Simultaneous detection of multiple lower genital tract pathogens by an impedimetric immunochip

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a b s t r a c t

Lower genital tract infections caused by both sexually and not-sexually transmitted pathogens in women are a key public health priority worldwide, especially in developing countries. Since standard analyses are time-consuming, appropriate therapeutic intervention is often neglected or delayed. Lab-on-chips and biosensors open new perspectives and offer innovative tools to simplify the diagnosis by medical staff, especially in countries with inadequate resources. Here we report a biosensing platform based on Electrochemical Impedance Spectroscopy (EIS) that allows multiplexed detection of *Candida albicans*, *Streptococcus agalactiae* and *Chlamydia trachomatis* with a single biochip, enabling a quick screening thanks to the presence of different immobilized antibodies, each specific for one of the different target pathogens.

1. Introduction

Female vaginal infections are common conditions, which carry higher risks in developing countries where the lack of economical and medical resources often delays diagnosis and treatment and may cause progression to more severe disease (Spence and Melville, 2007). Many women in childbearing age show an abnormal vaginal flora with the presence of mixed pathogens, although these do not necessarily cause symptoms, which rather result from the interplay amongst microbial virulence, numerical dominance, and the innate and adaptive host immune response (Lamont et al., 2011a). The most common causes of vaginitis are bacterial vaginosis (BV) and vulvovaginal candidiasis. BVs are typically caused not by a single entity, but by different bacterial strains (Lamont et al., 2011b).

Streptococcus agalactiae, often reported in vaginal swabs, is known to colonize the genital tract in 4–18% of healthy women and is frequently isolated from patients with vulvovaginal symptoms. Although its causal role has not been well defined, infection with *S. agalactiae* is of primary importance in pregnant women, since it may cause sepsis in the newborn (Shaw et al., 2003).

Chlamydia trachomatis is the most prevalent sexually-transmitted bacterial infection worldwide and, if left untreated, may result in serious complications (Mahilum-Tapay et al., 2007). This Gram-negative bacterium usually infects the cervix and fallopian tubes of women and the urethra of men. Chlamydia may cause a purulent vaginal discharge, but it is asymptomatic in 80% of women. Infection by Chlamydia is associated with an increased risk of HIV transmission, especially in the developing world (Van Dyck et al., 2001) where early detection is difficult and treatment is frequently neglected or delayed. This results in the spreading of the disease and in the occurrence of serious complications such as pelvic inflammatory diseases (PIDs) (Donders, 2010), infertility,

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ectopic pregnancy, preterm labor, and chronic pelvic pain. An early diagnosis is therefore necessary (Yang et al., 2012).

Candida albicans is another widespread pathogen, which leads to vulvovaginal candidiasis. It is a diploid sexual fungus commonly found in the human mouth and gastrointestinal tract that accounts for 85% to 90% of cases of vulvovaginal candidiasis (Zhang et al., 2014). Asymptomatic presence has been reported in 10% of women, compared to more than 70% with at least one episode of vulvovaginal candidiasis.

Usually symptomatology and standard analytical techniques are employed for the diagnosis of lower genital tract infections in women. In particular, microbial cultures have long been accepted as the gold standard. However, this classical approach has a number of drawbacks, such as low sensitivity, long duration (at least 72 h), and the need for culture media specific for the different strains, technically trained staff and appropriate instrumentation (Nelson and Helfand, 2001).

Recently, molecular techniques such as nucleic acid amplification tests (NAATs) and immuno-diagnostic assays have been developed for the identification of pathogens. For example, PCR-based tests for the detection of *Chlamydia* infections are frequently used in fertility centers. NAATs have proved efficient at identifying different vaginal infections and are now considered as the new gold standard for diagnosis. However NAATs are not suitable as point-of-care diagnostic methods because of their high costs, complexity, need of highly trained staff and complex equipment (Su et al., 2011).

To overcome these limitations new tools are required to provide a fast-response, low costs and high sensitivity. In particular, for point-of-care applications in developing countries, where specialized laboratories are few and far between, these platforms must be designed so as to be usable even by non-skilled people, hence be based on simple detection methods and on user-friendly, portable and disposable devices. Another important aspect is the possibility to test simultaneously for different pathogens. In this respect, Lab-on-chip (LOC) platforms can open new perspectives thanks to their great advantages in term of miniaturization, small sample quantities, portability, fast response and sensitivity.

Here we report an impedimetric LOC platform for multi-pathogen analysis that is designed to simplify the diagnosis of lower genital tract infections caused by *S. agalactiae*, *C. trachomatis* and *C. albicans* in women. We have selected Electrochemical Impedance Spectroscopy (EIS) as a sensitive, label-free detection method, and combined it with a microfluidic module for the delivery of the sample to four sensing areas (one for each pathogen plus one control chamber). The proposed approach achieves significant

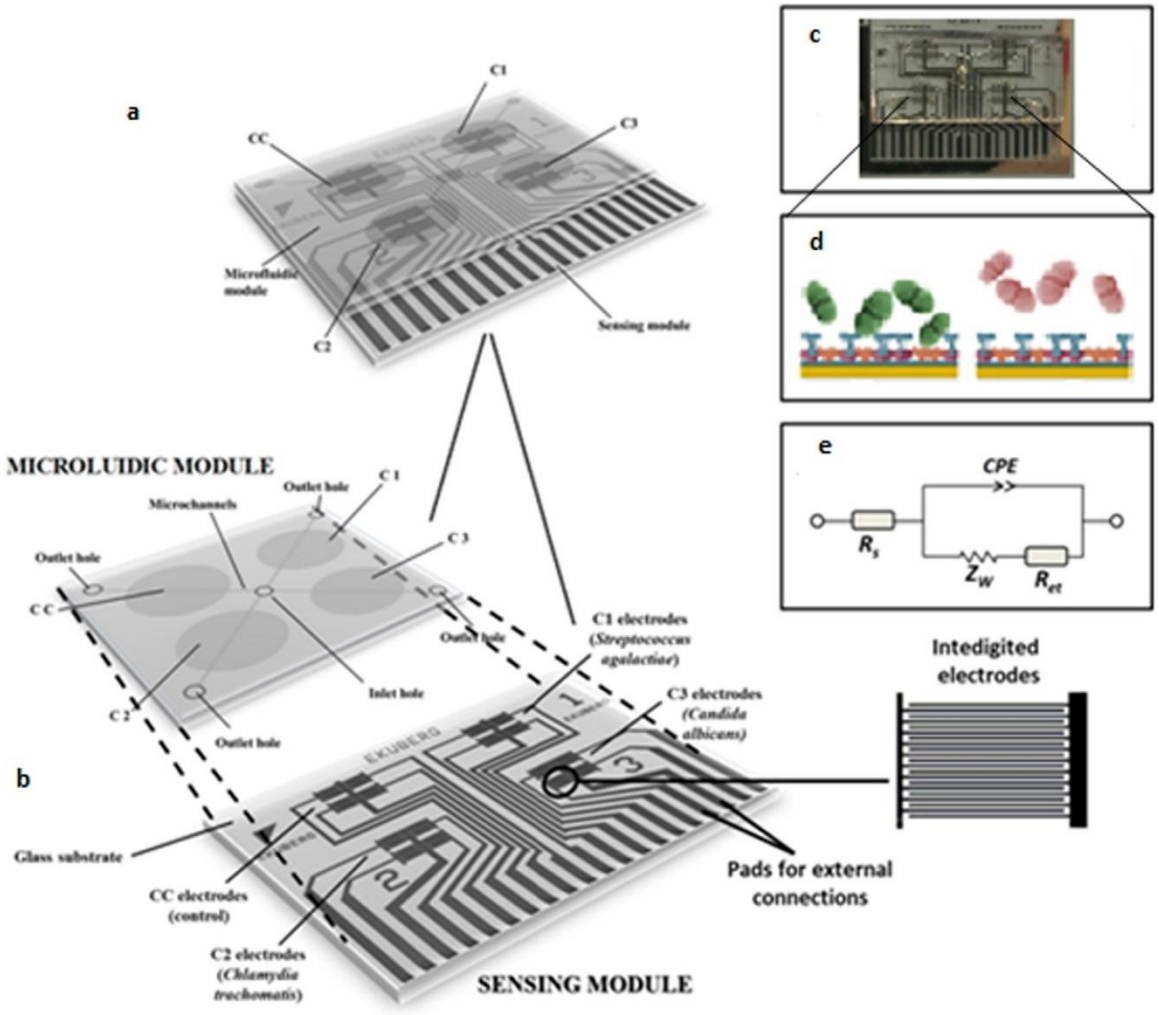

Fig. 1. Setup of the detection platform. (a) Final biochip with the integrated microfluidic module which allows the delivery of the sample solutions through the central hole to the (b) gold microelectrode arrays fabricated on a glass substrate. (c) The fabricated biochip with microelectrodes functionalized with specific antibodies. (d) Operating principle with the detection of target pathogens (green) while unspecific pathogens (red) are not bound. (e) Equivalent circuit for impedance spectroscopy transduction. The circuit includes the ohmic resistance of the electrolyte solution R_s, the Warburg impedance Z_w resulting from the ionic diffusion of the electrolyte, a constant phase element (CPE) and the electron transfer resistance R_{et}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

time-savings with respect to standard culture tests since it re- quires only one hour for the binding between the antigens ex- pressed on the pathogen membrane and the specific antibodies immobilized onto the electrodes. Furthermore, differently from others systems, our platform offers the advantage to handle samples without any pretreatment. Hence this portable device may become a useful tool for the gynecologist in an outpatient setting, even in precarious conditions as those often found in de- veloping countries.

2. Materials and methods

2.1. Chip fabrication and functionalization

The immunochip reported in this work (Fig. 1a and c) is based on a modification of our previously developed EIS lab on chip platform (Primiceri et al., 2010; Primiceri et al., 2011). Specifically, the biochip integrates a microfluidic module with four separate sensing areas, each one containing an array of 4 gold interdigited microelectrodes to perform repeated experiments and statistical analysis. The microelectrodes (with 10 μm spacing and width) were fabricated on glass substrates by optical lithography and lift- off, while the microfluidic PDMS module was realized by replica molding with 20 μL chambers with inlet and outlet microchannels for fluid handling (Fig. 1b).

Biosensing was achieved by means of specific functionalization (Fig. 1d) of the gold microelectrodes (Chiriaco et al., 2011) using maximum purity reagents from Sigma Aldrich. Specifically, the first step was a passivation of the silicon substrates based on a solution of polyethylene glycol (PEG 0.5% in ethanol) to prevent unspecific adhesion of bacteria on the surfaces and phenomena of cross-reactions. The next step was an overnight deposition of a mixed SAM of 11-mercaptoundecanoic acid (MUA) and 2-mercaptoethanol (2-ME) in a ratio of 1:5 (0.2 mM of MUA and 1 mM of 2-ME) followed by the activation of the COOH groups by incubation with N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in ultrapure water to form reactive N-hydroxysuccinimide esters. Electrodes were incubated sequentially in solutions of (i) protein A (50 mg/ml), to allow the oriented immobilization of antibodies (de Juan-Franco et al., 2013; Ingavle et al., 2015) through their Fc fragment, thanks to five homologous IgG-binding domains (Anderson et al., 1997; Babacan et al., 2000), (ii) ethanolamine (1 M) to block excess NHS and (iii) BSA (Bovine Serum Albumin, 5 mg/ml) to saturate remaining free electrode sites. The electrodes were then differentially functionalized with the following monoclonal antibodies from Santa Cruz Biotechnology, Inc. (1 ml/ml): chamber 1 with anti-*Streptococcus agalatctiae* antibody (sc-73072), chamber 2 with anti-*C. trachomatis* antibody (sc-51837), chamber 3 with anti-*C. albicans* antibody (sc-51811), while the last chamber was not functionalized with any antibody in order to provide a negative control (Fig. S1 in Supplementary information). The antibodies were chosen for their ability to recognize specific membrane proteins of the three above-mentioned pathogens. In this way each chamber gives information about the infection of a specific pathogen and co-infections can be detected as simultaneous signals coming from different chambers.

Functionalization procedure has been monitored by AFM and contact angle investigation as reported in SI (Fig. S2 and Tables S1 and S2).

2.2. On chip analysis of samples

Control samples for characterization experiments contained pathogens' cells derived from a colony picked up from selective culture plate and suspended in 1 ml of sterile PBS. As regards patient samples, the study was conducted with ethical approval from the Ethical Committee of ASL Lecce (No. EKU001CT). Vaginal swab specimens were obtained with the informed consent of patients, resuspended in 1 ml of sterile PBS and stored at − 20 °C until use. Anonymous numeric specimen codes were used to protect the identity of individuals. Diagnosis of vaginal infection was always confirmed by standard selective culture assays.

For our biochip analysis, control and test samples were incubated in the PDMS chamber for 1 h. Devices were then washed with PBS through the microfluidic channels to remove unspecific deposition. Successively, devices were filled with a solution of the redox couple potassium hexacyanoferrate (II/III) $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) (Sigma Aldrich) at a final concentration of 10 mM in PBS. Impedance spectroscopy measurements were performed with an Autolab PGSTAT30 in the frequency range between 0.1 and 10^5 Hz by applying a sinusoidal 15 mV AC voltage (Chiriacò et al., 2015, 2013).

The device can be modeled with a modified Randles circuit (sketched in Fig. 1e), including s constant phase element (CPE) and the electron transfer resistance R_{et}. The last term is very sensitive to the electrode modifications and increases progressively as consecutive molecular layers are deposited on the electrode surface (during the functionalization or detection phases). The circuit also includes a Warburg impedance Z_W related to the depletion of the redox species at the interface and a series resistance R_s accounting for the uncompensated solution resistance. Impedance spectroscopy is thus very effective for detecting biorecognition events on surface-modified electrodes (Katz and Willner, 2003).

Each plotted curve derives from the mean of at least five independent experiments and has been fitted on the basis of a simplified circuit in which the contribution of Z_w has been neglected. Each biochip was used for a single experiment.

3. Results and discussion

3.1. Impedimetric characterization of the immunochip

As a first step, a characterization of the antibody-functionalized electrodes was performed. After immobilization of *anti-S. agalactiae* antibodies, impedance values of around 34 kΩ were reproducibly measured. In contrast, anti-*C. thrachomatis* and anti-*C. albicans* gave impedance values of about 40 kΩ. Such variations may be attributed to some differences in molecular weight and surface affinity of the three antibodies (Supplementary information Fig. S1). The impedance after BSA immobilization (the last step before antibody deposition) was around 20 kΩ. All these values were assumed as the baseline for subsequent experiments with test samples (see Fig. S1 Supplementary Information) (Chiriaco et al., 2011).

The second characterization step required an evaluation of the biochip sensitivity and specificity (for example unspecific binding of *S. agalactiae to* anti-*Candida* or anti-*Chlamydia* antibodies). For this purpose, three suspensions were prepared, each containing cells picked up from a colony of the pathogen under investigation in 1 ml of PBS. These suspensions were separately injected into different devices and incubated for 1 h.

As shown in Fig. 2, an impedance increase from 34 kΩ to 113 kΩ was observed with a suspension of *S. agalactiae* in the chamber functionalized with the anti-*S. agalactiae* antibodies (red curve in Fig. 2A), whilst in the case of *C. albicans* the pathogen suspension caused an impedance increase from 38 kΩ to 92 kΩ in the chamber functionalized with the anti-*C. albicans* antibodies (green curve in Fig. 2B). In both cases, a negligible increment was measured for the three unrelated chambers, demonstrating very

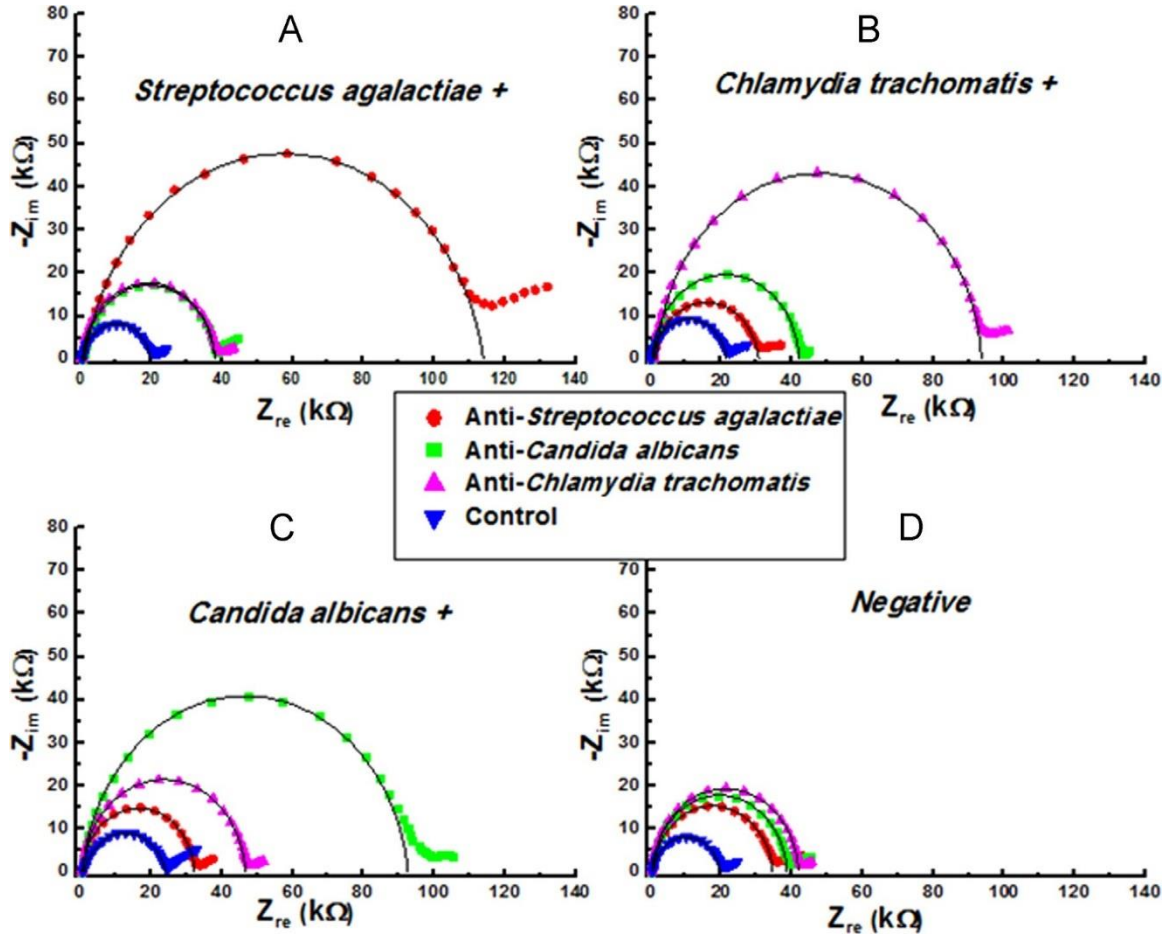

Fig. 2. Nyquist plots for immunochips characterization with a sample containing *Streptococcus agalactiae* (A), *Candida albicans* (B), *Chlamydia trachomatis* (C) and without pathogens (D). Black lines are the fitting curve on the basis of Equivalent circuit element (see Table S3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

low unspecific signals. With *C. trachomatis* (Fig. 2C), the impedance signal increased from 41 kΩ to 92 kΩ on the specific anti-*C. trachomatis* antibodies, with lower impedance increments in the other chambers. As a control, we tested the signals of all four chambers after incubating with a PBS solution in the absence of any pathogen (Fig. 2D). This produced the same values as obtained after functionalization (about 35 kΩ for anti-*S. agalactiae* and about 40 kΩ for anti-*C. trachomatis* and *anti-C. albicans)*, showing the absence of unspecific signals.

3.2. *On chip analysis of vaginal* fluid *specimens*

To demonstrate the diagnostic value of our biochip, a blind test on patient samples was performed. Specifically, 45 samples derived from vaginal swabs were resuspended in sterile PBS and analyzed by injecting 90 ml in the microfluidic module with a micropipette (or by an insulin syringe). Standard microbial cultures were performed in parallel as a control. Table 1 summarizes the results, comparing our biochip with the culture method. Detailed impedance data are reported in Table S4

Based on some preliminary tests on vaginal samples (data not shown) and considering the contribution of the biological matrix, we defined a result as positive when the impedance signals recorded form the respective chamber exceeded a threshold of 45 kΩ for *S. agalactiae* and *of* 65 kΩ for *C. albicans* and *C. trachomatis*. These values are higher than those obtained with PBS, to adjust for the presence of factors in the vaginal mucus.

The analysis of samples from 1 to 34 confirms the efficiency of our method for the diagnosis of *C. albicans* and *S. agalactiae*, since in all these cases biochip outcomes were confirmed by culture tests. In the cases highlighted with a yellow background, standard analysis revealed the presence of other pathogen species which however did not affect the biochip selectivity. In particular, microbial cultures identified a "Candida non albicans" infection in sample 28 which was not detected with our immunochip, thus confirming its high level of selectivity.

Samples 35–39 were collected for the diagnosis for *C. trachomatis*. In fact, unlike the vaginal swabs taken for *C. albicans* and *S. agalactiae* analysis, samples for *C. trachomatis* are collected with endocervical swabs. These contain an increased amount of mucus and blood (Newhall et al., 1999) that affect measurements, causing three false positives.

Our sensing platform was also able to detect co-infections such as those of samples 40–42, which contained both *C. albicans* and *S. agalactiae*: in these cases an impedance increase was detected from both the corresponding chambers, while no change was re- corded from the negative control and the anti-*C. trachomatis* chambers. Samples infected with other microorganisms were also tested, such as samples 43–45, which standard tests identified as a co-infection of *Staphylococcus* and *Enterococcus*. In these cases our immunochip reported a false positive outcome for *S. agalactiae*, probably due to an occasional cross-reaction which even PEG passivation in the functionalization process was not able to pre- vent (Newhall et al., 1999).

Table 1
Results from analysis carried out on 45 unknown samples from patients. All tests were performed blindly in parallel with standard analysis. In some cases, co-infections from two different pathogens were present.

	Streptococcus agalactiae		Candida albicans		Chlamidia trachomatis		agreement
	EIS	STD	EIS	STD	EIS	STD	
1	-	-	-	-	-	-	√
2	-	-	-	-	-	-	√
3	-	-	-	-	-	-	√
4	-	-	+	+	-	-	√
5	-	-	-	-	-	-	√
6	-	-	-	-	-	-	√
7	-	-	-	-	-	-	√
8	-	-	-	-	-	-	√
9	-	-	-	-	-	-	√
10	-	-	-	-	-	-	√
11	-	-	-	-	-	-	√
12	-	-	+	+	-	-	√
13	-	-	-	-	-	-	√
14	-	-	-	-	-	-	√
15	-	-	-	-	-	-	√
16	-	-	+	+	-	-	√
17	+	+	-	-	-	-	√
18	+	+	-	-	-	-	√
19	-	-	-	-	-	-	√
20	-	-	-	-	-	-	√
21	-	-	-	-	-	-	√
22	-	-	+	+	-	-	√
23	-	-	-	-	-	-	√
24	-	-	-	-	-	-	√
25	-	-	-	-	-	-	√
26	-	-	-	-	-	-	√
27	-	-	-	-	-	-	√
28	-	-	-	-	-	-	√
29	-	-	-	-	-	-	√
30	-	-	+	+	-	-	√
31	-	-	-	-	-	-	√
32	-	-	-	-	-	-	√
33	-	-	-	-	-	-	√
34	-	-	-	-	-	-	√
35	+	-	+	-	+	-	!
36	-	-	-	-	-	-	√
37	-	-	-	-	+	-	!
38	-	-	-	-	-	-	√
39	+	-	+	-	+	-	!
40	+	+	+	+	-	-	√
41	+	+	+	+	-	-	√
42	+	+	+	+	-	-	√
43	+	-	-	-	-	-	!
44	+	-	-	-	-	-	!
45	+	-	-	-	-	-	!

4. Conclusions

Although lower genital tract infections may be some of the most common disease in gynecological outpatient clinics, their diagnosis is difficult, since symptoms are not specific and classical assays are time-consuming. Here we report a new diagnostic tool to detect simultaneously the presence of three of the most com- mon microorganisms responsible for lower genital tract infections in women: 45 gynecological samples from unidentified patients were analyzed in blind tests, for a total of 180 individual analyses with the sensing platform. Most of the results were consistent with those obtained by the standard culture method, except for six cases. Some of these are false positive results for *S. agalactiae* in samples co-infected with *Staphylococcus* and *Enterococcus* probably due to occasional cross-reaction phenomena. The other 3 false positives are samples from endocervical swabs for the diagnosis of *C. trachomatis*, which were rich in mucus, blood and tissue debris (Newhall et al., 1999). To overcome interference by these factors and improve sensor responses in term of sensitivity and specificity, we plan to add a module for microfluidic sample filtration.

Our microfluidic platform is suitable as a multiplexed immunoassay overcoming the much longer analysis time and higher cost of standard techniques. Moreover our device may be readily improved by expanding the number of sensing areas and hence of detected pathogens within the same biochip, thus increasing its diagnostic power. This, in combination with portability, makes our

platform a promising *point of care* device for gynecologists and patients which would not only reduce the number of invasive examinations and tests, but also minimize analysis time and costs. These feature are likely to be of particular value in developing countries where lower genital tract infections are highly prevalent and the health care system lacks adequate medical and economical resources. .

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.11.100.

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