DEVELOPMENT OF A MICROFLUIDIC SAMPLE PREPARATION SYSTEM FOR BACTERIA MAGNETIC LABELLING AND CAPTURE IN CLINICAL SAMPLES

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Abstract

Hospital infections represent one of the novel health care challenges of the new century and rapid, economic and efficient technique for their detection are essential for rapid isolation of the patient and limited diffusion. Magnetic cytometry represents an emerging platform for bacteria detection, but complexity of the sample matrix and necessity of bacteria cell labelling require a preparative step. In the frame of a LoC (Lab on Chip) device PoC-oriented (Point of Care), such sample pre-treatment must be integrated in the cytometer and thus realized as a microfluidic component. Needed steps are bacteria labelling with magnetic particles and their concentration in a smaller volume, while unwanted components of the original sample, as other bacteria or residues, are discarded; labelling requires that recognition elements (here antibodies) specific for the target bacteria are immobilized on magnetic particles.

As concerning the following study, a bench-top assay for antibodies immobilization and bacteria capture and concentration has been optimized. A PDMS based microfluidic device, in two variants, have been produced and the parameters from the previous optimized protocol, such as ratios between antibodies, particles and bacteria have been applied to automatize sample pre-treatment through a new assay based on the device.

Devices ability on bacteria labelling and concentration have been evaluated and compared with results from bench-top assay, verifying if the former could be a substitute for the latter.

Microfluidic devices have been tested with spiked samples at different concentrations, as controlled solution of target bacteria in buffer, and with hospital samples, representing all the complexity of the real matrix. Efficiency in processing spiked sample is quantified and a mean capture efficiency of 86% ± 9% is obtained at a concentration of $2*10^5$ CFU/ml, while the lowest concentration capture with reasonable difference between positive and negative control is $2*10^3$CFU/ml. Processing of hospital sample offered limited results due to high variability in sample concentration and limited tests, even though capture is observed and qualitative results are promising.

The experimental work present in this thesis have been accomplished at Instituto de Engenharia de Sistemas e Computadores–Microsistemas e Nanotecnologias INESC-MN laboratories, in collaboration with Instituto Superior Técnico (IST), in Lisbon, Portugal.

Keywords: sample preparation; sample pre-treatment; microfluidics; hospital sample; labelling; capture; immunomagnetic separation

1. Introduction

In recent years, miniaturization and implementation of detection systems in portable device have grown, with a major impulse in chemical and biological detection [1]; innovative systems have been developed with the aim of discriminating the analyte of interest from its surroundings, with an attempt to ensure the lowest limit of detection while maintaining high accuracy and specificity. All with the aim to develop a device which guarantees what WHO (World Health Organization) defined as the goal for PoC-LoC (Point of Care - Lab on Chip) systems, namely the ASSURED criteria, or Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end users [2]. Such trend is not entirely mirrored in the area of sample pre-treatment, which contains all the sample processing required, previous to the detection, to ensure minimal interference with the latter. As a matter of fact, sample matrix, defined as all the components forming the sample except for the detection target, on numerous times may have a negative effect on the interaction between analyte and sensor, and their elimination is advised, if not essential.

One limiting factor is related to the tendency of many sample processing steps to be performed off-chip [1], thus hampering complete integration of a device, while representing a high-cost, time-consuming and equipment-dependent [2] feature. Sample pre-processing is typically represented by filtration, centrifugation, dilution, mixing, target amplification and extraction [1]; in addition, any sample treatment process must guarantee that analytes are present in a form compatible with the detection system, or alternatively, such approach limits the type of detection method that can be applied downstream. Besides, small volumes for sample and reagents, in the order of pL to uL are sought in most miniaturized systems [1].

In order to guarantee all the above mentioned characteristics, microfluidic implementation represents the best approach to achieve the development of PoC-LoC device which integrates sample preparation and target detection. As a matter of fact a microfluidic system generally provides reduced sample/reagents consumption, minor cost requirements per single analysis, reduced power consumption, low contamination risk, improved sensitivity/sensibility and high reliability [3]. Fabrication of the device can be achieved with well characterized photolithographic and soft-lithographic techniques, which include the formation of a mold that can be repeatedly used; PDMS is the most chosen material for the final device given, among others, properties as flexibility, optically transparency, and biocompatibility [3].

When designing a similar complete system, sample preparation and detection inter-dependence cannot be neglected, as a result their characteristics have to be taken into consideration in a choral approach.

Specifically, Soares et al. at INESC-MN [4] [5] have been studying and developing a magnetic cytometer platform, oriented to bacteria detection in hospital sample as part of a project for rapid hospital bacterial infection detection. The role of this work is thus to cooperate and support such platform, designing, producing and testing a coupled microfluidic device for sample preparation, applied to bacteria solutions and refined for resuspended rectal swab samples. This coupling defines the requirements the device must fulfil. Firstly, given the absence of natural magnetic activity of biological samples, target bacteria cells need to be labelled with magnetic particles. This effect is achieved in the device by mixing a solution of superparamagnetic particles covered with a specific recognition element, namely antibodies, with the solution containing the target, eventually represented by the resuspended swab. Secondly, the analyte requires to be separated by the complex matrix where it is contained and it also requires to be concentrated in a smaller volume. The retaining of magnetic particles, along with connected bacteria, is implemented to fulfil such demand.

Coupling together the sample preparation method here described with the detection method developed by Soares et al can potentially provide an answer for the demand of a fast all in one device able to detect bacteria infections. As for clinical samples, represented by blood, urine, saliva or different collected body fluids, a significative obstacle is represented by matrix complexity, whose components are able to affect the efficiency in detection or in other steps of the process. For example, contained lipids can interfere with antibody/antigen interaction. For such kind of matrix pre-treatment steps as sample collection and storage, separation, extraction and

concentration are generally required [2], as proposed by the discussed system. In addition, limited time is given for the detection, in order to achieve patient rapid isolation if needed.

Researched characteristics are thus 8h processing time (to be compared to the 24 to 72h needed with classical culture-based methods already in use), all in one approach with the ability to include sample pre-processing, Point Of Care (PoC) solution integrating all the step in a single Lab On a Chip (LoC) device, ability to deal with complex biological samples, high sensitivity (very low number of false negative) and specificity (reduced false positives, even if less relevant for the possibility of further, longer, detection method) and able to process sample volumes in the order of 500ul.

2. Materials and Methods

2.1. Materials

Benchtop protocol

Buffers: PB (pH=7.4; 0.1M; Disodium phosphate from FisherScientific), PB-T20 (pH=7.4;0.1M; Tween20 from FisherScientific), LB agar (40g/L_{H2O}, FisherScientific), LB broth (25g/L_{H2O}, FisherScientific), SuperBlock(PBS)T20 (FisherScientific), BSA5% w/v(pH = 6.5 to 7.5, FischerScientific, sterilized by filtration: Whatman GE healthcare 0.2um sterile)

Flow hood (Scanlab Mars from Labogene), magnetic column (dynamag-2 invotrogen), agitator (ika MS 3 basics), incubator (Heraterm by ThermoFisher)

Beads (Bio-Adembeads Streptavidin plus 0322 200nm, $3.8*10^{11}$ particles/ml)

Antibody stock: ab69468 Rb pAb to *Klebsiella spp* (biotin), ab68539 Rb pAb to *Pseudomonas aeruginosa* (biotin) from AbCam, both at concentration equal to $1.6*10^{16}$ Abs/ml.

Bacteria (kindly provided by Tecnophage): *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

Hard Mask production

Glass (50*50 mm^2, 0.7mm thick) covered in aluminium (composition: Al98.5Si1.0Cu0.5, thickness: 3000Å)

Lasarray DWL 2.0 (Direct Write Laser system, Heidelberg), Nordiko 7000, SGV track

PFR7790G27cP positive photoresist, TMA283WA PR Developer, TechniEtch Al 80 MOS Aluminium Etchant

Buffers: IPA (LabChem), Alconox(LabChem), Acetone(LabChem), DI water

SU-8 production

Silicon substrate (60*60 mm^2, 0.7mm thick)

Flow hood (Faster BSC EN2.6), Spin coater (Technology Corporation model WS 650MZ 23NPP lite), UV exposition setup (lamp from UV light technology), Hot plate (Torrey Pines scientific), degasser (Bel Arts Products model 1 800 4)

SU-8-50 negative photoresist (Microchem), PGMEA SU-8 developer (MW=132.16g/mol, SigmaAldrich), FDTS (SigmaAldrich)

Buffers: IPA (LabChem), Alconox (LabChem), Acetone (LabChem), DI water

PDMS production

DMS monomeric solution (Sylgard 184 Silicone Elastomer Kit, Dow Corning), Fossil 184 silicon elastomer curing agent)

Oven (Memmert model 100-800)

Punch pen for inlets/outlets (WPI 1.25mm plunge)

Glass substrate for sealing (50*50 mm^2, 0.7mm thick)

Buffers: IPA (LabChem), Alconox (LabChem), Acetone (LabChem), DI water

Microfluidic testing

2xMechanical pumps (NewEraPumpSystem model NE300), Vortex (FisherBrand)

1ml syringes (Codan), connectors LS20 20ga*1/2 inch (Instech), Tubing BTPE90: Polyethylene, internal diameter 0.86 mm, external diameter 1.27 mm (Instech)

BSA5% g/v (pH = 6.5 to 7.5, FischerScientific, not autoclavable, sterilizing filter: Whatman GE healthcare 0.2um sterile), Ethanol 70% v/v

2.2. Methods

Immobilization protocol

Immobilization refers to the process of covering the superparamagnetic particles with the proper amount of antibodies in order to be later exploited to recognize and capture the target bacteria. A specific ratio of antibodies per particle is needed to maximise the efficiency in interaction with the bacteria.

The immobilization has been performed on a variable number of samples per experiment, depending on the amount of beads required, and always under sterile conditions as guaranteed by the flow hood. For a single aliquot of 100ul particles covered with antibodies, needed for the

capture of 100ul of bacteria solution, 10ul of beads from stock are resuspended in 100ul PB-T20 in an Eppendorf tube, and further washed two time in the magnetic column, while supernatant is discarded. The pellet formed on the wall of the tube due to the magnetic force of the column is then resuspended in 100ul PB-T20.

After, antibodies are added to the particle solution, in a volume of 1ul from the stock.

Once antibodies are added, the solution is incubated at RT for 2h under 250rpm agitation.
Solution is later washed again and resuspended in 300ul SuperBlock™ or 5% w/v BSA solution in DI water; this represents the blocking step.

Follows incubation of the sample at RT for 1h under 250rpm agitation.

A final wash of the solution is then performed, with resuspension in 200ul PB added to 100ul SuperBlock™ or 5% w/v BSA solution in DI water. The solution is finally stored overnight at 4°C.

Before being used to perform a capture protocol in a bench-top assay or in the microfluidic device, particle solution is washed twice and resuspended in 100ul PB.

Bacteria inoculum preparation

In order to be able to test the efficiency in the prepared magnetic particle solution with immobilized antibodies to interact with bacteria, a solution of target bacteria at wanted concentration is needed. Bacteria are grown following standard laboratorial procedure. All the procedures are performed under sterile conditions, in a flow hood. Target bacteria stock is stored at 6°C in a Petri dish filled with LB agar media; one colony is moved from the stock to a falcon tube containing 5ml LB broth media and incubated overnight (16h to 18h) at 37°C under 250rpm agitation.
After first incubation, 155ul of the solution are added to 4845ul of LB broth media in a falcon tube and incubated a second time at 37°C under 250rpm agitation, for 1h in the case of *Klebsiella pneumoniae* and for 1.5h in the case of *Pseudomonas aeruginosa*.

The volume of 155ul is chosen as average between volume computed in different experiments in which OD_{600} was measured; this parameter, in fact, is related to the concentration of bacteria in a solution.

Protocol for bench-top capture

This protocol represents the bench-top assay performed as a comparison with the protocol performed in the produced microfluidic device, as presented later. It involves the mixing of particles with immobilized antibodies and bacteria solution and retention of the labelled bacteria, and are thus performed by the operator in a manual procedure.

Dilutions of the inoculum are prepared in order to obtain solutions at wanted concentrations, until a -7 dilution (or 10^{-7} dilution from original inoculum, with expected concentration of $2*10^1$=20 CFU/ml) is reached.

Beads solution with immobilized antibodies is washed and resuspended in 100ul of bacteria solution from the tested concentration, depending on the aim of the single experiment.

Sample is incubated at RT for 15min at 250rpm.

The solution is then washed 2 times, but supernatants are not discarded and are collected instead, as they represent the solution containing bacteria that have not been proficiently captured; at every wash, the pellet is resuspended in 100ul PB. After such washing step, three samples are obtained: the first supernatant, called SB, the second supernatant, called WI, and the resuspended pellet, called CM. If three washes are performed, an additional supernatant, called WII, is present.

Microfluidic device fabrication

The role of this section is to present to the reader the mean by which the microfluidic device production can be achieved, thus exploiting the already mentioned soft lithography technique.

This process involves three main steps, the production of a hard mask, the creation of a polymeric mold and finally the realization of the microfluidic device itself; this steps are preceded by another essential component of the process, the software device design.

Here a summarized version of the production protocol is presented and reported parameters refer to the final optimized protocol.

Hard Mask

All step reported in the section are performed inside a cleanroom facility *(class 10000 and class 1000)*.

Initially a glass slab of 0.7mm thickness is cut with dicer in order to obtain squares of $50*50mm^2$. After properly cleaned in Alconox (1h at 65°C under ultrasounds) and rinsed with IPA, DI

water and blow dried, metal (Al98.5Si1.0Cu0.5 wt%) is deposited on its top with a thickness of 3000Å in Nordiko 7000. Positive photoresist PFR7790G27cP is then spin coated at SGV track on it to obtain a ~1.45um thick layer. Follows a soft bake at 85°C for 1 min.

Substrate is after exposed and photoresist patterned with DWL(Direct Laser Writing) tool at a wavelength of 442 nm based on NeAr laser. Photoresist development is performed at SGV track and consists of a baking step at 110°C for 1 min, cooling for 30s and contact with developer TMA283WA for 1 min.

Once the pattern is developed and metal uncovered in selective areas, etching of the metal layer is performed. Substrate is immersed for 5 min in TechniEtch Al 80 MOS Aluminium etchant at RT and under manual agitation, then washed in DI water. To complete the procedure, photoresist removal is achieved by acetone wash (2 min at RT under manual agitation) and cleaning with IPA, DI water and blow dried.

SU-8 mold

Following steps are performed inside a cleanroom facility *(class 10000)*.

Silicon substrate 0.7mm thick is manually cut as a square of 60*60mm^2 and cleaned in Alconox (1h at 65°C under ultrasounds) and rinsed with IPA, DI water and blow dried. Surface is lately treated with oxygen plasma for 1min at a pressure of 800mTorr and with an RF power of 11W in Plasma Cleaner (model PDC 022 CE from Harrick Plasma) and moved out from cleanroom facility while properly stored in a clean plastic box.

Following steps are performed inside a flow hood (Faster BSC EN 2.6).

Sample is baked at 110°C for 5 min to be dehydrated on hot plate(Torrey Pines Scientific) and cooled down to RT. Follows negative photoresist SU-8-50 spin coating in order to reach a nominal thickness of 100um. Soft baking of the sample is performed at 65°C for 10 min and 95°C for 30 min on hot plate. After cooled down to RT, exposition takes place in proprietary tool from INESC-MN™ , based on a lamp filtered at wavelength of 365nm, exploiting previously produced hard mask; optimal exposure dose have been set to 166.5mJ/cm^2.

Post exposure bake is performed on hot plate at 65°C for 1 min and at 95°C for 10 min, following cooling down to RT.

In conclusion, sample is developed in PGMEA developer for 15 min at RT under manual agitation, rinsed with IPA to verify complete development and checked under microscope. If development is satisfying, sample is rinsed in IPA and blow dried.

PDMS production

PDMS is prepared by mixing DMS and curing agent in a proportion 10:1 (w/w) in a clean plastic cup, for an approximated mass of 15g of DMS and consequently approximately 1.5g of curing agent. The solution is manually mixed and degassed for 1h at low pressure in degasser (Bel Arts Products). When no air bubbles are present in the solution, it is manually poured in the previously fabricated SU-8 mold and baked at 70°C for 1h in oven (Memmert model 100-800).

Once removed from the oven, sample is let cool down to RT and manually peeled off from the mold with the help of a tweezer. A proper hole punch pen for desired measures is used to produce inlet/outlet holes in the device, which is later cleaned with DI water and blow dried to remove any residue.

A glass substrate of 50*50mm^2 area and 0.7mm thickness is cleaned in Alconox (1h at 65°C under ultrasounds) and rinsed with IPA, DI water and blow dried. Both glass substrate and PDMS are treated with oxygen plasma for one minute and finally put in contact with gentle manual pressure to achieve device sealing. Device is stored 24h before being tested.

Protocol for microfluidic capture

When the produced microfluidic device is tested, the following protocol is followed, depending if the sample tested is a simple solution of target bacteria in PB buffer at a controlled concentration, or if complex hospital samples are tested; in both cases, immobilization of antibodies on particles is performed as reported in previous section.

The following protocol are performed in sterile condition inside a flow hood.

Buffer based bacteria solutions

For device characterization, bacteria solutions in PB buffer at controlled concentration have been used as a sample, together with magnetic particle solution with immobilized antibodies, obtained as described before. Different sample concentration, as concerning bacteria, are tested.

At first, elution inlet is closed by a proper metallic rod, while mixing inlets and outlet are connected with suitable tubing. Tubes are then connected to syringes through proper connectors, which are inserted in the mechanical pumps. Figure 1 shows the discussed set up

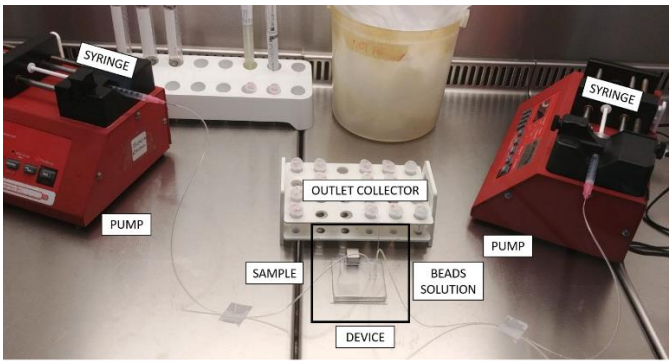

Figure 1 - Set up of the device during testing

The device is first sterilized with 200ul an ethanol solution 70% v/v in DI water at a flow rate of 50ul/min (per single inlet). After, a washing step is performed with 200ul DI water at flow rate of 50ul/min (per single inlet).

To conclude the preparation steps, system is primed with 100ul of a blocking solution, SuperBlock™ or 5% v/v BSA in DI water, at a flow rate of 50ul/min (per single inlet); volume is completely released and discarded before next step is started.

Mixing and capture/retain step are then performed. The magnet is positioned in the suitable location, close to the chamber, and 100ul of magnetic particle with immobilized antibodies are injected from one inlet, while bacteria solution sample is injected from the other, at a flow rate of 10ul/min. The outcoming volume is collected in an Eppendorf tube and marked as non-retained (NR), until the whole volume is released.

Once completed, elution step can take place; mixing inlets are closed with proper metal rods, while elution inlet is connected to a tube, and 40ul PB are injected in the microfluidic device at a flow rate of 10ul/min, filling the elution channel, chamber (where particles are retained) and outlet channel, as well as part of the outlet tube.

Magnet is now removed, and a time interval of 5 min is waited in order to allow particles to resuspend in the buffer.

The solution in the device is then released at a flow rate of 100ul/min to limit particles depositions at the outlet, and collected in an Eppendorf tube as retained(R).

Preparation steps comprising of sterilization, washing and priming of the device can be performed again if a new sample is wanted to be tested; the reusability of the device is simply for testing purposes, as final device applied to real samples is meant to be disposable, at least for the component discussed in this section.

Hospital samples

Hospital samples are stored at 6°C before being tested. The swab is stored in a gelatine-based matrix and thus needs to be resuspended in a liquid buffer before being tested in the platform. Resuspension is obtained in an Eppendorf tube filed with 500ul PB and the solution is then homogenized with vortexing at 1600rpm. 100ul of the solution are thus separated and will represent the tested sample. All further steps are equivalent to protocol reported in the previous section related to buffer based bacteria solution.

The microfluidic device is disposed after a single test and autoclaved to ensure sterilization.

Plating

After samples are collected from different washing steps, as regarding the bench-top assay protocol for bacteria capture, or as non-retained and retained solution, for microfluidic device testing, the concentration of bacteria in each sample have be assessed; at the same time, also the initial concentration of bacteria, present in the original sample which is the specific inoculum dilution or the resuspended hospital sample swab, depending on test, is wanted to be quantified.

Two plating methods have been used, here referred as “full dish” or “droplet method”; in the former 100ul from the sample are poured on the dish and distributed on the whole surface through a disposable spreader. In the latter, a 10ul drop from the sample is poured on the upper part of the dish, which is then inclined to allow the drop to flow vertically and spread along a vertical line; from 4 to 6 drops per dish can be plated, from different samples.

As for the “full dish” approach, a colony count comprised between 30 and 300 is considered reliable [6]; as for the “droplet method”, the writer accepted a colony number per drop from 10 to 100 as reliable, given comparison with example of “full dish” plates from the same sample.

In order to be able to guarantee that plated concentrations are in the range of reliability as explained above, different dilutions of the same

sample are always plated, commonly up to 5 dilutions, but depending on different prediction that can be done on the expected concentration in the sample. Serial dilutions are prepared mixing 20ul from higher concentration dilution into 180ul PB in an Eppendorf tube in order to obtain serial dilution of 1/10.

After plating procedure, Petri dishes are incubated at 37°C overnight (16h to 18h) with no agitation.

Depending on the experiment, duplicates or triplicates are produced to confirm results.

2.3. Simulations and designs

Simulations, performed on COMSOL Multiphysics™ software, are based on a simplified structure respect to the design that the devices will acquire; in fact only two inlet channels disposed in a Y configuration with a 90° angle, and a serpentine composed of 20 turns is studied, while obstacles occupies 3 or 4 turns only.

Figure 2 represents mixing of magnetic particles(red) and of bacteria(green) at the serpentine of the simulated microfluidic device. Distribution of the particles along the channel width in the area of obstacle concentration is represented; Figure2.A and C are characterized by particle segregation and their respective side of the channel, while an interesting effect, as predicted by Bhagat et al. [7], is verified in B and D, where obstacles modifies particles trajectories and deviates their flow to the center of the channel, thus increasing the chance of interaction between the two specie; in both two configurations, though, this tendency of concentrating at the center of the channel is not maintained along the serpentine and particles are still dispersed at the outlet.

This behaviour is amplified in the configuration in Figure 2.E, where the effect of the modified shape of the obstacle, with triangular edges substituted to curved one in order to avoid Coanda effect , is to force particle convergence to the center of the channel and reach the outlet in this configuration. This configuration could be efficient in increasing interaction events between the magnetic beads and the bacteria, thus promoting recognition and labelling.

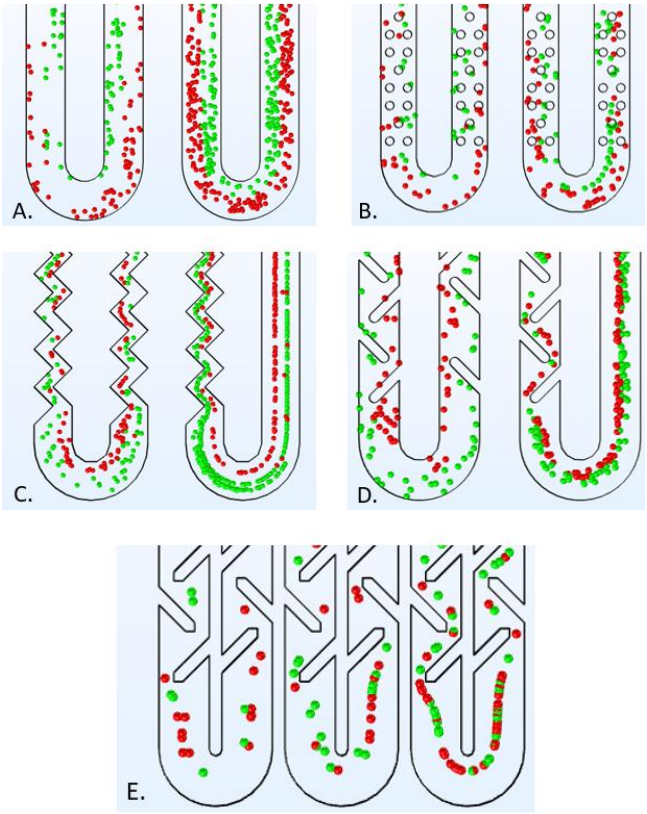

Figure 2 - Distribution of beads(red) and bacteria(green) along channel width when close to obstacles (A) No obstacles (B) Alternated cilindrical pillars (C) Triangular obstacles at the channel walls (D) Rectangular obstacles at the channel walls with curved end (E) Rectangular obstacles at the channel walls with sharped end

According to what discussed in the simulation and the information provided by previous studies found in literature [7] [8] [9], two designs are chosen to be verified by experimental tests, as reported in Figure 3 and Figure 3.

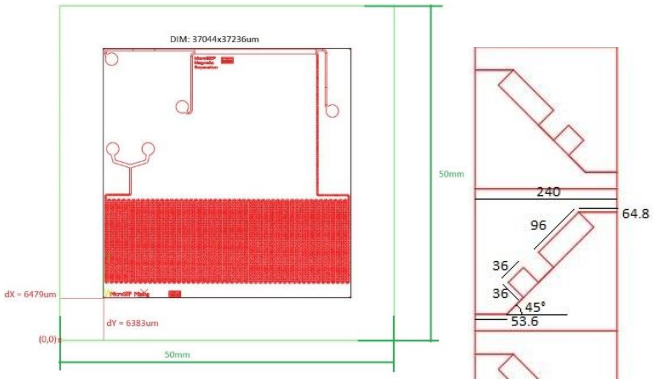

Figure 3-CAD file of the first design of the microfluidic device produced, on the right zoom on channel and obstacles. (all measures are in um if not differently specified)

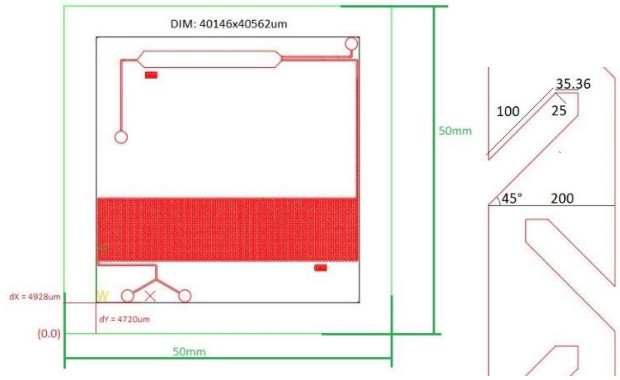

Figure 4 - CAD file of the second design of the microfluidic device produced, on the right zoom on channel and obstacles. (all measures are in um if not differently specified)

3. Results

3.1. Fabrication

Results on fabrication process are reported for the first design only, with similar conclusions for the second.

Figure 5 displays results obtained for the fabrication of hard mask, SU-8 mold and final PDMS device respectively, when optimized parameters have been used.

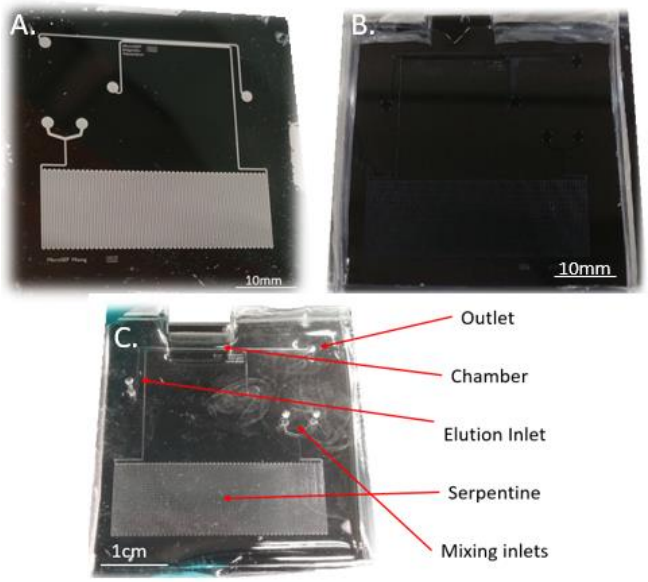

Figure 5 – A. Hard mask; B. SU-8 mold; C. final PDMS device;

Figure 6 shows microscope images representing cross section of the final PDMS device, with a focus on the obstacles. These in fact are the most delicate elements to be produced and here optimal results are commented; as seen, obstacles are well defined and their walls, as wells as the ones of the channel, are vertical and of approximatively the expected thickness, namely 100um.

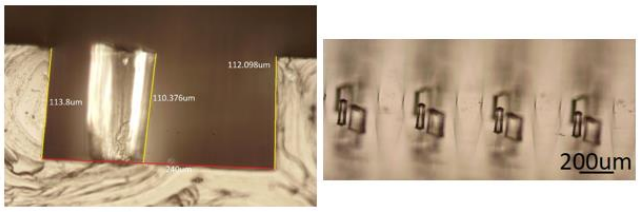

Figure 6 - Microscope images of PDMS device for the first design, sectioned transversally: left, inclined at 90deg respect to line of sight; right, inclined >90deg respect to line of sight

3.2. Microfluidic and Bench-top comparison

In order to evaluate the efficiency of the device, it was initially tested parallelly to bench capture, using same covered beads stock in order to exclude a priori differences in beads coverage that could be obtained based on a day to day variability.

By the modality the protocol is established the non-Retained volume is compared with the supernatant obtained in bench-top capture method, where a magnetic column was applied. Similarly, the retained volume consists of all retained beads and so is to be compared with the magnetic capture sample obtained in bench, as defined in methods section.

Table 1- Summarized results from comparison in capture efficiency for bench in 2 microfluidic design devices. High Beads Concentration (HBC) Is equal to $3.8*10^{10}$ beads/ml and Low Beads Concentration (LBC) is equal to $1.9*10^{10}$ beads/ml .

			Mean (%)	SD(%)
Bench		Positive (specific)-HBC	86.12	9.46
		Negative(Aspecific)-HBC	4.49	2.62
Microfluidics	Design 1	Positive (specific)-HBC	100.00	0.00
		Negative(Aspecific)-HBC	20.28	12.29
		Positive (specific)-LBC	65.48	14.84
		Negative(Aspecific)-LBC	1.08	0.49
	Design 2	Positive (specific)-HBC	98.57	1.27
		Negative(Aspecific)-HBC	4.91	2.23

Results from 3 independent experiments with 2 dependent replicas each verify that capture reached in the microfluidics is comparable, if not superior, to results in bench-top capture in bench. Mean values can in fact been compared with average capture value from bench capture as

showed in Table 1, reporting 86% ± 9% for positive samples and 4% ± 3% for negative samples, compared to 77%±21% and 99%±1% for positive samples and 7%±11% and 5%±2% for negative samples obtained in the microfluidic device.

3.3. Evaluation of Limit of Detection

Concentrations as low as 20 CFU/ml have been tested for the first design of the microfluidic device, in a volume always equal to 100ul, which guarantees in the lowest concentration samples the presence of 20 bacterial cells in average.

As regarding the protocol of the experiment, it was maintained equal to the one presented in the previous section.

As shown in Figure 7 capture efficiency decreases as the concentration of the sample decrease, behaviour expected as the reduced number of bacterial cells increases the probabilities that such few cells do not enters in contact with enough particles or for a long enough time, as well as the possibility that the few cells, even if surrounded by magnetic particles, are not retained by the magnet and escape the magnetic field imposed. In addition, even if limited, some loss of bacteria cell in the channel occurs, and when dealing with such reduced number, the probability that the few cells present do not reach the outlet is high.

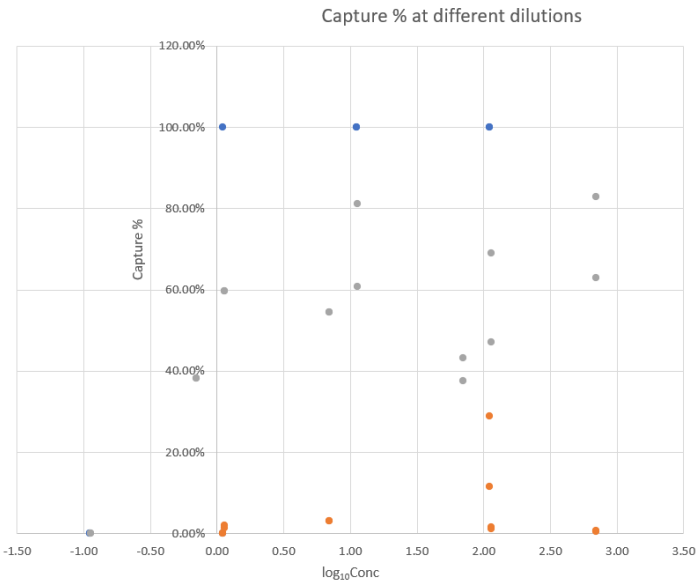

Figure 7- Graph on results for capture of bacteria Klebsiella pneumoniae at different dilutions; Concentration as CFU/ul. Blue dots: Positive high concentration beads; grey dots: Positive low concentration beads; orange: Negative

Even if at very low concentration as 200 CFU/ml capture is not efficacy, concentrations as low as 2 $*10^3$ CFU/ml shows, at least at samples constituted of a simple bacteria solution in a buffer, allows an appreciable capture, roughly quantified as above 50%. Such capture efficiency is present only when antibodies specific for the bacteria are immobilized on the beads, demonstrating specificity in the capture, where negative/aspecific samples shows consistently reduced capture efficiency.

3.4. Hospital samples

With the aim to evaluate the effect of the complex matrix and so deal with a sample that could represent all of the difficulties in real sample treatment, 5 rectal swabs have been tested and the ability to retain target bacteria evaluated, once again taking as model a device based on the first design.

First sample to be tested has been a spiked sample; a positive hospital sample have been verified through plating method to assess the presence of resistant *Klebsiella pneumoniae*, as expected, and eventually quantify it. Unfortunately, possibly due to long storage time, no resistant bacteria were evidenced, while a consistent amount of other bacteria were found when plated in LB agar media Petri dishes. Since the resuspended swab, even if lacking of resistant bacteria to be targeted, represented a realistic sample as for physical properties, equivalent to any other positive hospital sample, it was decided to spike it with one colony of resistant *Klebsiella pneumoniae* cultivated in a different dish filled with selective media, result of a previous experiment.

Result from processing of the discussed sample in the produced device are reported in Table 2; the low capture efficiency of 1% can be explained by an elevated initial concentration, out of the optimized range performed in previous section, which leads to a particle to bacteria ratio insufficient for correct labelling and capture.

Lately four more hospital samples covered the role of stabilizing the range of possible concentration to be expected; this demonstrated to be highly dispersed; all results are shown in Table 2

Table 2-Results from four hospital sample testing, as shown in ***Errore. L'origine riferimento non è stata trovata.*** *and* ***Errore. L'origine riferimento non è stata trovata.***

Sample	Measured initial concentration (CFU/ml)	Captured	Initial – Non Retained
Spiked	$1.41*10^9$	1%	-
1st	$2.3*10^7$	Impossible to quantify	77%
2nd	$7*10^2$	none	71%
3rd	0	none	0%
4th	0	none	0%

Given the different concentrations, unexpected in such a wide range, testing was not quantitatively efficient in identifying the results, but gave important qualitative information; approach on quantifying the results for new samples needs to evaluate a wider range of dilutions to cover all possible concentration.

4. Conclusions

Optimized fabrication process achieved to produce devices with required characteristics in term of thickness, obstacles definition and sealing.

The realization and testing of a microfluidic device for sample preparation in the role of bacteria labelling and concentration have been discussed, and results compared with a previously optimized bench-top assay. It was demonstrated that the device can proficiently substitute the bench-top protocol as comparable, when not superior, capture efficiencies have been verified.

When tested with hospital samples, with the need to process a complex matrix and a high concentration of non-target bacteria, even if limited, results are shown promising, with capture obtained at expected concentration range, even though a quantitative analysis resulted impossible and further tests are required.

To achieve full automation, microvalves and micropumps, as well as a system for magnet decoupling, are required to be implemented.

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