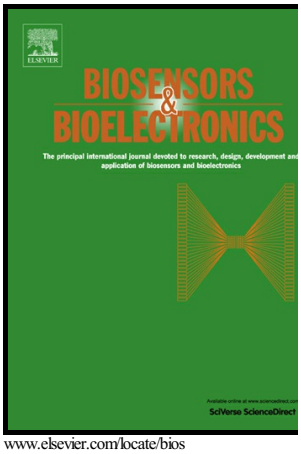

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A 3D µPAD BASED ON A MULTI-ENZYME ORGANIC–INORGANIC HYBRID NANOFLOWER REACTOR

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Abstract. This work reports on the development of a 3D microfluidic paper-based device (3D µPAD) for glucose detection using organic-inorganic hybrid nanoflower technology to immobilize the bi-enzymatic system (glucose oxidase and horseradish peroxidase). The system is based on nanoflowerssupported on cellulose paper (the microreactor zone) coupled to 3,3′,5,5′-tetramethylbenzidine (TMB) as the colorimetric probe in the detection zone. We used a digital camera for the quantitative analysis of glucose with the S coordinate of the HSV colour space as the analytical parameter. Under optimal operational conditions, linearity was observed for glucose concentrations up to 300 µM, with a detection limit of 15.6 µM. The biosensor is reusable and remains stable for 75 days in conventional storage conditions.

Keywords: GOx-HRP nanoflowers; Enzymaticmicroreactor; µPADs; Colorimetric detection; Glucose monitoring.

1. Introduction

Microfluidic paper-based devices (μPADs), based on the use of cellulose paper to generate flow by capillary action, have currently emerged as a very powerful platform in biosensing applications (Jeong et al., 2013), since they are extremely low-cost, disposable, rapid, technically simple, have high throughput and are low sample consuming (Cate et al., 2014; Hu et al., 2014). In particular, μPADs have been recognized as exceptional candidates for easy/ready-to-use analytical platforms for point-of-care (POC) diagnostic devices in health care and other sectors, so-called point-of-need (PON) testing (Petryayeva and Algar, 2015).

Of special interest are the μPADsmade up of various layers to form three-dimensional (3D) devices in which the fluid can run horizontally and vertically, opening the door to compact devices with increased functionality (Martinez et al., 2008). This approach increases the number of basic unit operations from its characteristic fluid operation, i.e. passive fluid transport via capillary forces, to many others combined and integrated by means of different production technologies such as paper stacking with double-sided adhesive pasting, paper stacking with spray adhesive gluing, paper folding and clamping, paper wax-printing and bookbinding, and paper stacking with toner lamination(Yetisen et al., 2013).

The different basic unit operations implemented in the 3D-μPAD platform include: fluid distribution and de-multiplexing (Martinez et al., 2010), fluid valving(Martinez et al., 2010), fluid wicking control (Chen et al., 2012), fluid metering (Noh and Phillips, 2010b), fluidic timer inclusion (Noh and Phillips, 2010a), fluidic battery inclusion (Wang et al., 2014b), preloading of reagents (Liu et al., 2011), washing (Wang et al., 2012), recognition reactions (Martinez et al., 2010; Rattanarat et al., 2014; Wang et al., 2014a), pH conditioning and masking reactions (Wang et al., 2014a), and redox state adjustment (Jayawardane et al., 2014).

One common operation is analyte recognition using enzymatic schemes. Enzymatic configurations range from simple one-enzyme to multi-enzyme devices, which expand, in the latter case, the range of potential applications for the detection of biological substrates, although this makes it difficult to manufacture and design these devices. The different strategies to create multi-enzyme assemblies that facilitate the substrate flow between cascade enzymes include the emerging concept of enzyme-embedded nanomaterials, in which enzymes are immobilized in nanostructured materials (Ariga et al., 2010). Recently, a method for preparing enzyme-inorganic nanocrystal complexes

with flower-like shapes was reported. The copper complexes of protein in the phosphate buffer serve as nucleation sites for primary crystals of copper phosphate and subsequent protein-copper phosphate hybrid nanoflower (NFs) crystallization in diffusion controlled conditions (Ge et al., 2012; Zeng and Xia, 2012). Different hybrid materials have been described, mainly containing $Cu_3(PO_4)_2 \cdot 3H_2O$ with enzymes such as horseradish peroxidase (Somturk et al., 2015), soybeanperoxidase(Yu et al., 2015), laccase(Ge et al., 2012; Zhu et al., 2013), trypsin(Lin et al., 2014); α-lactalbumin, carbonic anhydrase and lipase (Ge et al., 2012); $CaHPO_4$ with α-amylase (Wang et al., 2013) and $Ca_3(PO_4)_2$ with α-chymotrysin(Yin et al., 2015). These nanobiocatalist materials have a large surface-to-volume ratio when compared with that of bulk materials, as well as enhanced catalytic activity, stability and durability compared with the free enzyme (Zeng and Xia, 2012).

Some co-embedded flower-like nanomaterials have been described to catalyse reactions sequentially and provide accessibility to the target analyte and subsequent substrates. One example is glucose oxidase and horseradish peroxidase containing nanoflowers used to determine colorimetric glucose in solution (Sun et al., 2014). This multi-enzymatic nanomaterial can be prepared by one-pot synthesis resulting in randomly distributed multi-enzyme complexes (Sun et al., 2014) or sequentially resulting in a spatially confined distribution of enzymes (Li et al., 2014).

This paper presents a reusable enzymatic microreactor containing the hybrid GOx-HRP-$Cu_3(PO_4)_2 \cdot 3H_2O$ NFs synthesized in the presence of cellulose paper for the colorimetric determination of glucose.

2. Experimental

Sections related to reagents and materials, apparatus and instrumentation,image capture and processing, as well as glucose assay are included in Supporting Information.

2.1. Bulk synthesis of GOx-HRP -$Cu_3(PO_4)_2 \cdot 3H_2O$ nanoflowers

To prepare the crystalline hybridGOx-HRP NFs, a procedure similar to Sun et al. (Sun et al., 2014) was followed. In an optimized synthesis, 0.3 ml $CuSO_4$ 200 mM aqueous solution was added to 10 ml of 0.01 M phosphate buffered saline (PBS; pH 7.4) containing 0.25 mg/mL of both GOx (207 U/mg) and HRP (156 U/mg) followed by incubation at 37º C for 72 hours, after which NFs were obtained as a precipitate. After that, the reaction solution was centrifuged (3,000 rpm for 10 min) and the precipitate

rinsed twice with PBS buffer and stored reconstituted in PBS in a refrigerator at 4º C and in the dark.

For the synthesis of NFsin the presence of cellulose paper, a circle of 5 cm of filter paper was placed in a 5.3 cm Petri dish and a solution containing 0.25 mg/mL of HRP and GOx enzymes, 0.3 mL $CuSO_4$ 200 mM in 10 mL of PBS (0.01M, pH 7.4) were added. The Petri dish was incubated at 37ºC for 72 hours and the paper was rinsed twice with PBS and dried at room temperature. Finally, the paper containing the NFs was stored until use in a closed container in a refrigerator at 4ºC and in the dark.

2.2. Production of 3DµPAD

To make this portable 3DµPAD cost-efficient, simple and reproducible, we chose to produce the device usinga craft-cutting process. The pattern of the 3DµPAD was first designed using Illustrator software (Adobe Systems) and the design was exported as an FS file to the controller software of an X-Y knife plotter (Secabo GmbH, Wolnzach, Germany). Forthe production, a piece of paper was affixed using double-sided adhesive tape to a plastic sheet. To optimize the procedure and the substrate used, the 3D-µPAD elements were produced in 4x32 replicas by cutting the acetate-backed cellulose with an 87% rate of success, confirming the viability of the design.

The device consists of two layersprepared individually, as shown in Figure 1. The first and disposable layer is made up of two separates zones, one for sampling and the second for detection, where the chromogenic reagent is immobilized. Both parts were taped to a rigid plastic support using double-sided adhesive tape. The reusable second layer consists of a 5 mm-diameter disc of NF paper obtained with a holepunch and taped to a plastic sheet of the same size as the first layer with a hole that matches the sampling area.

To prepare the detection zone device, 1 µL of a solution prepared with 20 µL of 20 mM TMB, 20 µL of 0.5 mg/mL chitosan and 20 µL of pH 4.0acetate buffer was dispensed onto the detection zone, dried at room temperature for 5 min and stored in a dry environment at 4ºC in the dark.

To prepare the device for glucose analysis, two points of adhesion with double-sided adhesive tape were placed at both ends of the first disposable layer to obtain the best contact between the two layers once they overlapped and were correctly aligned, which allows optimal speed and pressure between them.

3. Results and Discussion

The development of hybrid materials by combining or reinforcing the properties of the components involvedis an interesting area. One of these hybrids is flower-like nanoparticles, both inorganic and organic (Ge et al., 2012). In the case of enzyme-embedded hybrid materials, the catalytic activity of the immobilized enzyme is enhanced as well as the stability and durability compared to the free enzyme (Ge et al., 2012). The co-immobilization of two enzymes catalysing multi-step reactions as organic–inorganic hybrid NFs(Sun et al., 2014) opens the door to the subsequent enhancement of the overall efficiency and specificity of the reaction (Schoffelen and van Hest, 2012).

The formation of multi-layered flower-like structures containing both GOx and HRP in copper phosphate crystals in paper can assist in the preparation of reusable 3DμPADs for the determination of glucose.

3.1. Immobilization of multi-enzyme co-embedded organic–inorganic hybrid nanoflowers

Two strategies were used to prepare the enzymatic microreactor with multi-enzyme NFs, first by depositing the previously synthesized NFs in paper from a suspension and second by growing the NFs directly in a cellulosic paper.

The enzyme–inorganic hybrid NFs were synthesized in one pot by the procedure designed by Sun et al. (Sun et al., 2014) with some modifications (higher PBS volume, 10 ml, and higher temperature, 37ºC). The prepared blue GOx-HRP-$Cu_3(PO_4)_2 \cdot 3H_2O$ precipitate has a spherical shape with a variable diameter ranging from 3 to 15 μm (Figure S1). The morphology of the NFs depends on the concentration of both enzymes, especially GOx, with the NFs being less compact and uniform as their concentration decreases, in good agreement withthe published results (Sun et al., 2014). Figure S1shows SEM images of NFs with a similar peony-like flower morphology and a high number of nanoplates. The EDX analysis of NFs shows the presence of atoms from $Cu_3(PO_4)_2$ and enzymes in the hybrid material (Figure S2). Interestingly, chloride atoms appear in the structure supporting Sun et al.'ssuggestion(Sun et al., 2014) about the essential role of chloride for the formation of NFs. Furthermore, the X-ray diffraction pattern of the NFs matched that of $Cu_3(PO_4)_2 \cdot 3H_2O$ (JCPDS card 00-022-0548) (Figure S3). The IR spectra corroborate the presence of phosphates by the asymmetric

stretching of the P–O bond at 1049 cm^{-1} and bending O–P–O vibrations at 560 cm^{-1}(Jastrz-Öbski et al., 2011) and proteins by the amide I band at 1637 cm^{-1}, amide II at 1560 cm^{-1}, amide III band at 1300 cm^{-1} as well as the OH and NH stretching that appears as a broad band centred at 3445 cm^{-1}(Barth, 2007) (Figure S4).

To prepare the reactor, 3 µL of a suspension of synthesized NFs of some 100 µg/mL were deposited on a 3 mm-diameter circle of filter paper and washed twice with PBS. The second procedure grows the NFs directly in the paper according to the described procedure and uses a 3 mm circle obtained with a holepunch in the 3D-µPAD.

Using SEM data, the microreactor prepared with the first method hasa lower density of NFs (1500 particles/mm^2on average) than with the second (2580 particles/mm^2on average) although the average size of the NFs is similar; in the first case 8.1±1.4 µm and 6.1±0.6 µm for the NFs synthesized directly in the presence of the paper. In the latter case, the size is more uniform and their distribution through the cellulosic membrane is more homogeneous (Figure S5). The difference between the average size of the NFs synthesized in solution and the size of same NFs in paper can be ascribed to the retention value of paper (10-13 µm), which cannot retain larger particles.

3.2. Study of the reaction of glucose in the 3D-µPAD

The purpose of this study is to prepare a µPAD device that can be reused, thus decreasing the cost whenever the user can easily reuse it. Of the systems proposed to prepare paper-based microfluidic devices, physical blocking of pores and two-dimensional shaping / cutting (Yetisen et al., 2013), we selected the latter technique because of its simplicity for this specific design. As a substrate to fabricate the µPAD, we selected a Whatman grade 1 type filter paper according to (Evans et al., 2014).

The chromogen used for the detection was TMB, although this has the drawback of inhomogeneous colourdistribution in the detection area (Evans et al., 2014). This common problem in paper-based devices (Yetisen et al., 2013) is overcome by using chitosan, which stronglyattaches to the cellulose paper by electrostatic adsorptionand hydrogen bonding(Orelma et al., 2011), slowing down the movement of the oxidized TMB and increasing the homogeneity of the ROI (Liu et al., 2014).

To reuse the device, the sampling area was separated from the detection area, so that the sample rises to flow through the cellulosic microcapillaries containing the NFs as the reaction space acting as a microreactor(Asanomi et al., 2011) and then flows up to the

detection area. The areal size, spatial separation between the areas, and linear layout of the 3D-µPAD are designed to accomplish the following: 1) to minimize colour reading errors (3 mm-diameter detection area; 6000 pixels); 2) to obtain a suitable collection of the sample (triangular reception area for 10 µl sample); 3) to minimize the impact of the separation between glucose recognition (microreactor) and TMB oxidation (detection area) (7 mm from the middle of the microreactor to the middle of the detection area; see Figure S6). With these conditions, the wicking time of the device is 16.6±0.6 s. The time required to complete the assay is 15 min and was determined by monitoring the colour (Figure S7) in agreement with literature (Sun et al., 2014).

3.3. Characterization of the glucose assay

The colour of the 3D-µPAD imaged with a digital camera and measured by the S coordinate of the HSV colour space increases with glucose concentration, exhibiting a Michaelis-Menten behaviour that is fitted well by a rectangular hyperbolic equation (eq. 1).

$$S = \frac{S_{max} C_{glucose}}{K + C_{glucose}} \quad \text{(eq. 1)}$$

where S_{max} indicates the maximum signal of saturation and K is a pseudo Michaelis-Menten constant equal to the concentration of glucose at which half of the maximum S value is achieved. A Lineweaver-Burk plot of the results shown in Figure 2 makes it possible to derive the K (193 µM) and S_{max} (36) values. The inset in Figure 2 shows the range between 0-300 µM where it is possible to have a linear relationship (R= 0.9443) between the value of S and the glucose concentration.

The limit of detection obtained using thestandard criteria was 15.6 µM and the limit of quantificationwas 21.3µM. The precision of the 3D-µPAD wastested using different devices for each measurement. Precision was studied at three concentrations, obtaining 5.5% (70µM), 5.2 (100 µM) and 3.4 (300 µM). The comparison with the µPAD procedures in the literature is presented in Table S1. Both calibration curves were quite similar when the same microreactor was used for calibration (see Figure S8).

Following the general procedure, the effect of potential interferences on signal was studied (D(+)galactose, D(+)mannose, D(+)trehalose, D(+)maltose, D(-)fructose, saccharose, $CaCl_2$, KCl and NaCl) showing good selectivity (Figure S9).

3.4. Stability of NF paper and reusability

The stability of the NF microreactor was checked over a period of 90 days with the glucose assay. Different NFmicroreactors were prepared and checked with 200 μM glucose for 3 months (Figure S9). There was a reduction in the response of some 75% 75 days after preparation, which indicates excellent durability.

Reusability is one of the major advantages of nanoflowers immobilized in μPAD. After investigating the stability, suitability and sensitivity of the nanoflowers deposited in the system for the colorimetric detection of glucose, we checked the capability of the biosensor to be reused.Figure 3 shows the reuse for 9 consecutive assays using the same microreactor, showing that it is possible to use it 6 times with no loss of activity. In the seventh cycle, the biosensor response decreased to 70%, and the response continued to fall to 50% in cycle 8 and to 10% in cycle 9. The activity loss was higher than that reported when only GOx and HRP were immobilized in a cellulosic matrix.

3. Conclusions

We have simultaneously synthesized and supported the coupled GOx-HRP-$Cu_3(PO_4)_2 \cdot 3H_2O$ as nanoflowers in cellulose and used it to prepare a 3D μPAD colorimetric biosensor for the accurate determination of glucose. The co-immobilized system maintained its activity compared to free enzymes, making it reusable, and was stable for 75 days under conventional storage conditions. All of these possible applicationsof the biosensor, together with the search for new strategies to improve reusability, long-term stability and low cost (an estimated cost of 20 cents per unit, taking into account reuse), are now under investigation and will be the subject of future work.

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Figure Captions

Figure 1.Picture of the 3D µPAD device showing the two layers. 1: disposable layer showing the two separates zones, sampling area and detection area; 2: Reusable microreactor made with NFs paper; 3) Disposable layer after reaction of the device with glucose.

Figure 2.External calibration for the glucose assay with digital camera. Data points are the mean of 3 trials. The datawerefit with eq. 1 and the inset shows the linear calibration at low glucose concentration.

Figure 3. Reusability of NFs microreactor of 3D µPAD device in the analysis of glucose (blue) compared to free enzymes on paper filter (red). The cycles of use were tested each 24 h.

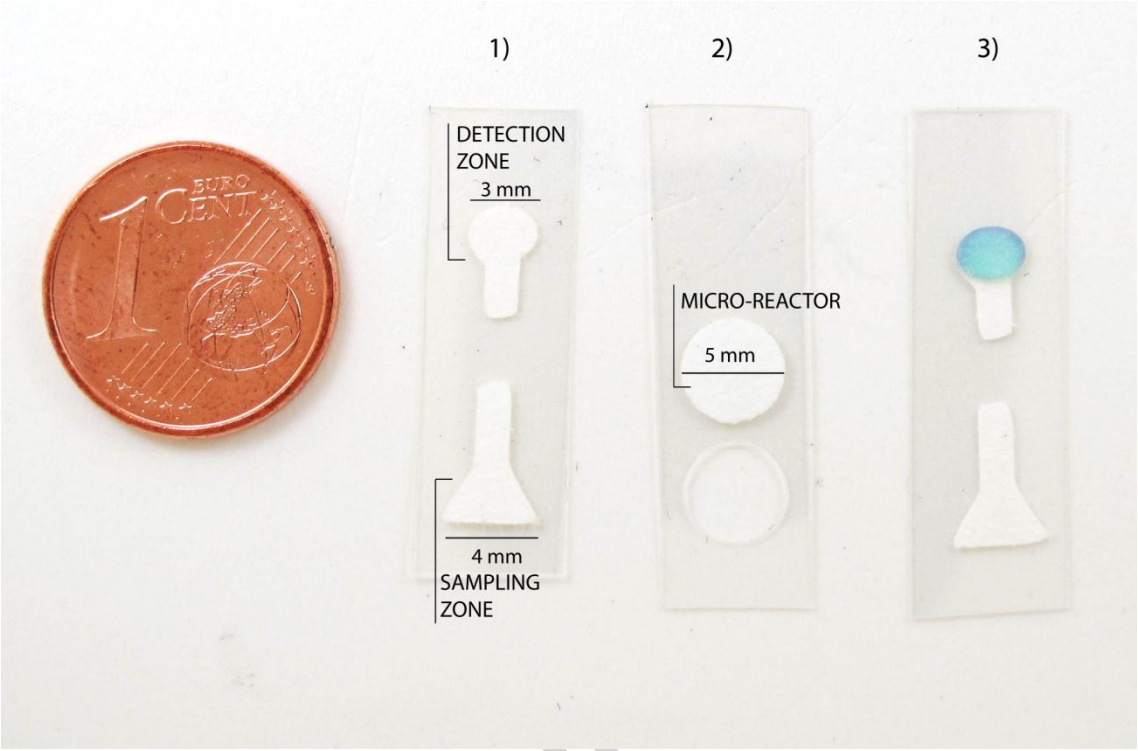

Figure 1

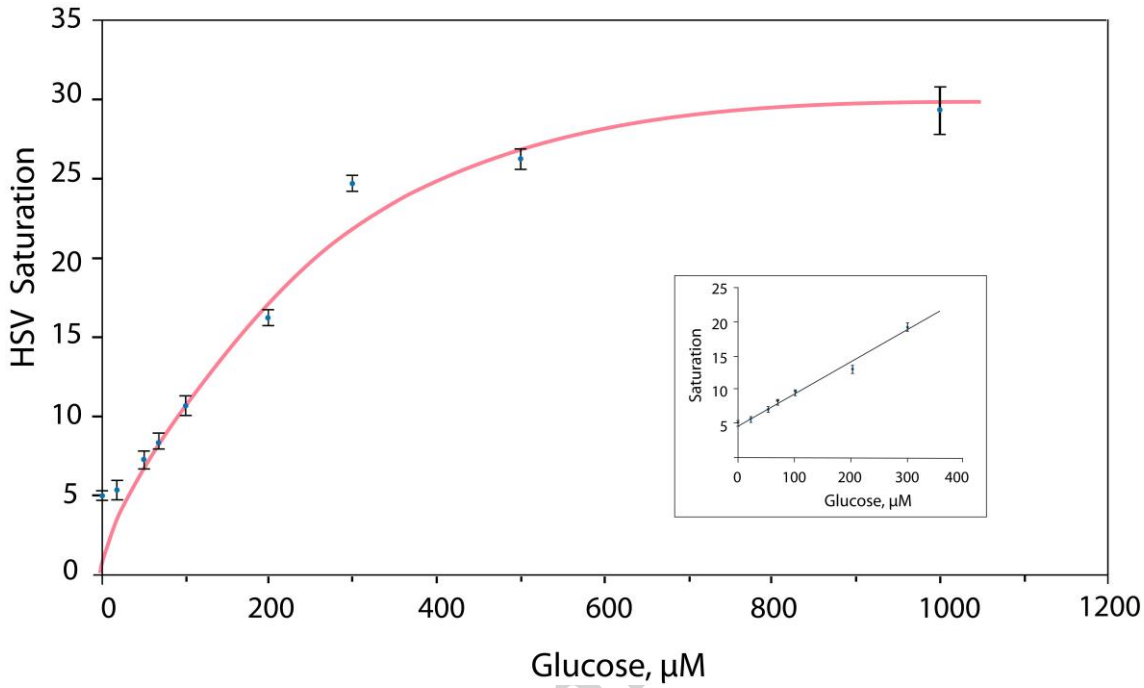

Figure 2

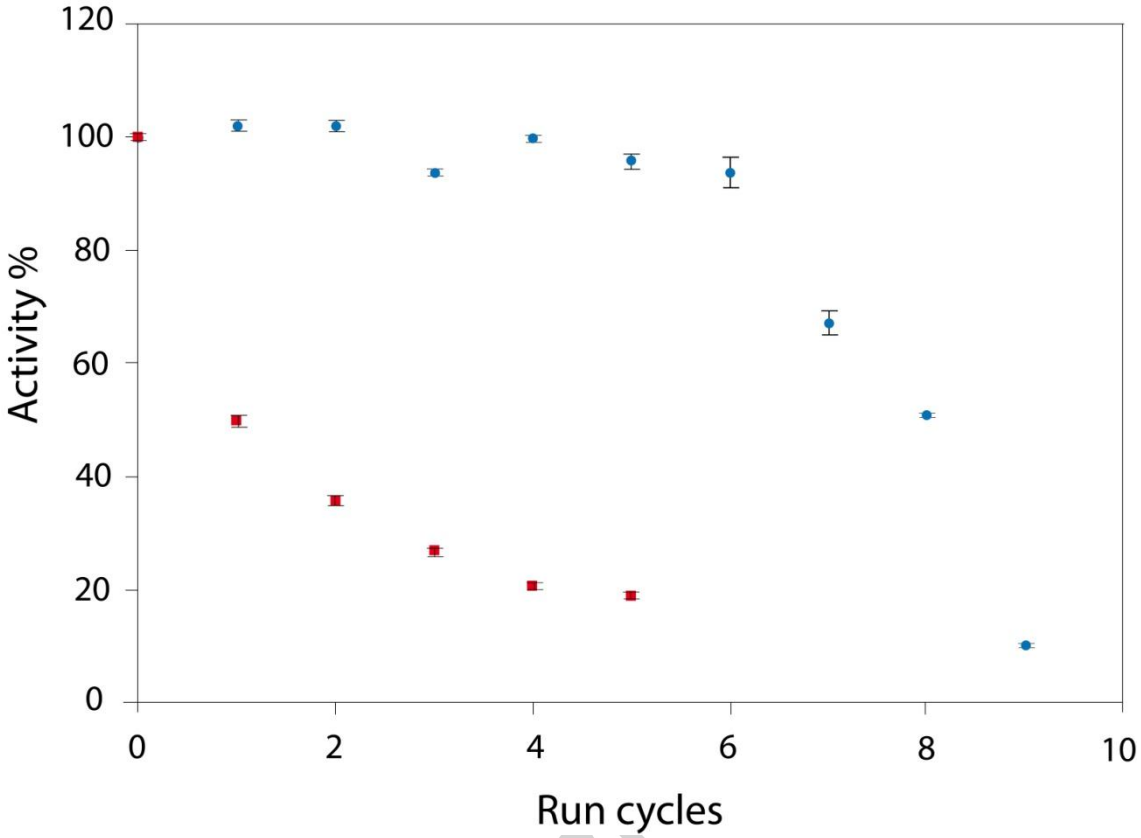

Figure 3

Highlights

- A 3D microfluidic paper-based device for glucose detection is developed.
- A reusable microreactor containing enzymatic nanoflowers is included.
- The biosensor shows quick response, reusability and long-term stability.
- A digital camera for the color-based quantitative analysis of glucose is used.