Screen-printed microsystems for the ultrasensitive electrochemical detection of alkaline phosphatase†

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Screen printing technique has been used to manufacture a microsystem where the graphite-based electrodes hold both a functional and an architectural task. The thick film manufacturing technique has proved valid to develop a very low volume (*ca.* 20 μL) device where different electrochemical operations can be very efficiently performed. Biomolecule immobilisation within the microsystem for biosensors applications has been explored by inducing and optimizing the *in situ* generation of a potential pulse polypyrrole electropolymerised film entrapping either glucose oxidase or glucose dehydrogenase. This biomodified microsystem was applied to the ultrasensitive electrochemical detection of alkaline phosphatase yielding limits of detection below 10^{-12} M for glucose oxidase and of 10^{-15} M for glucose dehydrogenase modified systems, within 15 min of incubation time. The results obtained showed the advantages of using low volume microsystems in combination with an optimised polypyrrole–enzyme film, which displayed a good immobilisation efficiency in conjunction with a good diffusion of species through. Ultrasensitive detection of AP in combination with a stable and reproducible surface modification for entrapping of biomolecules opens the window for new electrochemical detection platform with great potential for integrated biosensor applications.

1. Introduction

Screen printing as a manufacturing technology within the biosensors research area is mainly restrained to the fabrication of electrodes that are then integrated within complex systems that comprise electrode and separated microfluidics parts, the latter are mainly manufactured by the techniques such as PDMS or plastic injection moulding.[1–5] In these complex systems structural (fluidics) and functional (electrodes) are clearly differentiated. The concept presented in this work consists of the utilization of screen printing technology to combine both structural and functional aspects of the microsystem. In other words, using screen printing to fabricate three dimensional structures responsible for architectural and electrochemical tasks simultaneously, this simplifies greatly the manufacturing complexity of very low volume microsystems and provides a low-cost, reliable and versatile platform for biosensor applications. This concept has already been described by our group covering fabrication and functionalisation aspects,[6] as well as exploring a direct application of it.[7]

The utilization of screen printed electrodes in biosensors applications refers mainly to graphite based electrodes. Screen printed carbon electrodes are made from a matrix of graphite powder and an organic binder, are inexpensive, easily prepared, provide rapid responses and disposable. They also have rough and porous structure that generates a large surface area and provides high conductivity and low background current.[8]

Modification of screen printed electrodes surface for electrochemical biosensing often implies immobilisation of biomolecules. Different methods have been developed to facilitate biomolecule immobilisation including covalent binding, physical adsorption, thiol or diazonium derivation, polymer entrapment or surface (electro)polymerization.[9–11] In many electrochemical biosensor applications using screen printed carbon electrodes the biomolecule to be immobilized is an enzyme that acts regenerating the electrochemically active molecule responsible to generate the electrochemical signal on the electrode surface. Physical entrapment of enzymes within polymer films such as polyacetylene, polyaniline, polythiophene or polypyrrole is a common procedure.[12] This provides good access of the substrate to the immobilized biomolecules, but the contents of the biomolecules is relatively low and is normally localized at the polymer-solution interface, where it is adsorbed due to electrostatic interactions between the polycationic matrix of the oxidized polymer and the total negative enzyme charge. Drawbacks of this procedure are binding forces changing with pH or compromised lifetime and stability of the modified electrode due to the loss of enzyme due to washings or during subsequent measurements. Enzyme immobilisation by covalent binding, cross-linking and entrapment in gel or membranes techniques increases stability of enzyme electrodes but it generally provides low reproducibility and poor spatially controlled deposition.[13]

One-step entrapment by monomer electropolymerisation from a matrix containing the biomolecule of interest is considered a more than suitable technique because it provides an easy preparation methodology of the polymeric films containing enzymes in a rapid procedure, site directed control of the

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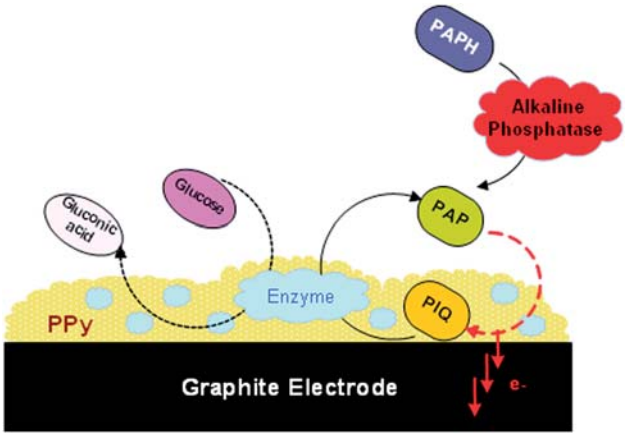
biomolecules on the electrode area and monitoring of the thickness of the polymer layer based on the measurement of the electrical charge passed during the electrochemical polymerization. These features make the biomolecule immobilisation by entrapment within electropolymerised films the most appropriate method when attempting the miniaturization of the sensing devices. An alternative to the one step method has also been developed, by which a preliminary adsorption of the monomer and biomolecule takes place on the electrode surface followed by *in situ* electropolymerisation. This variation has been reported and identified as most appropriate when the solubility of the biomolecules or monomer of interest is compromised in aqueous media, to avoid the need for high concentrations of monomer and biomolecule and also to be able to estimate the amount of biomolecule effectively immobilized on the surface.[14–17]

Most of the electrochemically deposited films for biomolecule immobilisation are to some extent conducting polymers, such as polyacetylene, polythiophene, polyaniline, polyindole and polypyrrole.[18–20] A large number of different biomolecules have been immobilised on electrodes for different biosensor applications,[12] however it appears that mainly polypyrrole and polyaniline based modified electrodes have been the most widely studied ones. In the case of enzymes, oxidases in particular, as the biomolecule to be entrapped on the electrode surface, polypyrrole is considered one of the most promising ones for biosensor applications due to its good conductivity, stability and efficient polymerization at neutral pH, which may not always be possible for other electropolymerisable aromatic monomers. Furthermore, the polymer is a good energy transducer an efficient protector of electrodes against interfering materials (namely the proteins present in real-life samples, such as blood and urine) and provides easy ways to immobilize biomolecules.[16] It is also an inherently biocompatible material since its high water content ensures minimum disturbance of the biologically active compound in the biosensor.[21,22]

Electropolymerisation is carried out normally by potentiostatic or galvanostatic means in order to obtain rather thin or thick films respectively.[23] In the case of biosensor applications, thin films are desirable to allow convenient access to the electrode surface of reacting molecules, hence potentiostatic methods are more commonly used. Within this anodic electropolymerisation chronoamperometry and cyclic voltammetry are common techniques. An alternative of these is the use of potential pulse techniques, which have been reported to introduce enhancements in the nanostructuring of the polypyrrole films during the electropolymerisation.[21,24] Potential pulse techniques increase the concentration of biomolecules entrapped by alternating: i) high anodic potentials that are responsible for the fast initiation of the pyrrole electropolymerisation while ii) lower (or even negative) potentials aid the replenishing of the diffusion layer in the vicinity of the electrode surface. The consequences in the morphology of the films produced and/or efficiency of the biomolecule entrapment might depend on the number of potential steps and potential profiles applied. Part of the work presented here describes how different potential steps and potential profiles are related to different performances of immobilised enzymes as amplifying agents of currents generated in electrochemical biosensor application.

Glucose oxidase (GOx) and glucose dehydrogenase (GDH) have been immobilised by potential pulse entrapment of pyrrole within a screen printed microsystem. The goal of immobilising such enzymes is to establish an enzymatic electrochemical amplification mechanism on the electrode surface to improve the limits of detection of another compound. (See Scheme 1). In this case the target compound to detect is alkaline phosphatase (AP). AP is a very attractive enzyme to take as a model because it is widely used as a label in immunoassays and in molecular biology. AP is widely used because of the low level of interferences associated with its reactions, it is highly stable, it has a high turnover, it is low cost and has a broad substrate specificity. The measurement of low concentrations of AP has also been shown to be of interest in the field of pathogen detection since the detection of traces of AP can be indicative of the presence of a pathogenic load in different natural and biological media.[25] Electrochemical determination of Salmonella by indirect measurement of AP has been reported by our group.[7] The progress made is in the area of improving the performance of the electrochemical detection *via* enhancement of the immobilisation technique to modify functional and structural components of a screen printed microsystem.



Scheme 1 Schematic of the enzymatic reactions on the polypyrrole-enzyme modified screen printed carbon electrode within the microsystem.

AP is able to hydrolyze a large variety of orthophosphoric monoesters into the corresponding detectable phenol derivative. p-Aminophenyl phosphate (PAPH) has long been widely used as a substrate of AP[26–28] because AP dephosphorylates p-aminophenol phosphate enzymatically to produce p-aminophenol (PAP), which is measured amperometrically at a low potential yielding p-iminoquinone (PIQ). Very sensitive methods for AP (electro)chemical detection have been reported however most of them require long incubation time with its substrate PAPH to detect sub-picomolar AP concentrations.[29–36]

The presented work combines the optimization of polypyrrole entrapment of enzymes (GOx and GDH) within a screen printed microsystem and its application to achieve the highest AP detection sensitivity, reaching femtomolar AP detection values in short incubation times.

2. Experimental

2.1. Reagents and apparatus

All the reagents, glucose oxidase (GOx) from *Aspergillus niger* (fluka); D-(+)-glucose anhydrous and potassium chloride extra pure (Sharlau); 4-aminophenol (PAP) (Riedel de Haën);

magnesium chloride hexahydrate (Acros); sodium carbonate (Sharlau); p-aminophenol phosphate (PAPH), pyrroloquinoline quinona (PQQ) and AP from *Escherichia coli* (Sigma-Aldrich) and glucose dehydrogenase (GDH) (SORACHIM) were used as received. Pyrrole monomer (Sigma-Aldrich) was distilled before use. Solution containing the monomer was prepared in 0.1 M phosphate buffer aqueous solution (pH 7.2) and stored under argon. All solutions were prepared using distilled water (18 MΩ.cm), obtained with a Milli-Q Plus. Voltammetry and amperometric measurements were performed with an Autolab PGSTAT10 coupled with a computer and controlled by Autolab GPES (General Purpose Electrochemical System) software. The inks used for the microsystem fabrication were 7102 and 5874 conducting pastes based on carbon and Ag/AgCl respectively (DuPont Ltd). The apparatus used for the screen printing-based fabrication of the microsystem was a DEK-248 (DEK International) coupled with an optical alignment system.

2.2. Procedures

2.2.1 Microsystem manufacturing. The screen printed microsystem produced consisted of a microchannel structure formed by two electrodes that acted as walls too, the working electrode was made of a graphite ink, and the reference/counter electrode was made of Ag/AgCl ink over a polyester film substrate, see Scheme 2. Screen printed carbon exposed area is *ca.* 0.1 cm^2 and the final volume of the microsystem in *ca.* 20 μL. More detailed description of the screen printed microsystem and fabrication methodology has been previously reported by our group.[6,7]

2.2.2 Microsystem electrochemical activation. In order to improve the electrochemical response, electrocatalytic activity, surface hydrophilicity and the reproducibility of the cyclic voltammetry of redox process on screen printed carbon electrodes, their activation was found to be a necessary step.[7] A single-step procedure was implemented: injection of a saturated Na_2CO_3 solution inside the microchannel and applying 1.2 V for 300 s. Cyclic voltammetry of 0.1 mM PAP was carried out before and after the activation to evaluate the improvement of the signal. A stable peak-to-peak separation of 80 mV between the oxidation and reduction waves was achieved (Fig. S1, ESI†).

2.2.3 Microsystem modification. Direct anodic electropolymerisation of pyrrole monomer entrapping GOx and GDH by potential pulse chronoamperometry was the procedure applied. Three anodic potentials were applied from within the range of pyrrole electropolymerisation potential range investigated by cyclic voltammetry (Fig. S2, ESI†). Anodic and resting pulses were applied consecutively for 1 and 5 s respectively (Fig. S3, ESI†). The solution used was 0.1 M pyrrole and 0.1 M potassium chloride in 0.1 M phosphate buffer at pH 7.2 containing different concentrations of enzyme (5 mg ml^{-1} of GOx or 0.5 mg mL^{-1} GDH with 12 mg mL^{-1} PQQ). The microsystem was filled with the solution containing enzyme 1 min before/after the pulse electropolymerization. PBS-Tween buffer was flushed through the microsystem to remove the non-trapped enzyme. The microsystem was stored in acetate buffer solution (pH 4.7) at 4 °C when not in use.

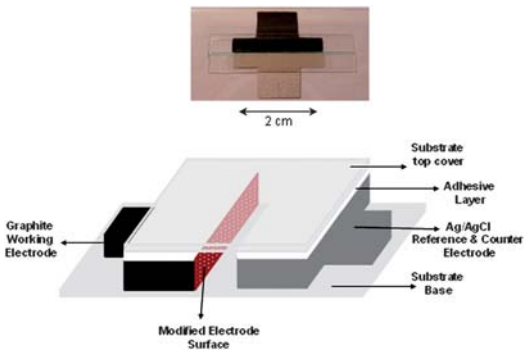
2.2.4 Amperometric measurements. GOx was used as enzyme in the experiments aiming to optimize the parameters for the polypyrrole-based enzyme entrapment. Solutions of different PAP concentrations with anhydrous glucose 1×10^{-2} M were prepared. Experiments to determine the AP concentration were carried out at a fixed 2×10^{-3} M concentration of PAPH as AP substrate.

Anhydrous D-(+)-glucose (1×10^{-2} M) was the substrate for entrapped enzymes (GOx and GDH), accompanied by 1×10^{-2} M of magnesium chloride to facilitate the substrate conversion.[37] All the solutions were prepared using acetate buffer 0.2 M pH 4.7 avoiding chloride containing solutions that could affect the stability with time of the Ag/AgCl reference/counter electrode. All the chronoamperometry measurements were carried out at 0.15 V *vs.* Ag/AgCl. The samples of AP with PAPH were incubated at 37 °C under mild shaking before injection into the microsystem. In all measurements the signals plotted had the background currents in absence of glucose subtracted.

3. Results and discussion

3.1 Enzyme entrapment: electropolymerisation optimization

Different number of pulses and anodic potentials were applied during the entrapment of glucose oxidase. Upon modification the microsystem was investigated for its electrochemical response to the oxidation of PAP amplified by GOx. For each case of anodic pulses and each potential applied a calibration plot like the one depicted in Fig. 1 was produced and the sensitivity of the electrochemical response expressed as the intensity of the oxidative current per molar unit of PAP.



Scheme 2 Schematic and real image of the screen printed microsystem.

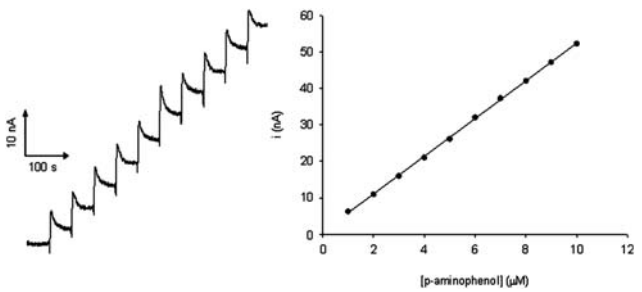


Fig. 1 Example of chronoamperogram and typical response of polypyrrole-GOx modified microsystem to the addition of 1 to 10 μM PAP measured at 0.15 V *vs.* Ag/AgCl ink counter reference electrode.

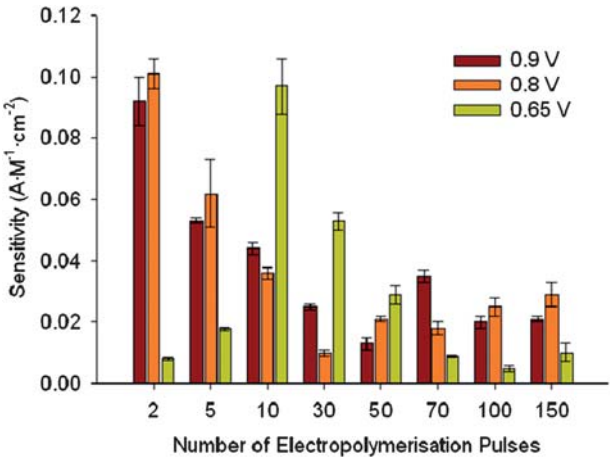


Fig. 2 Different sensitivities to the addition of 1 to 10 × 10^{-6} M of PAP as a function of the potential and pulses applied during the fabrication of the polypyrrole-GOx modified microsystems.

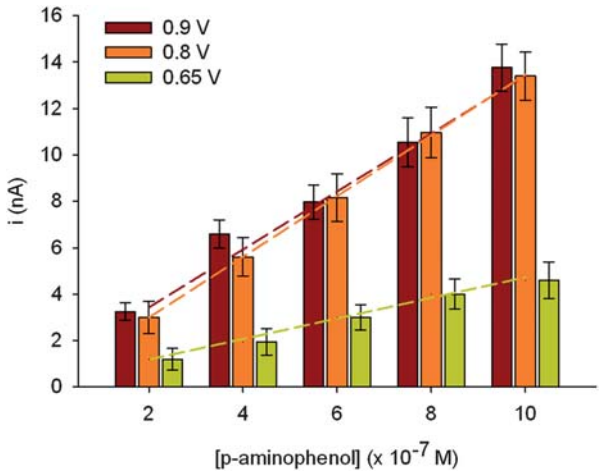


Fig. 3 Calibration curve response of different polypyrrole-GOx modified microsystems at different potentials upon addition of PAP concentrations.

The overall results (three replicates per potential and per number of pulses) of such evaluation summarising all the voltages and number of pulses applied can be observed in Fig. 2. There it becomes clear that thinner films of polypyrrole-GOx, still with lower loads of enzyme, yielded more sensitive responses; this was attributed to the fact that they might provide an easier mass transport through it both for the substrate (Glucose) and electrochemically active species generated near the electrode surface. Slightly lower anodic electropolymerisation potential, 0.65 V, required about ten pulses to reach the sensitivity levels recorded for electropolymerisations of just 2 pulses at 0.9 and 0.8 V. When evaluating polypyrrole film generated (analysing the deposited charges during electropolymerisation) it was observed that a similar number of coulombs were deposited for the best sensitivity achieved with each of the potentials, see Table 1, which corroborates that higher sensitivity levels are dictated by the thickness of the film. The sensitivity values achieved within the microsystem constitute a 5 to 10-fold enhancement with respect to other polypyrrole-GOx modified electrodes reported in the literature.[38,39]

Having explored the optimum conditions for a sensitive response of the polypyrrole-GOx modified microsystem, the different limits of PAP detection were also explored. See Fig. 3. Films generated displayed lower limits of detection for PAP, (24.9, 7.01 and 1.86 × 10^{-8} M for 0.65, 0.8 and 0.9 V respectively). This was attributed to possible different morphologies or other film characteristics associated to the different electropolymerisations.[40]

Table 1 Summary of the optimal conditions for the highest sensitivity achieved in the polypyrrole-GOx modification of the microsystem

Potential Applied/V	No Pulses Applied	Total Charge Deposited/C	Sensitivity/ A M^{-1} cm^{-2}
0.9	2	2.95 × 10^{-4}	0.044
0.8	2	3.10 × 10^{-4}	0.0505
0.65	10	2.89 × 10^{-4}	0.0485

Based in the results obtained during the optimisation of the entrapment of GOx within the polypyrrole film by anodic pulse electropolymerisation, it was decided that the optimum films would be fabricated by applying 2 pulses at 0.9 V. This modified and optimised microsystem was applied in the detection of AP.

3.2 AP electrochemical detection

Experiments aiming at evaluating the detection of AP were carried out using GOx and GDH as the immobilised biomolecules. First polypyrrole-GOx films were prepared by applying a 2-pulse anodic polymerisation potential of 0.9 V. Different incubation times of AP with PAPH as substrate were explored and the results outlined in Fig. 4. Values between 10^{-8} and 10^{-13} M were detected for the different times evaluated; 9.05 × 10^{-13} M (70 ng L^{-1}) for 20 min; 2.36 × 10^{-12} M (207 ng L^{-1}) for 30 min and 4.09 × 10^{-12} M (356 ng L^{-1}) for 40 min were the limits of detections estimated. Limits of detection were calculated from the current equivalent to 3 times the standard deviation of the zero concentration value.

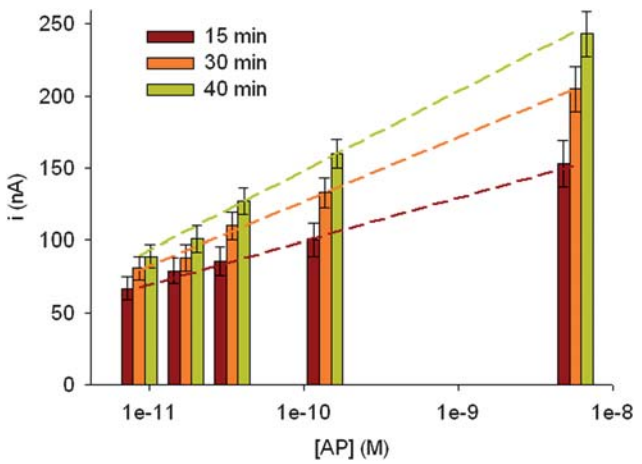


Fig. 4 Calibration curve responses of different polypyrrole-GOx modified microsystems after incubation for different times of 2 × 10^{-3} M PAPH in presence of different concentrations of AP.

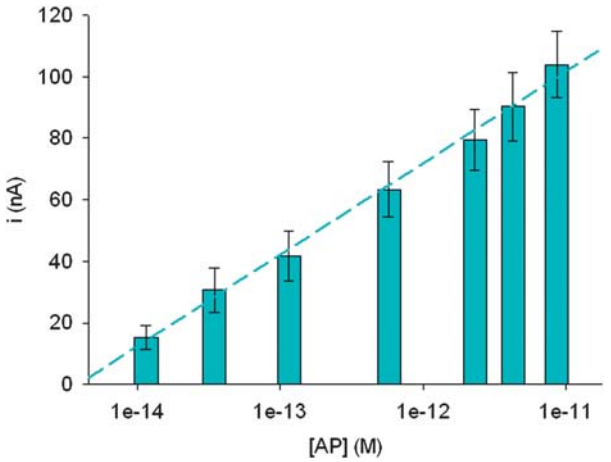


Fig. 5 Calibration curve response of different polypyrrole-GDH-PQQ modified microsystems after incubation for 15 min of 2×10^{-3} M PAPH in presence of different concentrations of AP.

These values constitute an improvement in the limits of detection reported using GOx as amplifying enzyme even in more complex systems.[41] It was observed how for higher incubation times the limit of detection did not really improve that already achieved for 20 min. The reported spontaneous degradation of PAPH directly to PAP meant that for longer incubation times the signal recorded from the blank experiment was more prevalent, effectively decreasing the estimated limits of detection.[31]

The evaluation of AP detection was also attempted by immobilising GDH. The results depicted in Fig. 5 clearly showed the enhancement induced by the use of GDH, which seemed to yield a better performance as polypyrrole-immobilised amplifying enzyme than glucose oxidase with incubation times of only 15 min. Apart from possible different immobilisation efficiencies it has to be considered that GDH (with PQQ cofactor) is an enzyme that does not have oxygen as electron acceptor (as it is the case of glucose oxidase).[42–45] This means that the regeneration of the amplifying enzyme can only be done by the electrochemically generated PIQ with no competition with the oxygen present in solution. As a result the limit of detection was much enhanced with a short incubation time, *i.e.* 9.11×10^{-15} M (0.80 ng L^{-1}), limit of detection calculated from the current equivalent to 3 times the standard deviation of the lowest concentration value. These result constitutes an improvement to previously reported electrochemical detections of ALP with simple carbon-based screen printed electrodes and even much more complex systems.[46,47]

The optimisation of the film formation, achieving very thin films of PPy immobilising the GDH-PQQ has shown to be a very efficient method to achieve very high amplification of the electrochemical signals, and the very low volume of the effective electrochemical cell that the microsystem constitutes, certainly contribute to the improvement of the optimum limit of detection achieved.

4. Conclusions

The fabrication of a low cost, easy to manufacture microsystem and its application to achieve ultrasensitive electrochemical detection of AP has been demonstrated. The values of AP detected have, at least, matched those of much more complex systems. The produced microsystems have shown a highly sensitive response to the oxidation of p-aminophenol, which shows the efficiency of the combination of a low volume microsystem and the pulse potential polypyrrole-GOx and GDH-PQQ modification procedure. That high performance was directly translated in a very sensitive electrochemical detection of AP. Given the relevance that AP has in many electrochemical sensing platforms (DNA, aptamer or immuno-based sensors) and the wide range of applications that these have in fields such as pathogen detection (work ongoing), food or environmental monitoring, the work described here can constitute by itself a clear example of an ultrasensitive, simple, low cost, integrated microsystem for straight implementation in many already existing applications.

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