spiked with DH5∝ bacteria (10^6 cell mL^{-1}). The stack pads were

placed above a 150 µL sample solution and measured with CCD camera 5 min after sample addition.

2.8. Sensitivity of the stack pad immunoassay

Our assay sensitivity to the DH5∝ cells target bacteria was compared with that obtained from ELISA test results. Both approaches were exposed to different DH5∝ cell concentrations (10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cells mL^{-1} and pure water). All immobilization procedures and pad preparation were previously described in section 3.4.1. The setup design was explained in section 3.6, where six blocking layers and a 1:2,000 anti-*E.coli*-HRP dilution were used. The stack pads were placed above a 150 µl bacterial sample solution and measurements taken with a CCD camera 5 min after sample addition.

2.9. Specificity of the stack pads biosensor

Five different bacterial strains, four *E.coli* (DH5∝, K12, B and NC1061) and one of *Salmonella Typhimurium* were used. Stack pad design and membrane preparation steps were the same as in the previous section. Each strain was diluted with PBST to 10^6 cells ml^{-1} prior to exposure to the stack pad sensor. 150 µL of the different bacterial solutions were added to the stack pad and then measurements taken with a CCD camera 5 min after sample addition.

2.10. Reproducibility of the system

For each development step, a minimum of 20 separate and different stacks, were exposed to the tested parameters. Reproducibility in the results can be estimated by the standard deviation values in the figures.

3. Results and discussion

3.1. Device setup

Lateral flow methods are set for an yes/no detection perceived by the naked eye, which is to date the most inexpensive and simplest to use. However, subjective judgment from operators and differences in the illumination settings can lead to a variation of reported results [29]. Depending on the application semi-quantitative or quantitative results may be required. Our technology is a new bioassay approach based on stacks of membranes allowing vertical migration of the measured fluid. This bioassay exploits the same common and cheap membranes as in the lateral flow system (e.g. absorption, conjugation, nitrocellulose and sample), but each one of them will be used in a different purpose (figure 1). Prior to fabrication, each of these membranes are treated and modified for the specific function intended, such as, when anti-*E.coli* antibodies were dried onto the conjugation pad, the substrate (H_2O_2 + luminol ratio 1:1) was dried in the absorption pad and bacteria were immobilized on the hydrophobic nitrocellulose membrane. In our approach, a hydrophobic nitrocellulose membrane served as a blocking layer, on which *E.coli* (the same that is supposed to be found in the samples) is immobilized. In the first step, the cells are collected and allowed to traverse through a sample pad until they reach the confined and dried immobilized anti-analyte capture antigens (here *E. coli*) conjugated with an HRP enzyme. Upon molecular recognition, a complex of immunoglobulins and bacteria is formed and, thereafter, it migrates up to the next layer, where a nitrocellulose membrane lies with pre-immobilized *E.coli* in wait. If the target analyte of interest (bacteria) is present in the sample, then the *E.coli*-antibody conjugate will not be able to interact with the pre- immobilized cells on this layer. Thus it will continue to move up to the next layer unaffected. In the "target-empty" samples (without target analyte (here the *E. coli*), migration of the non-conjugated antibodies will be stopped by their interaction with the corresponding bacteria on the nitrocellulose membrane. Analyte-positive complexes will migrate to the highest pad (laying in wait with dried substrate), where an enzymatic reaction occurs with the HRP previously linked on the E. coli specific antibodies in the immunoglobulin-bacteria complexes. The re-hydrated substrate will generate the measurable electrochemical signals. Thus, during the migration only the target antigen free-labelled capture antibodies will not form the complexes and therefore will be stopped, unlike the bacteria/antibodies complexes, which will migrate to the next pad (figure 1). The signal generation intensity will depend on the HRP concentration in the substrate layer. Thus, the bioreceptor linked to the enzyme (and attached to the target antigen) will reach the upper layer (with dried substrate) and oxidize the substrate to produce a quantitative light generated signal. This technology provides a single-step measurement, miniaturized (5 mm diameter for this first prototype) self-contained (without the need to provide additional reagents), rapid (5 minutes for analyte flow to the higher layers), reaction and signal generation (light production), with a quantitative approach.

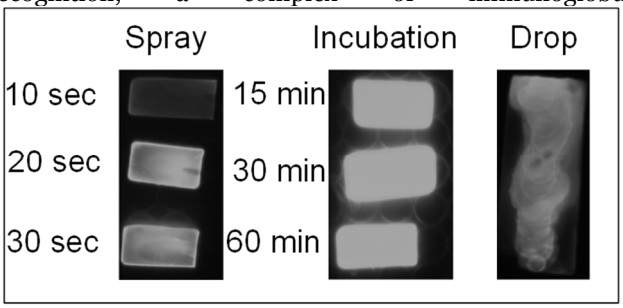


Figure 2: Optimization of the immobilization procedures

3.2. Optimization steps

The key novelty of the technology is the special blocking layer that not only simplifies the measuring processes, but also eliminates false responses of the system. As a result, the flow membrane carries the HRP-conjugated capture antibodies and cells under capillary forces to the highest layers, while target components, based on their affinity, are captured by the functionalized blocker layer (in the target-negative samples) or allowed flow to the highest layer (in the target-positive samples) for subse-

quent measurement. Thus, optimum functionalization of this layer is critical in the bioassay and measuring process. Two different parameters are supposed to be optimized in this case, first the efficiency of the target cell immobilization on the nitrocellulose membrane (to prevent false positives), and second, the putative leaching issues. Figure 2 shows different immobilization ap

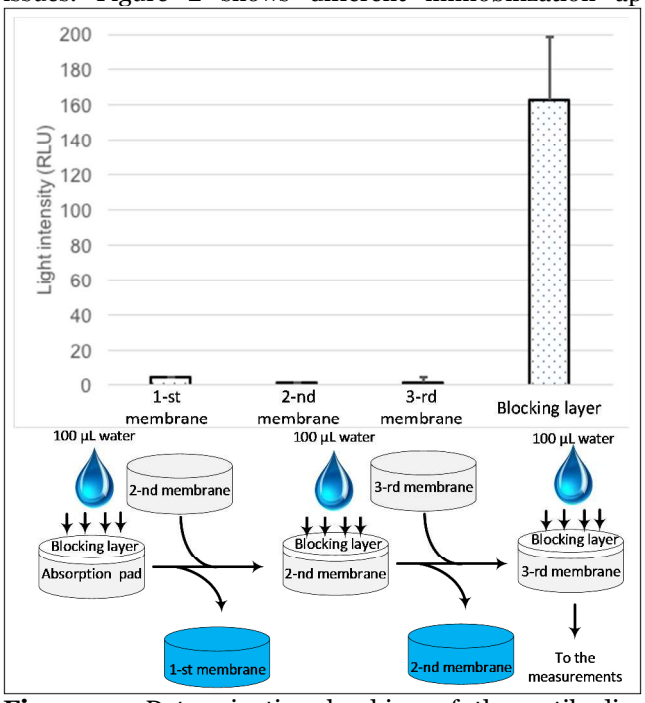


Figure 3: Determination leaching of the antibodies from nitrocellulose membrane

proaches used for determining the optimum fixing procedure. For all the experimental evaluations, we used (1:2,000 dilution) antibodies conjugated to HRP. In the first approach, antibodies were dispensed on the membrane by chromatographic spread at different durations (figure 2A). In the second approach, membranes were incubated in 10 mL antibodies solution and in the last approach, antibody solutions were slowly and carefully drop-deposited in the middle of the nitrocellulose sheet. All membranes were dried in a closed chamber at room temperature. A high uniformity blocking layer was achieved by incubating membranes within antibodies solution, and all tested incubation durations produced a similar uniform layer. Membranes treated with a dispenser provided the worst results (figure 2B). In this case, the treatment time influenced the immobilization efficiency, where longer exposure produce higher uniformity. However, even in the case of the highest tested time, the sprayed membrane demonstrated a lower immobilization efficiency, when compared to the incubation approach. Finally, antibody solutions from which drops were deposited at the center of the membrane, were shown to provide the worst results. During the drying phase, antibodies migrated from the drop site to the membrane borders (figure 2C). Indeed, the main purpose of the blocking layer is to prevent non-target analyte bound antibodies-HRP to move to the next upper levels of the assay, thus enabling to eliminate false positive responses. Thus, uniformity of the surface is crucial and therefore we will adopt the incubation approach for all subsequent experiments.

Another important issue in this important anti-analyte immobilization layer, is the possibility that fixed cells may leach away from the nitrocellulose layer during operation of the bioassay. This produces false responses, thus should be prevented. Figure 3 shows the possible leaching of the cells from membrane. There were light responses in the absorption membrane (1-st) after the first blocking layer, suggesting that diffusion of unbound cells or antibodies from blocking membrane to the next layer had taken place. For the next two absorption membranes (2-nd and 3-rd), no visible light response was recorded, suggesting strong cell linkage to the nitrocellulose membrane. Nevertheless, the light response from the first absorption membrane is still more than 100 fold lower than the blocking layer responses. Our results suggest that only a very few bacteria/antibodies or antibodies complexes were unbound during the immobilization processes. In terms of the long term stability of the dried capture E. coli layer and its antigenic stability over time, we have shown in this study that the epitopes remain intact during at least a week, however, we have not yet undertaken to check long term studies. One of the reasons being the new preferred scenario of future pad design that will have cross-linked immobilized bacteria directly on the membrane which will then be freeze dried to ensure long term storage and stability of the antigenic constituents of the bacterium. In addition, the data within the publication clearly show that several days after preparing the stacks, an immunoassay can be successfully done thus showing a proof-of-principle that all reagents and immune-reagents used can be stable at least over a short period. The authors will next study long term stability of the system in both the antigen preservation and substrate layer mix.

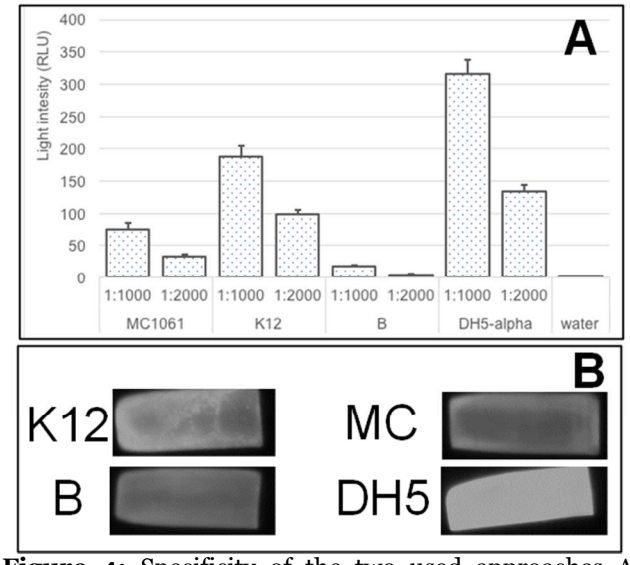


Figure 4: Specificity of the two used approaches A. ELISA B. Stack pad immunoassay to the anti-*E.coli* antibodies.

The next important optimization step that was tested involved the specificity of the antibodies to the target

bacterial strains. Four different *E.coli* strains were used, while the chosen capture antibodies were elicited against the DH5-α strain. Both of the used technologies (e.g., ELISA (figure 4A) and nitrocellulose membrane (figure 4B)) have shown a similar behavior with the tested microorganisms. A higher response was obtained for DH5-α, and its derivative K12 strains. *E.coli* type B had shown the lowest cross-reactive response, suggesting that the antibodies used were useful. In both techniques the tested *E.coli* strains showed varying degrees of cross-specificity (DH5-α >K12>MC1061>B) and that the immobilization procedure on the nitrocellulose membrane is useful as the blocking layer.

The issue of long-term storage of dried luminescence-triggering substrates is an open one. Attempts at freeze drying it exists however these have not been applied herein [30]. In the present study we have used a substrate, luminol (stable as a solid) and its iodo-phenol enhancer from a commercial kit which came in a liquid form, dried them within our paper pad and then used it as is. The longest storage tested as such was 4 days and the said dried substrate mixture worked as is without any problem. Not performed in this study is the use of the water soluble solid, that is a hygroscopic thorhombic crystalline and which can provide an anhydrous source of hydrogen peroxide, called sodium percarbonate [31]. This alternative method of providing a source of hydrogen peroxidase will be tested in the future and the mix of dried substrate and iodo-phenol related enhancer is a study in itself.

3.3. Setup tested

The next step in this study evaluates the entire immunoassay setup to confirm the capability of the blocking layer to prevent non-captured HRP-labeled antibodies from flowing through to the final membrane step. This was tested with two different samples, one positive with DH5-α strain cells at a concentration of 10^6 cell/ml and a negative control consisting of uncontaminated water. Figure 5, shows that the setup exposed to the negative control water sample had a 1,000 fold lower response, than with samples contaminated (spiked) with bacteria, thus confirming the important role of the blocking layer, where unbound antibodies are not able to bypass the immobilized bacteria in the nitrocellulose (blocking) layer. In the case of bacteria, in positive controls, that will conjugate to the HRP-labeled antibodies will migrate as a complex through the blocking layer unchallenged so as to migrate up to the substrate (upper) layer. The low background light values (in the negative control) suggested that few antibodies diffused through the blocking layer. If unbound antibodies or bacteria/antibodies complexes leach out of the nitrocellulose membrane, then a false positive would be obtained but these are not seen in our results (figure 3).

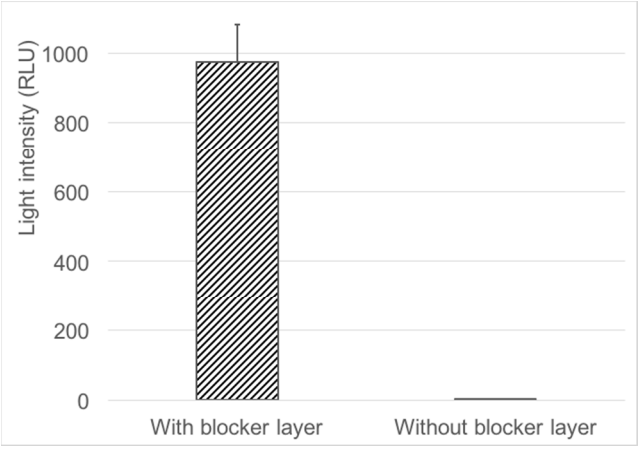


Figure 5: Effect of the blocking layer on the stack pad assay functionality.

3.4. How the number of blocking layers and antibody concentrations affect the immunoassay efficiency

The sensitivity of an immunoassay is usually determined by its efficiency in differentiating the signal output from the background values. The next step in this study was to reduce the background light levels, by adding more blocking layers and optimizing the antibody concentrations. Three different conformations were tested, with zero, three and six blocking layers. For each, three different anti-*E.coli*-HRP antibody concentrations (1:1,000, 1:2,000 and 1:5,000) were evaluated (figure 6). Each setup was exposed to uncontaminated water as the negative control, and bacterial DH5-α strain cells (10^6 cell/ml) as the positive control. In general, these results may be separated into two parts, high (1:1,000) or low (1:2,000 and 1:5,000) labeled antibody concentrations. For 1:1,000 antibody dilution, the experimental test setup response was not linear in some cases. Nevertheless, the output values in the negative controls were lower than the positive, and no significant difference was recorded between the different conformations exhibiting different number of blocking layers. A possible reason for this phenomenon may be due to the relatively high antibody concentrations. Oversaturation of antibodies resulted in an insufficient overall capture by *E.coli* of all the available antibodies, thus, a number of them ended up passing to the upper layers and produce false responses. Indeed, increasing the number of blocking layers decreased the signal values in water samples. Nevertheless, in all tested conformations, the signal output values of tests exposed to bacteria were higher than non-spiked water. In the case of lower antibody concentrations (e.g., 1:2,000 and 1:5,000) the blocking layers reduced non-conjugated antibody flow, while reducing false responses in the system. One must realize that there is direct correlation between antibody concentrations and the blocking efficiency of the system. At the lower tested concentration, the need for additional numbers of blocking layers was reduced. The optimal configuration between positive and negative controls was shown to be the presence of six blocking layers with a membrane modified by exposure to an HRP-antibody

dilution at 1:2,000. These parameters will be used in further experiments. Nevertheless, in all the aforementioned optimization steps, there were still background noises, which will ultimately need to be resolved.

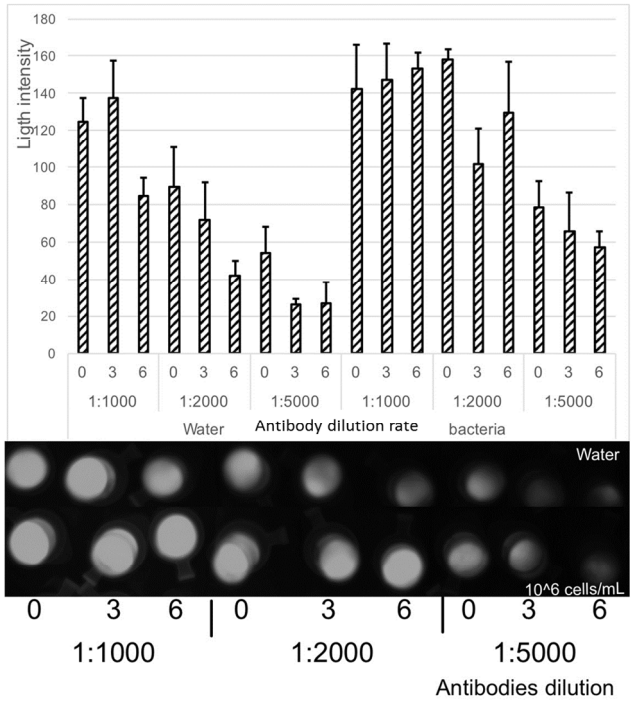


Figure 6: Effect of the blocking layers number and antibodies concentrations on the sensor false response generation

3.5. Correlation curve

ELISA [32] is commonly used in determining antibacterial antibodies or pathogen antigens [33] but has a relatively poor sensitivity when measuring whole cell microorganisms. Infectious doses of *E.coli* (10 cfu mL^{-1}), *Salmonellosis* and *Listeriosis* (1×10^3 cfu mL^{-1}) and *Campylobacter* (1×10^2 cfu mL^{-1}) are much lower than the detection limits of most known ELISA immunoassays (1×10^4 cfu mL^{-1} [34], 1×10^5 cfu mL^{-1} , 1×10^6 cfu mL^{-1} [35,36] and 1×10^4 cfu mL^{-1} [37] respectively). In this study, as seen in Figure 7 the threshold sensitivity of the immunoassay stack pads was 1×10^2 cfu mL^{-1}, about 100 times lower than ELISA performance in our hands. Nevertheless, the sensitivity of our test is still 10 folds higher than the lowest known infectious dose of pathogenic *E.coli*, albeit sufficiently sensitive to test for other infectious microorganisms (e.g., *Salmonellosis, Listeriosis, Campylobacter*). Furthermore, an interesting fact has appeared whereby our stack pads immunoassay has shown higher, or similar sensitivity, to otherwise usually accepted as exhibiting greater sensitivity biosensors including a high density microelectrode array (10^4 cfu mL^{-1}) [38], quartz crystal microbalance immunosensors (10^5 cfu mL^{-1}) [39,40], SPR (surface plasmon resonance) (10^6 cfu mL^{-1}) [41], acoustic wave immunosensor (10^6 cfu mL^{-1}) [42] and more. Similar sensitivity was achieved with magnetic nanoparticle clusters and optical nanoparticle probes [43] and higher sensitivity with polymerase chain reaction (PCR) procedures. However, the aforementioned technologies remain laboratory-based, requiring dedicated personnel and instrumentation. Most of these approaches suffer from long assay time and can only detect one pathogen at a time. Our stack pad immunoassay, on the other hand, can be easily modified to that of an array mode, we call a palette.

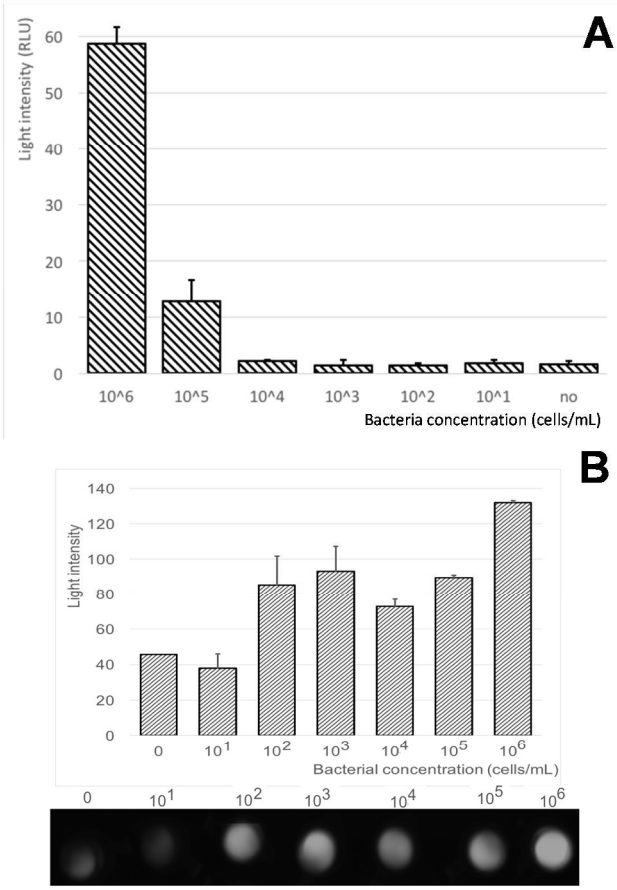


Figure 7: Correlation curve of the A. ELISA B. Stack pad assay to the different DH5 concentrations

3.6. Determining the specificity of the setup

The specificity of stacked pad immunoassays was confirmed by detecting *E. coli* DH5α, between five different bacterial strains (e.g., four different *E.coli ones* and one *Salmonella Typhimurium*) (table 1). The mode of measurement was the same as in previous steps, where 150 µl of a spiked bacterial solution (dissolved in water) was placed in a plastic holder with stacks, allowing the bacteria to migrate from the bottom to the upper layers. The highest signal was obtained with DH5α cells, the actual target strain that our immunoassay was targeted to, with the chosen specific immunoglobulins. For all the other *E.coli* strains, a much lower signal was recorded, despite usual cross reactivity. As expected, from the all tested bacteria, *Salmonella Typhimurium* [44] gave the lowest output signal. We however still recognize the presence of cross-reactive antibodies, including in the case of *Salmonella*, however this is a general limitation of immuno-

assays towards the detection of bacteria in general. Indeed, this exhibits high cross-reactivity due to their evolutionary shared macromolecular compositions. The pictures in Table 1 do not however instead show issues with flow as these are specific with the bacrerial strains and not, as shown in repeated experiments, different homogeinities in repeated experiments within a same strain [45].

Table 1 : Response of the stack pad assay to the different bacterial strains.

Bacteria strain		Signal generation	
E.coli	DH5-α	++++	
	B	+	
	K12	+++	
	MC1061	++	
S. Typhimurium	SA2386	-/+	
Water		-	

5. Conclusions

The main advantage of the proposed system is the simplicity of the measuring process. The pads are easily stored in the refrigerator, require a one-step action, by its exposure to test samples by placing very small volumes of the medium to test that may contain the putative analyte. The simplicity of this procedure enables personnel without technical or science background to use this device. The key novelty of our technology is the mode of sample flow and special blocking layers that not only simplifies the measuring processes, but also eliminates false responses. As a proof of concept, *E.coli* was chosen as the model bacteria. During the optimization steps, the efficiency of the blocking layer, leaching effects and specificity of the antibodies were tested and optimized. Sensitivity of the proposed immunoassay was 10^2 cfu mL^{-1}, that is 100 fold lower than that found in ELISA tests performed in our laboratory. Furthermore, due to its high sensitivity it may be used for the detection of various pathogenic bacteria, mostly in the case when no quantitation is required albeit we show herein semi-quantitation too.

6. Future perspectives:

The authors have proposed a new scheme for an immunoassay configuration that lends itself to the possibility of being of minimal cost. We see therefore definite prospects for this invention to be used and replace costlier POCT devices. In the mean time a lot of work remains to test the set-up with colorimetry and electrochemistry. In addition, real samples are needed to challenge the system and its robustness (substrate long term storage, capture layer covalent immobilization, epitope resilience upon drying, sample matrices interference) tested.

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Declaration of conflicting interests

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