Merging lateral flow immunoassay with electroanalysis as a novel sensing platform: Prostate specific antigen detection as case of study

Merging Lateral Flow Immunoassay with Electroanalysis as a Novel Sensing Platform: Prostate Specific Antigen Detection as Case of Study

Antonella Miglione[1], Fabio Di Nardo[2]*, Simone Cavalera[2], Thea Serra[2], Claudio Baggiani[2], Stefano Cinti[1,3]*, Laura Anfossi[2]

[1]Department of Pharmacy, University of Naples "Federico II", Via Domenico Montesano 49, 80131 Naples, Italy.

[2]Department of Chemistry, Università degli Studi di Torino, 10124 Turin, Italy

[3]BAT Center - Interuniversity Center for Studies on Bioinspired Agro-Environmental Technology, University of Naples "Federico II", 80055 Naples, Italy

* Corresponding author: stefano.cinti@unina.it; fabio.dinardo@unito.it

ABSTRACT: COVID-19 pandemic highlighted lateral flow immunoassay (LFIA) strips as the most known point-of-care (POC) devices enabling rapid and easy detection of relevant biomarker by non-specialists. However, these diagnostic tests are usually associated with qualitative detection of the biomarker of interest. Alternatively, electrochemical-based diagnostics, especially known for diabetes care, enables quantitative determination of biomarkers. From an analytical point perspective, the combination of the two approaches might be representing a step forward for the POC world: in fact, electrochemical transduction is attractive to be integrated into LFIA strips due to its simplicity, high sensitivity, fast signal generation, and cost-effectiveness. In this work, LFIA strip has been empowered by the combination with an electrochemical transduction, yielding an electrochemical LFIA (eLFIA). As proof-of-concept, the detection of prostate-specific antigen (PSA) has been carried out by combining printed-electrochemical strip with the traditional LFIA tests. The electrochemical detection has been based on the measurement of the Au ions produced from the dissolution of the gold nanoparticles previously captured on the test line. After having optimized both gold nanoparticles dissolution and the electrochemical parameters, the analytical performance obtained at LFIA and eLFIA were compared, highlighting how the use of differential pulse voltammetry allowed for a lower detection limit, respectively 0.38 and 0.15 ng/mL, but with the necessity of longer time of analysis. Although the correlation between the two architectures confirmed the satisfactory agreement of outputs, this technical note has been thought to provide the reader a fair statement with regards the strength and drawbacks about combining the two (apparently) competitor devices in diagnostics field, namely LFIA and electrochemical strips.

INTRODUCTION

The lateral flow immunoassays (LFIAs) satisfy all the criteria of an "ASSURED" POC device (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Delivered to the end-users)[1] and can easily address also the additional requirements recently introduced with the REASSURED criteria (real-time connectivity, ease of specimen collection, and environmental friendliness).[2]

Some of the key features of LFIAs are the possibility to 1) perform quick analysis, e.g. 5-30 min, 2) require one-step procedure without needing any additional equipment, and 3) be low-cost.[3] These attractive features have highlighted the use of LFIAs during the global pandemic of SARS-CoV-2 providing an additional tool for the pandemic control and management.

However, despite the appealing features, the simplest LFIAs only provide a qualitative result regarding the presence or absence of the target analyte (evaluating the test line signal), and their sensitivity and specificity is often worse if compared to laboratory-based tests, like the enzyme-linked immunosorbent assay (ELISA) and the reverse transcription polymerase chain reaction (RT-PCR).[4]

In the last decades, huge efforts have been made to improve the analytical performances of the traditional LFIAs. Several strategies have been studied and reported, such as the chemical enhancement to improve the signal/background ratio in the test line,[5–9] the use of readers and the adoption of different transduction systems, i.e. fluorescence,[10–12] chemiluminescence,[13–15] surface-enhanced Raman scattering (SERS),[16–18] photothermal,[19,20] etc. Recently, among these approaches, fluorescence and SERS detection have been largely reported in literature to boost LFIAs.[21]

It should be noted that, even if these strategies have clearly improved the traditional LFIAs, the resulting procedures have been made more complex (requiring the flow of additional (bio)reagents, washing steps, etc.), more time consuming (due to the additional steps and laborious data treatment) and more expensive (due to the specific label or to the additional reagents needed for the chemical/enzymatic enhancement, or to the use of specific detector).

As an example, the readout time of SERS-based LFIAs is quite long,[4] and Raman microscope system are highly expensive and bulky. Vi Tran et al. tried to partially face this issue developing a portable SERS reader for lateral flow assay.[22] However, to the best of our knowledge no SERS-based LFIA is commercially available yet. It is evident how additional procedures and equipment to make the analysis more quantitative often encounter practical drawbacks, both in terms of increased cost and time-consuming approaches. A major challenge is represented by the possibility to reach an optimal compromise for enhancing sensitivity without reducing the affordability of the whole detecting system. Among the various sensing and biosensing architectures, electrochemical ones represent promising candidates to be coupled with such naked-eye devices. In fact, the adoption of electroanalytical methods has highlighted the possibility to be coupled with several decentralized settings, from wearable to implantable devices.[23,24] The effectiveness of electroanalytical systems is associated with the easiness in miniaturization and the possibility to analyze colored complex matrices, i.e. blood, serum, soil etc.[25,26] The main advantage of electroanalysis is to be "blind" towards colored species that sometimes limit optical readouts. The most known (and sold) example worldwide is represented by the glucose strip for diabetes patients. It should be considered that less than a microliter of whole blood is sufficient to quantify the amount of glucose and, in addition, the required time for display visualization is less than 10-15 s. The combination of strips micro-fabrication, lamination and the manufacture of affordable readers, put the blood glucose test on top of the list of the desirable devices for self-healthcare monitoring. The growing interest towards the implementation of miniaturized and low-cost electrochemical readers, both for research and real-world settings, is pushing the application of portable electroanalysis far. Several companies are currently producing smartphone-based potentiostats that offer a wide range of techniques, with the adoption of user-friendly software. This is leading to the development of portable architectures, mainly based on screen-printing technology, that can be easily applied to a wide class of application, such as medical, pharmaceutical, environmental, agri-food, industrial etc.[27,28]

It should be noted that the electroanalytical systems, with their portability, would represent an excellent alternative to existing solutions (e.g. fluorescence, chemiluminesce, SERS, etc.) to be combined with LFIAs for making these more sensitive, without adopting complicated setups. However, despite the aforementioned unique features, electrochemical-supported LFIA (eLFIA) still represents a niche,[21] and recently published reviews have highlighted some applications towards this vision, by merging the advantages of a LFIA with those of the electrochemical detection.[29,30] Perju and Wongkaew schematized the existing principles to integrate electrodes to LFIA, including electrodes underneath the NC membrane, electrodes placed above the NC membrane with the electroactive surface facing downwards, NC membrane positioned in between the stacked electrodes, and ex-situ detection.[29] Among these, the so-called ex situ electrochemical detection, in which the detection is performed off-strip after cutting the test line zone, appears as the most feasible approach: the complete integration of electrochemical transducers to LFIA is still an open challenge mostly due to the requirements of complicated fabrication methods causing the uncertain reliability of the tests that hinders their future commercialization. With regards to the ex-situ analysis, different approaches have been reported including architecture based on the use of both biological and inorganic labels, respectively, horseradish peroxidase and quantum dots.[31–36] However, both the strategies are characterized by some drawbacks. For instance, the use of the enzyme, along with the antibodies, requires additional reagents (generally ortho-phenylenediamine and hydrogen peroxide) to exploit the catalytic activity, leading to an increase of the cost and experimental tasks at end-users. Regarding the strategy reported by Nian and co-workers, even if the CdS@ZnS quantum dots can be easily dissolved in hydrochloric acid allowing electrochemical sensing by measuring cadmium ions, this step entirely hides the fluorescence of labels and also involves heavy metal ions waste.[36]

In comparison with these attempts to include electrochemistry within the world of LFIA, the selection of gold nanoparticles (AuNPs) as a double colorimetric/electrochemical label would appear as an effective solution. In fact, among the traditional and commercially available LFIAs, AuNPs are the most studied, characterized and used labels.[21] Even by an electroanalytical point of view, many systems have been developed to detect AuNPs due to their increasing relevance and use as antimicrobial and therapeutic agents.[37,38] In particular AuNPs might be exploited both for colorimetric and electrochemical sensing, and this would lead to a novel concept of qualitative/quantitative analytical method within the same experimental setup. The proposed strategy would allow preserving the traditional naked-eye detectable qualitative output and, at the same time, offer the electrochemical quantification when needed. In fact, as reported by Khlebstov et al. the minimum number of AuNPs that can be visualized at the naked eye on the LFIA nitrocellulose membrane ranged from 1.5 x 105 to 6.7 x 107 particles/mm2 (depending on their size),[39] thus limiting the sensitivity of these devices. To overcome this physical limitation, our idea has involved the combination of the naked-eye detection with the electrochemical quantification by dissolving the AuNPs captured on the test line and then measuring Au ions at the working electrode. To date, a unique example has been reported in literature based on a similar dissolution of AuNPs from LFIA. However, has reported by authors the method involved the use of HBr–Br2 mixture to dissolve AuNPs and addition of phenoxyacetic to eliminate the excess of Br2 before the analysis. The use of traditional bulk electrodes was not compatible with a portable application, and the electrochemical detection only replaced the naked-eye one.

In this technical note, we developed an eLFIA exploiting he use of AuNPs both as colorimetric and electrochemical label coupling traditional LFIA to electrochemical analysis by differential pulse voltammetry, using a screen-printed electrode as the transducer. As the case of study, the proposed eLFIA has been applied towards the detection of the prostate specific antigen (PSA), an enzyme whose blood levels are commonly used for prostate cancer early-diagnosis and therapy management.[40] Herein, hydrochloric acid has been used to dissolve the AuNPs avoiding the use of complex mixtures and also reducing the number of experimental tasks. Both the detections have been

performed, analytically characterized and correlated, demonstrating the effectiveness of the combination, which does not replace the naked-eye visualization, but it added more value being complementary depending on the analytical need, improving the sensitivity. The entire system has been designed to be totally portable, the AuNPs-on-strip dissolution has been carried out in pre-acidified vials, without the necessity of additional equipment, and the smartphone-powered potentiostat ulteriorly enhanced the entire decentralization of the detection. The simplicity of the approach, supported by miniaturization, might represent the starting point for plenty of application,

maintaining the cost-effectiveness and the portability requirement for POCT.

EXPERIMENTAL SECTION

(Bio)reagents, chemicals, and materials

Gold (III) chloride trihydrate (ACS reagent), anti-mouse immunoglobulin G antibody produced in goat, boric acid, sodium tetraborate decahydrate, casein sodium salt from milk, sucrose, bovine serum albumin (BSA), hydrochloric acid, and gold nanoparticles from Sigma Aldrich were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tween® 20 and other chemicals were purchased from VWR International (Milan, Italy). Nitrocellulose membranes (CNPC-SS12) with cellulose absorbent pad and FR-1 sample pads were purchased by Advanced Microdevices Pvt. Ltd. (Ambala, India). Glass fiber conjugate pads were obtained from Merck Millipore (Billerica, MA, USA). The anti-PSA monoclonal antibody (mAb_1) used to form the test line were purchased from Fitzgerald Industries International (North Acton, MA, USA), while the anti-PSA mAb used for AuNPs conjugates and the PSA used to prepare the standard solutions were provided by NIB biotec Srl (Torino, Italy). SigmaPlot v.14.0 (Systat Software, Inc) was used to perform the statistical analyses. To obtain the colorimetric test line signal intensity the strips images were acquired by a benchtop scanner (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany) and processed by QuantiScan 3.0 software (Biosoft, Cambridge, UK). For the electrochemical detection a Sensit Smart (Palmsens, The Netherlands) small wireless potentiostat connected to a smartphone was employed.

Preparation and characterization of AuNPs and anti-PSA mAb-AuNPs conjugate

AuNPs with a mean diameter of 30 nm were synthesized through the tetrachloroauric acid reduction with sodium citrate, as previously reported.[41,42] Very briefly, 1 mL of 1% w/v sodium citrate was added to 100 mL of boiling 0.01% w/v tetrachloroauric acid under vigorous stirring. Finally, the AuNPs were cooled down to room temperature and stored at 4 °C for successive conjugation to anti-PSA mAb. AuNPs were characterized by UV-vis spectroscopy on a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, USA) and by transmission electron microscopy using a Jeol 3010-UHR (Jeol Ltd, Japan) high resolution transmission electron microscope (HR-TEM) equipped with a LaB6 filament operating at 300 kV and with an Oxford Inca Energy TEM 300 X-ray EDS analyzer. For TEM imaging, a drop of the AuNPs aqueous suspension was put on a copper grid covered with a lacey carbon film for the analysis. The AuNP resulted almost spherical in shape, with a sharp SPR band centered at 525 nm (Figure S1, Supporting Information).

The AuNPs-Ab anti-PSA (AuNPs-Ab) conjugate was prepared by passive adsorption of the anti-PSA mAb on the surface of the citrate-capped AuNPs. Briefly, the AuNPs pH was adjusted to ~8.5 with carbonate buffer (0.05 M, pH 9.6). Then, for each mL of AuNPs with optical density (OD) 1, the appropriate amount of the mAb was added and gently mixed for 30 min at 37 °C. Subsequently, 100 µL of blocking solution (borate buffer supplemented with 1% w/v casein) were added (for each mL of AuNPs) to block the unbound sites for 10 min at 37 °C. Finally, the AuNPs–mAb conjugate was recovered by centrifugation (10 min at 7100×g), washed twice with borate buffer supplemented with 0.1% casein, and reconstituted in borate buffer supplemented with 2% (w/v) sucrose 1% (w/v) casein, 0.25% (v/v) Tween® 20, and 0.02% (w/v) NaN3. AuNPs-mAb conjugates were stored at 4 °C until use. The appropriate amount of mAb to be added to obtain stable conjugates was evaluated through the flocculation stress test.[43,44] Very briefly, 250 µL of buffered AuNPs were placed in the wells of a microtitration plate and incubated for 30 min with increasing amount of mAb (from 0 to 2.5 µg). Then, 25 µL of aqueous NaCl (10% v/v) was added and reacted for 10 min to promote aggregation of unstable AuNPs. When the AuNPs are sufficiently shielded by the mAb, no salt-induced aggregation occurs. According to the flocculation stress test. The minimum amount to obtain stable conjugate was 1.5 µg of mAb (corresponding to 6 µg per 1 ml of AuNPs). The results are reported in Figure S2, Supporting Information.

LFIA strip manufacturing

The mAb_1 (0.5 mg/mL) and goat anti-mouse IgG (0.5 mg/mL) diluted in phosphate buffer (20mM, pH 7.4) were spotted onto NC membranes at 1 µL/cm by means of an XYZ3050 platform (Biodot, Irvine, CA, USA) to form the test and control lines, respectively. The conjugate pad was pre-saturated with borate buffer supplemented with 2% (w/v) sucrose 1% (w/v) BSA, 0.25% (v/v) Tween® 20, and 0.02% (w/v) NaN3 and dried at 60 °C for 1 h. Subsequently, AuNPs-Ab conjugate solution at OD 0.25 (80 µL/cm) was used to saturate the conjugate pad. Then, it was dried at room temperature for 3 h. The NC membranes were dried at 37 °C for 60 min under vacuum, layered with sample and conjugate pads, cut into 3.5 mm wide strips by means of a CM4000 guillotine (Biodot), and inserted into plastic cassettes (Kinbio, Shanghai, China) to fabricate the ready-to-use LFA device. Cassettes were stored in the dark in plastic bags containing silica at room temperature until use.

SPE manufacturing

Screen–printed electrodes have been manufactured by serigraphy. Autostat HT5 polyester sheets were used as the flexible support. Ag/AgCl (Elettrodag 477 SS) ink has been used to print the reference electrode, while graphite-based conductive ink (Elettrodag 421) has been used for printing both the working and counter electrode.[45] After each printing steps, the electrodes have been cured in the oven for 20 min at 80 ◦C. The area of the electrochemical cell has been defined by using an adhesive tape also avoiding samples reaching the electrical connectors at the potentiostat. The final diameter of the working electrode is 4 mm.

Assay principle

The principle of the proposed eLFIA is depicted in Figure 1.

Figure 1. Experimental setup of eLFIA architecture which includes: LFIA visualization, cutting of the test line, AuNPs dissolution in pre-acidified vials, and smartphone-based electrochemical detection.

The sensing concept is to exploit the AuNPs both for colorimetric and electrochemical detection to improve the sensitivity, depending on the analytical need. To start the assay, 70 µL of PSA standard solutions (concentration ranged from 0 to 20 ng/mL, prepared in in phosphate buffer 20 mM, pH 7.4 supplemented with 1% w/v BSA and 0.1% v/v Tween® 20) were applied to the sample pad. When the sample containing the PSA reach the conjugate pad, the enzyme interacts with the AuNPs-Ab forming a first immunocomplex, AuNPs-Ab-PSA. Then the immunocomplex continue to flow through the NC membrane encountering the capturing anti-PSA mAb in the test line, forming a colored line corresponding to the sandwich immunocomplex AuNPs-Ab-PSA-Ab. The excess of AuNPs-Ab continues to move to the control line, where it is captured by the goat anti-mouse IgG, forming a second colored line. Thus, in the presence of the target analyte the colorimetric response results in the formation of two colored lines where the color intensity of test line is directly proportional to the PSA content in the sample. Instead, in the absence of the PSA (or for concentration lower than the limit of detection), only the control line is visible. After the LFIA completion (15 minutes), both the test and control lines have been cut through the use of common scissors. Successively, the selected line has been introduced into a pre-loaded vial containing 100 µL of concentrated HCl. This step is essential to dissolve all the AuNPs that have been accumulated onto the chosen line. The process of dissolution takes 3', subsequently the acidic solution is 10-fold diluted with distilled water to a final concentration of 1 M HCl, and 100 microliters are placed onto the electrochemical printed strip to be analyzed with a portable potentiostat coupled to a smartphone. The measurement have been performed through the adoption of differential pulse voltammetry (DPV) using the following parameters: 1) E dep: 1.25 V, t dep: 120 s, E step: 0.01 V, E pulse: 0.2 V, t pulse: 0.02 s, Scan Rate: 0.05 V/s, and 2) E beg: 0.7 V, E end: 0 V, E step: 0.01 V, E pulse: 0.2 V, t pulse: 0.02 s, Scan Rate: 0.05 V/s.

RESULTS AND DISCUSSIONS

AuNPs detection at portable electroanalytical strip

The development of an electroanalytical-based methodology to be combined with lateral flow technology is based on the determination of the gold nanoparticles that are accumulated on both the test and control line on the strip. In agreement with literature, the electrochemical determination is usually performed in hydrochloric solution, as per metal sensing in general. To this regard, the first investigation was about the selection of the acidic media: hydrochloric acid at different concentration was compared with the use of aqua regia (hydrochloric acid/nitric acid in a 3/1 ratio), using a printed electroanalytical strip for sensing (Figure 2).

Figure 2. Selection of the acidic media to detect AuNPs. Measurements carried in presence of 1 µM of AuNPs in 1M HCl (blue line), 0.1 M HCl (green line) and aqua regia HCl/HNO_3 (3:1, v/v) (red line). The dashed lines are representative of the measurements in absence of AuNPs.

Among the 0.1 M and 1 M hydrochloric acid and aqua regia, the 1 M hydrochloric acid was consistent with an optimal recorded signal. In addition, the use of aqua regia might represent a drawback for the dissolution of the silver-based conductive ink used for the screen-printing process. Subsequently, some electrochemical parameters including the open circuit time and the deposition time of the differential pulse voltammetry have been taken into account. The open circuit time is about a short period of time where the sample drop is left onto the working electrode surface without applying any potential difference, and it is necessary to have gold ions physically accumulated. According to experimental results, 2 min appears as the optimal time to accumulate gold ions at the electrode, while an electrochemical deposition time of 3 min was chosen as the optimal one, also considering that 5 min did not represent a significant improvement of signal intensity to justify almost the double of time. Considering these preliminary observations, these results have been used as the basis to be applied towards the detection of gold nanoparticles accumulated onto a lateral flow strip.

Analytical performances of the eLFIA

Depending on the sensing architectures and settings, three different levels of detection can be provided to the end-user. Two main settings should be considered, namely LFIA and eLFIA. Among these, the LFIA-based detection is capable to offer two levels of sensitivity. In fact, the sensitivity can be estimated through the naked eye observation and by measuring the AuNPs signal intensities from the acquired strip images with the help of a reader (e.g. scanner). Instead, with regards the eLFIA architecture, sensitivity of the method is estimated through the electrochemical measurement of the Au ions produced from the dissolution of the AuNPs captured on the test line. As reported in Figure 3, the three methods have been reported by interrogating the portable platforms with PSA in the range comprised between 0.01 and 50 ng/mL.

Figure 3. A) Calibration curve obtained by measuring PSA in the 0.01-50 ng/mL range with the use of scanner for optical detection. Inset: LFIA strips showing the visual LOD (naked-eye detection) equal to 1 ng/mL; B) Calibration curve obtained by measuring PSA in the 0.01-50 ng/mL range with the use of smartphone-powered potentiostat, namely eLFIA. Inset: Voltammetric curves related to increasing level of PSA.

The visual limit of detection (visual LOD) was defined as the lowest PSA concentration resulting in a test line color visible to at least 5 different operators: as showed in inset of Figure 3A, it was equal to 1 ng/mL. With regards to the other two settings, namely LFIA aided by scanner reader and eLFIA, the LOD was calculated as follows: average of the signal at the lowest detectable PSA concentration + 3*standard deviation. The calculated LOD was 0.38 and 0.15 ng/mL, respectively, for LFIA and eLFIA. However, even if the eLFIA displayed the better LOD (using the same type of LFIA strips), some considerations need to be highlighted in order to make a fair comparison between

the two settings: although the electrochemical detection allowed for lower detection limit, it should be considered that the whole process (which also includes line cutting, dissolution and electrochemical protocol) makes the PSA measurements ca. 10 minutes longer with respect to the traditional scanner-aided LFIA ones. However, the two approaches should be seen as complementary in terms of needs of application/sensitivity: one can decide if the electrochemical reader would be preferred to the optical one, and vice versa, depending on the analytical need. In fact, as reported in Figure 4, the correlation between the two approaches has been evaluated: eight randomized strips, considering both control and test lines, have been measured with the two approaches (in triplicate) obtaining a satisfactory coefficient R of 0.985.

Figure 4. Correlation between eLFIA and LFIA measurements of eight randomized strips for PSA analysis, including both control and test lines (all measurements in triplicate).

CONCLUSION

A common LFIA test has been combined with the electrochemical detection, through the adoption of a printed-strip connected to a smartphone, namely eLFIA. Although the LFIA tests represent a powerful class of POC devices for the use of non-specialists in decentralized context, e.g. pregnancy, infectious diseases, COVID-19, etc., the aim of this technical note was to provide the reader an easy way to improve the "quantification" feature of these analytical tools. To do this, a smartphone-powered electrochemical assay has been integrated with a LFIA test, using PSA detection as a model study. The electrochemical visualization has been carried out through the use of printed-strip following the dissolution of AuNPs in pre-acidified vials. As per our findings, the addition of the voltammetric measurements allowed to enhance the sensitivity towards PSA down to 0.15 ng/mL with respect to the use of a scanner commonly employed for the optical visualization, characterized by a 0.38 ng/mL as the detection limit. Despite the enhancement of the analytical sensitivity, eLFIA's features can be summarized as follows: 1) cutting the test line and analyzing it into a pre-acidified vial is user-friendly, 2) the electrochemical settings is capable to obtain lower detection limit if compared to traditional LFIA, and 3) the electrochemical combination is characterized by additional 10 minutes of analysis, making the entire system slower than the traditional LFIA. However, eLFIA should not be seen as a replacement of LFIA, instead as a complementary tool: depending on the sensitivity requirement, one can choose to combine the LFIA to electrochemical transduction or to leave the LFIA test unchanged. The performance of the electrochemical methods might represent a step forward to obtain more quantitative tests, however this should not be synonymous of more-complex and time-consuming setup: the combination of LFIA, electrochemistry, paper-based fabrication, smartphone-based reader and nanomaterial might lead to new finding towards the development of novel class of ASSURED diagnostics, easily extendible to other fields, i.e. environmental, pharmaceutical, agri-food.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

brief description (file type, i.e., PDF)

brief description (file type, i.e., PDF)

AUTHOR INFORMATION

Corresponding Author

Stefano Cinti − Department of Pharmacy, University of Naples "Federico II", 80131 Naples, Italy; BAT Center Interuniversity Center for Studies on Bioinspired Agro- Environmental Technology, University of Naples "Federico II", 80055 Naples, Italy; orcid.org/0000-0002-8274- 7452; Email: stefano.cinti@unina.it

Fabio Di Nardo - Department of Chemistry, Università degli Studi di Torino, 10125 Turin, Italy; orcid.org/0000-0003-0497-4251; Email: fabio.dinardo@unito.it

Author Contributions

Antonella Miglione − Department of Pharmacy, University of Naples "Federico II", 80131 Naples, Italy
Simone Cavalera - Department of Chemistry, Università degli Studi di Torino, 10125 Turin, Italy
Thea Serra - Department of Chemistry, Università degli Studi di Torino, 10125 Turin, Italy
Claudio Baggiani - Department of Chemistry, Università degli Studi di Torino, 10125 Turin, Italy
Laura Anfossi - Department of Chemistry, Università degli Studi di Torino, 10125 Turin, Italy

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

The research leading to these results has received funding from AIRC under MFAG 2022 - ID. 27586 project P.I. Cinti Stefano.

Authors also acknowledge support from the Project CH4.0 under the MUR program "Dipartimenti di Eccellenza 2023–2027" (CUP: D13C22003520001).

REFERENCES

(1) Kettler, H.; White, K.; Hawkes, S. J.; Diseases, U. B. S. P. for R. and T. in T. *Mapping the Landscape of Diagnostics for Sexually Transmitted Infections : Key Findings and Recommendations*; TDR/STI/IDE/04.1; World Health Organization, 2004. https://apps.who.int/iris/handle/10665/68990 (accessed 2023-08-30).

(2) Land, K. J.; Boeras, D. I.; Chen, X.-S.; Ramsay, A. R.; Peeling, R. W. REASSURED Diagnostics to Inform Disease Control Strategies, Strengthen Health Systems and Improve Patient Outcomes. *Nat Microbiol* **2019**, *4* (1), 46–54. https://doi.org/10.1038/s41564-018-0295-3.

(3) Vázquez, M.; Anfossi, L.; Ben-Yoav, H.; Diéguez, L.; Karopka, T.; Ventura, B. D.; Abalde-Cela, S.; Minopoli, A.; Nardo, F. D.; Shukla, V. K.; Teixeira, A.; Tvarijonaviciute, A.; Franco-Martínez, L. Use of Some Cost-Effective Technologies for a Routine Clinical Pathology Laboratory. *Lab Chip* **2021**, *21* (22), 4330–4351. https://doi.org/10.1039/D1LC00658D.

(4) Liu, Y.; Zhan, L.; Qin, Z.; Sackrison, J.; Bischof, J. C. Ultrasensitive and Highly Specific Lateral Flow Assays for Point-of-Care Diagnosis. *ACS Nano* **2021**, *15* (3), 3593–3611. https://doi.org/10.1021/acsnano.0c10035.

(5) Anfossi, L.; Di Nardo, F.; Giovannoli, C.; Passini, C.; Baggiani, C. Increased Sensitivity of Lateral Flow Immunoassay for Ochratoxin A through Silver Enhancement. *Anal Bioanal Chem* **2013**, *405* (30), 9859–9867. https://doi.org/10.1007/s00216-013-7428-6.

(6) Xu, H.; Chen, J.; Birrenkott, J.; Zhao, J. X.; Takalkar, S.; Baryeh, K.; Liu, G. Gold-Nanoparticle-Decorated Silica Nanorods for Sensitive Visual Detection of Proteins. *Anal. Chem.* **2014**, *86* (15), 7351–7359. https://doi.org/10.1021/ac502249f.

(7) Rodríguez, M. O.; Covián, L. B.; García, A. C.; Blanco-López, M. C. Silver and Gold Enhancement Methods for Lateral Flow Immunoassays. *Talanta* **2016**, *148*, 272–278. https://doi.org/10.1016/j.talanta.2015.10.068.

(8) Panferov, V. G.; Safenkova, I. V.; Byzova, N. A.; Varitsev, Y. A.; Zherdev, A. V.; Dzantiev, B. B. Silver-Enhanced Lateral Flow Immunoassay for Highly-Sensitive Detection of Potato Leafroll Virus. *Food and Agricultural Immunology* **2018**, *29* (1), 445–457. https://doi.org/10.1080/09540105.2017.1401044.

(9) Panferov, V. G.; Byzova, N. A.; Biketov, S. F.; Zherdev, A. V.; Dzantiev, B. B. Comparative Study of In Situ Techniques to Enlarge Gold Nanoparticles for Highly Sensitive Lateral Flow Immunoassay of SARS-CoV-2. *Biosensors* **2021**, *11* (7), 229. https://doi.org/10.3390/bios11070229.

(10) Di Nardo, F.; Anfossi, L.; Giovannoli, C.; Passini, C.; Goftman, V. V.; Goryacheva, I. Y.; Baggiani, C. A Fluorescent Immunochromatographic Strip Test Using Quantum Dots for Fumonisins Detection. *Talanta* **2016**, *150*, 463–468. https://doi.org/10.1016/j.talanta.2015.12.072.

(11) Li, K.; Li, X.; Fan, Y.; Yang, C.; Lv, X. Simultaneous Detection of Gastric Cancer Screening Biomarkers Plasma Pepsinogen I/II Using Fluorescent Immunochromatographic Strip Coupled with a Miniature Analytical Device. *Sensors and Actuators B: Chemical* **2019**, *286*, 272–281. https://doi.org/10.1016/j.snb.2019.01.149.

(12) Qie, Z.; Yan, W.; Gao, Z.; Meng, W.; Xiao, R.; Wang, S. Ovalbumin Antibody-Based Fluorometric Immunochromatographic Lateral Flow Assay Using CdSe/ZnS Quantum Dot Beads as Label for Determination of T-2 Toxin. *Microchim Acta* **2019**, *186* (12), 816. https://doi.org/10.1007/s00604-019-3964-x.

(13) Sciutto, G.; Zangheri, M.; Anfossi, L.; Guardigli, M.; Prati, S.; Mirasoli, M.; Di Nardo, F.; Baggiani, C.; Mazzeo, R.; Roda, A. Miniaturized Biosensors to Preserve and Monitor Cultural Heritage: From Medical to Conservation Diagnosis. *Angewandte Chemie International Edition* **2018**, *57* (25), 7385–7389. https://doi.org/10.1002/anie.201713298.

(14) Hong, D.; Jo, E.-J.; Kim, K.; Song, M.-B.; Kim, M.-G. Ru(Bpy)32+-Loaded Mesoporous Silica Nanoparticles as Electrochemiluminescent Probes of a Lateral Flow Immunosensor for Highly Sensitive and Quantitative Detection of Troponin I. *Small* **2020**, *16* (44), 2004535. https://doi.org/10.1002/smll.202004535.

(15) Zangheri, M.; Di Nardo, F.; Calabria, D.; Marchegiani, E.; Anfossi, L.; Guardigli, M.; Mirasoli, M.; Baggiani, C.; Roda, A. Smartphone Biosensor for Point-of-Need Chemiluminescence Detection of Ochratoxin A in Wine and Coffee. *Analytica Chimica Acta* **2021**, *1163*, 338515. https://doi.org/10.1016/j.aca.2021.338515.

(16) Hwang, J.; Lee, S.; Choo, J. Application of a SERS-Based Lateral Flow Immunoassay Strip for the Rapid and Sensitive Detection of Staphylococcal Enterotoxin B. *Nanoscale* **2016**, *8* (22), 11418–11425. https://doi.org/10.1039/C5NR07243C.

(17) Blanco-Covián, L.; Montes-García, V.; Girard, A.; Fernández-Abedul, M. T.; Pérez-Juste, J.; Pastoriza-Santos, I.; Faulds, K.; Graham, D.; Blanco-López, M. C. Au@Ag SERRS Tags Coupled to a Lateral Flow Immunoassay for the Sensitive Detection of Pneumolysin. *Nanoscale* **2017**, *9* (5), 2051–2058. https://doi.org/10.1039/C6NR08432J.

(18) Serebrennikova, K. V.; Byzova, N. A.; Zherdev, A. V.; Khlebtsov, N. G.; Khlebtsov, B. N.; Biketov, S. F.; Dzantiev, B. B. Lateral Flow Immunoassay of SARS-CoV-2 Antigen with SERS-Based Registration: Development and Comparison with Traditional Immunoassays. *Biosensors* **2021**, *11* (12), 510. https://doi.org/10.3390/bios11120510.

(19) Qin, Z.; Chan, W. C. W.; Boulware, D. R.; Akkin, T.; Butler, E. K.; Bischof, J. C. Significantly Improved Analytical Sensitivity of Lateral Flow Immunoassays by Using Thermal Contrast. *Angewandte Chemie International Edition* **2012**, *51* (18), 4358–4361. https://doi.org/10.1002/anie.201200997.

(20) Wang, Y.; Qin, Z.; Boulware, D. R.; Pritt, B. S.; Sloan, L. M.; González, I. J.; Bell, D.; Rees-Channer, R. R.; Chiodini, P.; Chan, W. C. W.; Bischof, J. C. Thermal Contrast Amplification Reader Yielding 8-Fold Analytical Improvement for Disease Detection with Lateral Flow Assays. *Anal. Chem.* **2016**, *88* (23), 11774–11782. https://doi.org/10.1021/acs.analchem.6b03406.

(21) Di Nardo, F.; Chiarello, M.; Cavalera, S.; Baggiani, C.; Anfossi, L. Ten Years of Lateral Flow Immunoassay Technique Applications: Trends, Challenges and Future Perspectives. *Sensors* **2021**, *21* (15), 5185. https://doi.org/10.3390/s21155185.

(22) Tran, V.; Walkenfort, B.; König, M.; Salehi, M.; Schlücker, S. Rapid, Quantitative, and Ultrasensitive Point-of-Care Testing: A Portable SERS Reader for Lateral Flow Assays in Clinical Chemistry. *Angewandte Chemie International Edition* **2019**, *58* (2), 442–446. https://doi.org/10.1002/anie.201810917.

(23) Zhu, B.; Li, X.; Zhou, L.; Su, B. An Overview of Wearable and Implantable Electrochemical Glucose Sensors. *Electroanalysis* **2022**, *34* (2), 237–245. https://doi.org/10.1002/elan.202100273.

(24) Faheem, A.; Cinti, S. Non-Invasive Electrochemistry-Driven Metals Tracing in Human Biofluids. *Biosensors and Bioelectronics* **2022**, *200*, 113904. https://doi.org/10.1016/j.bios.2021.113904.

(25) Raucci, A.; Miglione, A.; Spinelli, M.; Amoresano, A.; Cinti, S. A Hybrid Screen-Printed Strip for Enhanced Electroanalysis towards Lead and Cadmium in Multi-Matrices. *Journal of The Electrochemical Society* **2022**, *169* (3), 037516. https://doi.org/10.1149/1945-7111/ac5c98.

(26) Miglione, A.; Lorenzo, R. D.; Grumetto, L.; Spinelli, M.; Amoresano, A.; Laneri, S.; Cinti, S. An Integrated Electrochemical Platform Empowered by Paper for Fast Nickel Detection in Cosmetics. *Electrochimica Acta* **2022**, *434*, 141332. https://doi.org/10.1016/j.electacta.2022.141332.

(27) Singh, S.; Wang, J.; Cinti, S. Review—An Overview on Recent Progress in Screen-Printed Electroanalytical (Bio)Sensors. *ECS Sens. Plus* **2022**, *1* (2), 023401. https://doi.org/10.1149/2754-2726/ac70e2.

(28) Lo Presti, D.; Di Tocco, J.; Massaroni, C.; Cimini, S.; De Gara, L.; Singh, S.; Raucci, A.; Manganiello, G.; Woo, S. L.; Schena, E.; Cinti, S. Current Understanding, Challenges and Perspective on Portable Systems Applied to Plant Monitoring and Precision Agriculture. *Biosensors and Bioelectronics* **2023**, *222*, 115005. https://doi.org/10.1016/j.bios.2022.115005.

(29) Perju, A.; Wongkaew, N. Integrating High-Performing Electrochemical Transducers in Lateral Flow Assay. *Anal Bioanal Chem* **2021**, *413* (22), 5535–5549. https://doi.org/10.1007/s00216-021-03301-y.

(30) Cheng, J.; Yang, G.; Guo, J.; Liu, S.; Guo, J. Integrated Electrochemical Lateral Flow Immunoassays (ELFIAs): Recent Advances. *Analyst* **2022**, *147* (4), 554–570. https://doi.org/10.1039/D1AN01478A.

(31) Inoue, K.; Ferrante, P.; Hirano, Y.; Yasukawa, T.; Shiku, H.; Matsue, T. A Competitive Immunochromatographic Assay for Testosterone Based on Electrochemical Detection. *Talanta* **2007**, *73* (5), 886–892. https://doi.org/10.1016/j.talanta.2007.05.008.

(32) Wang, L.; Lu, D.; Wang, J.; Du, D.; Zou, Z.; Wang, H.; Smith, J. N.; Timchalk, C.; Liu, F.; Lin, Y. A Novel Immunochromatographic Electrochemical Biosensor for Highly Sensitive and Selective Detection of Trichloropyridinol, a Biomarker of Exposure to

Chlorpyrifos. *Biosensors and Bioelectronics* **2011**, *26* (6), 2835–2840. https://doi.org/10.1016/j.bios.2010.11.008.

(33) Zou, Z.-X.; Wang, J.; Wang, H.; Li, Y.-Q.; Lin, Y. An Integrated Electrochemical Device Based on Immunochromatographic Test Strip and Enzyme Labels for Sensitive Detection of Disease-Related Biomarkers. *Talanta* **2012**, *94*, 58–64. https://doi.org/10.1016/j.talanta.2012.02.046.

(34) Ge, X.; Zhang, W.; Lin, Y.; Du, D. Magnetic Fe3O4@TiO2 Nanoparticles-Based Test Strip Immunosensing Device for Rapid Detection of Phosphorylated Butyrylcholinesterase. *Biosensors and Bioelectronics* **2013**, *50*, 486–491. https://doi.org/10.1016/j.bios.2013.07.017.

(35) Wang, Y.; Wang, L.; Wang, S.; Yang, M.; Cai, J.; Liu, F. 'Green' Immunochromatographic Electrochemical Biosensor for Mercury(II). *Microchim Acta* **2016**, *183* (9), 2509–2516. https://doi.org/10.1007/s00604-016-1866-8.

(36) Nian, H.; Wang, J.; Wu, H.; Lo, J.-G.; Chiu, K.-H.; Pounds, J. G.; Lin, Y. Electrochemical Immunoassay of Cotinine in Serum Based on Nanoparticle Probe and Immunochromatographic Strip. *Analytica Chimica Acta* **2012**, *713*, 50–55. https://doi.org/10.1016/j.aca.2011.11.028.

(37) Escosura-Muñiz, A. de la; Parolo, C.; Maran, F.; Mekoçi, A. Size-Dependent Direct Electrochemical Detection of Gold Nanoparticles: Application in Magnetoimmunoassays. *Nanoscale* **2011**, *3* (8), 3350–3356. https://doi.org/10.1039/C1NR10377F.

(38) Castañeda, M. T.; Alegret, S.; Merkoçi, A. Electrochemical Sensing of DNA Using Gold Nanoparticles. *Electroanalysis* **2007**, *19* (7–8), 743–753. https://doi.org/10.1002/elan.200603784.

(39) Khlebtsov, B. N.; Tumskiy, R. S.; Burov, A. M.; Pylaev, T. E.; Khlebtsov, N. G. Quantifying the Numbers of Gold Nanoparticles in the Test Zone of Lateral Flow Immunoassay Strips. *ACS Appl. Nano Mater.* **2019**, *2* (8), 5020–5028. https://doi.org/10.1021/acsanm.9b00956.

(40) Occhipinti, S.; Mengozzi, G.; Oderda, M.; Zitella, A.; Molinaro, L.; Novelli, F.; Giovarelli, M.; Gontero, P. Low Levels of Urinary PSA Better Identify Prostate Cancer Patients. *Cancers* **2021**, *13* (14), 3570. https://doi.org/10.3390/cancers13143570.

(41) Di Nardo, F.; Occhipinti, S.; Gontero, P.; Cavalera, S.; Chiarello, M.; Baggiani, C.; Anfossi, L. Detection of Urinary Prostate Specific Antigen by a Lateral Flow Biosensor Predicting Repeat Prostate Biopsy Outcome. *Sensors and Actuators B: Chemical* **2020**, *325*, 128812. https://doi.org/10.1016/j.snb.2020.128812.

(42) Cavalera, S.; Di Nardo, F.; Forte, L.; Marinoni, F.; Chiarello, M.; Baggiani, C.; Anfossi, L. Switching from Multiplex to Multimodal Colorimetric Lateral Flow Immunosensor. *Sensors* **2020**, *20* (22), 6609. https://doi.org/10.3390/s20226609.

(43) Guo, Y.-R.; Liu, S.-Y.; Gui, W.-J.; Zhu, G.-N. Gold Immunochromatographic Assay for Simultaneous Detection of Carbofuran and Triazophos in Water Samples. *Analytical Biochemistry* **2009**, *389* (1), 32–39. https://doi.org/10.1016/j.ab.2009.03.020.

(44) Cavalera, S.; Russo, A.; Foglia, E. A.; Grazioli, S.; Colitti, B.; Rosati, S.; Nogarol, C.; Di Nardo, F.; Serra, T.; Chiarello, M.; Baggiani, C.; Pezzoni, G.; Brocchi, E.; Anfossi, L. Design of Multiplexing Lateral Flow Immunoassay for Detection and Typing of Foot-and-Mouth Disease Virus Using Pan-Reactive and Serotype-Specific Monoclonal Antibodies: Evidence of a New Hook Effect. *Talanta* **2022**, *240*, 123155. https://doi.org/10.1016/j.talanta.2021.123155.

(45) Raucci, A.; Miglione, A.; Lenzi, L.; Fabbri, P.; Di Tocco, J.; Massaroni, C.; Presti, D. L.; Schena, E.; Pifferi, V.; Falciola, L.; Aidli, W.; Di Natale, C.; Netti, P. A.; Woo, S. L.; Morselli, D.; Cinti, S. Characterization and Application of Porous PHBV-Based Bacterial Polymers to Realize Novel Bio-Based Electroanalytical (Bio)Sensors. *Sensors and Actuators B: Chemical* **2023**, *379*, 133178. https://doi.org/10.1016/j.snb.2022.133178.