Direct bacterial cell detection in a microfluidic chip

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Abstract

Direct capture and detection of bacterial cells is one of the aimed goals in the therapeutics field. One of the reasons behind this search is the increasingly incidence of Antibiotic Resistance, occurring due to an uncontrolled antibiotic prescription and incorrect use of this drug. Conventional diagnostic methods are cost and time consuming since, in the majority of the cases, rely on cultures and amplification approaches. In microfluidics there is the opportunity to miniaturize and mimic biological processes, promising the development of systems low-cost, with high sensitivity, ease of transport and time saving. In the development of this project, a microfluidic chip to perform direct capture and detection of Staphylococcus aureus was to be achieved using a porous bead-based system.

PDMS microchannels were the basis of the microfluidic system, fabricated using a soft-lithography technique and sealed against a PDMS membrane. Aptamers were acquired to be used as probes in order to perform a capture-detection sandwich, resourcing a biotin to allow immobilization and a fluorophore to detect. Different bead types were tested and one selected to assess the aptamers binding efficacy. Distinct dyes were used in the matter of evaluating if cellular viability was somehow compromised. A final assessment was established regarding the sensibility of physically capturing cells using solely the microfluidic chip packed with beads.

Keywords: Microfluidic system, beads, *Staphyloccus aureus,* aptamers, detection, capture.

Introduction

Antibiotics are considered one of the biggest discoveries that came across Medicine's history. In the majority of human existence, infectious diseases threatened its health and well-being [1]. Antibiotics arrived to change that path of the history's course.

Between 1950 and 1970, the majority of antibiotic classes were discovered and after this golden era, there were not others to be found, solely modifications of already existent ones, which gave space to bacteria resistance emergence and rise.[2][3].

Antibiotic Resistance (AR) is the designation attributed to the phenomenon of infectious pathogens to overcome the effect of drugs that were design to kill them in first instance, developing the ability to resist antibiotic's action. As all organisms, also bacteria are prone to evolve in order to adapt to the surrounding environment, gaining different mechanisms to sustain life and allow the species proliferation. [3][4][5].

The problem is antibiotic resistance has been having an increasingly higher incidence the past few years, existing more and more species that persist the drugs usage, on a rate that overcomes the natural evolutionary adaptability. This speeded up raising is converting antibiotic resistance in a true crisis, which can lead to the re-emergence of harmful bacterium, that were considered as trivialized upon antibiotic use, gaining ability to be successful in their goal. [3][6]. The reasons behind this are the overuse and misconception of antibiotics, which played and still play the alarming role on the crisis emergence.

It is natural to assume that the use of antibiotics contributes to its resistance, by eliminating certain bacteria and those who survive proliferate as result of natural selection, but when this use is uncontrolled, the resistance reaches critical levels [3][4].

There are various organisms characterized as multi-drug resistant, having the ability to survive to antibiotics or other antibacterial drugs action. Thus, a higher importance is given in trying to identify these organisms upon a infection or infectious disease, to set the correct course of action, avoiding ineffective treatments that can be time and cost consuming.

Nowadays, routine cultures are performed, from blood, sputum and urine. The samples are collected and cultured, allowing bacteria to growth and expressing their featured proteins. From that, a possible profile can be obtained in order to identify the pathogen/s present in the sample. Nevertheless, the need to culture the cells is a time burdening, considered an essential counter back in the patient's course treatment. The use of polymerase chain reaction (PCR) in order to amplify the samples DNA can also be used to identify a bacteria type and genus. Although it overcomes the time consumption issue from the cultured approaches, its sensitivity is not as ideal. [7][8][9]

Current approaches focus on phenotypical and genomically-based tests, used to achieve the goal of bacteria identification, such as Biochemical testing, Fluorescence In Situ Hybridization, PCR Techniques as already mentioned, Microarrays, among other.

The idea of having a mechanism able of avoiding the negative aspects of nowadays approaches is enticing and is also the main reason behind a vast range of studies.

The Microfluidic field has the potential to miniaturize several procedures avoiding large consumption of reagents and other materials involved on these processes. Even the more complex approaches can be scaled down. Microfluidics brings advantages, like cost and time efficiency adding the fact of be possible to use as a device, bringing ease to the user and to the means of transportation. This field can bring another view, perspective and innovative features to Antibiotic Resistance thematic and somehow be the tool to overcome some of the disadvantages of the current practices. [10]

Being multifaceted, there was the need of Microtechnology to be qualified according to its aim. It was in the 1980s that the field of Microelectro-mechanical systems (MEMS) began to emerge, but only later, in the 1990s, medical, biological and even chemical applications took their place in the field. Through fluidic manipulation under artificial conditions, where there is a scaled down to the range of micrometers. Microfluidics studies "… flows that are simple or complex, mono- or multiphasic...", according to Patrick Tabeling, on the "Introduction to Microfluidics" published by Oxford Press. [11][12][13]

Microfluidics present innumerous advantages that make its use suitable for a vast range of applications. Besides MEMS, there are also perks in the fields of electrical sensors and Lab

on a chip technology. Through its use it is possible to have a restrict control in the consumption of reagents, samples and materials needed on those applications, adding to low-cost fabrication, it should be easier for the user in terms of employment and transportation. For analyses and assays, adds the advantage of low time consumption face other techniques [10][11][14].

Current techniques in the field of microfluidics, base their detection into two distinct methods. Detection by immune-sensing, where a probe acts to target a specific molecule characteristic of a certain bacteria. In this case a probe can be selected and can be used to perform the bound antibody-antigen or aptamer-target. Another method is based on the detection resourcing nucleic-acid identification, other specific type of targets.

The use of probes, such as antibodies or aptamers as an agent of detection can also be used as means of capture and immobilization of the intend target, creating a "sandwich" ('capture molecule'-'target'-'detection molecule'), like the known ELISA approach. Regarding mechanisms to perform the detection, there is a vast range that include optical, electrical or electro-chemical impedance, cantilever, quartz crystalline microbalance,surface plasmon resonance (SPR), and magnetoresistivity, in means of the immune-sensing and resource to PCR or RT-PCR can be used if the intention is the amplification of the target nucleotides, increasing the signal to detect [16]. The use of fluorescence probes is shown to be very appealing due to the high specificity, which can be performed adding the promising sensibility of these methods [17]. Xiang et al. reported a microfluidic system for detection of Escherichia coli using laser-optical fiber fluorescence detection and Meagher et al. detail an integrated microfluidic platform fluorescence-based detection of Shiga toxin I (Shigella dysenteriae) and Staphylococcal enterotoxin B (Staphylococcus aureus) [18]. Electrical-based methods of detection are also appealing in what concerns ease-to-fabricate features, for instance Boehm et al. elucidates the presence of Escherichia coli in suspension by measuring its impedance [19]. Innovative new approaches are also keen to emerge. Terekhov et al. combined the use of a droplet system with FACS detection technique to develop a single-cell activity selection in the matter of cell screening, common to a "Single-cell microscopy of suspension cultures using a microfluidics-assisted cell screening platform", reported by Okumus et al. [20][21].

Antibodies (Abs) are molecules that belong to a family of globular proteins, nominated immunoglobins that are considered heterodimeric proteins. This class is characterized by having two pairs of chains, heavy and light and can also be divided and classified into different domains, variable and constant. Antibodies naturally belong to the Human's immune system and act as a defense against pathogenic agents. These proteins type are characterized by having a Y shape that acts as an advantage to be used as a detection mechanism. Its variable domain can recognize and bind to specific proteins present in the pathogens surface membrane, called antigens.[21][22]

Aptamers are sequences of nucleotides, that are able to target specific molecules and can be considered as single or double strands of DNA or RNA. Aptamers are part of a new approach in the therapeutic field, where nucleotides are used as functioning not only to store genetic information but also as enzymatic catalysis or even to bind to other molecules, proteins and cells acting similarly featured to the antibody-antigen interaction [21]. Thus, are passible to be used as to replace antibody's functions on the

therapeutic field, overcoming some of the challenges that are being faced, and also, used as mean to capture and detect pathogenic agents.

The objective of this project is the development of a microfluidic device capable of direct capture and detect bacterial cells from the bacterium model Staphylococcus aureus (SA) resourcing microbeads to increase the immobilization area. The capture aims to function due to the integration on the used beads, in the microfluidic chip, of labeled aptamers that in other approach are used as a detection tool together with fluorescence and imaging techniques.

Materials and Methods

Microfabrication: Soft Lithography

To fabricate Polydimethylsiloxane (PDMS) structures by a soft lithography technique it was necessary to use a mold with simple columns engraved on it. The master mold (SU-8) was already available for usage.

Regarding the elastomer fabrication, it was necessary to mix the PDMS base and the curing agent from the elastomer kit (Sylgard 184 silicon elastomer kit, Dow Corning), on a ratio of 10:1. The mixture should have an opaque white color with a bubbly consistence. Ready to be gazed-out, the mixture was left on the Vacuum desiccator (Bel-Art Products , South Wayre, NJ/USA) during 45 minutes. The pre-polymer was then ready to cured and should be poured in the Petry dish that had the SU8 mold, already available, that contained the columns were the assays were to be performed. An extra attention should be taken into consideration, of not leaving bubbles on the PDMS mixture, which can appear due to the pouring action. Next, the Petry dish was left in the oven (Memmert, Schwabach, DE), during 90 minutes at an average temperature of 70ºC. Cured, the PDMS structure was cut down and the inlets and outlets were open with different size needles. For the inlet, 20Ga blunt needles (Instech Laboratories, Inc., Plymouth Meeting, PA/USA), were used and for the outlet, 17Ga blunt needles (Instech Laboratories, Inc., Plymouth Meeting, PA/USA) ones. With the performed holes, it was possible to flow solutions inside the column, so, the remaining step was to seal the channels.

The structure was sealed against a 500 µm PDMS membrane, where the pre-polymer preparation was proceeded equally as above. Once having it, the mixture was poured onto a silicon wafer and spin coated at 250 rpm during 25 seconds. This is the suitable program to achieve the desired thickness. The PDMS was then ready to be cured and the wafer was left on the oven, during 90 minutes at 70ºC, previously identical. From the wafer, several membranes could be cut down with shape and size able to cover the fabricated structure.

To seal the structure against a membrane, both needed to be oxidize, which was possible to achieve with the use of a plasma cleaner (Harrick Plasma, Ithaca, NY/USA) during 60 seconds at medium power. After the removal from the equipment, the structure was placed on the membrane and Si-O-Si covalent bonds were created.

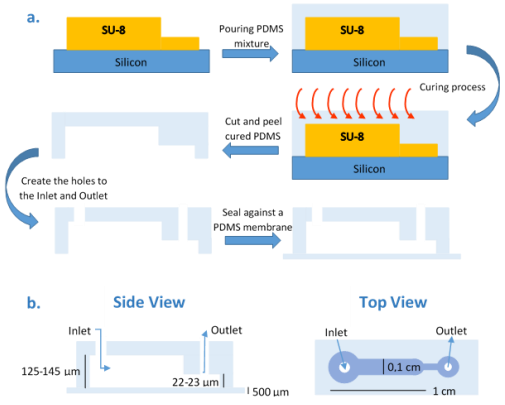


Figure 1 a. Schematic of the Soft Lithography technique fabrication: PDMS structure. b. Side and top view of the final design with structure dimentions. Dimentions not at scale.

The plasma treatment allowed the sealing and it marked the end of the fabrication process. It is of relevance to point out that on this stage, the remaining surface of the PDMS is yet oxidized, and a rest period of 24 hours should be regarded before the structure use. The process is schematized on figure 1.

Bead Packing

Within the different beads, the packing protocol had always the same features. The pumping system was set to pull at 5.5 µL/min flow rate, during approximately, 3 minutes, which gave a volume margin to avoid air entering the column. Regarding the washing step, 20 µL of Phosphate buffered saline (PBS) (P4417, Sigma-Aldrich, USA) were flowed with the same features as the packing process.

Staphylococcus aureus assays

Within the different working concentrations, the cells protocol had always the same features. The pumping system was set to pull at 2.5 µL/min flow rate, during, 20 minutes.

Aptamers

SA17 and SA61 are named according the intended function as capture and detection aptamers, respectively. When purchased, in addition to their DNA sequence an extra element was requested to be added to each one, in order to be possible to either function as a detection or capture aptamer. Table1 has the specific DNA sequence of SA17 and SA61 and their additional features.

Table 1 Features of the acquired aptamers. The detection aptamer is entitled as SA61 and the capture one as SA17.

Aptamer Nomination - Function	Purchase details	Oligosequence 3'-5'	5' Modification
SA61 - Detection	STAB Vida, (Portugal)	TCCCTACGGCGCTAACCTCCCAACCGCTC CACCCTGCCTCCGCCTCGCCACCGTGCTACAAC	Spacer C12 + Atto 430LS
SA17 - Capture	STAB Vida, (Portugal)	TCCCTACGGCGCTAACCCCCCCAGTCCGTCCT CCCAGCCTCACACCGCCACCGTGCTACAAC	Spacer C12 + Biotin

SA61 protocols

Regarding SA61's folding technique, the cooling down of the solution was performed in one hand, resourcing a rapid and drastic dropping of the temperature, placing the Eppendorf® in water with ice blocks after it had been 10 minutes at 95ºC. In the other hand, the folding was performed gradually, controlling the temperature dropping until it reached 37ºC where it was maintained during 15 minutes. In this last approach, the solution was heated initially until 95ºC but only kept at it during 2 minutes, before beginning the cooling down process. As for the incubation step, the adding of the SA cells to the SA61 solution was set to be prior the flowing through the column, where the mix was 30 minutes under agitation at 750rpms. In this case, on the packet column the mixed solution was flowed at 2.5 µL/min during 20 minutes following by a washing step of 3 minutes at 5.5 µL/min with PBS. The other type of incubation was achieved inside the column, sequentially, where the SA solution was flowed first using the same concentration at a 2.5 µL/min flow rate during 20 minutes followed by SA61 solution at 1 µL/min during 15 minutes, ending the flowing process by washing the column with identical features as the first protocol.

Dyes – *Staphylococcus aureus* viability assay

A specific viability test assay took motion in order to assess the cellular integrity of Staphylococcus aureus cells. With that aim, two different dyes were selected to perform an alive/dead cell test. EvaGreen™ (31000, Biotium Inc., USA) and Hoechst 33342 (ThermoFisher, USA) gathered certain features that shown to be useful in the mentioned assessment. Hoechst 33342 is able to stain every cell, regardless of its viability, having the ability to cross an intact cellular membranes, by other words can be used as an accountant for the total number of cells in solution. EvaGreen™, however, is not passible of crossing a viable cell membrane. This dye fluoresces when bound to ssDNA and its action is only allowed upon the membrane disruption thus, acts as disrupted cell stain. . Hoechst 33342 measurement acquisition is based on the use of UV Olympus florescence microscope filter, emitting on the blue color, on the other hand EvaGreen™ needs the use of the Blue filter to be excited which results visually on a green staining.

To analyze the channel images different approaches were taken into consideration. Nevertheless, the entire analysis set was performed resourcing ImageJ software and for the results presentation, Origin 2020 software

Bright Field Measurements

Existing the need to perform an analysis across different colored images, two types of ImageJ tools were selected. Grayscale measurements assess the change in color intensity translated to shades of gray across a defined region. In this case, a middle line crossing the inside's channel was defined to measure the changes across the column. The second approach uses ROI manager tool from the software to output a mean RGB intensity value of certain sectional area. This analsys consisted in selecting a oval shape form inside the column and a squared one outside (background). Afterwards, the absolute value from inside the channel was achieved by performing a subtraction between the background average intensity and the measurments acquired from the selected channel region. Figure 2

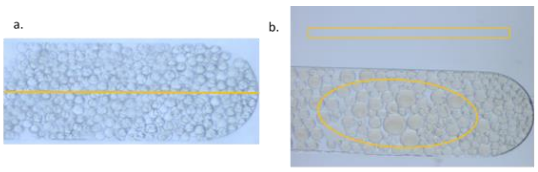


Figure 2 Section used for signal measurements in ImageJ software: a. Grayscale analysis; b. Mean RGB signal intensity

Fluorescence Measurements

An identical aproach to figure 2.15b was carried to perform fluorescence measurments. ImageJ allows the splinter of the images into the three color channels: red, green and blue. In the majority of the assays, the fluorophores used, when excited, colored on green, so, that was the channel used to perform the measurments. This way, the albsolut value for the mean fluorescence intensity was achieved by, once again subtracting the selected region inside the column by the background signal. Figure 2.b

Results and Discussion

Beads assay

Four different bead types were tested, aiming to understand if exists a particular characteristic that could influence, positively, the capture of *S. aureus (SA)* cells. Nevertheless, it was intended

to selected one of those options in order to understand if the aptamers were binding to the cells.

C_{18} beads both grayscale and Mean bright field intensity signal had dubious results. Besides the non-coherence across the measurements, the truth is, during the multiple attempts of performing this experiment, several side backs were encountered. Air entering the channel was the main problem, due to the beads hydrophobicity and the high cell concentration, there were complications in flowing the solution properly. To avoid this situation, C_{18} beads were excluded from the selection.

The grayscale analysis from SiO_2 bead test had quite positive results in which concerned the physical trapping of *staphylococcus aureus* cells. Although showing a progression of the cellular content across the flowing time, the truth is SiO_2 beads were able to sustain SA cells in part of the column, slowing down its passage. Apart from these, NH_2 beads also showed pretension to sustain cellular content across the column. Although having a positive outcome, the trapping effect by NH_2 beads was less accentuated when compared to the action of SiO_2 beads, tending to allow SA cells to flow with less retention. Nevertheless, regarding the signal analysis using the ROI tool, both of the above bead types have shown good results among the all set tested. Although NH_2 beads presented a higher signal, SiO_2 beads were selected to continue the development of this project. Positively charged beads, like NH_2, can present an advantage in the *Staphylococcus aureus* attraction, due to the cell surface properties, but they also would have influence near the flow of the aptamer since is a DNA sequence and this side back was to be avoided.

SA61 binding efficacy test

After choosing the best bead type to use and according to the results obtained in the previous section, it was possible to perform an essay where SA61 binding capability was tested. The idea of having a strategy capable of concentrating the cells in the channel was an optimal path to assess the detection aptamer efficacy. SA61 was tested using two different protocols of DNA folding and incubation.

The experimental image of all four protocols have not showed any relevant fluorescence in the entire set. By analyzing figure 3, the signal values were not featured to be labeled as a positive result, however, there was a particularity that was interesting and should be pointed out. Observing the signal difference between the darker and lighter sites of the beads in the channel, it was constant thought all four protocols that the signal measured onto the concentrated cell's zone was slightly higher. This could mean that a small portion of the aptamers could have been being withheld, but not at the cell's concentration proportion.

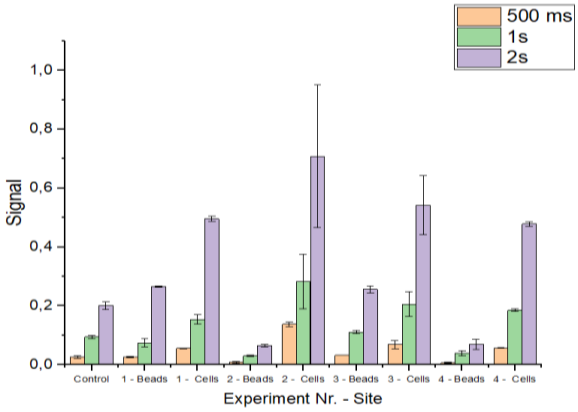


Figure 3 Mean fluorescence intensity signal measured on a section of the column after the washing step. Values from protocols 1-4 on the concentrated cell site (Cells) and outside that zone (Beads). (Aquisition: Olympus Microscope CHX41, exposure time: 500ms, 1s and 2s, gain: 0 dB, magnification:10x.)

Considering the information mentioned above, there was the need to perform a trouble shooting of the situation and there was the need

to understand how the performed protocols affect cell viability not allowing the binding process.

Staphylococcus aureus viability assay

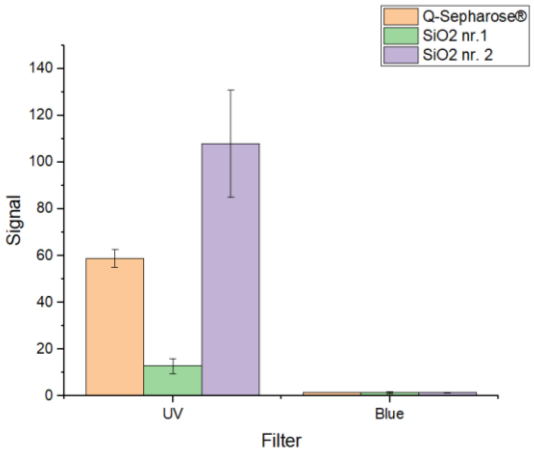
The performed protocol on the assay with SA61 aptamer, had different actions that could have jeopardized SA viability and must be counted in, such as high cell concentration, centrifugation steps, pumping flow rates and the bead's physical characteristics.

To this end, two different dyes were used: EvaGreen™ that colors the cell if there is a disruption on the membrane, meaning that its viability was compromised; and Hoechst 33342, that can pass through the cell wall even if it is intact, in other words, it will stain all cells (alive or dead).

Taking into consideration the entire set of results, what was intended to understand was if the protocol followed on SA61 assay was somehow compromising SA viability ending up jeopardizing the aptamer binding potential. From the experimental images and analytical results was possible to ascertain that although there was live cellular content posterior to SiO_2 beads experiment, there was also *Staphylococcus aureus* cells being affected due to the protocol features. This could have happened due to the fact that cells were highly concentrated, which required centrifugation action that end up jeopardizing the cells. In addiction, SiO_2 beads are heavily dense, and the applied flow rate against them could also play a role in the cells death. The following approach was to reduce the concentrated SA solution in order to skip centrifugation steps. Adjusting SA cellular concentration, from $8*10^9$cells/mL to $4*10^8$cells/mL.

Analyzing the analytical outcome from figure 4 Nevertheless, with the new established SA solution concentration, no death was occurring and SA61 aptamer could once more be tested in order to access its binding efficacy.

Figure 4 Mean flourescence intensity signal measured on a section of the column after the washing step. Values from a $4*10^8$cells/mL SA solution on Q-Sepharose® beads and Spherical silica® beads; Hoechst 33342 and EvaGreen™ were used simultaneously on Q-Sepharose® and SiO_2 nr.2 experiments and sequentially on SiO_2 nr.1. (Aquisition: Olympus Microscope CHX41, exposure time: 2s, gain: 0 dB, magnification:10x.)

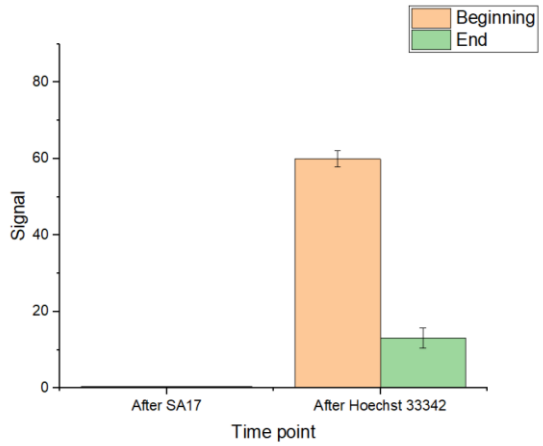


In spite of the new protocol features, SA61 was still not binding to *Staphylococcus aureus* cells.

SA17 binding efficacy test

The failure of using SA61 as a detection aptamer propel the search of an alternative, as so, the strategy was to use SA17, the capture aptamer, as a mean for both functions. Converting this aptamer (suitable to function as a detection tool) was proceeded by adding to it, a molecule of streptavidin-FITC. SA17 aptamer has a biotin incorporated in the end of the DNA sequence and the bound biotin-streptavidin has an extremely high efficacy rate.

Figure 5 does not show any visible fluorescence as should be expected if SA17 was bound to *Staphylococcus cells.*



Mean fluorescence intensity signal measured on a section of the column after the washing step. Values from a $4*10^8$cells/mL SA solution on Spherical silica® beads; First time point – After SA17-streptavidin-FITC solution – related to the Blue filter acquisition and the secont time point – After Hoechst 33342 – related to the UV one. The Beginning and End labels are refereed to the different measured sites in the column. (Aquisition: Olympus Microscope CHX41, exposure time: 2s, gain: 0 dB, magnification:10x.)

Beads capture method: Sensibility

In spite of the binding assays failure, the truth is it was possible to observe some degree of cellular capture by the beads action. Although occurring due to physical interaction, the following approach was to test the sensibility of the method. In one hand, SiO_2 beads are small and highly dense, that concentrated SA cells in the beginning of the column, in the other hand, Q-Sepharose® beads are positively charged and could have some type of interaction on the bacterium cell surface, ending up attracting them.

To perform this sensibility test, columns were packed, individually, with both bead types and a certain *Staphylococcus aureus* cell concentrated solution (Table 3.2) was flowed through the channel. Afterwards, Hoechst 33342 dye was also passed through to assess the cellular presence in the column.

From that analysis, was possible to ascertain that the physical capture by Spherical silica® beads had a registered sensibility that stopped around $8*10^7$cells/mL concentration.

For Q-Sepharose® beads, a plausible detection could be performed when lowering SA concentration until $4*10^7$cells/mL.

Conclusion and Future outlooks

Bacterial detection plays an important role in therapeutically approaches and pathology identification. The need to assess the main origin of certain clinical manifestations is the pillar to set the appropriated course of treatments. Although existing innumerous technics in the matter of bacterial detection, these are prone to a wide variety of drawbacks. Current practices are time and cost consuming and rely on methods that increase quantitatively the target of the sample in order to reach an appealing sensitivity. The use of microfluidic chips is considered to be an innovative tool in the means of screening, promising unique advantages that are keen to overcome the burden on current applications. Regarding bacteria detection, the microfluidic field as the possibility of assemble a technique to directly detect certain pathogens without the need of DNA acquisition and amplification.

On the course of the development of this project, a microfluidic chip was manufactured, aiming to direct capture and detect bacterial cells from the testing model of Staphylococcus aureus (SA). The chip basis was set to be composed of nano-porous beads, used as a capturing surface, allowing an enhancement of the capture area when compared to the micro-bare-channels. This capturing surface would act as an

immobilization platform for a specific S. aureus targeting molecule, creating a selection-specific-approach that could be used among other desired targets. Upon capturing, other molecule target-specific, containing an identification marker, would set to be flowed through the beads to bind to the capture target, creating a molecule-target-molecule sandwich-type method.

To the present work, aptamers were chosen to act as molecule-targeting probes. Two aptamers were selected from the literature, where is stated their optimal binding efficiency to *S.aureus* cells [23] to attempt accomplishing the described approach. SA17 was purchased with a biotin molecule to allow the bead immobilization and SA16 with Atto 430LS molecule, a fluorophore to be able to be detected.

From the results analysis, Spherical silica beads® were selected, thus were prone to slow down the cells flow through the column, acting as a temporary capturing agent. The beads test results, showed that this trapping-effect was set time enough, that SA61 could be flowed and detected. On this assessment assay, the fluorescence measurements show no significant signal, although cellular content was clearly present in the channel. This gave rise to multiple questions. To continue the development of the project, one of those questions gained the need to be clarified and so, a viability assessment assay was performed resourcing two different dyes as probes. Hoechst 33342 and EvaGreen™, are able to stain every cell (alive or dead) and only compromised targets, respectively, creating an Alive/Dead cell evaluation test. From the results analysis, different conclusions could be obtained. The signal acquisition among distinct approaches of the dyes usage, led to the assumption of a binding competition between the dyes. Nevertheless, the initial question was able to be assessed and in fact, the high concentrations of S. aureus solutions and the need to perform centrifugation steps to achieved such concentration values, was indeed bursting the cells

After the reach of this solution, the natural path action was to re-try the binding efficacy tests on SA61 detection aptamer, using this new concentration, which showed to be enough to still slow down the SA cells flow through the microfluidic channel, using Hoechst's dye to confirm cellular presence upon the aptamers passage. Unfortunately, the binding was not yet successful and thus not related to viability issues. The following approach was to perform the same assessment for the capture aptamer type, SA17, modified to act as a detection molecule by the conjugation of a fluorescence fluorophore, aiming to avoid the negative results by using SA17 on both functions. Nevertheless, also this aptamer showed no binding efficacy against SA cells, which lead to the emergent questioning about the correct folding of both aptamers.

Threre was still a visible capturing method that was followed across the entire project's development: physical capture by the bead-packed columns. As a final approach, two different bead-types were assessed in their physical effects retaining bacterial cells in the column, using Hoechst 33342 to confirm cellular presence. Q-Sepharose® and Spherical silica® beads were packed prior to specific SA cells solutions concentrations to be flowed. Q-Sepharose® beads showed an higher sensitivity in comparison, where the lowest detection was set on ~ $4*10^7$ cells/mL solution. This number is extremely far from being considered an ideal detection tool, as was already expected, nevertheless it was a mean of addressing the sensibility aspect of techniques.

Due to the faced issues during the course of the work hereby described, the need to continue addressing the intended goal is keen to be a path that follows what has been set so far. The attempt of clarifying the encountered binding issues and assess feasible features on why aptamers were not binding, is somehow appealing. For instance, assessing the folding processes trying distinct protocols and buffers, upon literature study, that differ either in composition or physiological properties can be an approach to continue the binding trouble shooting. Other approach seen as future work could be using the “sandwich” initial idea, changing the targeting molecules from aptamers to antibodies. Although aptamers assemble a wide range of stated advantages over antibodies, the truth is, these last are an extremely sensitive approach in the matter of targeting and bounding. Thus, antibodies can be specific selected and also be keen to perform an antibody-target-antibody interaction, since can also be used as immobilization and detection tools, resourcing feature addition (such as a fluorophore).

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