Microfluidic paper-based analytical devices (µPADs) can be considered as the latest, and maybe, the most fast-growing technology for low-cost detection of analytes and pathogens. In this report, we present three examples of applications presenting variety of designs (lateral flow and microzone plates), chemistry of detection (nanoparticles and dye chemistry), type of analytes (organic compounds, small inorganic anion, and bacteria) and type of assays (colorimetric, enzymatic, and immunoassays).

KEYWORDS: microPADs, AuNPs, nitrite, P-ELISA.

INTRODUCTION

Microfluidic paper-based analytical devices (µPADs) are a promising technology to develop simple, low-cost, portable, and disposable diagnostic platforms for resource-limited settings. This technology is based in colorimetric bioassays for direct detection of contaminants, metabolites, drugs, diagnostics of disease, etc. Then, the analysis of image is evaluated directly from scanners, or even from cell-phone cameras for telemedicine applications.

We report examples of the fabrication of µPADs in cellulose membrane of Whatman #1 chromatography paper by wax printing [1], in which we apply for i) detection of adulteration of protein content with melamine and ii) analysis of nitrite in saliva for periodontitis, using a lateral flow µPAD, and iii) immunoenzymatic assays for *Toxoplasma gondii* detection in pregnant women, using a 96-microzone paper plate.

EXPERIMENTAL

**Patterning Paper by Wax Printing:** We designed patterns of hydrophobic barriers as black lines on a white background using CorelDraw software. The patterns were printed on Whatman #1 chromatography paper using the solid ink printer. The printed paper was placed on a hot plate set at 150 °C for 120 s, and the wax melted and spread through the thickness of the paper.

**Melamine Assay:** Colorimetric detection of melamine is achieved when gold nanoparticles is functionalized with thiol cianuric acid derivative (MTT) for specific interaction with melamine and to produce a visible color change on the µPADs. The hydrogen-bonding recognition between melamine and MTT results in the aggregation of gold nanoparticles (AuNPs), and the wine red color of the gold colloid is accordingly changed to a blue color. Due to its strong surface plasmon resonance (SPR) at 519 nm visual detection is possible. In each microPAD detection zone was spotted 0.5 µL of AuNPs-MTT; we used 10 µL of melamine standard (1 – 250 ppm) for each µPAD device.

**Nitrite Assay:** Nitrite levels were measured in artificial salive using a mixture of 50 mM sulfanilamide, 330 mM citric acid and 10 mM n-(napthyl)ethylenediamine in methanol. This mixture reagent reacts with nitrite and produces a purple azo dye end product. The sample consisted of a known amount of sodium nitrite (5 – 75 µM) dissolved in artificial saliva [2]. In each detection zone of the µPAD, we spotted 0.5 µL of the mixture reagent, while for the experiment we used 6 µL of sample for each device. Scanned images were analyzed in CMYK mode using the magenta channel in Adobe Photoshop® for quantitation.

**P-ELISA Assay:** Microzones were made on paper using a wax printer that prints a pattern that mimics a microplate ELISA, with 96 wells, and then the thermal process reflows the wax across the thickness of the paper [3]. The microzones were coated with a diluted protein extract from the sediment of *Toxoplasma gondii*, after sonication. The proteins from the extract adsorbed on the paper for 20 min. After washing with PBS with 0.075% Tween-20 (PBS-T), we used 10% skim milk powders for blocking during 20 min. The serum samples, diluted 1:25 in PBS-T, were incubated for 20 min, followed by four washing steps with PBS-T. After the washing, anti-human IgG conjugated to peroxidase at a dilution of 1:200 in PBS-T plus 1% milk powder is added for development of the assay. After 20 seconds the microzones were washed with PBS-T and TMB substrate added, and after 5 min the reaction was stopped with 0.1 M HCl. The paper plate was scanned and analyzed. The assay in paper zones was confronted with the regular colorimetric ELISA assay, and to a commercial chemiluminescence assay.
RESULTS AND DISCUSSION

We demonstrated the versatility and the performance of the optimized sensors for colorimetric detection in µPAD for melamine, nitrates, and bacteria.

Different amounts of melamine (1 – 250 ppm) were added to an aqueous solution of AuNPs-MTT in eppendorf vials. The same experiment was developed on the µPAD device, showing the change color from red to purple in less than 1 min. These levels of melamine detection are within the safety limit (2.5 ppm in the USA and EU; 1 ppm for infant formula in China). See Figure 1 and 2 for the comparative test between solution vs. paper-based assay and a lateral flow version of the assay, respectively.

![Figure 1: Comparison with AuNPs functionalized with MTT for melamine analyses in aqueous solution (A) and µPADs devices (B). Melamine concentrations (1) 1 ppm; (2) 10 ppm; (3) 50 ppm; (4) 100 ppm and (6) 250 ppm.](image)

![Figure 2: Lateral flow µPADs for AuNPs functionalized with MTT for detection of melamine (50 ppm)](image)

Nitrite assays were developed in a µPAD device, which was adapted to a different layout in order to obtain samples saliva directly from the dental crevices. The salivary nitrite levels are associated to periodontitis disease, and moderate and advanced nitrite levels between 6 and 16 µM for clinical parameter diagnostic.

![Figure 3: µPADs for nitrite salivary assays. Testes for artificial saliva spiked with different concentrations of nitrite. A) µPADs devices with nitrite levels of 5 µM and 15 µM in artificial saliva. B) Analytical curve for nitrite in the range of 5 – 75 µM.](image)

The validation and comparison of the immunoenzymatic assay was carried out in three different platforms. The results for P-ELISA were compared to conventional ELISA assay with spectrophotometric detection in plastic microtiter plates, and also using chemiluminescence detection, provided by a commercial kit in a clinical laboratory setting. Seven samples and one
negative control (serum with zero count in the chemiluminescence assay) were subjected to the ELISA and P-ELISA assays. Figure 4A shows the readings in the three systems and the direct correlation of P-ELISA (with detection using a desktop scanner) and ELISA (with detection using a commercial plate reader) are evident in Figure 4B.

**Figure 4.** Comparative studies of immunoenzymatic assays using serum samples from pregnant women tested positive for *T. gondii*. A) Direct read out from the three methods for control (negative serum) and seven samples. B) Correlation of results between ELISA (O.D.) and P-ELISA (CMYK read out).

**CONCLUSIONS**

In all three examples of new applications for µPADs, we successfully achieved the determination of the analytes tested, within the desirable analytical range, with simplicity of the assay and with low cost. In all cases, the devices are being used in real applications. Fabrication of µPADs using the wax printer provides the most flexible and inexpensive process.

**REFERENCES**