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IMPROVING THE ANALYTICAL PERFORMANCE OF A DIGITAL LAB-ON-A-CHIP FOR DETECTION AND QUANTIFICATION OF IG-E

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Digital microfluidics, electrowetting-on-dielectric, superparamagnetic nanoparticles, ELISA

ABSTRACT

This paper describes the optimization of the analytical performance of an aptamer-based ELISA on a digital lab-on-a-chip for the detection and quantification of IgE. In this digital lab-on-a-chip platform nanoliter droplet manipulations are executed by means of electrowetting forces. The high level of automation makes this digital platform perfectly suited for the implementation of an aptamer-based bioassay in which different reagents need to be transported, diluted and mixed. However, the bio-assay under study here involves several droplet manipulations which require accurate control of droplet size and chemical composition. Therefore, in a first phase, the main operational droplet actuation parameters were optimized – using a logistic regression approach – to assure monodisperse droplets throughout the bioassay. In a second phase, the three elementary manipulations – droplet transport, splitting and dispensing – were evaluated in the presence of magnetic beads. Actions including bead retention and re-suspension were successfully applied on the digital platform. In the final phase, an aptamer based ELISA for the detection of IgE was implemented on the digital lab-on-a-chip, using the optimized droplet manipulation parameters, as a proof-of-concept. The IgE molecules were first captured by monoclonal antibodies linked to superparamagnetic nanoparticles. After magnetic separation of the bead complexes, fluorescent labeled anti-IgE aptamers were added to quantify the amount of captured IgE which was monitored by means of an inverted fluorescence microscope.

1. INTRODUCTION

Among the different diagnostic techniques, the enzyme-linked immunosorbent assay (ELISA) is one of the most versatile analysis techniques today. The success of this technique is reflected by the numerous applications in the field of clinical diagnostics, biochemistry, food and feed analysis and environmental analysis [1]. Despite its great potential, the ELISA technique, typically executed in a microtiter plate, is not truly efficient since a vast number of preparative steps are necessary before quantification of the analyte is obtained. In addition, the chances on handling errors increase and assay time is typically prolonged up to a few hours due to mass transport limitations.

Digital lab-on-a-chip technology, involving the manipulation of nanoliter droplets on the microscale, offers some interesting advantages and is therefore a perfect candidate to improve the performance of the ELISA. Because droplets, containing the different reagents, are controlled on a drastically reduced scale, for instance by means of electrowetting-on-dielectric (EWOD), the whole analytical procedure is accelerated because the molecular diffusion time is significantly reduced compared to analysis in ordinary macro devices [2,3]. Furthermore, droplets are manipulated individually resulting in a high degree of assay automation.

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Finally, due to the nanoliter size volume, the assay cost is considerably reduced from the microliter scale to the nanoliter scale [4].

The most popular ELISA configuration is the sandwich-based assay in which the target specific antibody is immobilized on a fixed surface and in which a second labeled (e.g., fluorescent labels, enzymes) antibody is used to quantify the analyte. To avoid complex microfabrication and biofunctionalization steps with respect to the immobilization of the capture antibody to the chip surface, superparamagnetic micro- and nanoparticles offer an interesting alternative. They are characterized by a high surface-to-volume ratio and due to their magnetic core they can be easily attracted and isolated from the solution by means of an external magnetic field making them ideally suited as a solid phase carrier for the target specific antibody [5]. The literature on the use of magnetic nano- and microparticles in digital lab-on-a-chip systems is rather limited. Shah and Kim increased the efficiency of magnetic microparticle concentration with assistance of the interfacial tension of the droplet [6]. Sista et al. demonstrated the use of a digital microfluidic platform to quantify human insulin and interleukin-6 (IL-6) by implementing a magnetic microparticle-based heterogeneous immunoassay [7]. In particular, they isolated the magnetic microparticles from the rest of the droplet to remove excess reagents and any species adsorbed non-specifically on the bead surface. Besides using magnetic particles as a solid phase for immobilization, they can be used as sensitive labels as well. Schaller et al. detected 92.5 nm particles with a multi-core cobalt ferrite ($CoFe_2O_4$) by the (external) integration of a Superconducting Quantum Interference Device (SQUID) gradiometer on an EWOD-based microfluidic device [8].

In this work we integrate the concept of superparamagnetic nanoparticles as a solid phase support on a digital lab-on-a-chip platform to carry out an immunoassay for the detection of IgE. In a first phase, three elementary droplet manipulations are optimized with respect to droplet size variability. The use of monodisperse droplets is crucial to obtain the highest possible immunoassay performance. In a second phase, the effect of the magnetic particles on the different droplet manipulations is studied: the effect of droplet dispensing on the statistic dispersion of the particles inside the droplets is validated and the enrichment efficiency after isolation of the magnetic nanoparticles is investigated. Finally, a proof-of-concept of the implementation of the ELISA based immunoassay using superparamagnetic nanoparticles in combination with fluorescent labeled aptamers is presented and strategies to increase the performance of this assay are formulated.

2. EXPERIMENTAL METHODS AND MATERIALS

2.1 Lab-on-a-chip fabrication and setup

The digital lab-on-a-chip is fabricated by means of standard photolithographic techniques in the cleanroom facilities of MICAS-ESAT (Katholieke Universiteit Leuven). The bottom part of the chip consists of a photolithographically patterned aluminum layer (50 nm), deposited on a glass wafer *via* magnetron sputtering. Before coating with a 2.8 µm parylene-C layer *via* chemical vapor deposition, the substrates were cleaned in oxygen plasma and primed with silane A174 to improve adhesion of the dielectric layer. Finally, a 300 nm Teflon layer was spin-coated in order to render the surface hydrophobic. The top plate of the digital microfluidic chip was fabricated by spin-coating a Teflon layer (300 nm) on ITO-coated glass slides (150 nm).

Droplets were sandwiched between the two plates and are surrounded by a very thin shell of silicon oil [9]. Tape of about 80 µm was used to separate both plates. Droplets were manipulated by controlling the DC actuation voltage, activation time and relaxation time by means of homemade programs in MATLAB (The MathWorks, Inc.) and LabView (National Instruments Corp.). For the optimization of the elementary droplet manipulations (dispensing, transport and splitting), images were taken by a CCD camera (JVC TK-C1360B Color Video Camera, JVC) mounted on a stereomicroscope (SMZ1000, Nikon). Fluorescent images of the droplets were taken by an EM-CCD camera (C9100-13, Hamamatsu) mounted on an inverted fluorescence microscope (IX-71, Olympus). Intensity profiles were obtained by a homemade image analysis program in MATLAB.

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2.2 Chemicals

Phosphate buffer saline (PBS) (pH = 7.4; 165 mM) and 2-(N-morpholino)ethanesulfonic acid (MES) (pH = 5.5; 50 mM) buffer components were purchased at Sigma-Aldrich (Belgium). Every solution was prepared with deionized water purified with a Milli-Q Plus system (Millipore, USA). Pluronic F-68 (3M, USA) was added to the PBS buffer up to a final concentration of 0.05% w/v to prevent biofouling on the hydrophobic chip surface. Superparamagnetic nanoparticles of 30 nm were produced at the Department of Physics at the Katholieke Universiteit Leuven. 1-Ethyl-3-[3-dimethyl aminopropyl]carbodiimide hydrochloride (EDC) was obtained from Pierce Biotechnology (USA). Goat anti-human immunoglobulin E (IgE) and bovine serum immunoglobulin G (IgG) were supplied by Sigma–Aldrich (Belgium). Yellow fluorescent protein (Venus) was kindly donated by the Department of Chemistry at the Katholieke Universiteit Leuven. FAM-labeled aptamer against IgE, based on the sequence of Wiegand et al. [10], was purchased from Integrated DNA Technologies (Belgium).

2.3 Superparamagnetic nanoparticle functionalization

Both IgG and Venus labeled superparamagnetic nanoparticles were prepared according to the same protocol. Before functionalization, the superparamagnetic nanoparticles were washed with MES buffer before they were finally suspended in MES buffer to a concentration of 0.5 mg/mL. Protein (60 nM) was immobilized on the nanoparticles by adding the zero length linker EDC (0.5 mM). After 30' of incubation, the functionalized particles were washed in PBS buffer and concentrated up to 1 mg/mL.

2.4 Superparamagnetic nanoparticle attraction and enrichment

A disk magnet with a pull force of 0.68 kg (model no. S-06-02-N) was obtained from Supermagnete (Germany). A droplet of 600 nL containing Venus functionalized magnetic particles was sandwiched between the two plates and surrounded by an oil-shell as described above. The disk magnet was manually positioned on top of the counter plate right above an electrode. To collect the settled aggregates together, the droplet was transported underneath the magnet back and forth three times. Finally the droplet was split into two equal daughter droplets.

2.5 ELISA-based immunoassay on-chip

From a stock solution of 6 µM IgE in PBS buffer, several working solutions ranging from 20 to 200 nM were prepared. Just before use, the stock solution (1 µM) of FAM-labeled aptamer was shortly heated at 95°C to restore the natural conformation of the aptamer. A reaction mixture of 0.5 mg/mL IgG functionalized superparamagnetic nanoparticles and 500 nM FAM-labeled aptamer was prepared. One reservoir of the digital lab-on-a-chip was filled by pipetting 2 µL of the IgE stock solution on the dispensing electrodes. Similarly, a second reservoir was filled with reaction mixture and a third reservoir was filled with PBS buffer solution.

After dispensing and merging a droplet with the reaction mixture and a droplet containing IgE the mixture was incubated for 2 minutes while the droplet was shuttled back and forth in order to obtain sufficient mixing. After incubation, the magnetic complexes were isolated and enriched in a daughter droplet according to the aforementioned procedure. Finally, the complexes were subsequently washed 3 times with buffer solution by alternating dispensing a buffer droplet, merging with the complexes, mixing and isolating the complexes. The fluorescence intensity of the complexes was monitored using an inverted fluorescence microscope, with the chip placed in a specially designed holder (Fig.1).

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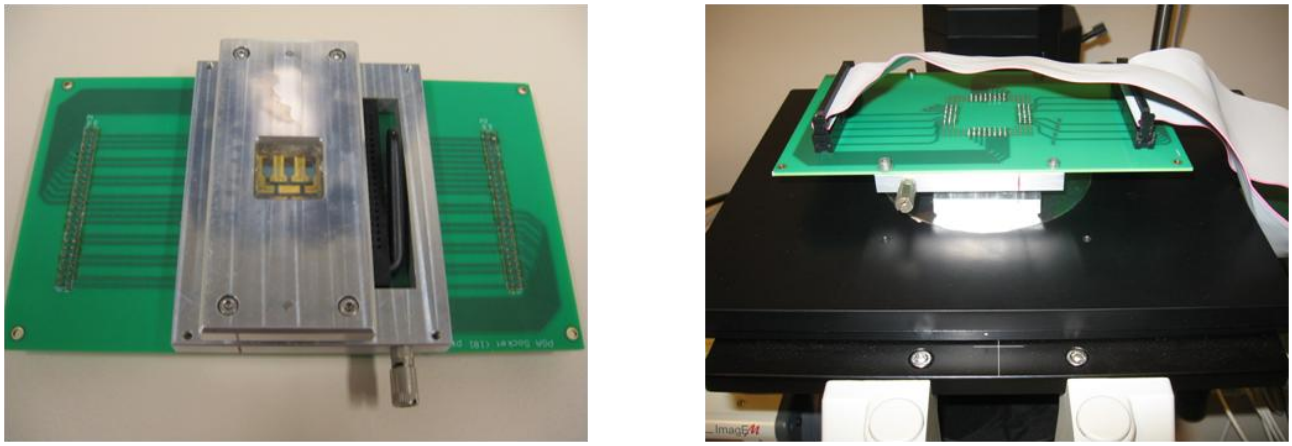

Figure 1: Image of the digital lab-on-a-chip placed in the specially designed chip holder (left) suitable for use on the inverted fluorescence microscope (right).

3. RESULTS AND DISCUSSION

3.1 Optimization of elementary droplet manipulations

To assure the execution of a high performance immunoassay, controlling the droplet size variability during the on-chip analysis is crucial. The overall assay variability is mainly determined by the variability in droplet size, caused by two elementary droplet manipulations: droplet dispensing and splitting. In order to keep this variability between narrow constraints, we developed a software based approach allowing the optimization of easy-to-control actuation parameters. Using logistic regression analysis techniques, three parameters were optimized with respect to droplet size variability: actuation time – i.e. time an electrode is activated –, relaxation time – i.e. time between two consecutive electrode activations – and the actuation voltage. Response data were analyzed by logistic regression, classifying the outcome of the droplet manipulation as good or bad. In the case of droplet dispensing multiple droplets were dispensed from a single droplet in the chip reservoir and when the droplet size variability was below a threshold of 5% CV the outcome was considered as successful. As seen from Fig.2a the droplet size variability decreases as the relaxation time and activation time increase. Voltage was not found to be statistical significant for the tested range (80 – 100 V).

An identical approach was followed to investigate the droplet splitting operation: multiple droplets were split and the same threshold was used to discriminate between good and bad splitting. Analogue to the dispensing operation, it was found that the activation time had a positive effect on the droplet size variability (Fig. 2b). The voltage had a positive influence as well. To make the model more significant an additional parameter was introduced in the regression analysis: electrode size. It was observed that splitting was more successful if the size of one electrode was maximal one third of the size of the droplet.

Based on these findings, an optimal set of actuation parameters was established and kept constant throughout the analysis: an actuation voltage of 100 V, an actuation time of 300 ms and a relaxation time of 30 ms.

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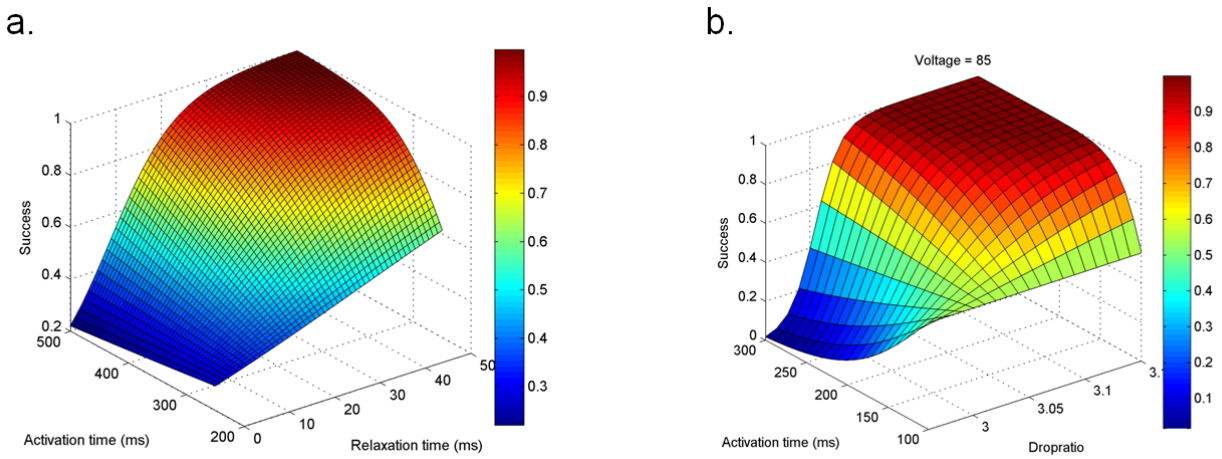

Figure 2: Output of two logistic regression models for droplet splitting (a) and droplet dispensing (b). The red and blue color corresponds to a successful and an unsuccessful event respectively. The 'Dropratio' parameter in b) expresses the ratio of droplet size to electrode size.

3.2 Droplet manipulations with superparamagnetic nanoparticles

Before the implementation of the immunoassay on the digital lab-on-a-chip the acquired set of optimized droplet manipulation parameters were verified and extended with solutions containing superparamagnetic nanoparticles. The first issue is to generate uniform droplets containing equal amounts of particles. It was demonstrated by Fouillet et al. that droplet dispensing caused a bias on microparticle concentration [11]. The variability on the microparticle (10 µm) concentration in the dispensed droplets increased when the initial particle concentration decreased. In order to explore the influence of droplet dispensing on the variability of superparamagnetic nanoparticle concentration, droplets were dispensed from a reservoir droplet containing different concentrations of fluorescently labeled (Venus protein) nanoparticles. As shown in Tab.1, the deviation of the particle concentrations remained constant irrespective of the initial particle concentration. This can be explained by the colloidal nature of the particles: the charge of the particles as well as the Brownian motion cause the dispersion to be stable.

Nanoparticle Concentration	Average Intensity (RFU)	Stdev (RFU)	CV (%)
1 mg/mL	1492	7,91	0,53
0,8 mg/mL	1299	62,46	4,81
0,7 mg/mL	1265	39,89	3,15

Table 1: Effect of initial nanoparticle concentration on the variability of nanoparticle concentration calculated for three dispensed droplets. Note that there is no statistical difference between the average fluorescence intensity of the two lowest particle concentrations because the fluorescence intensity was close to minimum detectable amount of fluorescence.

A second issue involves the washing efficiency on the digital lab-on-a-chip. In order to remove excess reagents and non-specifically adsorbed species, several washing steps need to be included. In these steps, nanoparticles have to be separated and enriched in the droplet. By applying an external magnetic field by positioning a magnet on top of the counter plate, nanoparticles aggregated at the boundary of the droplet near the magnet. Although a significant portion of the particles were attracted, some aggregates were settled down at the Teflon surface (Fig.3a). To collect these remaining particles, the droplet was shuttled forth and back in the presence of the magnet which resulted in the "sweeping" of all the magnetic nanoparticles together (Fig.3b). Shah and Kim pointed out that the van der Waals forces between the remaining aggregates and the Teflon surface can only be overruled by the much stronger interfacial force of the droplet boundary [6]. Once the particles were concentrated, the droplet was split thereby creating a daughter droplet enriched with magnetic nanoparticles (Fig.3c) and a daughter droplet depleted from magnetic nanoparticles. The separation

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efficiency of Venus functionalized particles is summarized in Tab.2. Besides the difference in fluorescence intensity of the two daughter droplets, the difference in amount of nanoparticle aggregates could be used as an indication of separation efficiency as well. This is due to the fact that the daughter droplets were not thoroughly shuttled after splitting which prevented some particles to resuspend. As can be seen from Tab.2, the overall enrichment efficiency is more than 95%, which is comparable to earlier reports [7,12]. Nevertheless, in order to fully assess the sense of magnetic nanoparticle enrichment in immunoassays, further studies are necessary to investigate the isolating efficiency of a target molecule with magnetic nanoparticles.

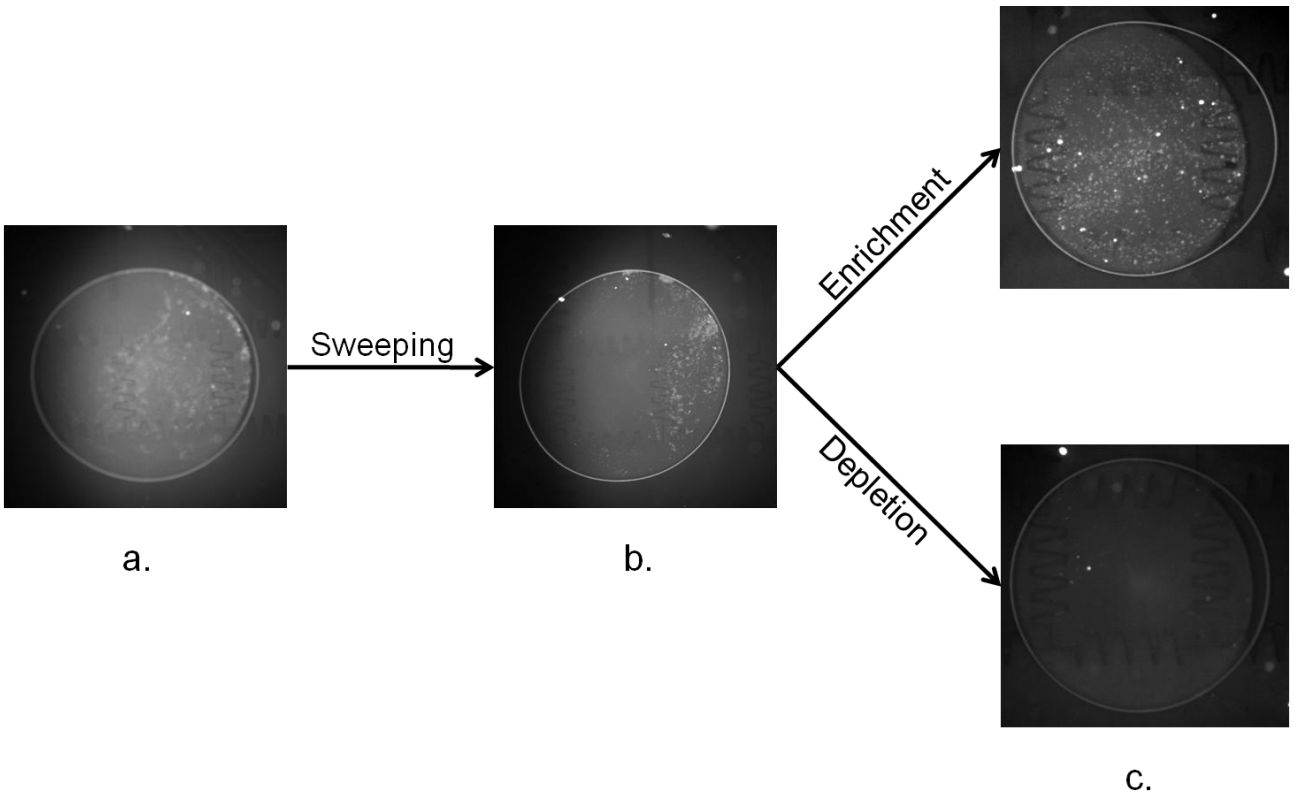

Figure 3: Magnetic nanoparticle enrichment procedure. After positioning the magnet on top of the counter plate, the particles started to aggregate and move towards the magnet (a). To collect all the aggregates, the droplet was shuttled back and forth so the droplet interface could sweep the aggregates together (b). Splitting the droplet resulted in the enrichment and the depletion of magnetic nanoparticles in the daughter droplets (c).

	Intensity ratio	**Aggregates ratio**
Run 1	96,16%	95,76%
Run 2	96,54%	96,39%
Run 3	96,24%	96,51%

Table 2: Enrichment efficiency of Venus functionalized superparamagnetic nanoparticles expressed as the fluorescence intensity ratio and aggregate amount ratio of the two daughter droplets.

3.3 IgE immunoassay

The potential of using superparamagnetic nanoparticles as a solid support in on-chip immunoassays is demonstrated by the implementation of an ELISA-based assay against IgE on a digital lab-on-a-chip. The main differences with a regular ELISA are the use of magnetic particles as solid supports for the capture antibody and the substitution of the second antibody by a FAM-labeled aptamer against IgE. The advantage of using aptamers over antibodies is the improved affinity and ease-of-use. In a proof-of-concept study the

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lowest detectable amount of IgE was established. After the execution of all the different reaction steps, the fluorescence intensity distributions for the different IgE concentrations were compared with the fluorescence intensity distribution for a blank sample. The measured fluorescence intensity resulted from both the magnetic complexes and the remaining amounts of unbound fluorescent labeled aptamer. A significant shift in peak intensity was observed for droplets containing at least 150 nM IgE proving the successful detection of IgE on the digital lab-on-a-chip, involving magnetic nanoparticles (Fig.4).

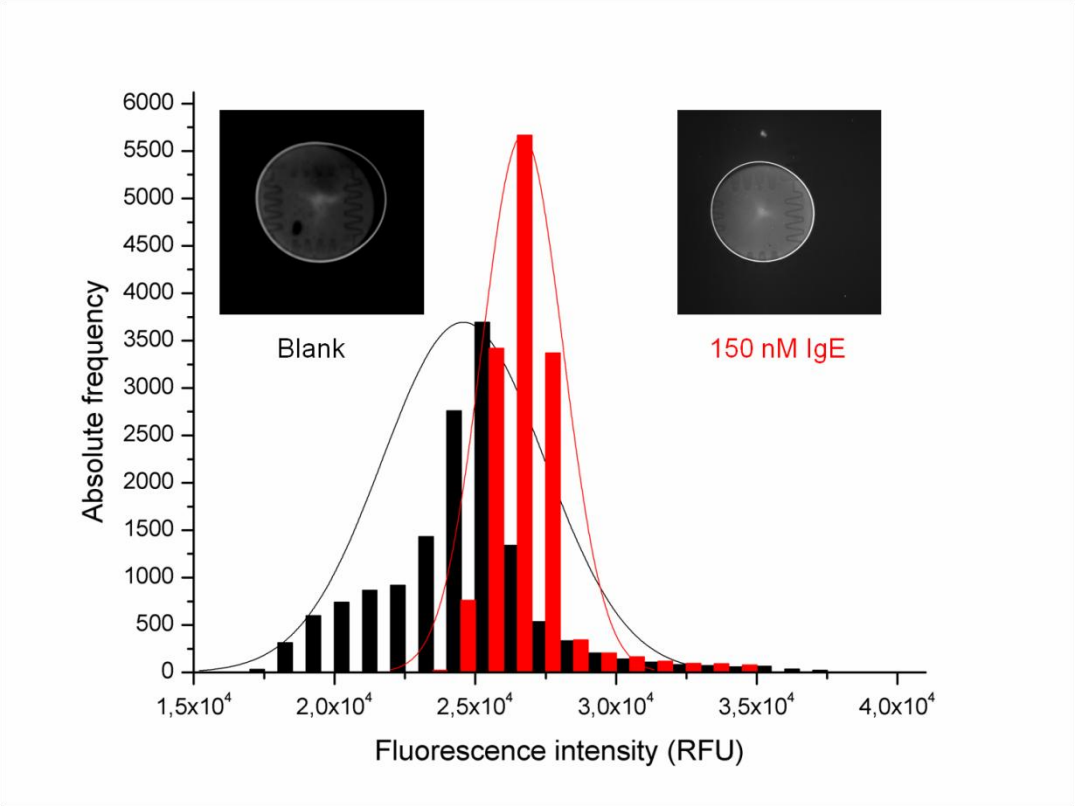

Figure 4: Overlapping distributions of the fluorescence intensity measured from a droplet containing 150 nM IgE (right distribution) and a blank droplet (left distribution). A shift in peak intensity is observed, indicating the successful detection of IgE with the on-chip ELISA-based assay.

Despite the successful implementation, several steps have to be undertaken to improve the analytical quantification of biological relevant concentrations (0.1 nM). First, the magnetic bead washing was still insufficient to discriminate between narrow ranges of IgE concentration (results not shown). Taken into account the limited amount of washing buffer present on the chip (no openings are present in the counter plate to add extra liquid), only three washing steps were possible in the current protocol. Because the droplet size remained constant throughout the whole sequence, this resulted in a 2^3 dilution of the unbound aptamer. Increasing the number of washing steps by decreasing the droplet size should be considered as a possible strategy to overcome this problem.

Secondly, the detection capabilities of the fluorescent labeled aptamer are insufficient compared to the detection limit obtained with a commercially available ELISA-kit for IgE (ALPCO, USA) (Fig.5). The calibration curve for the commercial kit indicates that IgE concentrations down to 0.01 nM can be detected (Fig.5a). In contrary, the lowest detectable concentration of aptamer was a hundredfold larger (Fig.5b). Given the 1:1 stoichiometric ratio between the fluorescent labeled aptamer and the captured IgE, the detecting capabilities of the assay are limited to a concentration of 1 nM. In order to compete with the commercial available ELISA kits, improving the detection limit is imperative. The use of alternative fluorescent labels like fluodots – i.e. fluorophore doped nanoparticles – will be investigated to improve the analytical performance of the method.

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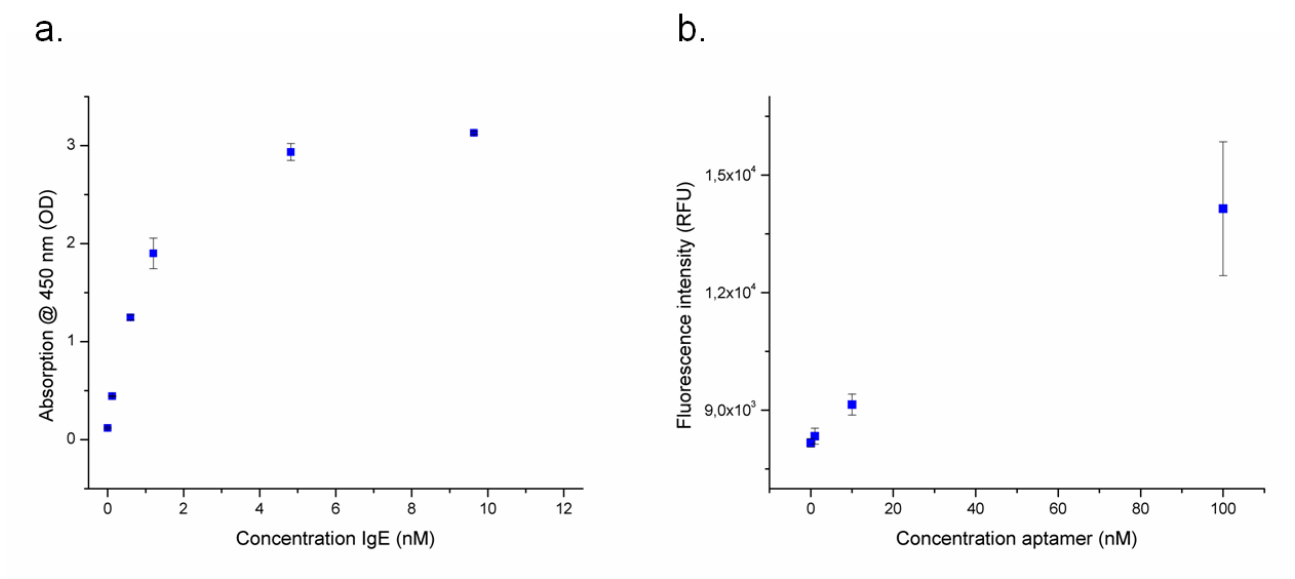

Figure 5: Comparison of the (A) calibration curve obtained from a commercial available IgE ELISA kit and (B) the calibration curve for different concentrations of FAM-labeled aptamer. Note that the absorption signal of the ELISA kit starts to saturate at 5 nM while the dynamic range of the assay is a tenfold larger.

4. CONCLUSIONS

In this paper a proof of concept study was presented involving the implementation of an ELISA-based immunoassay using superparamagnetic nanoparticles on a digital lab-on-a-chip analysis platform. Theoretically, the use of these particles should enhance the reactivity of the immunoassay due to the high surface-to-volume ratio. In addition, the nanoparticles are a perfect candidate for use as a solid phase carrier in digital lab-on-a-chip immunoassays, eliminating the need for complex surface functionalization of the chip. A first requirement to assure a high analytical performance of the immunoassay is the accurate control of droplet size variability. This was achieved by a software-based approach optimizing droplet actuation parameters like activation time, relaxation time and actuation voltage by means of logistic regression analysis. Using these parameters uniform droplet sizes throughout the analysis were assured with a maximal coefficient of variation of 5%.

Subsequently, the set of elementary droplet manipulations was verified for manipulations involving magnetic nanoparticles. It was found that the interfacial tension of the droplet was ideally suited to collect aggregated magnetic nanoparticles in the presence of an external magnetic field. The subsequent enrichment of the particles by splitting the droplet into two equal daughter droplets showed an efficiency of more than 95% (irrespective of the initial nanoparticle concentration). With respect to the dispensing operation, equal amounts of nanoparticles were retrieved in the multiple dispensed droplets, indicating a lack of bias in droplet dispensing.

Finally, it was shown that samples containing significant amounts of IgE (150 nM) were differentiated from blank samples with the integrated immunoassay based on IgE functionalized magnetic nanoparticles and a fluorescent labeled aptamer on the digital lab-on-a-chip platform. Nevertheless, improvements in washing protocols and sensitivity are imperative to detect biological relevant concentrations. Work is in progress to use fluodots as alternative detection labels while the washing efficiency is increased by scaling down the microfluidic device.

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