Inkjet Printed Nanopatterned Aptamer-Based Sensors for Improved Optical Detection of Foodborne Pathogens

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The increasing incidence of infectious outbreaks from contaminated food and water supply continues imposing a global burden for food safety, creating a market demand for on-site, disposable, easy-to-use, and cost-efficient devices. Despite of the rapid growth of biosensors field and the generation of breakthrough technologies, more than 80% of the platforms developed at lab-scale never will get to meet the market. This work aims to provide a cost-efficient, reliable, and repeatable approach for the detection of foodborne pathogens in real samples. For the first time an optimized inkjet printing platform is proposed taking advantage of a carefully controlled nanopatterning of novel carboxyl-functionalized aptameric ink on a nitrocellulose substrate for the highly efficient detection of *E. coli* O157:H7 (25 colony forming units (CFU) mL⁻¹ in pure culture and 233 CFU mL⁻¹ in ground beef) demonstrating the ability to control the variation within ±1 SD for at least 75% of the data collected even at very low concentrations. From the best of the knowledge this work reports the lowest limit of detection of the state of the art for paper-based optical detection of *E. coli* O157:H7, with enough evidence (p > 0.05) to prove its high specificity at genus, species, strain, and serotype level.

1. Introduction

The increasing incidence of infectious outbreaks from contaminated food and water supply continues imposing a global burden for food safety, creating a market demand for on-site, disposable, easy-to-use, and cost-efficient devices. Despite of the rapid growth of biosensors field and the generation of breakthrough technologies, more than 80% of the platforms developed at lab-scale never will get to meet the market. This work aims to provide a cost-efficient, reliable, and repeatable approach for the detection of foodborne pathogens in real samples. For the first time an optimized inkjet printing platform is proposed taking advantage of a carefully controlled nanopatterning of novel carboxyl-functionalized aptameric ink on a nitrocellulose substrate for the highly efficient detection of *E. coli* O157:H7 (25 colony forming units (CFU) mL⁻¹ in pure culture and 233 CFU mL⁻¹ in ground beef) demonstrating the ability to control the variation within ±1 SD for at least 75% of the data collected even at very low concentrations. From the best of the knowledge this work reports the lowest limit of detection of the state of the art for paper-based optical detection of *E. coli* O157:H7, with enough evidence (p > 0.05) to prove its high specificity at genus, species, strain, and serotype level.

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With a consistent growth of the biosensors market expected to reach 21.1 billion by 2020, the demand for on-site, disposable, easy-to-use and cost-efficient devices continues to arise, positioning the food-safety as the second main segment of the market only surpassed by medical devices.[8] As a natural effect, the research field of biosensors currently shows a rapid expansion, generating valuable insights about novel materials, effective detection mechanisms, and breakthrough practical applications,[9] exploring novel and sophisticated technologies that promise to mitigate the impact of infectious diseases worldwide.[10]

It is not a surprise how paper-based assays have gained such big attention due to their versatility, easy use, and low-cost. During the last few decades, a vast variety of paper-based biosensors have been successfully developed at the laboratory scale demonstrating an enormous potential looking for solving global issues such as food and environmental pathogen
contamination. *E. coli* O157:H7 as the most common shiga toxin enterotoxigenic (STEC) foodborne pathogen serves as a model for proof of concept, resulting in a state of the art with remarkable improvement over the years gaining more sensitivity, and specificity by taking advantage of novel nanomaterial properties and alternative capture biomolecules. Recent reports of lateral flow modifications excel with enhanced response detecting concentrations as low as 537 colony forming units (CFU) mL\(^{-1}\) in ground beef by using immunochromatographic assay with gold nanoparticle-assisted enzyme signal amplification,\(^{[8]}\) 200 CFU mL\(^{-1}\) (in pineapple juice) by immunomagnetic sample concentration,\(^{[9]}\) and 100 CFU mL\(^{-1}\) (in phosphate buffer) using a traditional lateral flow assay with controlled size and dispersion of the gold nanoparticles.\(^{[10]}\) When the complexity of the real food matrix tested increases, the limit of detection (LOD) tends to dramatically decrease due to the noise to signal ratio, posing an obstacle for validation under real conditions; In 2017, Jin and collaborators presented gold decorated polystyrene beads as a novel label material for immunocapture, achieving a limit of detection of 500 CFU mL\(^{-1}\) in ground beef without enrichment.\(^{[11]}\)

The advancement of biosensors development at lab-scale is very promising. However, the fact that more than 80% of the platforms developed never get to meet the market is not yet an open topic of discussion within the field. There is evidence of a significant gap in-between the lab-bench technologies and manufacturing processes, where scaling-up challenges, economic viability, and inherent technology limitations have been ignored at early development stages, preventing a big number of novel technologies from ever been commercialized.\(^{[12]}\) This restriction is mainly imposed by the cost-efficiency, stability of the biomolecules under harsh conditions, and high-scale reproducibility of the novel biosensing platforms.

Inkjet printing techniques have emerged during last decades as a strong approach for paper-based biosensor fabrication, aiming to digitally control the variables affecting the sensing performance, controlling with nanoscaled precision the printing parameters, allowing to optimize the process since early stages in development. All this under the premise of low-cost and high throughput for validation purposes. From a variety of types of available inkjet printers, the piezoelectric approach is gaining attention as a suitable strategy for biomolecules patterning. This device applies a piezoceramic plate to create the droplets, whose size is governed by the voltage strength, pulse duration and diameter of the orifice.\(^{[13]}\) Since the print head works at room temperature, and it is compatible with a wide range of ink’s compositions, this method has served as a promising strategy to overcome the high susceptibility of biological molecules to harsh environments, while improving both, the reproducibility and stability of the printed platforms. There is evidence of successful applications for the detection of a diverse nature of targets and development of new printing approaches such as a sol-gel based method,\(^{[14]}\) patterning of microfluidic channels for immuno-based inkjet printing,\(^{[15]}\) photoassisted printing of antibodies on cellulose\(^{[16]}\) and wax micro patterning, followed by inkjet printing active enzymes.\(^{[17]}\)

According to literature reports, most of the authors describe the incorporation of antibodies or catalytic proteins as bioactive inks for inkjet printing applications\(^{[13,16,18]}\); it is well known that immuno-based strategies are the gold standard for biosensing because of antibodies’ high affinity and specificity; unfortunately, these biological molecules have considerable drawbacks mostly related with production costs, stability, shelf-life and the limitation imposed by the need to interact with the target by immune response.\(^{[12,19]}\) These conditions might affect the biosensors global performance and harm the chances to accomplish future scaling up processes.\(^{[20]}\) As an alternative for biorecognition, aptamers have arisen, demonstrating the potential to provide equal or even excel the performance showed by antibodies. Aptamers, are short single stranded sequences of DNA or RNA highly specific for selected targets, and obtained through randomized selection by SELEX method.\(^{[21]}\) Aptamers possess interesting features such as their small size (increased surface coverage), flexible structure (enhanced penetration), and ability to recognize small molecules that fail in providing immune response; from the manufacturing point of view the most relevant facts are: they are inexpensive and chemically synthesized at high scales under controlled and standardized conditions.\(^{[22]}\)

To date, thousands of aptamers have been selected and successfully integrated to biosensors for detection of several targets ranging from small ions such K\(^+\)\(^{[17]}\) and small molecules as BPA,\(^{[18]}\) to complex whole cell targets as bacteria.\(^{[24,25]}\) The use of aptamers in a lateral flow assay for detection of *E. coli* O157:H7, was reported by Wu and collaborators, using a DNA aptamer as primary capture molecule followed by strand displacement amplification pushing the LOD down to 100 CFU mL\(^{-1}\) in milk and apple juice.\(^{[26]}\) Regardless of the promising results, this limit of detection was achieved by DNA amplification rather than whole cell detection, adding a complexity layer to the detection mechanism by imposing a rigorous sample preparation (cell lysis, DNA amplification) and undesired instrumentation.

Despite the inherent potential of the incorporation of aptamers as biorecognition agents, to date there is not a single report of an aptamer-chromatographic application for whole cell detection of *E. coli* O157:H7 as well as any description of nanopatterning of aptamer-based inks for inkjet printed platforms. Considering the global impact that infectious diseases continue imposing, and the described limitations for scaling-up initiatives of lab-scale biosensors, this work aims to demonstrate the viability of using Carboxyl-functionalized aptamers, for nanocontrolled inkjet printing patterning of a low-cost chromatographic platform for the sensitive and highly specific detection of *E. coli* O157:H7, as illustrated in Figure 1, reporting from the best of our knowledge the lowest limit of detection for this foodborne pathogen in complex real samples.

2. Results and Discussion

2.1. Detection Mechanism

Taking advantage of the stability provided by the aptamers as capture biomolecule and the opportunity to implement by the first time their use in a scalable manufacturing process, this work explores the fabrication of highly stable test strips by incorporation of DNA-aptamer, and DNA complementary sequences as “bioinks” by a noncontact Inkjet printing
technique. Figure 2a shows the proposed mechanism for detection of E. coli O157:H7. 1) The sample is dropped into the sample pad, where the flow starts moving into 2) the conjugation pad where highly affine Au-Ps label particles interact and migrate by capillarity through the test strip, getting in contact with both 3) test and 4) control lines and providing a color response when appropriate. When the target is present in the sample tested, it is recognized with high affinity and specificity by the aptameric sequences immobilized on both surfaces: the test line and the label particles. First, the label particles will bind to the bacteria cell surface by physical interaction triggered by a secondary conformational change, whereas flowing along the strip. When the target-labels complexes reach the test line (3), the inkjet patterned capture aptamer (carboxy/DNA aptamer) interacts with the target as a secondary immobilization point following the same secondary conformational binding, avoiding further movement as a conventional sandwich-type assay where the accumulation of label particles in the test line is responsible to show a colored line confirming the target presence. Finally, any unreacted label particle is trapped in the control line (4) by DNA base to base complementarity, showing a second colored line. 5) All the remaining liquid is collected in the absorbent pad where the flow dynamics are controlled. After the reaction is completed, the sample is let to dry. Then, the image is captured by a cell phone camera, following with an image analysis that accounts for the color intensity and provides a correlation with the cell concentration (Figure 2b). The test interpretation for the proposed approach is illustrated in the Figure 2d, where a positive test will always show two colored lines, and the negative test will show only the control line. Any other result is considered as an invalid test.

Figure 1. Illustration of nanopatterned inkjet printed platform. Dark blue spheres: bioinks. Double-stranded pink DNA: the composition of the bioink deposited on the substrate as the control line (showing the interaction by complementarity with the capture aptamer assembled on the label particles). Single-stranded purple DNA: aptameric bioink deposited as test line. Gray substrate: nitrocellulose membrane. Light yellow section: representing the absorbent pad near to the control line. Gray object in the upper mid-section: Inkjet print head.

Figure 2. Graphical illustration of the aptamer-based detection mechanism: a) Image acquisition, b) analysis, and c) test interpretation.
The associative interaction within the test line was confirmed by bilayer interferometry (BLI). The results presented in Figure S1 (Supporting Information) demonstrate the successive binding events during the detection process. First, the biotin-modified aptameric sequence \((200 \times 10^{-9} \text{ M})\) was heat-annealed \((90 \, ^\circ \text{C}/2 \, \text{min})\) and allowed to slowly cool down in order to provide optimal folding. The biotin-modified aptameric sequence was immobilized on streptavidin coated biosensors, showing a wavelength shift \((\Delta \lambda)\) of 0.152 nm \((110 \, \text{s})\) as presented in Figure S1a (Supporting Information). After baseline stabilization, two additional association events were identified as target cell binding with a \(\Delta \lambda\) of 0.626 nm \((576 \, \text{s})\) (Figure S1b, Supporting Information), following with a baseline/washing step that resulted in a slight dissociation most likely explained by an excess of weak-bonded cells been removed from the biosensor surface. Figure S1c (Supporting Information) presents evidence of the association of label particles (PEI-Au-Ps) with a \(\Delta \lambda\) of 1.168 nm \((1030 \, \text{s})\). The described results are in full agreement with recent reports characterizing aptamer-cell and aptamer-protein interactions demonstrating the correlation between \(\Delta \lambda\) and the optical thickness of the sensor layer as a function of time\([27,28]\). The results provide evidence of the proposed detection mechanism by a sandwich-like interaction between the immobilized aptamer, the associated cells and the label particles.

2.2. Gold-Polystyrene Label Particles Fabrication and Optimization

The potential use of Au-Ps particles conjugated with a polyclonal antibody as labels for colorimetric detection of \(E. \, \text{coli} \, \text{O157:H7}\) was first described by Jin, and collaborators in 2017\([11]\). Based on their findings, there is enough evidence to prove the significant effect of a controlled optimization of Au-Ps size and coverage on the detection performance, achieving an outstanding LOD of 500 CFU mL\(^{-1}\) in ground beef and improving by 80-fold the test sensitivity when compared with naked gold nanoparticles (AuNPs) \((20 \, \text{nm})\). Therefore, the present work will discuss for the first time, the effect of the incorporation of aptamers as capture biomolecules on the sensitivity performance for whole cell detection, and the potential enhancement provided by a controlled and repeatable technique for novel chemical immobilization of the “bioinks” on the nitrocellulose membrane.

To design Au-Ps label particles with enhanced optical density (OD) and stability, an optimization process was carried out by analyzing the effect of two different citrate excess ratios on the gold reduction, and the extent of agglomeration or destabilization of the fabricated particles when kept under room temperature storage for 5 weeks. Differences between molar ratios (gold: citrate) were evaluated by UV–vis spectroscopy and transmission electron microscopy (TEM) imaging. Figure 3a

![Figure 3. Stability evaluation—optical density (OD): effect of the a) molar ratio Au: citrate (1:15) and b) optimized ratio (1:3.5) over the OD of Au decorated PS particles. The stability was tested “as fabricated” (blue line), and after 5 weeks of storage at RT (red line). c) Optical density of the fully fabricated label particles (green line) and stability after 10 weeks of storage (red line /gray dashed line). All the stability tests were carried out by UV–vis spectroscopy and TEM imaging.](image-url)
presents the optical response of particles fabricated using a higher gold:citrate ratio (1:15) according to a previous report,\cite{31} revealing an initial absorbance within the visible spectrum region with a $\lambda_{\text{max}}$ at 525 nm (as-fabricated), and a dramatic drop on absorbance after 5 weeks of storage not only affecting its optical density (represented by the red line in Figure 3a) but redshifting to $\lambda_{\text{max}}$ at 540 nm, out of the visible spectrum demonstrating an irreversible agglomeration among the gold clusters reduced on the polystyrene particles surface. TEM images of the particles under room temperature storage confirmed that Au-Ps particles after 5 weeks of storage (1:15) showed the presence of large aggregates on the surface, possibly composed of several unstable spherical gold clusters and particle agglomeration by the compression of the double layer which might be promoted by the high concentration of sodium ions.\cite{29,30} The ratio 1:3.5 was selected from literature as the optimal for small and quite monodisperse synthesis conserving the same stability obtained from the optimized fully-fabricated label particles (gray dotted line). On the other hand, the TEM images, confirmed the presence of agglomeration on the surface. These results confirm the high stability obtained from the optimized fully-fabricated label particles.

2.3. Materials Characterization

The core polystyrene beads (430 nm) were decorated with gold nanoparticles with average size of 20 nm; later, the surface was modified by self-assembling of branched polyethyleneimine (B-PEI) to incorporate a positive net charge providing available amine groups for further aptamer conjugation. In order to achieve specificity, a truncated aptameric DNA sequence (a-aptamer)\cite{32} highly specific for *E. coli* O157:H7 was conjugated to the fabricated particles, taking advantage of the polymeric (B-PEI) surface with available linker points for well-oriented conjugation.\cite{33} In order to mimic the peptide bonding naturally occurring during antibody conjugation, a carboxyl-linker was functionalized to the DNA sequence and conjugated to the label particle by NHS/EDC covalent chemistry. The proper assembling was confirmed by a redshift in the UV–vis spectrum that corresponds to the B-PEI functionalization and a slight final redshift which demonstrates for the first time, the successful conjugation of a carboxyl functionalized aptameric sequence to the surface of modified AuNPs (Figure 4a) in agreement with the TEM image that presents the microstructure of the self-assembled PEI-Au-Ps particles with the controlled growth of GNPs on the core material surface (Figure 4b). In line with the UV–vis results, the $\zeta$ potential presented significant changes providing confirmation of each assembled layer (Figure 4c). The bare polystyrene particles showed a negative relative surface charge of $-33.3 \pm 6.41$ mV which relates with the anionic nature of the initiator during the chain-growth polymerization,\cite{33} when gold was reduced on the core material surface the apparent negative $\zeta$ potential slightly increased to $-43.1 \pm 10.6$ mV, due to the presence of negative citrate ions stabilizing the particles.\cite{34} Finally, the apparent $\zeta$ potential became positive ($+33.4 \pm 5.82$ mV) in presence of a layer rich in amine groups provided by the B-PEI grafting.\cite{35}

X-ray photoelectron spectroscopy (XPS) experiments were carried out in order to provide enough evidence of the successful immobilization of the carboxyl/ss-DNA sequence on the PEI-gold decorated polystyrene particles (PEI-Au-Ps). Figure 4d presents the survey spectrum for the as-fabricated label particles showing evident signals due to C and N from the core polystyrene and PEI overlayer, as well as an O signal arising from the peptide bond created between the as-fabricated particles and the carboxyl functionalized ss-DNA. All details on the experimentally determined binding energies obtained are presented in Table S1 and Figure S3 (Supporting Information). In addition, Cl and Na were detected suggesting the presence of reduction residues most likely introduced by excess of AuHCl$_4$ and C$_6$H$_9$Na$_3$O$_9$; this fact is supported by previous reports demonstrating that hydroxyl present in the PS surface might serve as an additional reducing agent leading to the reduction of AuCl$_4^-$ anions into Au(0).\cite{36} A weak signal of Au was identified suggesting the presence of a low amount of Au on the particle surface. The Au4f signal (Figure S3a, Supporting Information) was assigned to reduced Au(0) at 87.5 eV which is in line with previous reports.\cite{37}

High-resolution (HR) XPS results presented in Figure 4e–g show the calculated composition after signal normalization by Phosphor. P2p signal was used as reference for positive identification of DNA due to its rich phosphate-sugar backbone composition and following the method described by Mankos and collaborators.\cite{38} After fitting, the composition of C and N resulted in >60% of the surface charge. The C1s peak was decomposed in four subpeaks, where C1 was assigned to aliphatic and aromatic interactions from the PS, overlapping with adventitious C contamination at 284.6 eV. The N1s peak was decomposed in four subpeaks at 398.9, 399.6, 399.9, and 401.3 eV (Figure 4e), corresponding to the amine-rich PEI layer deposition as described by Louette,\cite{39} and amide groups. The peptide bond formed after carboxyl/ssDNA immobilization was confirmed by the amide and carbonyl peaks (Figure 4f) at
401.34 and 530.5 eV respectively which is in line with the typical energies of assembled peptides.[40] The elemental molar ratios experimentally obtained support the conclusion that more than 63% of the total N corresponds to the ss-DNA aptamer which is in total agreement with the theoretical molar ratios calculated (Table 1).
Table 1. Theoretical calculated molar ratio ss-DNA Aptamer.

<table>
<thead>
<tr>
<th>N/P</th>
<th>C/P</th>
<th>O/P</th>
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<tr>
<td>4.023</td>
<td>4.767</td>
<td>1.930</td>
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Theoretical stoichiometric ratio of P versus N, C, and O (ss- Aptameric DNA) (A/T = 1.2, G/C = 1.1 and A + T/G + C = 1.1).

3. Platform Fabrication–Inkjet Printing

Most reported sensors and biosensors fabricated at laboratory level use manual deposition of label particles and/or recognition molecules. Alternatively, inkjet printing was used for this approach as a rapid, inexpensive and efficient strategy to immobilize the DNA-based biocapture molecules on the nitrocellulose substrate with high precision by controlling the drop ejection. A piezoelectric dispenser was selected for this work due to its ability to jet a wide diversity of fluids in a very controlled fashion, by providing precise and variable droplet sizes (down to 1.5 pL) and allowing a careful control of the density of ink droplets reaching the substrate. The printing system was carefully tuned, by standardizing the nozzle diameter, jetting voltage and stand-off distance aiming to optimize the droplet size and control the satellites formation for enhanced resolution.

The patterning strategy was carried out applying a multilayered overlapped pattern as illustrated in the Figure 5a, allowing the immobilization of biocapture molecules whose primary composition is illustrated in Figure 5b favoring the immobilization and physical adsorption on the nitrocellulose membrane and forming two clean lines. Following along with the proposed goal of developing an overall efficient biosensing platform, two parameters were optimized to avoid any undesired material waste.

Figure 5d shows the optimization for the number of printed layers by comparing the color intensity response (Sum DeltaE from test lines) of printed strips reacted with a fixed concentration of E. coli O157:H7 (10³ CFU mL⁻¹).

Multilayers of bioink were tested in a range from 4 to 15 layers for both test and control lines. The results demonstrated that nine printed layers provide an optimal signal response and improve the variability ratio among the independent measurements. This fact is explained by the increasing availability of capture biomolecules from four to nine layers, improving the probability of having successful detection events. However, when the layers are increased above the optimal value, the mean intensity response does not change drastically, but the variation quickly rises most likely because of the “crowding effect” leading to the signal repression by avoiding the predicted conformation change of the aptamer (adaptive folding) (Figure 5c), resulting in a lower intensity response and high variation.[41] These results are supported by the captured and binary images presented in Figure 5d.

After fixing the parameter to nine layers, the effective printed area was optimized. As shown in the Figure 5e, three printed areas were tested: from 1 × 1 to 1 × 3 mm. The plot of color intensity response versus printed area shows a positive and direct relationship between the two variables, performing an optimal resolution at 1 × 3 mm. This result is explained by the fact that E. coli O157:H7 cells (~2 μm long and 0.25–1 μm diameter) represent a big and complex target, requiring abundance of functional capture molecules to fully immobilize the cells on the test line against the flow and provide a color response[42]; otherwise the cells accumulate in the absorbent pad, preventing a visible evidence of detection[43,44] According to the analytical data, the smaller printed area presents a mean signal response which is not significantly different from the background (p = 0.078), above that value, the signal response increases as a function of printed area. This assumption is supported by the results obtained from the captured and binary images presented in the Figure 5d.

4. Limit of Detection and Repeatability Assessment

4.1. Performance in Pure Culture

Repeatability of the measurements is one of the main problems encountered with manually fabricated biosensors. Here, a preliminary correlation between gray intensity response (Sum DeltaE) and increasing concentrations of E. coli O157:H7 (10⁰ to 10⁶ CFU mL⁻¹) was performed (Figure S7, Supporting Information), providing evidence of a positive and linear interdependence of the variables with a correlation coefficient of 0.98896 among the low concentrations tested (10⁰–10³ CFU mL⁻¹). After a careful optimization of the printing parameters, the same experiment was carried out by testing low concentrations of E. coli O157:H7 (10¹ to 10³ CFU mL⁻¹), analyzing their correlation with the obtained gray intensity values, and providing a robust validation of the repeatability by the inkjet biosensor fabrication approach. For the following results at least 10 independent measurements were collected for each tested concentration. All the results are reported as obtained without discarding any data point regardless of their performance. Due to the current ruling directive of “zero tolerance policy” for this foodborne pathogen by the USDA[7,45] we dedicated our attention on the lower range of concentrations to determine the effective limit of detection in pure culture.

Figure 6a presents a linear positive correlation between the gray intensity response (Sum DeltaE) and increasing concentrations of E. coli O157:H7 (10¹ to 10³ CFU mL⁻¹) with a correlation coefficient of 0.9953 and a slope value of 8.9 × 10³, all this in line with the proposed detection mechanism, where the carboxyl-aptamer interacts with the target cell by induced folding, immobilizing the label-target complex on the printed test line and providing a color response that can be quantified by image analysis. The real images captured show visible test lines by naked-eye at concentrations as low as 253 CFU mL⁻¹ (Figure 6b), and there is evidence of detection events after image binarization processing, at concentrations as low as 25 CFU mL⁻¹ (Figure 6c).

Although the fact that this limit of detection may be taken at first sight as a risky claim, our main interest is discuss the repeatability of the above results and the implications it might have for potential scaling up purposes and on-site applications.

According to the results presented in Figure 6d-f, the error distribution changes among the experimental conditions tested, and it is inversely related with the target concentration. When we analyze the response variation at bacterial concentration of
2.51 × 10^3 CFU mL^-1, the gray intensity response shows a normal distribution (p = 0.314) with 83% of the data contained within ±1 SD from the mean value of gray intensity; when decreasing the target concentration to 251 CFU mL^-1 the normal distribution is conserved (p = 0.955) with the 75% of the data distributed within ±1 SD from the mean gray intensity value. These results represent
a reliable detection approach with a potential repeatable performance, which provides enough statistical evidence to assume that the data is sampled from a population normally distributed. Nevertheless, when dropping the concentration to 25 CFU mL$^{-1}$ the data collected is not normally distributed ($p < 0.001$) with a negative excess of kurtosis, meaning that there is a high probability for outliers. This result agrees with the distribution presented in Figure 6d, where 82% of the data collected is located within ±1 SD from the mean gray intensity value which is provided by the background noise. For this last concentration, despite the fact that we get to detect values as low as 25 CFU mL$^{-1}$, the detection events are predicted to behave as outliers, leading to a poor performance in terms of repeatability. This claim is strongly supported by the sample partitioning challenge, due to the low concentration and small volumes sampled (<100 μL) which leads to a high probability of reporting false negative results when the sample concentration is less than 10$^2$ CFU mL$^{-1}$.

4.2. Real Sample Testing

The reliability of our printed aptamer-based platform for the effective detection of *E. coli* O157:H7 in real samples was tested by recovery experiments. We selected ground beef as a real...
food matrix because of its natural complexity, with high composition of fats and proteins that are expected to act as interferents for the signal collection in real on-site testing. The samples were spiked with known concentrations of the target. Figure 7a presents the performance of the proposed platform under real conditions by maintaining a linear positive relationship between the color intensity response (Sum DeltaE) and cell concentration, with a correlation coefficient of 0.9888 and a slope value of \(1.6 \times 10^3\).

The reduction of the slope when compared with the performance in pure culture represents the expected reduction of the signal: noise ratio, due to the added noise provided by the complex matrix. Images captured in Figure 7b and binarized images (Figure 7c) provide evidence of the lack of detection for the lowest concentration tested (23 CFU mL\(^{-1}\)) and increased optical response as a function of concentration.

Table 2 summarizes the recovery performance of the proposed platform when compared with the initial spiked concentration, achieving recovery efficiency values from 83 to 109\% \((n = 3)\). In addition, Figure 7d presents the validation of the proposed platform versus the conventional plate counting method, with recovery efficiency values from 93 to 98\% \((n = 3)\). The successful implementation of our inkjet printed fabrication approach for patterning of aptameric ink, is demonstrated by retaining the aptamers’ molecular functionality and accomplishing a stable physical interaction with the target despite of the natural interference coming from the food matrix.

After a rigorous analysis of the platform efficiency, it showed an enhanced performance under real sample conditions, when compared with the strategies reported to date for chromatographic detection of \(E.\ coli\) O157:H7, as summarized in Table 3. We report a limit of detection of 233 CFU mL\(^{-1}\) in ground beef, pushing down the LOD by 2.15-fold compared with the lowest reported in literature.

5. Specificity

To complete this study, a very detailed specificity analysis was conducted, evaluating 10 different microorganisms as possible bacteria interferents. Testing was performed for genus, species, and serotype specificity. High concentrations of all the tested microorganisms were evaluated (10\(^6\) CFU mL\(^{-1}\)) along with a negative control (background) as a reference for image analysis and signal

Figure 7. Real sample testing. a) Correlation plot: gray intensity (Sum DeltaE) versus Cell concentration. b) Selection of real images acquired by an EPSON1000XL flatbed scanner. c) Gray-level and binary images obtained. d) Recovery test for the proposed platform (d) and e) validation of the proposed approach compared with the conventional plate counting technique.
quantification. The results in Figure 8a present the quantified color intensity response by all tested microorganisms under the same conditions.

The signals collected from potentially interferent bacteria failed to provide enough evidence \( (p > 0.05) \) of a significant difference between the response intensity and the background signal after image capture and binary analysis for signal quantification represented in Figure 8b,c. However, one \( (1/5) \) of the independent replicates of \( E. coli \) O121:H19 showed a weak signal \( \approx 10^2 \) CFU mL\(^{-1} \) (Figure 8c) increasing the mean signal value response and pushing us to conclude that there is significant difference between this specific signal and the background \( (p < 0.05) \). We decided to conserve the data point as an outlier that could be explained at some extent by the close genetic relation between these serotypes, possibly linked with the variable lipopolysaccharides (LPS) of membrane that act as major targets of the human immune system\(^ {46,47} \) creating a crossed reaction. Finally, a group of test strips was blocked with 20 \( \mu L \) of complementary DNA solution \( (10 \times 10^{-6} \text{ M}) \) in order to discard the possibility of nonspecific interaction between the primary aptamer sequence immobilized on the test line and \( E. coli \) O157:H7. The results confirmed the high specificity of the detection events, demonstrating that the hybridization with the cDNA resulted in destabilization of the secondary conformation and further inhibition of the physical interaction with the target as consistently reported before for truncated aptameric sequences\(^ {48,49} \).

### 6. Conclusions

This work has demonstrated a versatile, rapid, reliable, and cost-effective fabrication strategy to enhance the overall performance of a novel paper-based chromatographic platform. In summary, we present the optimization of an aptamer-based label particle for optical whole cell detection of \( E. coli \) O157:H7, which proved to be highly specific, sensitive (LOD 233 CFU mL\(^{-1} \) in ground beef), and extremely stable over time storage (10 weeks) at room temperature. Taking advantage of aptamers’ versatility, we introduced a novel modification (Carboxyl-functionalized DNA aptamers) to effectively graft the aptamer on the label particles surface (peptide bonding) and to achieve the stable aptamer printed deposition on nitrocellulose; we introduced by the first time the inkjet printed patterning of aptamer-based inks, for colorimetric platform fabrication and the successful analytical detection \( (r^2 = 0.988) \) of \( E. coli O157:H7 \) at the lowest concentration reported to date \( (233 \text{ CFU mL}^{-1}) \) in real complex samples, with demonstrated specificity against 10 interferent bacteria strains. The results presented in this work provide with fundamental understanding of the statistical significance of the inkjet-printed approach and a basis for further scaling-up processes. This work might be considered as a baseline towards future applications, by translating the biopatterning developed for aptamer-based functional inks as part of alternative dispensing methods aiming to achieve the scaling up of

### Table 2. Recovery performance of the printed aptamer-based platform for detection of \( E. coli \) O157:H7.

<table>
<thead>
<tr>
<th>Ground beef samples ((n = 3))</th>
<th>Spiked concentration ([\text{CFU mL}^{-1}])</th>
<th>Found concentration ([\text{CFU mL}^{-1}])</th>
<th>Recovery [%]</th>
<th>SD [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground beef samples ((n = 3))</td>
<td>Spiked concentration ([\text{CFU mL}^{-1}])</td>
<td>Found concentration ([\text{CFU mL}^{-1}])</td>
<td>Recovery [%]</td>
<td>SD [%]</td>
</tr>
<tr>
<td>Ground beef samples ((n = 3))</td>
<td>Spiked concentration ([\text{CFU mL}^{-1}])</td>
<td>Found concentration ([\text{CFU mL}^{-1}])</td>
<td>Recovery [%]</td>
<td>SD [%]</td>
</tr>
<tr>
<td>[2]</td>
<td>(2.3 \times 10^2)</td>
<td>(1.95 \times 10^2)</td>
<td>83.3</td>
<td>8.1</td>
</tr>
<tr>
<td>[3]</td>
<td>(2.3 \times 10^3)</td>
<td>(1.98 \times 10^3)</td>
<td>84.6</td>
<td>6.4</td>
</tr>
<tr>
<td>[4]</td>
<td>(2.3 \times 10^4)</td>
<td>(2.15 \times 10^4)</td>
<td>91.8</td>
<td>3.9</td>
</tr>
<tr>
<td>[5]</td>
<td>(2.3 \times 10^5)</td>
<td>(2.56 \times 10^5)</td>
<td>109.4</td>
<td>4.2</td>
</tr>
</tbody>
</table>

### Table 3. Comparison of performance for paper-based optical detection of \( E. coli \) O157:H7.

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>Capture biomolecule</th>
<th>Recognition structure</th>
<th>Sample tested &amp; Complexity ((\text{+}))</th>
<th>LOD ([\text{CFU mL}^{-1}])</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA- assisted enzyme</td>
<td>Polyclonal antibody; enzyme</td>
<td>Whole cell</td>
<td>Ground beef ((+++))</td>
<td>537</td>
<td>[8]</td>
</tr>
<tr>
<td>Conventional LFA #2</td>
<td>Polyclonal antibody</td>
<td>Whole cell</td>
<td>Ground beef ((+++))</td>
<td>500</td>
<td>[11]</td>
</tr>
<tr>
<td>Immunomagnetic separation &amp; Lateral flow assay</td>
<td>Polyclonal antibody</td>
<td>Whole cell</td>
<td>Pineapple juice ((+))</td>
<td>200</td>
<td>[9]</td>
</tr>
<tr>
<td>Conventional LFA #1</td>
<td>Polyclonal antibody</td>
<td>Whole cell</td>
<td>Phosphate buffer ((+))</td>
<td>100</td>
<td>[10]</td>
</tr>
<tr>
<td>Lateral flow – strand displacement amplification</td>
<td>Aptamer/ complementary sequences</td>
<td>PCR product</td>
<td>Milk &amp; apple juice ((++))</td>
<td>100</td>
<td>[26]</td>
</tr>
<tr>
<td>Inkjet printed -aptamer chromatographic test</td>
<td>Aptamer</td>
<td>Whole cell</td>
<td>Phosphate buffer ((+))</td>
<td>25</td>
<td>This work</td>
</tr>
<tr>
<td>Inkjet printed -aptamer chromatographic test</td>
<td>Aptamer</td>
<td>Whole cell</td>
<td>Ground beef ((+++))</td>
<td>233</td>
<td>This work</td>
</tr>
</tbody>
</table>
highly stable paper based colorimetric detection devices, able to remain fully functional under industrial harsh environments, and providing predictive variation for a wide variety of targets.

7. Experimental Section

Materials: Gold chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), tri-sodium citrate dihydrate (USP testing specifications), polystyrene beads (PS) with mean diameters of 0.46 μm (10% solid, 2 mL, LB5), and B-PEI (average $M_w \approx 25000$) were from Sigma-Aldrich. The carboxyl-functionalized truncated DNA aptameric sequence [5’Carboxyl/ATCAAATGTGCAGATATC-AAGACGATTGTACACATCATGCTGAGGTGGTCATAGCTGATCCTACC], the DNA biotinylated aptameric sequence [5’BiotinTEG/ATCAAATGTGCAGATATC-AAGACGATTGTACACATCATGCTGAGGTGGTCATAGCTGATCCTACC] (binding assay), and its complementary sequence [5’BiotinTEG/TTTGGTAGGATCAGCTATGACCA] were synthesized by Integrated DNA Technologies (Skokie, IL, US). All glassware was from VWR Intl. (Batavia, IL, US). It was cleaned with aqua regia HCl: HNO₃ (3:1) and washed with deionized water before use. Phosphate buffer solutions (PBSs) used in this work were PBS standard solutions from Sigma-Aldrich Co. (St. Louis, MO, US) (10 × 10⁻³ M, pH 7.4).

Instruments: Bright-field TEM images of the microstructure of the PEI-Au-Ps particles were acquired using a Tecnai G2 20 TEM (ACC V: 200 kV) (FEI Corp., Hillsboro, OR, US). PEI-Au-Ps particles zeta potential data were obtained using a Zetasizer Nano Z (Malvern Instruments Ltd., Westborough, MA, US). Optical densities were measured using a SpectraMax Plus 384 (Molecular Devices, L.L.C., Sunnyvale, CA, US). The surface chemical composition was determined by XPS (SPECS) with a PHOIBOS 150 1D-DLD analyzer and monochromatic Al Kα radiation (1487 eV) operated at 50 W. The high-resolution XPS spectra were recorded with a pass energy of 20 eV and a step size of 0.01 eV. A charge compensation was achieved by a low-energy electron flood gun (5 eV cathode voltage and 40 mA emission current). Adventitious C1s core level line was selected as reference to calibrate the energy scale. The spectra were analyzed using a Gaussian–Lorentzian blend (GL 20–30%) and a Shirley type background subtraction.

The binding assay was performed by bilayer interferometry (BLI) using an Octet RED 384 instrument equipped with streptavidin (SA) biosensor tips from ForteBio (Menlo Park, CA, USA).

The bioinks (DNA-based) were printed on the substrates (Nitrocellulose strips) using a piezoelectric inkjet printer PipeJet (BioFluidIX, Freiburg, Germany) with deposition of single droplets in the range of 2–70 nL.

Lateral-Flow Test Strip Fabrication: The nitrocellulose strips were fabricated according to Lin et al., with dimensions of 58 mm × 4 mm. [30]

Figure 8. Specificity test. All interferent bacteria were tested at a high concentration (10⁶ CFU mL⁻¹). The results presented correspond to the average response of at least five independent measurements. The error bars show the standard deviation from the mean value.
Each part was overlapped by 3 mm to ensure sample transport between pads. For the nitrocellulose membrane, HF 075 and HF 120 were tested and the HF 075 was selected as the optimal membrane because of the better flow performance and decreased trapping of particles in the conjugation pad.

Microorganisms: Escherichia. coli O157:H7, Salmonella Typhimurium, Staphylococcus aureus, Listeria monocytogenes, Staphylococcus aureus, Salmonella Typhimurium E. coli JM109, E. coli BOZO 2.7, E. coli N5428, E. coli BHB2688 strains, E. coli O103: NM, E. coli O121:H19, and E. coli O55:H7 were provided by the Food Science Department-Purdue University. Fresh cultures were first plated in LB agar (Thermo Fisher Scientific, Waltham, MA), allowing overnight growth. Then, a single colony of each microorganism was transferred to sterile LB broth and cultured in a temperature controlled orbital shaker (MaxQ HP Incubated Tabletop) at 35 °C and 160 rpm for 24 h. Cell enumeration of all the microorganisms tested was conducted by a specific spread-plate method using MacConkey agar (for E. coli strains), Modified Oxford agar (MOX), Brain heart infusion agar (BHI), and XLT-4 agar (BD Difco, Lake NJ) for specific growth of L. monocytogenes, S. aureus, and S. Typhimurium respectively. Plates were incubated at 35 °C for 48 h and cell concentration in the stock culture was calculated by plate colony counting.

**PEI-Au-Ps Complex Synthesis:** Gold decorated polystyrene (Au-Ps) particles were prepared by citrate reduction method, using gold chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), and trisodium citrate dihydrate (USP testing specifications) at a molar ratio 1:3.5. To achieve an Au-Ps coverage of 10%, using the method previously reported by Kim et al. [23] Briefly, the gold chloride trihydrate was dissolved in the polystyrene particle suspension and later mixed with the tri-sodium citrate (Au: citrate, 1:3.5). The mix was stirred vigorously for 30 min at room temperature then, heated up to 100 °C for 20 min. Excess of reactants was washed away by two consecutive cycles of centrifugation (1.4 rcf for 15 min) and resuspension in DI water using an ultrasonic digital benchtop cleaner (Cole Parmer, Ct Vernon Hills, IL, US). After the last washing cycle, the Au-Ps particles were resuspended in 8-PEI (13% wt) and stirred for 4 h. Finally, two washing cycles were completed by centrifugation (1.4 rcf for 15 min) and resuspension in DI water.

**Aptamer Conjugation:** Carboxyl-aptamer sequence (a-aptamer) [26] was conjugated with the PEI-Au-Ps particles by covalent peptide bonding. The reaction was carried out in 2-(N-morpholino) ethane sulfonic acid (MES, 99%, Sigma-Aldrich) buffer (pH 6), in the presence of N- Hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) both purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). N-Hydroxy succinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) both purchased from Thermo Fisher Scientific, Inc. (Waltham, MA) were mixed with 80 µL of bacteria culture and incubated at room temperature for 15 min. After incubation time the mix was loaded in the sample pad of each test strip. The strips dried completely before image acquisition and analysis. The same process was followed to assess the platform specificity by testing potential interferents at high concentration (bacteria interferent, 10⁵ CFU mL⁻¹). The proper blocking of the reporter complex surface was evaluated by using PBS buffer as a negative control (0 CFU mL⁻¹). All tests were run at least in triplicate. Repeatability measurements were carried out with at least 12 replicates.

**Real Sample Testing:** Standard sample preparation was followed. Ground beef (25 g) was homogenized in 225 mL of sterile PBS (pH 7.4), the samples were loaded into a sterile stomacher bag (400 mL, Gosselin Sterile Polyethylene Blender Bags) and homogenized using a stomacher 400 circulator (Seward Ltd., England) for 30 s. Samples were spiked with E. coli O157:H7 suspensions until reaching known bacterial concentrations. Each sample was subjected to the exact same procedure described in the above section.

**Image acquisition:** Images were collected from dry test strips in a Macbeth SpectraLight II light booth (Gretag Macbeth, New Windsor, NY, USA) to provide a daylight environment using a cell phone camera (iPhone X) with dual-lens 12 megapixels primary camera. The images presented in Figures 6b and 7b were acquired by using an EPSON1000XL flatbed scanner (Epson America Inc., Long Beach, CA).

**Image Analysis:** The threshold for image binarization was calculated by Otsu’s, and four points connected component method [31] to convert the gray-level images to a binary image. The boundary delimiting the dot was described by projecting the binary images. Finally, the number of nonzero pixels was calculated, providing a quantitative grayscale value for further analysis. For enhanced results, the background was collected from two different areas from each test strip and the average value was subtracted accounting for the noise.

**Data Analysis:** The color intensity analysis and correlation were made in Origin 2018, MATLAB, and j-image. Statistical analysis was conducted by Means comparison and Shapiro-Wilk test, using right tailed normal distribution (statistical package JMP).

**Supporting Information**
Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**
The authors declare no conflict of interest.
Keywords

aptamers, biofunctional patterning, foodborne pathogens, inkjet printing, whole cell detection

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