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Master Thesis in Bioengineering

Development and optimisation of point-of-care optofluidic devices for the rapid detection of genetic material

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Abstract

Nowadays, molecular analysis plays a major role in healthcare, being important for the diagnosis and clinical decision making of various disorders, such as cancer and infectious diseases. However, the current technologies available (*i.e.* qPCR, NGS and microarrays) have several associated bottlenecks such as high costs and poor sensitivity limiting their application for point of care (POC) diagnostics.

In this thesis, an optofluidic biosensor that combines surface-enhanced Raman scattering (SERS) spectroscopy (ultra-sensitivity), and microfluidics (automation) was developed and tested for the detection of nucleotide sequences. In this device, a Raman reporter is drawn away from a plasmonic substrate when a specific target sequence hybridises with the corresponding molecular probe leading to a decrease in SERS intensity.

At a first stage, flexible SERS substrates based on gold nanostars assembled on a PDMS surface were fabricated and tested to be potentially used as SERS substrate of the biosensor. However, these did not yield satisfactory SERS performances. As such, optofluidic devices were fabricated using an alternative substrate based on silver coated gold nanorods, previously developed within the Medical Devices Group of INL.

As proof of concept the optofluidic platform was used for the detection of the gene *RdRp* from SARS-CoV-2 gene (RNA) which is causing the current COVID-19 pandemic and *BRAF V600E* (DNA), a mutation commonly associated to several cancer types such as melanomas and colorectal cancers. However, even if promising, the results obtained in optofluidic tests did not properly correlate with the target sequence concentration. Notice that a limited number of target concentrations could be tested, and additional studies will be necessary to optimise the capabilities of this technology.

Aiming to optimize the design, and achieve an efficient POC device, multiphase flow simulations were successfully used to guide the design and to test *in silico* a microfluidic chip for droplet hydrodynamic trapping. After experimental validation, this system is meant to be used to fabricate controlled nanoparticle assemblies in the shape of microarrays to improve the homogeneity and sensitivity of the SERS substrates.

Resumo

Atualmente, análise molecular é fundamental em clínica. Sendo, importante para o diagnóstico e tomada de decisões em diferentes patologias, como cânceros e infecções virais. No entanto, as tecnologias disponíveis (*i.e.* qPCR, NGS, e microarrays) tem vários constrangimentos associados como custos elevados e baixa sensibilidade, o que limitam a sua aplicação para testes em *point-of-care* (POC).

Nesta tese, um biosensor optofluídico que combina espectroscopia SERS (do inglês *surface-enhanced Raman scattering*) (ultra-sensível) e microfluídica (automação), foi desenvolvido e testado para a detecção de sequências de nucleótidos. Neste dispositivo, um repórter Raman é afastado de um substrato plasmônico quando uma sequência hibridiza com uma sonda molecular complementar, levando a uma diminuição na intensidade SERS.

Numa etapa inicial, substratos SERS flexíveis baseados em nanoestrelas de ouro organizadas numa superfície de PDMS foram fabricados e testados para serem potencialmente usados como substrato SERS do biossensor. Contudo, estes substratos não mostraram performances SERS satisfatórias. Dessa forma, os dispositivos de optofluídica foram fabricados usando um substrato alternativo baseado em *nanorods* de ouro revestidos em prata previamente desenvolvido no grupo *Medical Devices* do INL.

Como prova de conceito, a plataforma de optofluídica foi utilizada para detecção do gene *RdRp* do vírus SARS-CoV-2 (RNA), causador da atual pandemia COVID-19 e *BRAF V600E* (DNA) uma mutação associada a vários tipos de cancro como melanomas e cânceros do colórectal. Contudo, apesar de promissores, os resultados obtidos nos testes, não se correlacionaram de forma adequada com a concentração de sequência alvo. Repare-se que um número muito limitado de concentrações de alvo pode ser testado e estudos adicionais serão necessários para otimizar as capacidades desta tecnologia.

Para otimizar o design e obter um dispositivo de POC eficiente, simulações para fluido multifásico foram utilizadas com sucesso para guiar o design e testar *in silico* um chip de microfluídica para aprisionamento hidrodinâmico de droplets. Depois de validação experimental é pretendido que este sistema seja usado para fabricar aglomerados controlados de nanopartículas na forma de micro-matrizes para melhorar a homogeneidade e sensibilidade dos substratos SERS.

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List of abbreviations

μ TAS: Micro total analysis systems
1NAT: 1-napthalenethiol
2019-nCoV: Novel coronavirus
A: Adenine (nucleotide)
AA: Ascorbic acid
Abs: Absorbance
Abs₄₀₀: Absorbance at 400 nm
Abs_{max}: Maximum value of absorbance
Ag: Silver (metal and atomic element)
AgNO₃: Silver nitrate
APTES: 3-aminopropyl)triethoxysilane
Au: Gold (metal and atomic element)
Au@AgNRs: Silver-coated gold nanorods
AuNRs: Gold nanorods
BRAF: V-raf murine sarcoma viral oncogene homolog B1
C.V.: Coefficient of variation
C: Cytosine (nucleotide)
CE: Capillary electrophoresis
CFD: Computational fluid dynamics
cfDNA: Cell-free nucleic acids
CMV: Cytomegalovirus
COVID-19: Coronavirus disease-19
CSF: Cerebral spinal fluid
CTAB: Cetyltrimethyl ammonium bromide
CTC: Circulating tumour cell
ddPCR: Droplet digital PCR
D_f: Dilution factor
DFS: Disease-free survival
DLS: Dynamic Light Scattering
DMF: N,N-dimethylformamide
DNA: Desoxyribonucleic acid
EGFR: Epidermal growth factor receptor

EM: Electromagnetic
EtOH: Ethanol
EVA: Ethyl Vinyl Acetate
FAM: Carboxyfluorescein
FDA: Food and Drug Administration
FDM: Finite difference method
FEM: Finite element method
FISH: Fluorescence *in situ* hybridization
G: Guanine (nucleotide)
GNSs: Gold Nanostars
H₂O₂: Hydrogen Peroxide
HAuCl₄: Chloroauric acid
HCl: Hydrochloric acid
KCl: Potassium chloride
KETs: Key enabling technologies
KRAS: Kirsten rat sarcoma viral oncogene homolog
LAMP: Loop-mediated isothermal amplification
LB: Liquid Biopsy
LIF: Laser-induced fluorescence
LS: Level-set
LSPR: Localized surface plasmon resonance
MFDGs: Microfluidic droplet generators
MgCl₂: Magnesium Chloride
MPTMS: 3-Mercaptopropyl)trimethoxysilane
N₂: Atmospheric nitrogen
NaBH₄: Sodium borohydride
NaCl: Sodium Chloride
NaOH: Sodium Hydroxide
NGS: next-generation sequencing
NIR: Near-infrared region
NPs: nanoparticles
NSCLC: Non-small cell lung cancer
OS: Overall Survival
PBS: Phosphate-buffered saline

PBST: Tween 20 (0.05%) in PBS solution
PCR: Polymerase chain reaction
PCR-HMR: High Resolution Melt
PDE: Partial Differential Equation
PDMS: Polydimethylsiloxane
PVP: Polyvinylpyrrolidone
qPCR: Quantitative polymerase chain reaction
RdRp: RNA-dependent RNA polymerase
RNA: Ribonucleic acid
RT-PCR: Reverse transcription PCR
RT-qPCR: Quantitative reverse transcription PCR
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEM: Scanning electron microscopy
SERS: Surface-enhanced Raman Scattering
Si: Silicon (metal and atomic element)
SPR: Surface plasmon resonance
T: Thymine (nucleotide)
TEM: Transmission electron microscopy
Tris HCl: Tris(hydroxymethyl)aminomethane hydrochloride
UV-Vis: Ultraviolet and visible
VOF: Volume-of-fluid
WHO: World Health Organisation

List of symbols

[Au]: Concentration of gold

μ : Absolute viscosity

μ_c : Absolute viscosity of the continuous phase

μ_d : Absolute viscosity of the dispersed phase

a. u.: Arbitrary units

Abs: Value of absorbance

Abs₄₀₀: Value of absorbance at 400 nm

AUC: Area Under Curve

AUC_{norm}: AUC normalized value(s)

Ca: Capillary number

F_{st}: Surface tension force

L: Characteristic linear dimension

p: Pressure

Q: Volumetric flow rate

Q_c: Flow rate of the continuous phase

Q_d: Flow rate of the dispersed phase

Q_t: Total flow rate

R: Molar ratio between chloroauric acid and gold nano seeds

Re: Reynolds number

t: Time

v: Fluid velocity

γ : Reinitialization parameter

ε : Parameter controlling interface thickness

ρ : Density

ρ_c : Density of the continuous phase

ρ_d : Density of the dispersed phase

$\sigma(X)$: Standard deviation of X

Φ : Level Set Function

Introduction

A. Molecular techniques for nucleotide sequence analysis

The capability of detecting specific nucleic acids is important and greatly beneficial to many fields such as environmental monitoring, food control, and forensics.[1]

In health in particular, genetics is of extreme importance to medical practice as it provides a definitive diagnosis for many clinically heterogeneous diseases, such as genetic disorders (e.g. cancer), and infectious diseases. Additionally, genetic testing enables a more accurate disease prognosis and provides guidance towards the selection of the best possible options of care for the afflicted patients. Much of its current potential stems from the capacity to evaluate the human genome at various levels ranging from chromosomal or chemical modifications to single-base alterations.[1,2]

Some of the most common technologies for DNA/RNA detection studies include: gel and capillary electrophoresis (CE), DNA microarrays, fluorescent *in situ* hybridization (FISH), polymerase chain reaction (PCR) chain reaction and its variants, and sequencing technologies, such as Sanger sequencing.[1,3]

PCR is the most widely used DNA amplification technique and it is, at present, the basis for all commonly used genotyping methods. In this procedure a nucleic acid sequence is amplified exponentially via a reaction with specific primes at 2-3 different temperatures, thus providing enough material for downstream analysis procedures.[3,4] PCR can be used for diagnosis of many human diseases, and it is an important confirmatory diagnostic tool for conditions such as tuberculosis, HIV, CMV (cytomegalovirus), mycoplasma, hepatitis, syphilis, fungal and protozoal diseases, cancers, malaria, staphylococcal bacteremia, and tuberculosis.[5]

PCR variants include reverse transcription PCR (RT-PCR) to allow RNA detection, quantitative polymerase chain reaction (qPCR), droplet digital PCR (ddPCR), allele specific PCR (ASA-PCR), and high-resolution melt (PCR-HRM).[6]

Among the different PCR variants, qPCR currently dominates the general diagnostic market at 70 %. A simple and graphic explanation of a conventional qPCR reaction protocol is shown in Figure 1.[6]

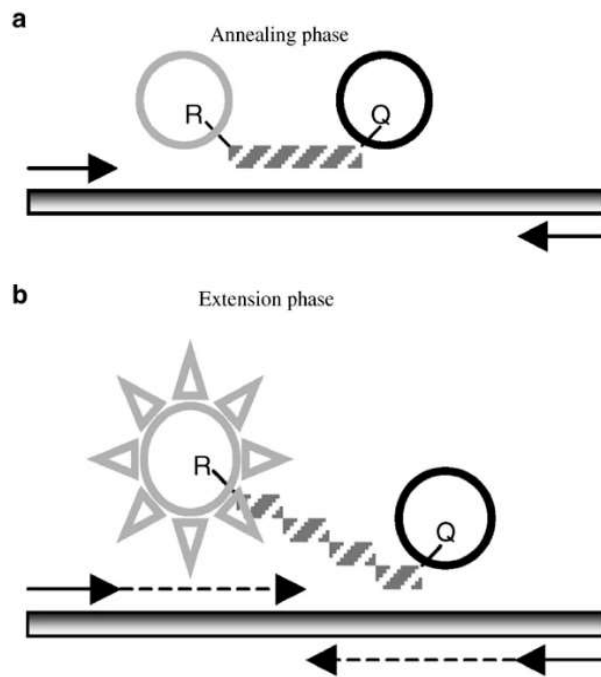


Figure 1- Principle of qPCR detection using hydrolysis probe (TaqMan™). (a) The bi-fluorescent probe, with both a reporter (R) and quencher(Q)), hybridizes, during the annealing phase on the complementary nucleotide sequence of the template during the PCR reaction. (b) The probe is degraded during the extension phase by a DNA polymerase enzyme resulting in an increase in fluorescence which is detected by the qPCR machine. Reproduced from reference 6.

Even though, qPCR dominates the current landscape, false positive or negative results tend to be introduced limiting their use to study single-base variations.[7] On the other hand, qPCR requires expensive DNA intercalating fluorescent dyes or probes (short DNA fragments) with intercalating dyes attached, which significantly increases the cost of testing. Additionally, qPCR can only be used to detect a limited number of known mutations.[3]

Isothermal DNA amplification techniques, such as loop-mediated isothermal amplification (LAMP), are becoming popular as alternatives to PCR. They have the advantage of eliminating the need for PCR thermal cycler thus representing less-demanding and affordable alternatives to PCR.[1] However, despite being promising strategies, several obstacles remain to be addressed, such as the amplification bias and nonspecific amplification inherent to exponential methods.[8]

In the 60s, the pioneer work made on DNA sequencing allowed for the development of a technique that opened new possibilities for DNA analysis, the Sanger sequencing method. However, since its launching novel and more advanced technologies

for DNA sequencing were introduced, based on the developments in nanotechnology and informatics, which are collectively known as next generation sequencing technologies.[9]

Next generation sequencing, or NGS, refers to the deep, high-throughput, in-parallel DNA sequencing technologies developed a few decades after the Sanger DNA sequencing method first emerged in 1977. NGS procedures provide massive parallel and extremely high-throughput analysis (millions to billions of nucleotides) of multiple samples much faster and at much lower costs compared to Sanger sequencing.[9]

The standard steps for NGS technologies are represented in Figure 2 – General workflow of NGS techniques and their main steps. Reproduced from reference 10., and they consist on library preparation, clonal amplification, parallel sequencing and analysis of the data obtained.[10]

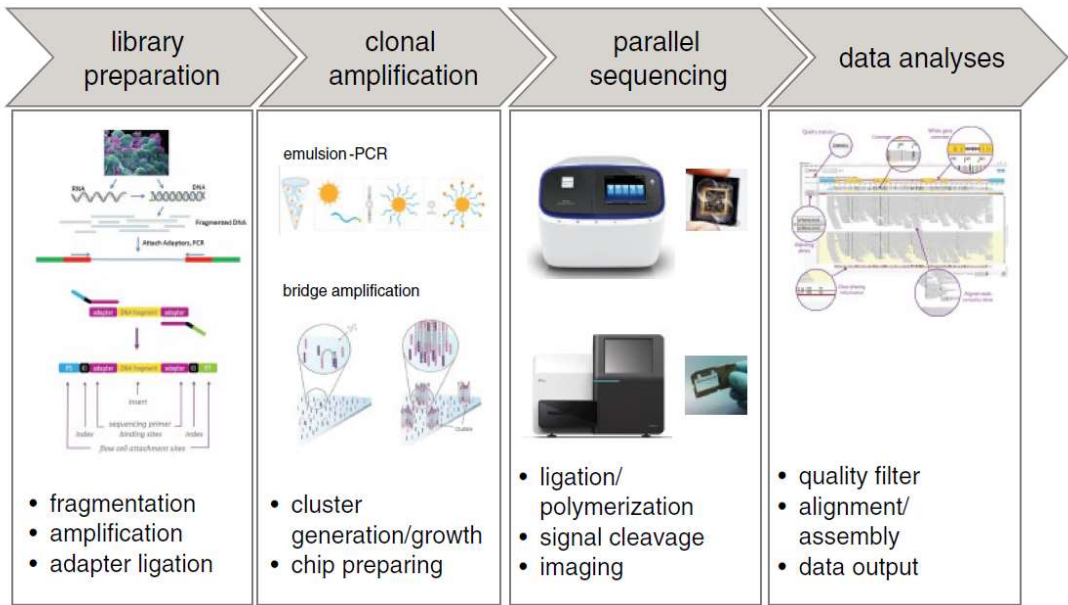


Figure 2 – General workflow of NGS techniques and their main steps. Reproduced from reference 10.

Compared to multiple “single-gene” approaches, NGS allows for a more efficient use of cells and tissues, it offers a more comprehensive assessment of clinically relevant mutations, it offers a more uniform workflow, and, depending on the number of genes under study, a lower testing cost per-gene.[11]

However, the prevalent practice of NGS has been limited by the high cost and long times of analysis (up to 2 to 3 weeks in a real-world clinical scenario), which makes it improper for single-base detection or urgent testing.[12] Furthermore, the still relatively poor sensitivity, the stability of the clone or subclones used, similarly to what happens with the

qPCR-based assays, and the difficulty to interpret the output data are the main barriers for clinical uptake associated to this technology.[13,14]

By converting a population of RNA molecules to a library of cDNA fragments, it is also possible to adapt this technologies of NGS for RNA sequencing (RNA-Seq).[15] RNA-Seq technologies allow for relatively rapid, precise, quantitative measurement of gene expression largely replacing the old-fashioned microarrays. However, they suffer from the same problem as NGS such as difficulty to interpret the data generated, and the still prohibitive costs to many laboratories. Additionally, since reverse transcription procedures are required, artifacts can be introduced during cDNA library construction and sequence alignments.[16]

Besides the traditional issues pointed out related to detection limits, selectivity, and reliability, the one common trend in the new nucleic acid detection methods is to make them suitable for POC (point-of-care) diagnostics.[1] Devices that can perform fast analysis and accurate diagnostics in close proximity to the patient are known as POC tests.[17] The ideal POC test should be user-friendly and as simple as possible so any user, even without any type of medical or laboratory knowledge, can use it and understand its response. POC tests should also be low cost to ensure that the device is easily acquired by everyone and anywhere. Additionally POC tests should be sensitive (very few false negatives), specific (very few false positives) and to enable on-site treatment.[17,18]

B. COVID-19 global pandemic: a worldwide emergency

In December 2019, an increasing number of pneumonia cases started to be registered in Wuhan, Hubei State in China. Dedicated in-depth studies, using high throughput sequencing revealed this was a cause by a new beta-coronavirus that was called 2019 novel coronavirus (2019 – nCoV). In January 2020, the WHO renamed the virus to SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) and the disease it caused as COVID-19 (coronavirus disease 2019). SARS-CoV-2 belongs to the betaCoV genera, it is a highly contagious virus, that tends to spread by the inhalation of respiratory aerosols, direct human contact, and contact with contaminated objects.[19]

SARS-CoV-2 infections may be manifested by several symptoms, ranging from asymptomatic/mild symptoms to severe illness and death. Some of the most common symptoms of SARS-Cov-2 infection consist in cough, fever, and shortness of breath.

Other reported symptoms include weakness, malaise, respiratory distress, muscle pain, sore throat, loss of taste and/or smell.[20,21]

Despite the attempts to limit the spread of the virus within the city, it rapidly disseminated to other states in China, and within weeks it had spread to many other countries throughout the world. The WHO (World Health Organization) by taking into account the increasing elevated number of cases reported worldwide, on the 12th March of 2020, it announced COVID-19 to be a global pandemic.[22]

The COVID-19 pandemic represents a sanitary, social and economic challenge at a global level.[23] It has been estimated that approximately one in five people worldwide could be at increased risk of severe COVID-19 disease if they become infected, due to underlying health concerns.[25] Moreover, since the City of Wuhan in China declared a lockdown on the 23th January of 2020, about a third of the world's population had to follow the same policies of restriction and isolation, imposed by the their governments, to reduce the spread of the disease and so prevent the collapse of the health system. However, with these actions great costs were involved since the lockdown measures led to the cessation of industrial and commercial production in most sectors, with job reductions and layoffs, causing unprecedented economic distress. In fact, it is predicted that the interruption of economic activities due to the lockdown directives and the reduction of movement, will lead to a total of approximately 420 million people to absolute poverty with incomes of less than 1.90 U.S. dollars per day.[23]

On the 11th of October, 2020 at 21:31 GMT, COVID-19 has affected more than 37 million people worldwide in 214 different countries and killed more than a million people.[24]

B.1 Molecular diagnosis of SARS-CoV-2: Challenges and Opportunities

Coronaviruses (e.g. SARS-CoV-2), are group of viruses composed of an enveloped, positive-sense, single RNA strand in a nucleocapsid. Coronaviruses (CoVs) present a crown-like appearance when observe under electron microscopy, hence the name (corona is the Latin word for crown).[21,22] Similar to other coronavirus, at present, RT-qPCR (quantitative reverse transcription polymerase chain reaction) testing, usually performed on nasopharyngeal swabs for the detection of the RNA of the virus, is the gold standard procedure to diagnose COVID-19 disease.[21,25]

Several conserved or abundantly expressed genes have been identified and are considered appropriate targets for SARS-CoV-2 RT-qPCR testing, such as the structural genes *N* and *E* and the nonstructural RNA-dependent RNA polymerase (*RdRp*) as well as the *ORF 1a/b* genes. Traditionally, two molecular targets should be included in the RT-PCR assay to avoid potential cross-reaction with other CoVs, and potential genetic drift of SARS-CoV-2. Among the several assays developed, the *RdRp* assay exhibits the highest sensitivity (3.8 RNA copies/reaction at 95 % detection probability). The *RdRp* assays have been used in over 30 laboratories in Europe. In the USA, the CDC (Centers for Disease Control and Prevention) recommends assays targeting two nucleocapsid proteins (N1 and N2), while the WHO recommends an initial screening with the *E* gene assay followed by a confirmatory assay using the *RdRp* gene. Interestingly, the *RdRp* assays were designed and validated using synthetic nucleic acid technology (*in vitro* transcription) in the absence of SARS-CoV-2 isolates or patient specimens.[21]

Although RT-qPCR assays are highly specific, their sensitivity is relatively low. False negatives often rise due to insufficient viral material in clinical specimens and laboratory errors related to the quality of the kits used, sample collection or test performance. Therefore, further optimization to shorten the high rate of false-negatives verified in SARS-CoV-2 testing is a crucial goal of high priority.[21,26] Additionally, RT-qPCR testing requires specialised laboratories with advanced equipment and highly trained personnel, making the process quite time consuming. Depending on the country, the time to get results ranges from several hours to 2 or 3 days.[22,26] The cost per testing by RT-qPCR is quite high reaching 135 euros in high income European countries and in many low to middle-income countries COVID-19 testing cost exceeds government per head health expenditure.[22] Thus RT-qPCR testing is not suitable for on-field and rapid diagnosis and screening of patients, being often limited by costs and laboratory capacity.[26]

LAMP and microarrays-based methods are other molecular assays which were reported to have been applied for the successful detection of genetic material from SARS-CoV-2. However, the high cost of microarrays inevitably limits its application in the detection of SARS-CoV-2. And, even though LAMP can often detect small numbers of DNA or RNA templates within roughly an hour, the requirement for high temperatures still limits its applicability.[27]

POC tests for COVID-19 were identified by the WHO expert group as the first of eight research priorities in response to the COVID-19.[28] As such, one of the most

important attributes desired for any novel COVID-19 test is to fit the requirements of POC diagnostics.[29] POC testing accelerates clinical decision making, allowing for effective triage and timely therapeutic and infection control interventions. In addition, POC tests enable testing in community settings, which in turn reduces pressure on the currently overburdened centralized labs.[28]

Hence, highly reliable, accurate diagnostic POC testing devices with high sensitivity, specificity, positive predictive and negative predictive values are urgently required in order to help combat the COVID-19 pandemic currently unfolding worldwide.[29]

As such, we propose the creation of an easy to use and point-of-care device for the sensitive detection of the *RdRp* RNA of SARS-CoV-2. With this it is intended to offer new opportunities and perspectives for the detection of COVID-19, and so contribute to controlling the current pandemic. Moreover, the platform proposed can be also be adapted for other clinical situations such as cancer medicine.

C. Cancer

According to the WHO, cancer is the second most common cause of death, worldwide, being responsible for about 1 in every six deaths.[30] In 2018, there were 18.1 million new cases of cancer, and there were 9.6 million cancer-associated deaths worldwide.[31] And the future only seems bleaker since the number of new cancer cases are expected to rise to 29.4 million in 2040, mainly due to the generalized growth and aging of the world's population.[32]

Cancer, a generic term for a large group of diseases, is characterised by rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs.[33] Cancer cells are characterized by the presence of six unique biological capabilities known as the hallmarks of cancer (Figure 3). Genome instability underlies these hallmarks, since it generates the genetic diversity required to acquire all six.[34]

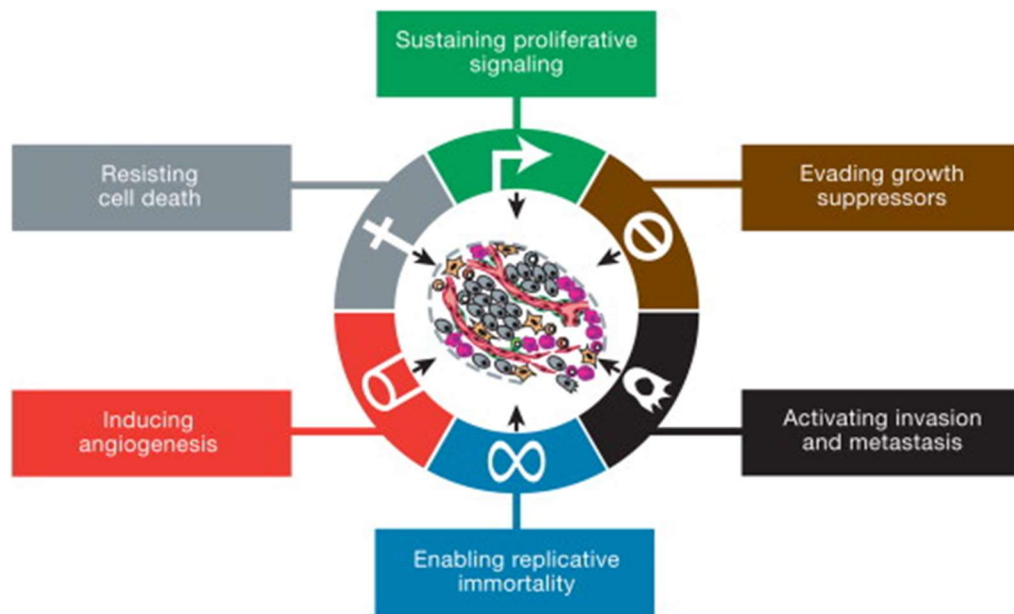


Figure 3 - The traditional six hallmarks of cancer. Reproduced from reference 32.

Cancer metastasis is the general term used to refer to the spreading of cancer cells from the primary tumour to surrounding tissues and to distant organs.[35] In fact, it is estimated that 90 % of all cancer-related deaths are due to metastasis, making it the primary cause of cancer morbidity and mortality.[35,36]

As seen in Figure 4, the metastatic process (for solid tumours) can be divided into five major steps: (1st) invasion by tumour cells of the basement membrane and subsequent cell migration; (2nd) intravasation into the surrounding vasculature/ lymphatic system; (3rd) survival in the circulation as circulating tumour cells (CTCs); (4th) extravasation from circulation to secondary tissues; and finally, (5th) colonization at secondary tumour sites.[37]

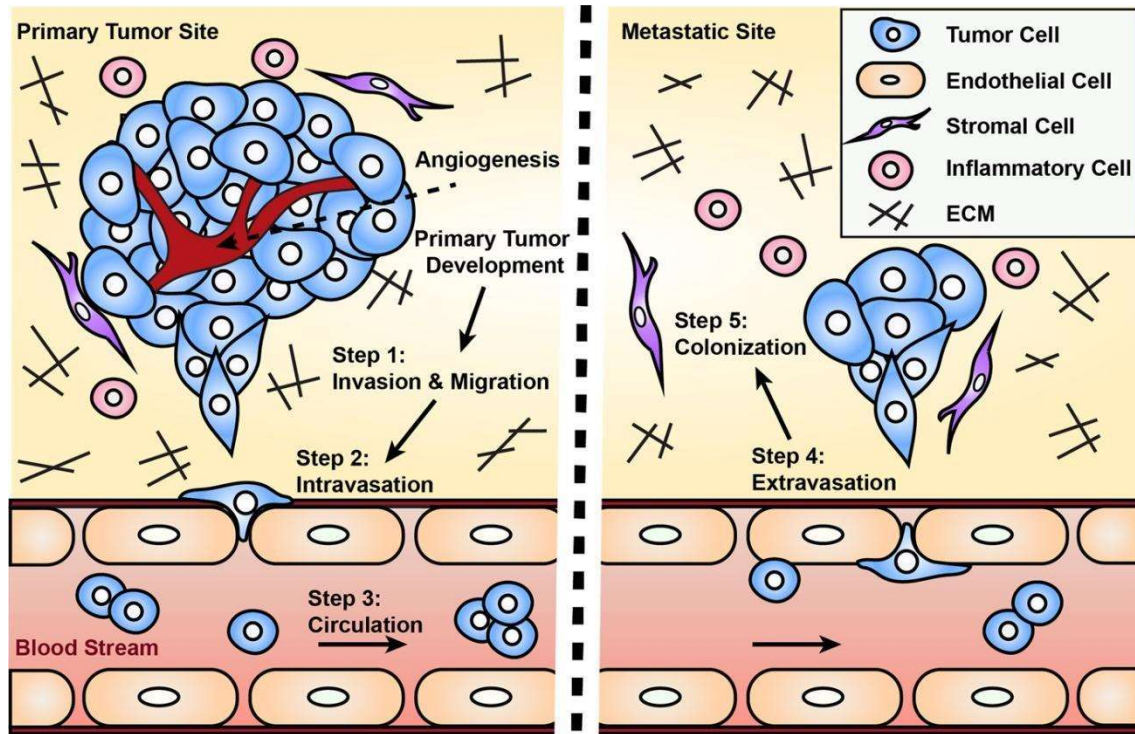


Figure 4- Representation schematics of the essential steps in the metastatic cascade. Step 1: cancer cells invade through basement membrane and migrate through the tumour stroma; Step 2: intravasation into vasculature; Step 3: survival in the circulation as circulating tumour cells; Step 4: extravasation through the endothelial barrier; Step 5: Colonization in the metastatic secondary organs and tissues. Adapted from reference 37.

C.1. Molecular techniques for mutation profiling in cancer

In the recent years, several multi-omics endeavours have unveiled a multiplicity of genomic and epigenetic components which are altered in tumours, such as DNA mutational profile, chromosomal copy number, coding and non-coding gene expression, promoter methylation, and metabolic activity.[38] These advances are largely owed to the vast improvements in massive parallel DNA/RNA sequencing technologies and computer analysis, which have allowed for the molecular profiling of thousands of tumours.[38]

Analysis of intercellular mutations has demonstrated to help assisting the prediction, diagnosis, and prognosis of cancer. Moreover, mutation identification can be used to find suitable therapeutic targets in patients, opening the way for a more personalised and efficient therapy, further assisting in reducing the mortality and morbidity associated with this type of diseases.[7] In fact, omics-based therapy has become a key component of precision cancer medicine, being a leap forward from the classical cytotoxic chemotherapy.[39] One example is the mutation testing for the

epidermal growth factor receptor (*EGFR*), which is routinely done in clinical setting to guide therapy in lung cancer patients.[39] Other case is the *KRAS* oncogene, which is an important biomarker in many types of adenocarcinomas, has frequent single base mutations associated with early stages of tumourigenesis. Moreover, 90 % of the activating mutations in pancreatic cancer are found in codon 12, which in wild-type- is GGT, being G to A the most frequently observed transitions.[40]

A bona fide “driver oncogene” is the *BRAF* gene. The *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) gene is located on the long arm of chromosome 7 (7q34) and encodes for an 18-exon cytoplasmic protein, a serine/threonine protein kinase (BRAF) which is recruited to the membrane upon stimulation by growth factors. The wild type *BRAF* gene is a downstream effector within the ERK/MAPK signalling pathway, which regulates growth, proliferation, differentiation, and apoptosis in human cells. When mutated, BRAF actively phosphorylates MEK, which leads to uncontrolled cell growth, as it can be seen in Figure 5.[41,42]

Mutated BRAF is a major driver gene alteration in cancers of multiple tissue origins. Almost 60 % of melanoma patients, 60 % of thyroid cancers and 46 % of colorectal cancer patients are reported to be *BRAF* mutant. Moreover, mutations in this gene are also known to be associated to non-Hodgkin’s lymphoma, NSCLC (Non-small cell lung cancer), glioblastoma as well as inflammatory diseases.[38]

Thus far, more than 40 BRAF mutations have been identified, the majority of which result in changes of the kinase domain and the P-loop of the protein. Interestingly, nearly 80 % of these genetic alterations correspond to the hotspot T1799A which causes the V600E activating mutation, which originates from the substitution of valine with glutamic acid at the amino acid (aa) 600. Presence of *BRAF V600E* mutations are associated to more aggressive phenotypes. In addition, since pharmacological inactivation of mutant BRAF often induces cancer cell toxicity, the U.S. FDA approved two inhibitors of BRAF V600E, vemurafenib for melanoma and Dabrafenib for melanoma and NSCLC. Therefore, detection of *BRAF V600E* mutation is useful for both prognostication and to guide clinical decisions for example regarding treatment.[41]

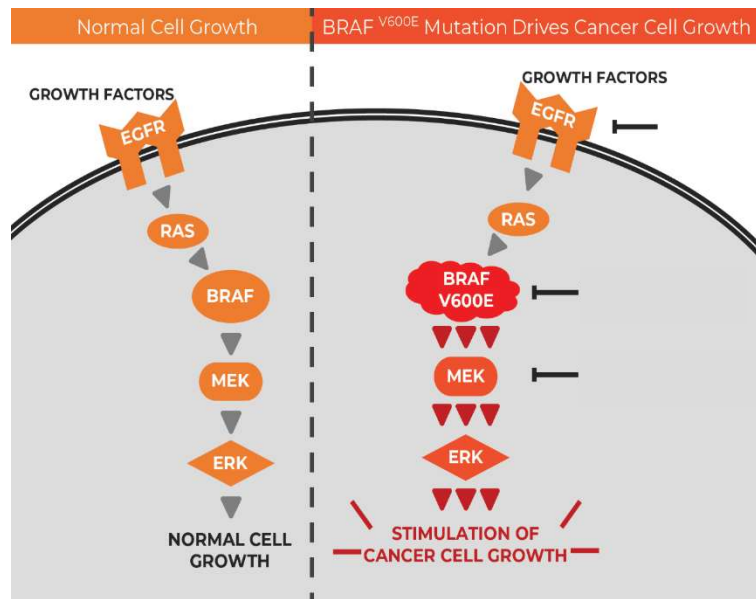


Figure 5 - Representation of the normal cascade RAF-BRAF-MEK-ERK (left), which leads to normal cell proliferation and differentiation, when activated. However, BRAF V600E causes over-activation MEK and ERK stimulating tumour cell-like growth (right). Selective inhibition of the mutated BRAF (blunt black arrow) reduces MEK and consequently ERK activation reducing tumour proliferation. Adapted from reference 42.

Molecular techniques for clinical use of cancer mutations can be divided mainly into PCR-based methods and DNA sequencing methods (e.g. next generation sequencing (NGS)).[7] However, as mentioned above both approaches suffer from limitations that severely impair their application for POC testing. The accuracy of PCR based techniques is highly dependent on the quality and quantity of DNA available, and so false positive and negative results tend to be introduced. Additionally, these approaches are only capable of detecting a limited number of known mutations.[3,7] Finally, PCR methods are costly and require adequate facilities, instrumentation (e.g. thermocyclers) and trained personnel.[5]

On the other hand, sequencing methods (mainly NGS technologies) are multi-staged, time-consuming, expensive and complex procedures, which are not practical for single-base variation detection.[3,7]

D. Liquid biopsy: a novel diagnostic concept

Recently, the need in cancer therapy for non-invasive molecular profiling tools has grown substantially due to the increasing demand and improved understanding over genomic alterations and novel personalised treatment options.[43] Traditional strategies

rely on tissue biopsies to access for specific biomarkers and at present they are essential to tumour characterisation, staging, and to genomic profiling which is key for evaluating disease progression and response to therapy. However, this approach presents several major caveats.[44,45] On the one hand, tissue biopsies are highly invasive making them not convenient for repeated sampling. Frequently, for example in lung cancer, tissue biopsies are not clinically possible to due to the high risk of bleeding, nerve injury or disease spreading it would present to the patient.[46] On the other hand, neoadjuvant treatment may shrink the tumour to undetectable size, leaving no tissue left for further analysis.[47] Moreover, these procedure only represents a single site of the tumour under evaluation, since cancers are recognise to exhibit spatial molecular heterogeneity, tissue biopsies do not properly reflect a tumour's complex molecular profile.[46,48]

Liquid biopsy (LB) is a novel diagnostic concept which is receiving a lot of attention these past few years for their potential to offer simple, fast and cost efficient monitoring of disease status and treatment response.[46,49]

LB refers to procedures where disease makers present in blood or other bodily fluids (e.g saliva, urine and cerebrospinal fluid (CSF)) are identified, measured, and characterised.[50] These markers are most commonly cell-free nucleic acids (cfDNA) and CTCs, but it might include others such as circulating tumour miRNAs and extracellular vesicles present in body fluids such as peripheral blood.[51] Analysis of these components can be used for early cancer detection, to help determine cancer stage, to assess prognosis and for real-time monitoring of drug resistance and minimal residual disease (MRD), emphasising the potential of liquid biopsies in improving cancer management.[49]

LB brings several advantages compared to traditional tissues biopsies. Perhaps the most critical is that they are much less invasive compared to traditional tissue biopsies since fluids like blood are much easier to access and retrieve.[52] LBs are less resource intensive, not requiring highly expert and trained personnel for the acquisition of clinical samples.[53] Due to their minimally invasive nature, liquid biopsies may be performed and used more frequently, while carrying fewer negative effects for the patients than conventional biopsies. Additionally, samples obtained through liquid biopsy are less likely to be contaminated with unwanted tissues/cells or other unwanted contaminants (e.g. chemical preservatives).[44]

The information obtained from a single tissue biopsy constitutes only a limited snapshot of the tumour and fails to reflect its heterogeneity. In contrast, LBs can be used

to track the evolutionary dynamics and heterogeneity of the tumour in real time and provide a genetic overview of tumour lesions and information on the dynamic genome evolution.[49] On the other hand, general imaging screenings such as tomography, scans and magnetic resonance imaging scans are expensive and scarce in many clinics.

Therefore, LBs can potentially serve in the future as an important tool to complement existing technologies (e.g. tumour imaging), critically improving detection, prognosis, monitoring and therapy guidance for many tumour types.[53]

Unfortunately, despite the increasing importance of molecular and genetic studies in numerous tumour-types, the usage of molecular and genetic methods currently clinically available, such as NGS, are generally inappropriate for liquid biopsy since they suffer from low sensitivity, high costs and/or cannot be used in all patients.

Thus, innovative, more sensitive, and cost-effective molecular techniques are required to make better use of all the knowledge accumulated and novel targeted therapies which are related to the molecular mechanisms underlying tumourigenesis and metastasis. Such solution would significantly promote a more appropriate and improved diagnosis, patient classification, stratification, and monitoring.

Interestingly, it has been reported the detection of SARS-CoV-2 in biological samples such blood, saliva and stools.[54,55] The preferred specimens for testing are obtained from the upper respiratory tract, collected with a flocked nasopharyngeal (NP) swab placed into a universal transport medium (UTM). However, the COVID-19 outbreak caused the disruption of the global supply chain of NP swabs and UTM.[56] Additionally, collection of NP specimens is a controlled act that requires a trained health care professional in full PPE (personal protective equipment), and a full change of PPE needs to be performed between each individual sample collection. As such the collection of swabs is resource intensive both in terms of personnel as well as in PPE.

Unfortunately, neither staff nor PPE or NP swabs are in adequate supply for responding to the current global pandemic situation, limiting the number of individuals being tested. Moreover, the collection of NP specimens can be uncomfortable for patients and thus not ideal for certain populations such as symptomatic children in the outpatient setting or those requiring repeated sampling. Therefore, new alternatives are being sought,[57] and so the diagnosis of COVID-19 disease can benefit from a biopsy technology that can detect accurately low levels of disease in alternative fluids like blood and saliva.

In this work it will be introduced a liquid biopsy solution for several tumour types, that can be applied for COVID-19 detection as well. The technology herein presented is expected to achieve high sensitivity in detecting molecular biomarkers present in peripheral blood and other fluids.

E. Microfluidics

Key enabling technologies (KETs) such as nanotechnology, microfluidics and nanophotonics, currently provide the basis for industrial and economical innovation, and so KETs and their support is a major priority for the European commission.[58,59]

Microfluidics, also referred to as micro total analysis systems (μ TAS), or lab-on-a-chip systems, is the science and technology of systems that process and/or manipulate extremely low amounts of fluids (10^{-9} to 10^{-18} L), using channels in the order of tens to hundreds of micrometres.[60] It is particularly interesting because the behaviour of fluids in channels at the micro-scale diverges from those of macroscopic fluids. In microfluidics, surface-related effects are much more significant, viscous forces tend to dominate over inertial ones and mass and heat transfer are highly efficient.[61]

Microfluidics is a field that has evolved rapidly in the recent years and it is expected to revolutionize in the future the medical, pharmaceutical, healthcare, and chemical industries.[62,63]

By substantially reducing the scale of the experiments, microfluidics offers unique advantages compared to more conventional approaches. For example, microfluidics devices offer faster reactions times, automation, and the possibility of making high throughput analysis with high resolution and sensitivity in a shorter time. Microfluidic devices are also a much cheaper alternative to conventional approaches. Since the energy and time required to fabricate and operate them and the amounts of samples and reagents necessary is significantly lower.[62–65]

The most widely used material in the fast prototyping of microfluidic devices is polydimethylsiloxane (PDMS). This elastomeric material allows to produce devices that can be easily replicated from low-cost photolithographically fabricated masters without the need for an expensive clean room facility. Moreover, PDMS-based devices support a wide range of applications because of their good biocompatibility, optical transparency, and flexibility.[60,62]

E.1. Droplet microfluidics

Conventionally, microfluidic devices can fall into one of two categories continuous or droplet based.[63]

In continuous microfluidic devices, as the name indicates, a continuous stream of fluid is enclosed in a microchannel. These systems can be easily made and controlled normally with resource to valves and micro pumps. However, they present some drawbacks such as low energy efficiency and low reliability of their components.[63] In alternative, in droplet microfluidics discrete droplets are generated and manipulated by using immiscible multiphase flows inside microchannels.[66]

The main advantages of droplet-based microfluidics can be summarized in three words: miniaturisation, compartmentalisation, parallelisation.[61]

Miniaturisation because, since the droplets are extremely small in size (sub nanolitre scale), they can be used for tightly controlled single-cell or molecular analysis.

On the other hand, each droplet is a unique singular entity. Therefore, they can be manipulated independently and serve as individual units for reactions (compartmentalisation). And finally, parallelisation can be achieved, since droplets are monodisperse and identical, hence providing large-scale quantitative reaction platforms for high-throughput analysis.

Moreover, droplet microfluidics also offers other advantages such as rapid mixing within the droplet at low Reynolds numbers and high surface-to-volume ratio. Therefore this technology has high value for a lot of industries such as those associated to photonics, cosmetics, molecular biology and pharmaceuticals since it offers a powerful alternative to perform typical laboratory operations, with a much smaller volume of reagents in significantly less time.[61,67]

Droplets can be produced by active or passive methods. In active methods, droplet generation is activated or assisted by external forces which can be of electrical, magnetic, thermal, and of mechanical nature. On the other hand, passive methods, also called pressure driven flow methods, use devices with specific geometries, also known as microfluidic droplet generators (MFDGs), to create monodisperse emulsions of two immiscible fluids and so, in other word, droplets. In MFDGs, droplet formation occurs when an immiscible dispersed and continuous fluids meet at a junction and the shearing forces of the continuous phase overcome the surface tension of the dispersed phase.[61,68]

Passive droplet production in microfluidics can be done through the use of T-junction, co-flow, or flow-focusing MFDGs (Figure 6).[69] However, flow-focusing geometries are more desirable than the remaining geometries since they offer a wide range of droplet sizes, improved generation rates and lower coefficients of variation (i.e., C.V.) in droplet size.[70] In this geometry, the dispersed and continuous phases flow coaxially through a contraction region and generate an elongation filament that eventually breaks into droplets.[61]

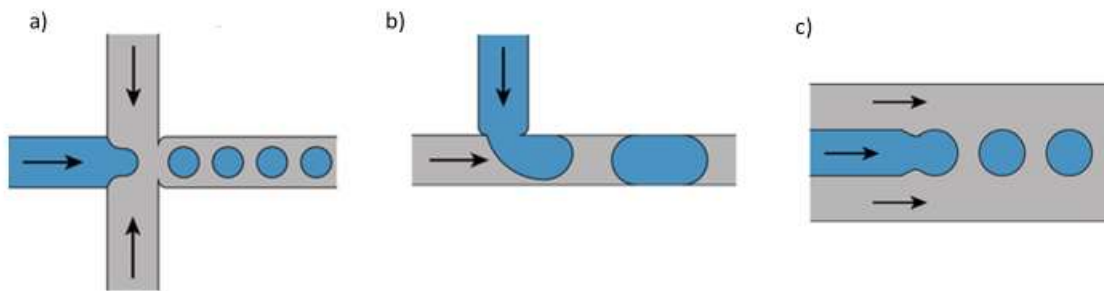


Figure 6 - Typical geometries of MFDGs for passive droplet generation. a) flow-focusing; b) T-junction; c) co-flow. Adapted from reference 69.

E.1.1. Droplet trapping

Sometimes it is useful for specific operations to locate and fix droplets in a specific location, this is called droplet trapping.[61] As with droplet generation, both passive and active methods for droplet trapping have been reported. More specifically, droplets can be passively trapped in discrete chambers using either hydrodynamic trapping or surface energy anchors. Active droplet trapping can be done using electrodes, lasers, or sound waves, which allow to on demand trap and relapse specific droplets.[71] However, passive approaches are particularly attractive because they offer better scaling with increase in system size.[72]

Some uses of droplet trapping include monitoring, detection, and incubation of reaction processes or of encapsulated biological material.[71]

Two of the main forms of hydrodynamic trapping described in literature so far were first described by Huebner *et al.*[73], and by Tan and Takeuchi[74]. As seen in Figure 7, the traps, described by Huebner *et al.*, have a small gap that allows the flow of the continuous phase when the traps are empty. However, if a droplet enters the trap, it gets stuck and by doing so it blocks the flow in the aperture, stopping other droplets from

entering. This design also allows to easily release the trapped droplets by simply reversing the fluid flow of the continuous phase. However, since the design consisted of the traps arranged in arrays on large chambers, the droplet capturing is random, and so the trapping efficiency is quite low.[73,75]

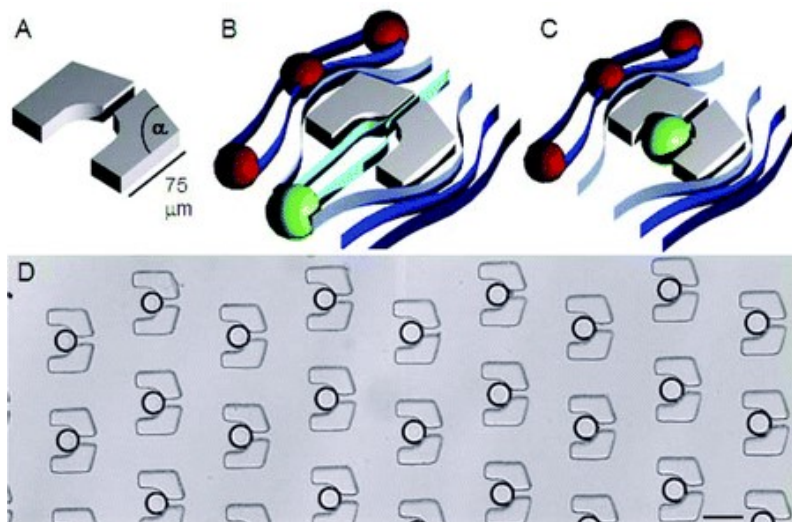


Figure 7 - Droplet trapping arrays described by Huebner *et al.* (A) Design of an individual trap (with a scale bar indicating the dimensions) The rear of the trap was drawn to give a 110° angle (α). (B) Schematic flow profile for droplets approaching a trap. Liquid enters through the opening in the center of the trap. (C) Schematic flow profile when a droplet is residing in the trap. As soon as a trap is occupied by a droplet, additional droplets pass outside the trap. This mechanism ensures that only a single droplet is trapped per feature. (D) Image of a trapping array containing uniformly aqueous droplets trapped in each unit. Reproduced from reference 73.

On the other hand, Tan and Takeuchi described a system of trapping and releasing system for microbeads which has been used and adapted by several authors to perform experiments where droplet trapping plays a crucial a role.[4,74–77] This system consists in an array able to store microfluidic droplets by passive means. More specifically, it allows for the creation of a train of confined monodisperse droplets into a fluidic network by using a repeated sequence of loops as shown in Figure 8.

Each loop contains a hydrodynamic trap where a droplet can be immobilized. Since the hydrodynamic resistance of the trap (path 1) is smaller than the hydrodynamic resistance of the loop branch, the first droplet in the train enters the trap and it gets captured in the hydrodynamic trap as shown in Figure 8. The subsequent droplets choose the loop-branch (path 2) because of the increased hydrodynamic resistance generated by the trapped droplet inside the hydrodynamic trap.[72] Remarkably this system was reported to be able to store droplets for up to 33 days, and to support complex reactions, such as LAMP.[4,76]

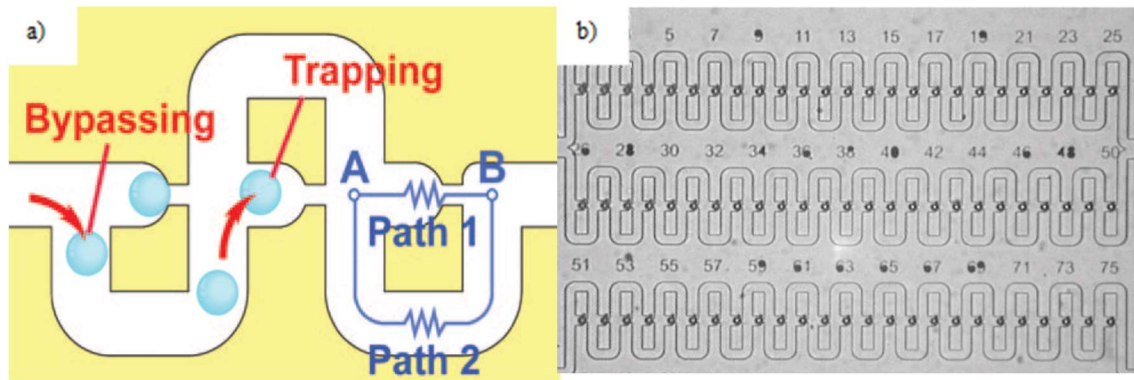


Figure 8 - Original hydrodynamics traps presented by Tan and Takeuchi. a) Schematics the underlying mechanism of trapping. b) Trapping array formed by multiple equivalent traps. Reproduced from reference 74.

E.2. Computational Fluid Dynamics

Scientists routinely rely on their expert knowledge and resort to manual calculations, simplifications, as well as assumptions to design a microfluidic device often ending up with situations where they cannot predict the consequences of their decisions. Therefore, prototypes are usually fabricated to test and validate the constructs. In case the results desired are not achieved, the designer must go back, revise the design, and repeat the prototyping. This often results in multiple iterations of prototyping (even dozens of iterations can be necessary depending on the complexity of the microfluidic network), leading to high costs (time and financially).[65]

Hence, there is a growing trend among engineers to utilise CFD (computational fluid dynamics) to analyse flow and performance of the design of new equipment and processes.[78] CFD makes microfluidics more accessible, it reduces the costs of the design process, and it allows for a faster time-to-market.[79] Indeed, because experiments are often expensive, time-consuming or sometimes even not possible, "virtual prototyping" is now the standard method to develop novel products.[78]

CFD is a branch of fluid mechanics that uses computers which perform numerical methods and algorithms to solve and analyse problems which deal with fluid flows.[78] CFD has already been applied with success in many areas of fluid mechanics (e.g. aerodynamics of cars, hydrodynamics of ships, flow through pumps and turbines) and it has applications in many important areas such as civil engineering, environmental and chemical studies, energy and weather forecasting.[80] Furthermore, several CFD tools

are currently available such as COMSOL Multiphysics, Ansys, OpenFoam, Fluent, CFX, FLOW-3D and Phoenixics.[65,78]

In CFD, equations that govern fluid motion (mathematical models), which are generally a set of partial differential equations, are solved by numerical methods such as the finite difference method (FDM) or the finite element method (FEM). Besides having the written equations, it is also necessary to input several parameters, define boundary conditions and to build a computational mesh. A mesh splits the computational domain into a set of discrete cells and it is in its nodes that the fluid parameters are determined.[80]

CFD is considered to be the most accurate way to simulate fluid flow. For example, with CFD it is possible to simulate turbulences and effects such as droplet deformation and splitting. However, to achieve such precision a highly complex setup is required (e.g. building a suitable mesh) and the related computational costs are very high due to the equally elevated levels of detail. Therefore, this approach is still limited to small designs and single components, not being appropriate to large-scale/complex microfluidic networks.[65]

One of the most important equations in CFD is the Navier-Stokes equation. This equation governs fluid motion, by representing the conservation of momentum and as such is considered as Newton's second law of motion applied to fluids. The Navier-Stokes equation is always solved together with the continuity equation, which represents the conservation of mass. Solving both the Navier-Stokes and continuity equations, for a specific set of boundary conditions (such as inlets, outlets, and walls), predicts the fluid velocity and its pressure in a given geometry, and together both are at the heart of fluid flow modelling.[81]

A fluid is considered as incompressible if the density variations it suffers are very small. This is true for liquids (unless under significant temperature variations) and gases under moderate pressure and temperature variations.[82]

For incompressible fluids and accounting for surface tension effects the Navier-Stokes equations and continuity equation can be written as:[83,84]

$$\rho \frac{dv}{dt} + \rho(v \cdot \nabla)v = \nabla \cdot [-pI + \mu(\nabla v + (\nabla v)^T)] + F_{ST} \quad (1)$$

$$\nabla \cdot v = 0 \quad (2)$$

In these equations, ρ denotes the density (kg/m^3), μ equals the dynamic viscosity (Ns/m^2), v represents the fluid velocity (m/s), p denotes the pressure (Pa), t represents time (s) and F_{st} is the surface tension force (N/m^3) acting at the interface.[84]

One of the most important numbers in fluid mechanics is the Reynolds number (Re). This dimensionless number corresponds to the ratio between inertial forces and viscous forces in the fluidic system and it is given by:

$$\text{Re} = \frac{\rho v L}{\mu} \quad (3)$$

Where ρ (kg/m^3) and v (m/s) are the density and velocity of the fluid respectively, L (m) is the characteristic linear dimension of the system and μ is the dynamic viscosity (N/m^2) of the fluid.[62]

In microfluidics, Re is generally small (≤ 1) due to the small channel sections and relatively small volumetric flow rates. Hence, the laminar flow regime is verified and the inertial effects (e.g., gravity and turbulence) are negligible.[65]

Microfluidic applications, however, involve rather often the flow of multiple fluid phases.

In immiscible liquid–liquid flow, the phases are separated by an interface. The interface is deformable, and the two-fluid flow may undergo topological changes due to breakup and coalescence. One typical example of this is in microdroplet generation and manipulation.[85] Therefore, numerical modelling for multi-phase flows and in particular for droplet generation and trapping is a rather challenging task since it involves moving fluid–fluid interfaces and the model created needs to be capable of handling complex geometries and topological changes.[67]

There are several numerical methods and models to simulate interfacial two-fluid flows such as the Lattice Boltzmann method, front tracking method, volume-of-fluid (VOF), the Level Set (LS) and phase field methods. Hence, it is important to select the most appropriate model and to tightly adjust the relevant parameters that affect it to accurately predict multi-phase fluid behaviour.[86]

At the microscale, the interfacial tension and viscosity effects dominate the behaviour of multiphase flows, while gravitational and inertial forces do not play as an important role as they do at the macroscale.[87] Therefore, a crucial parameter that affects

droplet dynamics in microfluidics is the capillary number (Ca), which represents the relative effects of surface tension and viscosity acting across an interface between two immiscible liquids and it can be defined as:

$$Ca = \frac{\eta_c \cdot U_c}{\sigma} \quad (4)$$

where η_c and U_c is the viscosity and the fluid velocity respectively of the continuous phase fluid while σ is the interfacial tension between the two immiscible fluids.[67]

Depending on the capillary number, the droplet formation process can occur in different modes which offer a different size range and polydispersity.

Figure 9 represents the three main modes of droplet formation described for a flow-focusing geometry: squeezing, dripping, and jetting regimes.[88]

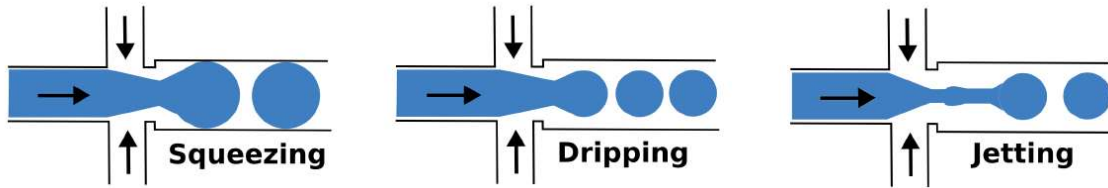


Figure 9- The three main droplet formation regimes for a flow-focusing geometry usually considered. The regimes from left to right are squeezing, dripping, and jetting. The arrows represent the flow directions of both the continuous and dispersed phases. Reproduced from reference 88.

In the squeezing regime in flow focus devices the dispersed phase penetrates the channel downstream, leading to the blockage of the perpendicular flows of the continuous phase. The pressure in the continuous phase occurring due to the blockage of its flow squeezes the dispersed phase and eventually pinches off a droplet.[89]

In the dripping mode, droplets are formed due to the shearing that the continuous phase exercises on the dispersed phase, when shear stresses overcome the interfacial tension effects droplet breakup occurs.[88]

Finally, the jetting regime is characterised by the formation of long threads of the dispersed phase, which eventually become unstable and break down in droplets due to instability.[88]

Flow-focusing MFDGs have been shown to produce monodisperse droplets at low capillary numbers when droplets are produced in a highly stable breakup process (i.e., squeezing and dripping regimes).[90]

F. Raman Spectroscopy and Surface Enhanced Raman Scattering (SERS) spectroscopy

Despite all the benefits of microfluidics, due to the small volume involved, highly sensitive techniques need to be integrated to perform analysis and detections.[91]

Optofluidic technologies are the ones that combine both microfluidics and optics at the micro/nano scale. These technologies enable the manipulation of fluids to detect samples precisely, overcoming the caveats of both approaches. Therefore, optofluidic is rapidly being applied for the real-time detection of biochemistry samples on chip.[92]

There are other several high-throughput detection techniques that have been successfully combined with microfluidic platforms, the most popular and representative being laser-induced fluorescence (LIF).[91] LIF is an easy to use, ultra-sensitive and low-cost technique. However, LIF presents some limitations such as mandatory fluorescent labelling, fluorescence peaks that often overlap making multiplex detection difficult, and sensitivity to *photobleaching*.

An alternative technique that has been showing promising results when combined with microfluidics is SERS.[91]

Raman spectroscopy and its variant surface-enhanced Raman scattering (SERS) spectroscopy have been growing strong in the recent years as two of the most powerful analytical techniques established. Despite the discovery of Raman spectroscopy at the start of the 20th century and SERS during the 70s, their application in the real world only started blossoming in the last years, mainly due to the recent advances in nanotechnology. Their potential as sensitive analytical tools has been demonstrated in numerous fields such as pharmacology, food, biology, environment, medicine, forensics, arts, and homeland security.[91]

Raman spectroscopy is a scattering technique and relates to vibrations of the molecules under study.[93] When a monochromatic electromagnetic radiation strikes a sample, it interacts with the molecules in the sample, scattering light in all directions. Most of the scattered radiation has a frequency which is equal to the frequency of the incident radiation, this is called the Rayleigh scattering. However, a small fraction of the scattered radiation has a frequency different from the incident radiation (Raman scattering). These Raman-scattered radiation can be used to build a spectrum. A Raman spectrum is the result of inelastic collisions between the incident monochromatic

radiation and the molecules present in the sample. It is presented as an intensity versus wavelength shift spectrum.[93] Raman spectra provides a vibrational fingerprint of the studied samples, which is in turn related to its constituents, symmetry, and environment. Raman spectroscopy can therefore be used to determine chemical components, molecular structures/conformations, and interactions between different molecules.[94]

Raman spectroscopy has several important advantages including non-destructive analysis; minimum preparation of samples required; measurements can be carried out in biological fluids, since the water signal is rather weak; simultaneous detection of different analytes is possible (multiplexing) since Raman peaks are usually narrow and very characteristic and finally portable instruments offer the possibility of performing on-site analysis.[95]

Raman spectroscopy can be applied for both qualitative and quantitative purposes. Measuring the frequency of scattered radiations provides qualitative information of the sample. While, quantitative analysis can be performed by measuring the intensity of the scattered radiation, since it is proportional to number or concentration of the molecules present in the sample.[93,96] However, compared to fluorescence, Raman scattering is six to ten orders of magnitude less efficient.[95] Since, only about 1 in every 10^6 - 10^8 photons experiences this effect at typical conditions (very low cross section).[97] As such, the sensitivity of Raman scattering is intrinsically low, which limits its wide application in the biomedical field.[94]

One way to overcome this is by using metal nanoparticles or other nanostructures to enhance Raman efficiency. These constructions work as *nanoantennas*, by enhancing the Raman signal of molecules that are in their vicinity.[91,97], known as SERS. More specifically, when the incident laser light impinges at a metal and/or dielectric interfaces, the electromagnetic wave can drive the delocalized conduction electrons, known as surface plasmons, of the metal nanostructures into collective oscillation.

When the frequency of the incident light matches the inherent oscillation frequency of the surface plasmons, in the metal the surface plasmon resonance effect (SPR) is verified. In metal nanostructures, the SPR can be highly localized to a specific position, so it is termed localized SPR (LSPR).[75][94] In this resonant state, free electrons transiently accumulate on the nanostructure surface, greatly increasing the density of surface charges, which in turn leads to tightly confined and highly enhanced electric field on the surface's close vicinity, which enhances several optical processes including Raman scattering.[94] It is important to note that the resonance frequency

depends on the size, shape, dielectric environment, and electron density of particles, the effective electron mass, etc.[3,96]

Thanks to the recent advances in nanoscience it is now possible to synthesize metallic nanoparticles with high control of size, morphology, and composition, providing elevated uniformity and tuneable optical properties which are crucial in SERS.[96]

The nanoparticles that are able to generate a strong LSPR effect are called plasmonic nanoparticles, and normally they are usually either made of silver (Ag) or gold (Au), because these noble metals show strong SPR in the visible to near-infrared region.[94]

SERS measurements are performed on a Raman microscope, in which the sample is in contact with what is known as a SERS substrate that provides the optical enhancement.[96]

SERS substrates can in general be of one of two types: nanoparticle dispersions or assemblies of nanoparticles on a solid support.[96]

Nanoparticle dispersions are low cost and convenient to fabricate and use. However, reproducibility of such method is not ideal due to difficulty in controlling the amount and extent of random aggregation, and the electromagnetic enhancement of single isolated nanoparticles is usually too low for practical applications.[96,98]

When two metal nanoparticles are within 1 to 5 nm of each other, a powerful electromagnetic coupling occurs, which leads to a high intensity of the electric field in the gap, and therefore enhancements. These regions where the enhancement is highest are called hotspots and it is where potentially single molecule detection is possible.[96]

Typical SERS hotspots include regions related to nanotips, interparticle nanogaps or particle-substrate nanogaps. Molecules present in these regions tend to dominate the spectrum since it is where the EM field and therefore SERS enhancement are at its highest.[99] So, a typical strategy to improve SERS signal is to use closely packed nanoparticles assemblies over a solid substrate to create hotspots and therefore greatly increase enhancement.[96] However, high-quality solid SERS substrates should meet the following requirements: high detection sensitivity, uniform enhancement across large areas and finally good reproducibility of the SERS measurements from batch to batch.[100][96]

Single nanoparticles can also display remarkable SERS performance, for example, star-shaped gold nanoparticles (nanostars) show strong LSPR effects at the sharp tips of its spikes, offering great SERS enhancement.[101]

In recent years, other assets procured for SERS substrates are high good transparency and flexibility. By replacing rigid substrates with flexible and transparent ones, the applications of SERS could be largely extended. The high flexibility of the SERS substrates means that they could be easily bent or twisted without breaking, making them useful in many applications, such as flexible electronics or photonics. The use of highly transparent substrates makes it possible to excite and collect the signals from both front and back sides of the substrates. Similar to rigid substrates, analytes can be adsorbed onto flexible SERS substrates for qualitative or quantitative detections. Moreover, flexible SERS-active films, can be attached onto many surfaces of interest, even curved ones, to reveal its chemical information. In case that the surface of interest is solid, the films can be easily detached after measurements, introducing no destruction to the detected surface. SERS-active films can also potentially be reused for molecular detection by attaching it again onto other surfaces. Owing to these advantages, flexible and transparent SERS-active films have been used for chemical mapping of fingerprints, helping crime investigation and determination.[102]

Due to the high Raman scattering cross-sections involved, SERS is an advanced analytical technique that can be used for the ultrasensitive detection of several analytes, with astonishing low detection limits, even up to a single molecular level, while offering the same benefits as conventional Raman scattering, such as the possibility of multiplexing, fingerprinting and non-destructive analysis.[95,103]

Interestingly, in conventional Raman spectroscopy, fluorescence of some analytes can strongly interfere with measurements. However, the metal surfaces used in SERS can provide a way of quenching any possible fluorescence which enables the measurement of fluorophores with visible excitation.[104] The temporal and spatial resolution of SERS is remarkably high as well, down to milliseconds and to sub micrometre respectively.[96]

SERS, however, like any other analytical technique has some limitations, which it is important to keep in mind.

For the SERS effect to occur, the analyte is required to be adsorbed onto or in very close proximity to the substrate, and the magnitude of the enhancement is very dependent on several parameters such as the choice of metal substrate and laser frequency.[104]

On the other hand, the spectra obtained are dependent on the orientation of the molecule on the metal surface, and vibrations with little to no intensity in normal Raman scattering can become relatively intense in some cases, and this can make it difficult to

identify the analyte positively.[104] Additionally, in some cases, a direct interaction between analyte and metallic surfaces can lead to a change in the structure of the analyte.[3]

Laser excitation of the substrate produces strong electric fields and considerable heat which combined with oxygen can lead to the degradation of the target or fluctuations in signalling.[96] For example, enhanced organic molecules, like DNA, RNA or proteins, can be burned under laser beam impact, causing an additional peak from amorphous carbon to appear on the spectrum, making analysis difficult.[3] Therefore, it is crucial to optimise laser power in order to allow the measurement, to protect precious sample and to obtain the best signal to noise ratio.[96]

One major obstacle to overcome that greatly hampers the implementation of SERS in clinical practice is the frequent lack of reproducibility of SERS signals. Since the intensity of the measured SERS signal is sensitive for example to the nanoparticles size and shape, a little variation in the characteristics of the SERS substrate causes signal changes, which prevents its widespread use.[3]

Lastly, the presence of contaminants is a persistent issue verified when working with SERS.[96] Since this technique is very sensitive, it is possible that very small amounts of a contaminant can be detected in the spectrum, and if the analyte to be considered does not adsorb efficiently or does not give a strong SERS signal, the contaminant can easily dominate the SERS spectrum.[104]

The problems mentioned limit the application of SERS as a quantitative analytical method. However, they can be overcome at least in part for example by combining it with microfluidics to create remarkable optofluidic platforms. Since microfluidic offers automation, it allows for greater reproducibility when compared to bulk SERS experiments. On the other hand, microfluidic assays, due to the small volumes involved, can greatly benefit from SERS due to the potentially ultra-high detection limits of this sensing technique.[91,104]

F.1. SERS-based DNA/RNA biosensors

Most common methods for nucleic acid detection rely on fluorescent outputs.[1] However, even though the use of fluorescence is well established, it suffers usually from strong background interference common in biological environments and *photobleaching*. Moreover, with fluorescence high-level multiplexing is hard to achieve and it is only possible to work in the visible region.[105]

Since SERS allows for the molecular-level identification of samples, and the sample preparation and operation are usually simple and non-destructive, SERS has been widely utilised, as an alternative to fluorescence, to detect biomolecules at trace level, such as nucleic acids and proteins.[105,106] SERS is particularly well-suited for DNA/RNA detection since it offers ultra-high sensitivity, possibility of multiplexing due to the narrow spectral bands and the abundant fingerprint information normally associated.[106] However, specific DNA/RNA label-free identification is something very hard to accomplish with SERS alone since DNA/RNA molecules are formed by only four distinct bases with different relative contents and sequences.[94]

Even though, label-free detection offers experimental simplicity; with complex samples, the signal may be indistinguishable and too low for the reliable detection of, for example, one base mutations.[3] Therefore, Raman reporters (molecules with an exceptionally large cross section for Raman scattering), are often included in SERS-based DNA/RNA experiments for the indirect detection of these molecules. The greatest advantage of this methodology is the much higher cross section of the Raman scattering of the reporter molecules compared to label-free nucleotides.[3]

In indirect strategies, when a DNA/RNA fragment with a given sequence is present in the analysed sample, it induces the displacement of the Raman reporter changing its distance to plasmonic nanostructure, which in turn induces a change in the intensity of the measured SERS signal of reporter. Essentially, the detection of the DNA/RNA fragment with a specific sequence is based on the intensity change of the SERS bands of the Raman reporter when the said fragment is present.[3]

Nonetheless, for any SERS based nucleotide biosensor to achieve ultra-trace detection of biomolecules, the Raman signals need to be greatly enhanced through either preparing highly efficient substrates or increasing the number of Raman signal molecules, for example by PCR.[106]

Several research groups were able to adapt it for nucleic acid analysis by combining SERS with the PCR technique. However, some impressive research work has also been done in amplification free SERS DNA/RNA sensors.[51]

One such work was published in 2019 by Wu and colleagues.[7] In this work a DNA biosensor was developed to detect mutated human *KRAS*. This biosensor showed remarkably low detection limits, as low as 50 fM (50×10^{-15} M), by combining both SERS and microfluidics. Microfluidics technology offers automation and parallel analysis while SERS is a great technique to study DNA mutations, thanks to its extraordinarily low detection limits, and usually.[3,7]

This simple yet elegant system consisted on a hairpin on-off type of DNA biosensor, where an oligonucleotide hybridization probe, a molecular beacon, is designed to spontaneously form a hairpin (stem-loop) structure due to their complementary ends (C/G sequences). The 3' end of the molecular beacon is modified with a thiol group (-SH) for covalent binding to the gold/silver plasmonic substrates and the 5' end is labelled with a Raman reporter. In normal conditions, in the absence of the target DNA, the self-hybridisation of the molecular beacon led to the attachment of the Raman reporter at the 5' end of the molecular beacon to the surface of the plasmonic surface, allowing the reporter to provide a strong Raman signal ("SERS on"). However, the presence of the target DNA, disrupts the hairpin by hybridising with the central portion of the molecular beacon, creating a DNA linear duplex. The Raman reporter is pulled away from the SERS substrate leading to a huge decrease in the intensity of the Raman signal from the reporter molecule ("SERS off"), since the reporter is now more distant from the SERS substrate. A schematic of this mechanism is shown in Figure 10.

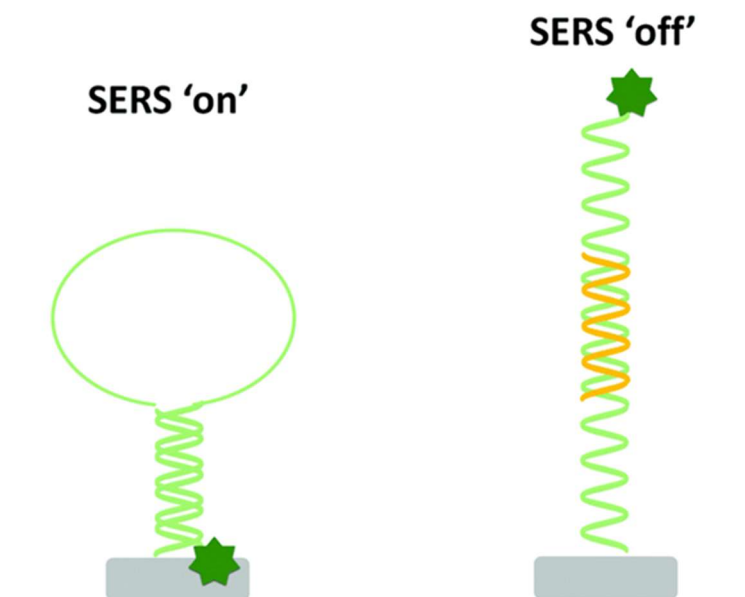


Figure 10 - Work principle of the biosensor presented by Wu *et al.* in 2019. The plasmonic substrate is represented by gray rods, while the Raman reported is represented by green stars. The molecular beacon (in pale green) spontaneously forms a hairpin structure that makes the Raman reporter to be in close contact with the plasmonic substrate ("SERS on"). However, the stem-loop structure is disrupted if a complementary sequence is present (yellow), which forces the Raman reporter to be pulled away from the plasmonic surface, causing a significant decrease in the SERS signal associated to the reporter. Adapted from reference 7.

The mutation detection is carried out on a microfluidic chip, which is fabricated by bonding the glass slide with pre immobilised nanoparticles and molecular beacons to PDMS microchannels. In these microchannels the DNA samples are flowed through in order to interact with the molecular beacons. Then, by acquiring the SERS signal, where the DNA samples meets the plasmonic substrate with the molecular beacons it is possible to detect the target DNA by evaluating if there is hybridisation with the molecular beacon (lower SERS intensity given by the reporter). The amount of target DNA present in the sample was shown to negatively correlate with the intensity of the SERS signal. Meaning, the higher the content in the DNA targeted by the molecular beacon, the lower the intensity of the SERS signal. This concept was expanded in a further work of the same group where they demonstrated the ability of this system to perform multiplex analysis using clinical samples.[107]

Despite this technology being promising, unfortunately, so far, not a single SERS DNA sensor has been applied in clinical practice, which is mainly due to the still insufficient sensitivity of SERS DNA sensors, as well as due to the uncontrolled inter-batch reproducibility of SERS substrates. In real clinical samples used to detect mutated DNA (for example, samples of blood used to detect a characteristic circulating free tumour DNA), usually only a very small amount of the mutated allele is present in the

sample (about 0.1 %–0.01 %). This often means that the difference between the Raman signal generated in experiments with samples containing such a small amount of mutated DNA and in samples without mutated DNA is very small, and thus indistinguishable.[3]

Therefore, to incorporate SERS DNA sensors in actual clinical practice, the development of new, highly active, stable and very reproducible plasmonic substrates for SERS measurements is necessary.[3]

Objectives

The objective of this thesis is to design, fabricate and optimise a universal optofluidic platform for nucleic acid detection (for both RNA and DNA), which combines both SERS and microfluidic technologies into a single medical device.

Due to the combination of SERS and microfluidics it is expected for the platform to offer fast, ultra-sensitive and specific detection at low cost, making it appropriate for POC applications. If such criteria are met the solution here envisioned will largely outperform the standard technologies for nucleotide selection currently on the market.

The platform is based on the DNA biosensor described by Wu *et. al.* in 2019, and so the applications of this technology are expanded, and its robustness is increased.

To achieve this ambitious objective, different configurations of SERS substrates will be explored. Ideally this process should lead to the development of flexible plasmonic structures that are reproducible and offer particularly high enhancements in a cost-effective manner, that are beneficial to be included in the platform.

Additionally, multi-phase flow CFD simulations will be implemented to guide the design and to test *in silico* a microfluidic chip for passive hydrodynamic droplet trapping. Such construct it is intended to be used to create, by self-assembly, array-like plasmonic structures that can be incorporated in the optofluidic platform.

As proof-of-concept of the developed biosensor, the technology will be applied for the detection of mutant *BRAF V600E*, which is associated to several types of cancer, and *RdRp* RNA associated to the virus SARS-CoV-2 which is known to cause severe respiratory problems. By detecting both RNA and DNA samples associated to very clinically distinct situations the versatility of this method for nucleic acid detection will be demonstrated.

Materials and Methods

A. Materials and reagents

Hydrogen peroxide (H_2O_2) 30 % solution, absolute ethanol (EtOH) and isopropanol (2-propanol) were purchased from Honeywell. PDMS was acquired from Ellsworth. Glass slides were obtained from Thermo Scientific. The synthetic nucleic acids related to the *BRAF* gene were ordered from Biobasic, while for the *RdRp* gene were purchased from IDA, Integrated DNA Technologies. The full DNA/RNA sequences used can be found on List A1 in the Appendix.

All other chemicals were purchased from Sigma-Aldrich.

B. Methods

B.1. Nanoparticle synthesis

Gold nanostars (GNSs) were synthesised by using a modified version of the seed-mediated growth method described previously by Kumar *et al.*[108].

First, gold citrate stabilized 15 nm nanoparticles were first produced by the Turkevich method to be used as seeds.[109] Briefly, 5 mL of 1 % solution of sodium citrate dihydrate was added to 100 mL of boiling chloroauric acid (HAuCl_4) solution, at 0.5 mM under vigorous magnetic stirring. Upon the addition of the sodium citrate, the solution was kept at boiling point under magnetic stirring until its colour turned from yellow to dark red. Then, citrate was replaced by polyvinylpyrrolidone (10 K (MW = 10,000 g/mol), targeting at 60 molecules of PVP per nm^2 . This was done by adding 1 mL of PVP (56 mg/mL) to 25 mL of gold nanospheres (0.557 mM $[\text{Au}]$) and leaving it overnight under magnetic stirring. The resulting solution was centrifuged for 90 min at 7000 rpm, re-dispersed in 25 mL of isopropanol, and stored in the fridge until further use. For the seed-mediated growth of the GNSs, typically, 25 g of PVP-10K were dissolved in 250 mL of N,N-dimethylformamide (DMF) for 30 min by ultrasonication. To this solution it was added an aqueous solution of 0.126 M HAuCl_4 , and vigorously magnetically stirred for 2 min, to allow the Au^{3+} to be reduced to Au^{+1} . Afterwards, 1.3 mL of 1.8×10^{-3} M $[\text{Au}]$ Au@PVP particles were added to the reaction flask and left under magnetic stirring for 1 hr. During this process, the colour shifted from wine red to dark bluish grey, indicating the reduction of the Au^{+1} to Au^0 and the subsequent GNSs

formation (Figure A1). The volume of HAuCl_4 added in this procedure was different according to the ratio (R) desired of this concentration in relation to the [Au] seed concentration in the volume of DMF added (Equation 5).

$$R = \frac{[\text{HAuCl}_4]}{[\text{Au PVP seeds}]} \quad (5)$$

In this work, the R values of 2.5, 5, 10, 20 and 45 were tested. The respective values of concentrations for the different R values can be found in Table A1. The newly formed GNSs were washed for five times (4000 rpm, 30 min) with isopropanol to remove excess PVP and DMF molecules and stored in isopropanol, protected from light, until further use.

Au@AgNRS (gold silver coated nanorods) were synthesized by coating gold nanorods (AuNRs) with a silver shell. This process was done by other members of the group in the laboratory according to the protocol described by Wu *et al.*, 2019.[7] The initial AuNRs were synthesised by the seed mediated growth method previously described in literature with some modifications. Typically, 1.5 mL of 1 mM HAuCl_4 solution was mixed with 2.5 mL of 0.2 M CTAB (Cetyltrimethyl ammonium bromide). Subsequently, under vigorous stirring, 600 μL of 0.01 M iced NaBH_4 solution was quickly added to the mixture. Within 2 min, the colour of the solution turned to dark brown, which indicated the formation of the Au nanoseeds. Meanwhile, the growth solution was prepared by sequentially adding 180 μL of 15 mM AgNO_3 , 1.25 mL of 15 mM HAuCl_4 and 11 mL of Milli-Q water into 12.5 mL of 0.2 M CTAB solution under vigorous stirring.

Then, 0.1 M ascorbic acid (AA) solution was slowly added drop by drop. After the colour of the solution became transparent, one third more of the total number of the drops was added. Finally, 20 μL of the seed solution was mixed with the growth solution. After 15 s stirring and 1 hr reaction without stirring, the colour gradually turned to dark purple, which signified the formation of AuNRs. The prepared AuNRs were purified by centrifugation once at 9000 rpm for 20 min.

Au@AgNRS were produced by adding 0.0728g of CTAB, 4 mL of Milli-Q water, 130 μL of 0.1 M AA, 140 μL of 15 mM AgNO_3 , 240 μL of 0.1 M NaOH to 2 mL of the purified AuNRs under vigorous stirring. After 5 min reaction, the obtained Au@AgNRS

were purified once by centrifugation at 7500 rpm for 20 min and stored protected from light until further use.

B.2. Nanoparticle optical and morphological characterisation

The synthesised GNSs were characterised in all cases by ultraviolet-visible (UV-Vis) spectrophotometry (SHIMADZU, UV-2550, Kyoto, Japan) and transmission electron microscopy (TEM) (JEOL-2100, Kyoto, Japan). UV-Vis absorption spectra ranged from 300 to 900 nm and they were acquired using 1.5 mL polystyrene disposable cuvettes with an optical path of 1 cm.

The gold concentration, [Au], of the samples of GNSs was determined by exporting the UV-Vis data to excel format and use the formula:

$$[\text{Au}] = \frac{\text{Abs}_{400}}{2.4} \cdot D_f \quad (6)$$

Where, [Au] is expressed in mM, Abs₄₀₀ is the absorbance of the sample at 400 nm and D_f is the dilution factor. An example of calculations using equation 6 can be found on Example of Calculus A1. UV-Vis spectra were normalised for the wavelength of 400 nm and plotted using the GraphPad Prim software version 8.

TEM samples were prepared by drop casting 10 µL of the NP suspension onto 400 mesh copper grids (Ted Pella, INC, Redding, CA, US). TEM images were acquired at 200 kV. Nanoparticle dimensions were determined by measuring the length of the largest segment that joins opposite tips, using the analysis software ImageJ, whose interface can be found on Figure A2.

A minimum of 100 nanoparticles (NPs) were measured per batch to allow for sufficient sample representation. Origin Pro v 9.5.1 was used to plot the size distribution histograms and respective normal curve.

To acquire the SEM images of the assemblies of NPs on solid substrates, the Scanning Electron Microscope QUANTA 650FEG was used.

The Au@AgNRs used were previously characterized by Wu *at al.*, and their effectiveness, when assembled on glass, as plasmonic substrate was previously comprehensively documented.[7]

B.3. Fabrication of SERS substrates

The plasmonic substrates were fabricated by assembling Au@AgNRs on thiol group modified glass slides or GNSs to thiol-group modified PDMS layers.

To functionalise the glass slides with the Au@AgNRs, the protocol described Wu *et al.*, 2019 was used.

Briefly, microscope glass slides were washed with piranha solution for 1 hr at 80 °C to create silanol bounds on the surface (Si-OH) of the glass thus activating it for the next steps. A picture of the setup used for the piranha glass treatment can be found on Figure A3. Then, the glass slides were rinsed 3 times with Milli-Q water and dried with pure nitrogen gas (N₂), before being treated with MPTMS ((3-Mercaptopropyl)trimethoxysilane) 10 % solution in pure EtOH, previously prepared under a fume hood, for 4 hr at room temperature. Then, the slides were rinsed with pure EtOH three times and dried with N₂ gas and left to dry and complete to MPTMS binding at 65 °C for 1 hr in a sealed container. After functionalisation, the different Au@AgNRs suspensions (on NaCl aqueous solutions) were poured over the functionalised glass slides to bind to its surface.

The use of NaCl solution to disperse the NRs for the glass functionalisation was shown previously to improve assembly and density of the substrate by reducing the repulsive power between the NPs, and by disrupting the CTAB bilayers around the particles making them bind, via thiol groups, to the glass slide more easily. However, excess NaCl leads to severe aggregation of the NPs, which in turn reduces the quality and efficiency of the assembly. Wu *et. al* for the Au@AgNRs were able to achieve an optimal NaCl concentration of 4 mM. This concentration was shown to offer the highest uniformity, density and SERS signal intensity.[7] The NP dispersions were confined and kept in place with the help of hand-crafted PDMS drilled piece firmly pressed against the glass slides (reversibly bounded).

After overnight incubation to allow the chemical assembly of the nanoparticles to the glass slides via thiol bounds, the glass slides were washed 3 times with Milli-Q water to remove any unbound or weakly bounded nanoparticles.

The protocol presented above for the glass slides was adapted as well for PDMS since its structure and composition shares similarities to the one of the glass. Only GNSs were assembled on PDMS. First a PDMS flat piece of a few millimetres of thickness contained in a plastic Petr-dish was washed 3 times with pure EtOH and dried with a N₂.

Then, its surface was activated with plasma treatment for two minutes using the Harrick Plasma Cleaner PDL-002 CE. Immediately after activation, the PDMS surface was treated with 10 mL of 10 % MPTMS + 10 % acetic acid solution in pure EtOH. The MPTMS treatment typically lasted for 2 hr at room temperature. Similar to the glass slides, after treatment the PDMS was rinsed three times with pure EtOH and dried with N₂. To complete the reaction and remove completely all EtOH, the PDMS was placed on an oven to dry, at 65 °C, in sealed container for 1 hr. Finally, the GNSs were re-suspended in the desired solvent, sonicated, and poured over functionalised PDMS, with a mould previously placed on and left for reaction overnight.

The GNSs were re-suspended in either Milli-Q, isopropanol or NaCl solution at different concentrations to test the best conditions leading to a homogeneous assembly. An image which includes all conditions tested can be found on Figure A4. For all cases, after treatment the plasmonic surface was washed three times with Milli-Q to remove unbound particles.

B.4. Nucleotide detection in optofluidic device

Glass slides functionalised with Au@AgNRs were used as the SERS substrate component of the optofluidic devices. An image of this substrate can be found on Figure A5.

After the initial production of the plasmonic substrate, molecular probes for either the *RdRp* gene or *BRAF V600E* mutation were attached to the metal substrate via thiol bounds. Then, a PDMS replica with the microfluidic channels was bonded via oxygen plasma treatment to each glass slide to form an optofluidic chip ready for analysis.

An illustrative representation of the main steps of the optofluidic platform creation and usage can be found in Figure A6.

A more detailed description of every step taken for the optofluidic fabrication process can be found below.

B.4.1. Immobilisation of the molecular beacon

First, the nucleotide sequences were heated to 95 °C for 10 min, and then gradually cooled down to form a hairpin structure. Then, 100 µL of 0.5 µM nucleotide, in 10 mM PBS mM solution, was added to the nanoparticle-MPTMS-modified glass slides and left for reaction at room temperature overnight. After, the glass slides were then treated with

the same volume (100 μ L) of 0.1 M NaCl (in PBS) for 12 hr to better immobilise these nucleotides. Finally, the glass slides were washed 3 times with Milli-Q water and dried with N₂ gas.

B.4.2 Production of the optofluidic chip

The molecular beacon immobilized glass slides were bounded to PDMS microchannels to create a microfluidic chip. The PDMS microchannels were produced by replica moulding, as previously described.[110]

Briefly, the PDMS prepolymer (Sylgard 184 silicone elastomer kit), was prepared by mixing the base and curing agent at a weight ratio of 10: 1, then it was poured onto the PMMA (poly(methyl methacrylate)) mould, placed under vacuum for 15–20 min to remove the air bubbles and cured in the oven at 65°C for 1 hr. After cooling down at room temperature, the PDMS replica was peeled off from the mould with the assistance of a scalpel. The design of the PDMS mold and a sample PDMS replica utilized in the optofluidic devices can be observed on Figure A7.

For the chip bonding, the microchannels were cleaned with N₂ and treated with oxygen plasma for 60 s to be irreversible bonded to the glass. After plasma treatment the PDMS portion was pressed gently against the functionalised glass slides. To ensure a strong and efficient bonding, the devices were pressed with a heavy weight for at least 1 hr before use.

The glass slides with the Au@AgNRs and molecular beacons were not at any part of these procedures exposed to oxygen plasma to ensure the integrity of the SERS substrate and molecular components.

B.4.3 Reaction with target DNA/RNA

The target DNA/RNA were diluted to the desired concentrations with the reaction buffer (0.01 M Tris-HCl buffer with 0.1 M KCl and 1 mM MgCl₂, pH=8). Then both Tris-HCl buffer (blank) and the target containing solution were injected into different microfluidic channels, at 5 μ L min⁻¹ for 5 min. NewEra syringe pumps equipped with 1 mL, 20G Terumo syringes connected to the microfluidic chips by EVA (Ethyl Vinyl Acetate) tubing were used for the controlled injection of the desired solutions. An image of the used setup can be found in Figure A8. After injection, the chip was kept at 50 °C for 30 min to allow the reaction to occur. Finally, the microfluidic channels were washed

with PBST (0.05 % Tween 20 in PBS) solution at $5 \mu\text{L min}^{-1}$ for 5 min to remove unbound target nucleotide sequences.

B.5. SERS measurements

A Witec Alfa300 R confocal Raman system was used for all SERS measurements.

To test the enhancement of the used substrates, the Raman reporter 1-naphthalenethiol (1NAT) was used. The most prominent peak (highest in relative intensity) is at about 1370 cm^{-1} , relative to the ring stretching vibrational mode of 1NAT molecule.[111]

Briefly, to test the SERS enhancement in solution of GNSs, dilutions of GNSs and 1NAT stock solution were prepared so the final 1NAT concentration would be 10^{-4} M , 10^{-5} M , 10^{-6} M . Then the dispersions were incubated for 2 h in an orbital shaker at 25 rpm protected from light, before observation at the dispersion on the Raman microscope inside a small container.

To test the SERS enhancement of solid substrates different dilutions of 1NAT were prepared to fully immerse the substrates for 2 h at room temperature. After the 2 h the substrates were rinsed once with Milli-Q water and dried with N_2 before Raman evaluation.

For the GNSs the laser 785 nm was deployed as the excitation source, with a power typically ranging from 20 mW to 100 mW in the sample position. The value of 1200 cm^{-1} was chosen as the spectral centre in these cases. The acquisition time was typically 1s with 10 accumulations for each spectrum, and each final spectrum was the average of 5 acquired in the same conditions at different locations of the sample.

For the Au@AgNRs the laser 532 nm was used as the excitation source with a power in the sample position of 2mW. The centre wavelength of 605 nm was considered in these cases. Single acquisitions for the 1NAT signal were taken using two accumulations with an integration time of 5 s.

Raman maps were constructed using the Large Area Scan – Continuous Scan function of the confocal Raman microscope. The mapping area used for testing homogeneity of the glass substrates with the Raman reporter 1NAT was $100 \mu\text{m}^2$, with a step size of $2 \mu\text{m}$ and acquisition time of 0.5s for each scan. Raman map acquisition made for genetic studies was made in area of $100 \mu\text{m}^2$ with a step of $4 \mu\text{m}$ with an acquisition time of 1s for each spectral unit (scan).

To build each visual map a sum filter was applied on the spectral area centred at 1360 cm⁻¹ for the 1NAT and centred on 1640 cm⁻¹ for the nucleotide detection studies. The sum filters covered a width of 80 cm⁻¹ and a background subtraction of 4 units in both sides was considered. Unless otherwise stated the Raman objective used was the 10x.

All the resulting spectra were processed with the WiTec Project Five v5.1 program to remove Cosmic Rays, attribute individual baselines and perform the average of the spectra when acquired on the same conditions.

Plotting of the processed spectra for the calculation of peak values was made using the Origin Pro v9.5.1 software.

Area under the curve (AUC) values were calculated, using the Spectragryph v1.2 software, for all the spectra obtained by mapping of the plasmonic substrates with the molecular probes. The calculation was done from 1620 to 1670 cm⁻¹ and an individual baseline was considered for each spectrum.

B.6. CFD simulations

The CFD Simulations for multi-phase flow in this master thesis's project were performed using the COMSOL Multiphysics® software v5.5, a potent FEM-based simulation environment.[78] The user-interface in COMSOL can be found in Figure A9.

All CFD simulations performed were two-dimensional, since 3D simulations would require far greater computational power than the one that was available.

The model employed to simulate multi-phase phase flow was the conservative LS method proposed by Olsson and Kreiss[112], coupled with the Navier-Stokes equations (Equation 1) and continuity equation (Equation 2) for laminar fluid flow.

The LS model equation used is given by:

$$\frac{\partial \phi}{\partial t} + \mathbf{v} \cdot \nabla \phi = \gamma \nabla \cdot \left[\varepsilon \nabla \phi (1 - \phi) \frac{\nabla \phi}{|\nabla \phi|} \right] \quad (7)$$

Where, ϕ is the LS parameter, t is the time (s), $\mathbf{v}$ is the fluid velocity (m/s), and γ and ε are numerical stabilization parameters. More specifically, ε corresponds to the parameter controlling interface thickness (m), and γ corresponds to the reinitialization parameter (m/s).

In this model, the LS parameter varies from 0 to 1. So, the value of 0 in this case was attributed to the continuous phase, and 1 in turn was assigned to the dispersed phase. Values of ϕ equal to 0.5 give the position of the interface between the two phases.

As represented in Figure 11, for the mesh area where $\phi < 0.5$ it is considered to be occupied by the continuous phase, on the other hand, if $\phi > 0.5$ then the mesh is occupied by the dispersed phase. So, by estimating the values of ϕ for a certain mesh over small time increments it is possible to track over time the interface between the two fluids considered.

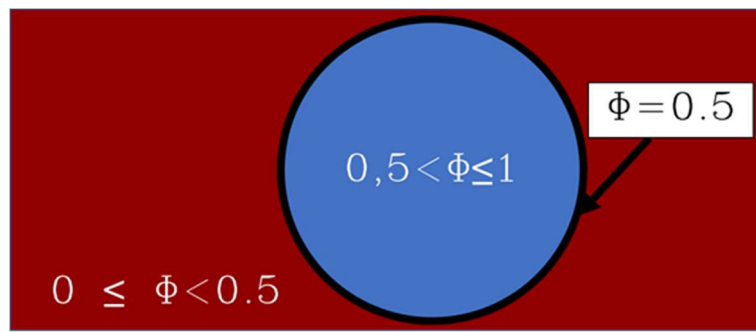


Figure 11 – The determination of the values of the level set function, ϕ , allow the determination of the phase at any point in the mesh-occupied geometry. The continuous phase is represented by Dark reddish brown. While the blue color represents the discontinuous phase. Values of ϕ between 0 and 0.5 represent the continuous phase, while values between 0.5 and 1 represent the discontinuous phase. The interface between the two fluids is defined by the region where ϕ equals 0.5.

The geometries used in the simulation were drawn with the computer aided design software AutoCAD® 2018 from AutoDesk, and they constituted a simplified version of the several parts of the device (MFDG and trapping unit). The interface of AutoCAD® 2018 can be found in Figure A10.

All fluids were considered for the purpose of the simulation as incompressible.

For both experiments and simulations, the continuous phase, with $\phi = 0$, was either Novec™ 7500 fluorinated oil or mineral oil. For the fluorinated oil, the values of absolute/dynamic viscosity and density were extracted from Sphere Fluidics Pico-Surf™ technical sheet and inserted manually on COMSOL. The properties for mineral oil were taken from Bai *et. al* simulation paper.[113] The values of the density and viscosity for both oils can be found on Table A1.

Water was considered to be the dispersed phase, $\phi = 1$, in every case and its properties were directly added from the built-in COMSOL's material library. To simulate droplet trapping, typically one to two droplets were created by drawing ellipses on the

desired model, with diameters ranging from about 120 to 150 μm . One inlet for the continuous phase was created for each system at the left end and one outlet at the right end of the main channel to control the fluid flow of the continuous phase.

The MFDGs were composed of flow focusing junctions with two inlets for the continuous phase, one inlet for the dispersed phase and finally one outlet at the right end of the channel opposed to the ones with the inlets. For all cases, the depth of the channel was assumed to be 75 μm . The outlets corresponded to a condition of pressure 0 in relation to the reference pressure (1 atm). The flow rates at the inlets for both the MFDGs and traps were defined case by case according to the needs and developments made over time and ranged from 25 $\mu\text{L/hr}$ to 2000 $\mu\text{L/hr}$.

The flow rate, Q , for a given channel with a specific cross section area of A , can be defined as the fluid volume crossing A per unit of time (t). Mathematically, this translates to:

$$Q = \int_A \vec{V} \cdot d\vec{A} \quad (8)$$

Where, $\vec{V}$ is the average velocity magnitude, and $d\vec{A}$ the element of area.[114]

For the level set model, the parameter controlling interface thickness was in general 2.5 μm , while the reinitialisation parameter was 0.1 m/s. Both values were chosen based on a trial and error approach.

A user-controlled mesh with free triangular geometry was created for every study. A maximum element of 10 μm was considered initially by the rule of thumb that the maximum element size should revolve around one-tenth of the droplet expected size.[115] However decreasing the maximum element size to 5 μm showed visible better results.

All the experiments were performed with a wetted wall condition using a contact angle of π radians, to represent a zero interaction between the water droplets and the channel walls.

From a thorough search in the literature, it was concluded that 5 mN/m was the best representative value of the surface tension between the continuous phase and water, either the first being HFE 7500 oil with Picosurf 2 % or mineral oil .[113] [116]

All other parameters and conditions were kept as default.

The time interval considered for the CFD studies typically ranged from 0 to 0.2 s up to 1s with a time step of 0.001 s, depending on the conditions of simulation.

Animations were created for every simulation using the built-in animation tool from COMSOL. The pictures displayed in this thesis were made by exporting in .png format the plots for the Volume-of-fluid 1 for the most relevant time points.

B.7. Microfluidic droplet trapping chip design

The new microfluidic chip design made consisted of one flow-focusing MFDG connected to, an adjacent droplet trapping array composed of 50 hydrodynamic traps. In between, an oil accelerator meets the channel linking the MFDG to the trapping array.

This design was created, as well, in the Computer Aided Design software Autodesk AutoCad 2018®.

The width of the channel crossing at the flow focusing junction was defined to be 80 μm .

Channels associated to the oil accelerator and trapping array have width of 120 μm .

The traps were defined to be of rectangular shape with an area of 120 μm (height) x 150 μm (length).

Three inlets were created, two to supply the MFDG, (one for the continuous phase and other for the disperse phase), and one last was created to feed the oil accelerator. All inlets have a built-in filtering structures adapted from Mazutis *et al.* microfluidic device for single cell-sorting and analysis.[117] At the end of the trapping array one outlet was placed to allow the flow direction to be from the MFDG/accelerator to the traps.

Results and Discussion

A. Optimisation and fabrication of the SERS substrates

As previously explained one technology promising in the field of detecting nucleotides is SERS due to its low detection limits and in general quantitative results. However, to achieve amplification free SERS-based DNA/RNA detection it is necessary to develop and utilize highly active, stable and reproducible plasmonic substrates.[3]

In the original version of the biosensor considered, Au@AgNRs were used for the SERS substrate since these NPs have shown to offer better enhancements when compared to bare AuNRs. With Au@AgNRs an enhancement factor of 1.85×10^6 was experimentally achieved.[7] Even though, this substrate offered great results the SERS performance still shows room to improvement since enhancements of up to 10^{14} order of magnitude can be reached with the current technologies available.[118]

Gold Nanostars (GNSs) are a plasmonic nanomaterial that have received considerable attention in numerous fields including biosensing, photothermal therapy, and surface-enhanced spectroscopies. GNSs typically consist of a central core and several protruding arms with sharp tips.[119] And, in SERS, these NPs are attractive plasmonic substrates, since even single GNSs offer great SERS enhancements in the order of $\sim 10^7$ for molecules located at its tips.[101]

Several methods have been reported to synthesize GNSs of tuneable LSPR wavelengths from visible to NIR (near-infrared region). Even though, seedless methods have been reported in literature, the mainstream protocol in the research community has been the seed mediated growth method with seeds of different shapes and sizes.[119]

Due to offering excellent yield (100 %), to being the most well described method to synthesize GNS in literature, and to offer GNSs with a well-defined and tuneable optical response, it was the method of choice in this Master Thesis to synthesize GNSs.[119,120]

Typically, the seed-mediated growth method is performed in a solution containing DMF and PVP. In Figure 12, the proposed mechanism associated for this process is presented.[119]

Briefly, in the seed mediated growth method, Au^{3+} (in our case provided by dissociation of chloroauric acid, HAuCl_4) present in the solution is, first, reduced incompletely by DMF to Au^{1+} , then PVP further reduces Au^{1+} to Au^0 with the PVP coated

seeds as the nucleation sites, leading to anisotropic growth of the NPs and the formation of tips. PVP is crucial for the growth of nanostars working both as a stabilizer and reducing agent in the presence of DMF.[119]

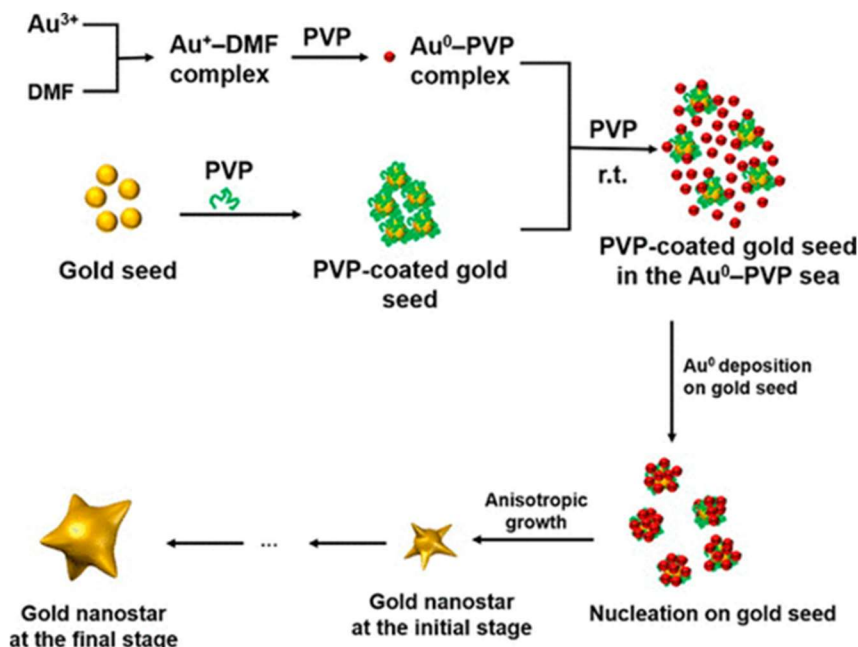


Figure 12- Proposed mechanism for the growth of GNSs produced by the Seed-Mediated Growth method using DMF and PVP. Adapted from reference 119.

One of the most interesting features of this method is that changing $[\text{HAuCl}_4]/[\text{Au seeds}]$ ratio, R , allows to easily tune the morphological and optical properties of the GNS, and it is one of the main reasons this method was chosen.

In general, it has been observed that smaller R values lead to not only smaller particles, but also to shorter and a smaller number of spikes. These changes in morphology and number of spikes cause the LSPR position and intensity to change. If R decreases, the main LSPR band (corresponding to the tip localised plasmon mode in the NIR) blueshifts and gets narrower while the band/shoulder corresponding to the core plasmon mode becomes more intense.[120]

Au nanospheres produced by the classical Turkevich method[109] were used as seeds for the GNSs synthesis. TEM analysis (Figure 13) revealed that the average size of the seeds used was 15~16 nm, which is in accordance to what has been previously reported.[109]

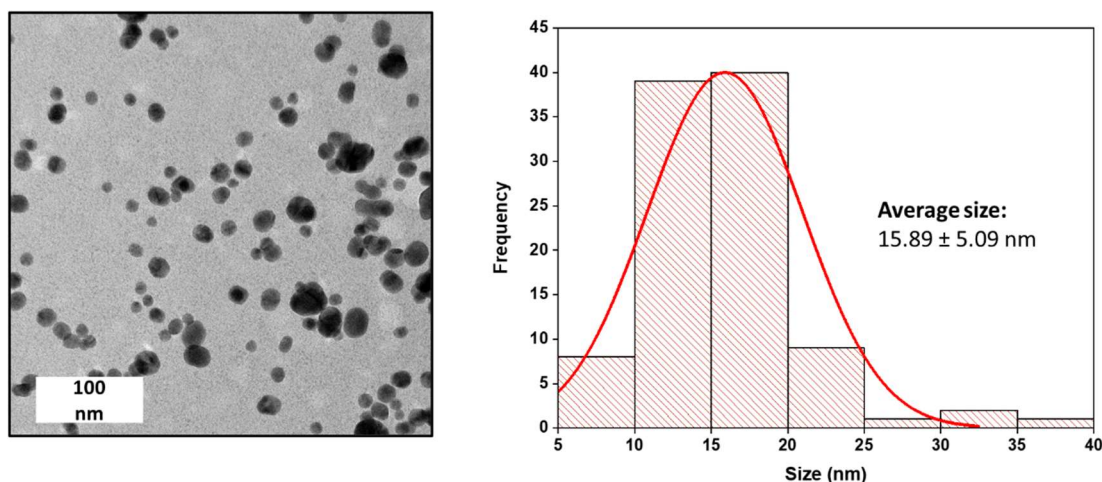


Figure 13 – Morphology and size distribution of the gold seeds used to synthesize the GNSs. TEM image (on the left), shows a round morphology of the nanoseeds. The size distribution (on the right) shows that the average size is around 15 nm but the polydispersity is quite significant, being the standard deviation (~ 5 nm) around one third of the total size (~ 15 nm).

It is important to note that in this case the polydispersity of the seeds is quite significant as showed visually by the TEM picture and size distribution histogram of Figure 13. Moreover, the shape of the nanospheres is sometimes oval and not spherical (aspect ratio higher than 1). Both occurrences are undesirable since it will hamper the homogeneity of the GNSs produced from these seeds and consequently the homogeneity of the SERS signals for them provided will be poorer. Therefore, a quality check of the reagents and conditions used to produce the seeds should be addressed in the future to improve their quality.

It is known that SERS enhancements are highly dependent of the optical properties of the plasmonic metal nanostructures, which in turn are dependent on their material(s), size geometry and surrounding environment.[96] Therefore different R values were tested for the GNSs synthesis to optimize their optical properties and SERS performance. These values can be found on Table 1. With this we expect to find ideal values of R that allow the production of GNSs with strong and appropriate LSPR effect in the NIR region.

Table 1 - Different ratios of [HAuCl₄] with [Au seeds PVP], R, and most relevant properties associated. LSPR values marked “-” could not be experimentally determined.

R Value	[HAuCl ₄] M	[Au seeds] M	Size (nm)	Main LSPR Peak (nm)
2.5	6.25E-05	2.50E-05	43.69 ± 12.70	595
5	1.25E-04	2.50E-05	56.87 ± 11.01	598
7.5	1.88E-04	2.50E-05	76.15 ± 14.08	794
10	2.50E-04	2.50E-05	94.39 ± 15.03	832
20	5.00E-04	2.50E-05	115.25 ± 14.75	-
45	1.13E-03	2.50E-05	119.30 ± 19.87	-

In general, in this work, it was experimentally verified that increasing the value of R used in the synthesis leads to an increase in the overall size, which happens in a linear to exponential fashion for low values of R. However with increase in R values the change in size starts to be less significant ultimately reaching a plateau at high values of R (>30), as observed in the graphic of Figure 14, which plots the sizes of the GNSs produced in function to their respective R value.

TEM pictures and size distribution for the various GNSs batches produced with different R values can be found in Figures A11 to A16.

Using this knowledge, with the concrete values of particle size it is possible to create a one phase association model that it is usually employed to describe this kind of behaviour.

The typical equation of a one phase association model with Y as the dependent variable X as the independent variable is,

$$Y = Y_0 + (P - Y_0) \cdot (1 - e^{(-K \cdot X)}) \quad (9)$$

Where is Y₀ is the Y value when X is zero, P is the Y value at infinite values of X (plateau), and finally, K is the rate constant, expressed in reciprocal of the X axis units. In this case, Y₀ will be associated with the seed-size, P will be associated to the highest size possible for the GNSs produced by this protocol and K will be a unitless constant similar to R.

The values that best fit the model are 9,989 nm for Y₀, 121,8 nm for P, and 0,1265 (unitless) for P. As such, the Equation 9 can be converted to:

$$\text{Average Size(nm)} = 9,989 + 111,811 \cdot (1 - e^{-0,1265 \cdot R}) \quad (10)$$

The model given by Equation 10 has a coefficient of determination (R^2) of 0.9843, which is quite satisfactory and confirms the good quality of the fit which is perceived by visual observation of Figure 14. However, Y_0 in theory should correspond to the average seed size (around 15 nm) and not ~10 nm, this is probably mainly due the polydispersity of the seeds used for synthesis. More monodisperse seeds and additionally R values should be tested to further optimise and validate this model and the experiments supporting it.

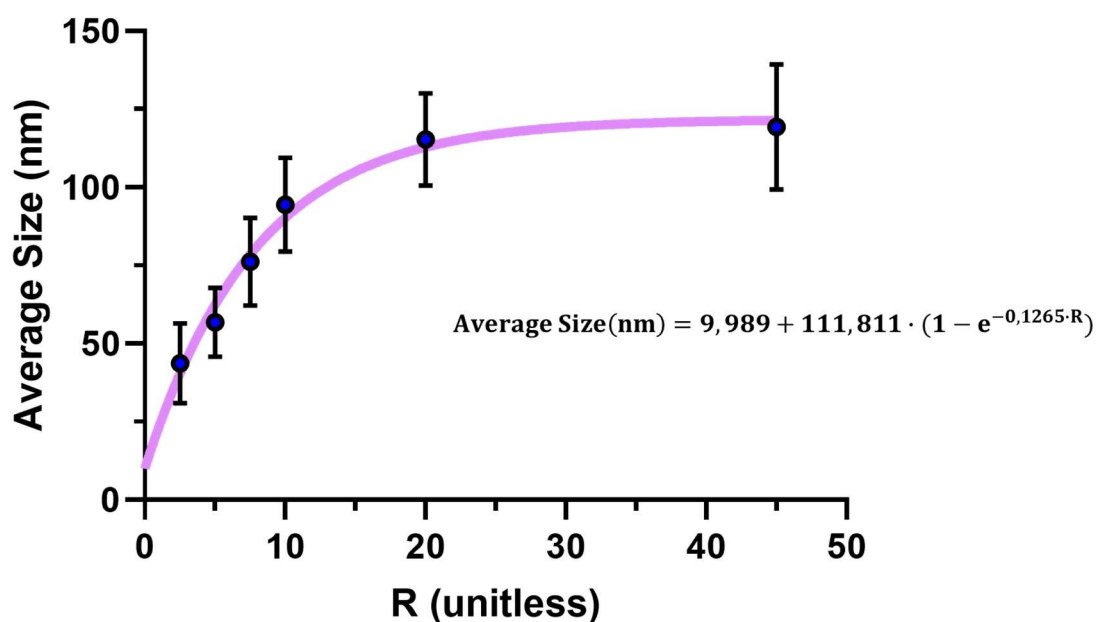


Figure 14 - Average size of the GNSs increases with R until it reaches a maximum value. The following plot represents the average size of GNSs (dark blue circles) as function of the R value. The black error bars correspond to the size (in nm) standard deviation. The pink curve corresponds to the curve of the model used to fit.

To measure the optical properties of the different batches of GNSs, the absorbance spectrum given by an UV-Vis spectrometer was obtained for every batch, which can be found on Figure 15.

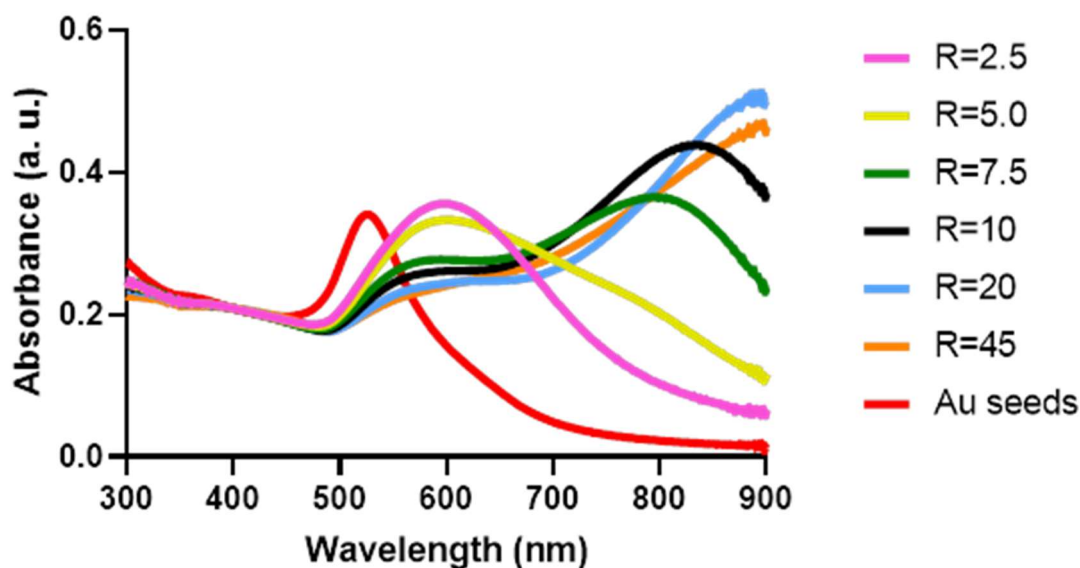


Figure 15 –Au 15 nm seeds and GNSs UV-Vis signature from 300 to 900 nm for the different R values tested. GNSs are characterised by two LSPR modes: one shoulder/peak corresponding to central core and the main one at the NIR region corresponding to the tip localized LSPR. Higher R values cause the LSPR of the tips of the GNSs to become more significant and to increase in wavelength.

As seen in the UV-Vis spectra of Figure 15, for smaller R values, as expected, the wavelength of the tip plasmon mode, in the NIR, gradually decreases while the core LSPR becomes the main band. The UV-Vis spectra is also useful to have an idea of the size and polydispersity of the GNSs prior to TEM, since the bigger the size and tips (higher R values), higher is the tip LSPR band wavelength, moreover more monodispersed GNSs batches have sharpest and more well defined LSPR bands.

Below $R = 7.5$, the contribution of the spikes is so small (most of the particles present usually just have few spikes with very small size), that their signal is negligible and only a single plasmon band, associated to the central core is observed, which becomes apparent when comparing the UV-Vis data of Figure 15 to the TEM images in Figure 16.

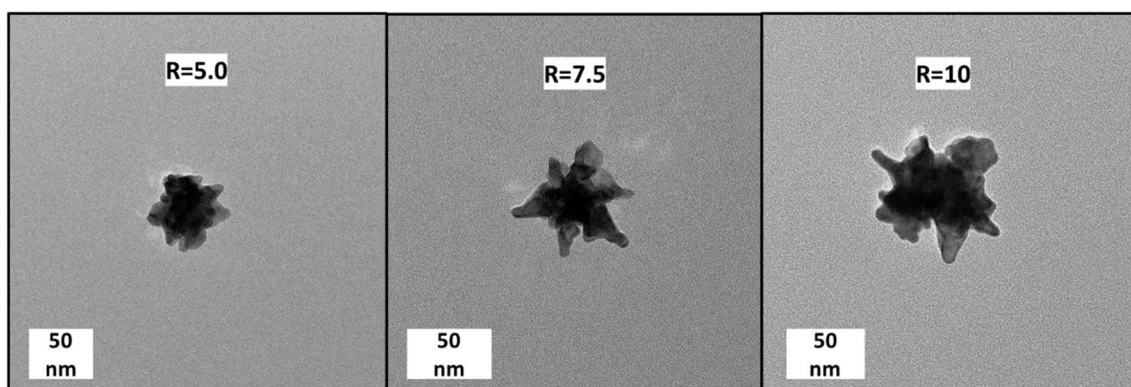


Figure 16 - Single particle TEM of GNSs with different R values. Increasing the R value from 5 to 7.5 causes the appearance of very sharp, long and well defined, which is demonstrated by the appearance of a second LSPR mode at the NIR, which becomes more significant than the one respective to the core and remain present for higher R values.

Additionally, with UV-Vis spectroscopy it is possible to determine the gold content of the sample easily by Equation 6, if the absorbance value at 400 nm is known. However, comparing the gold content of different type of batches of GNSs with different characteristics is not reliable. For example, when the GNSs are smaller the amount of Au atoms per particle is smaller as well, so it does not translate directly in particle number. In other words, for a sample with a smaller [Au] we can have a higher number of NPs, if these particles are smaller when compared to a sample with a higher Au content which has bigger but less numerous Au NPs. An alternative method such as DLS (Dynamic Light Scattering) could have been used instead to determine particle concentration and allow more reproducibility between studies with different GNSs. However, and unfortunately, such method was not readily available to the lab, so this alternative was used instead to have a notion of the concentrations.

The general practice in Raman spectroscopy is to use an excitation wavelength slightly blue shifted from the LSPR peak to optimise both the excitation and radiation efficiencies and maximize enhancement.[96]

As seen in the UV-Vis spectra of Figure 15, the GNSs synthesized display its main LSPR effect in the NIR. Since the three wavelengths available for the Raman excitation laser corresponded to 532 nm 633 nm and 785 nm, the appropriate laser for Raman excitation corresponds to the one with the wavelength 785 nm, closer to the NIR region (800 to 2500 nm). In turn, the LSPR of GNSs used should be just slightly higher than 785 nm to match the laser frequency chosen. Therefore, the GNSs suitable for SERS application should have an R value of 7.5 or 10 (Table 1). Knowing this information, the

next step is to test the SERS performance of these GNSs in solution before attempting to assemble them on a solid substrate.

To test the enhancement of the all substrates in this work, the Raman reporter 1NAT was used, since it is a small molecule with strong affinity to Ag and Au due to its thiol group.[121] And its Raman signature has been thoroughly documented.[111]

The most prominent (highest in relative intensity) SERS peak of 1NAT is at around 1370 cm^{-1} relative to its ring stretching vibrational mode.[111]

A Raman spectrum produced *in-house* previously by other colleagues was used as reference for this Raman reporter, and it can be found in Figure 17.

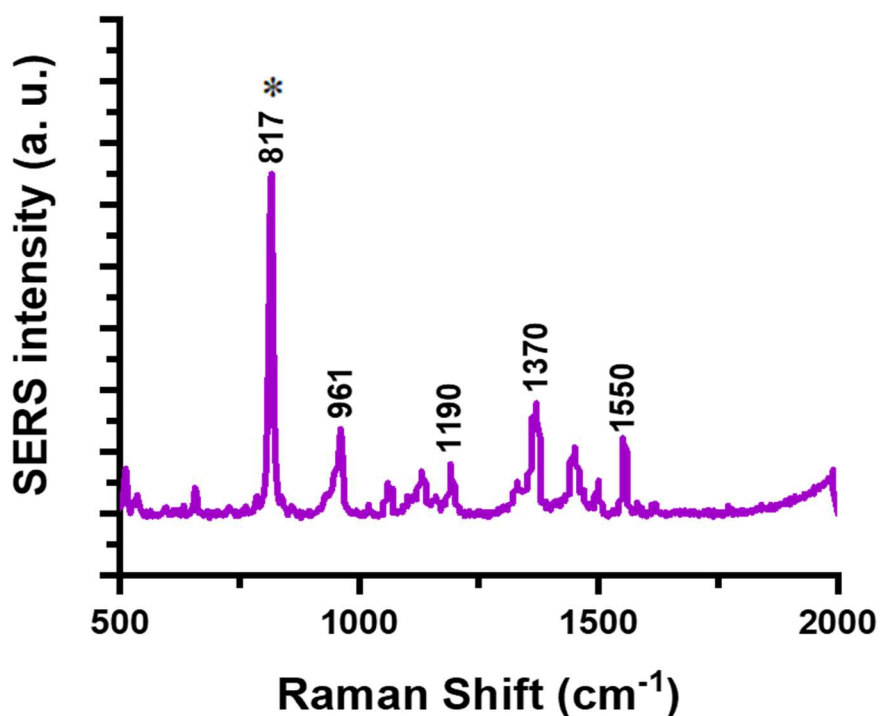


Figure 17 – Raman signature of 1NAT Raman reporter. Peaks used as reference for future studies are marked with its corresponding Raman shift values (cm⁻¹). The peak marked with an asterisk sign does not correspond to 1NAT but instead it is the characteristic peak of isopropanol used as solvent.

The reference in Figure 17 was deemed the most appropriate and accurate since many factors including instrumentation can affect the Raman signature. The peaks chosen as reference were 960 cm^{-1} , 1190 cm^{-1} , 1370 cm^{-1} , and 1550 cm^{-1} . These were chosen based on their relative intensity and distance from other reference peaks.

Raman studies with the Raman reporter 1NAT revealed that the synthesised GNSs for a R value of 7.5 and of 10 can detect reporter concentrations as low as 10^{-6} M when dispersed in a liquid phase (isopropanol at room temperature), as seen in Figure 18, and Figure 19 for the R values of 7.5 and 10 respectively.

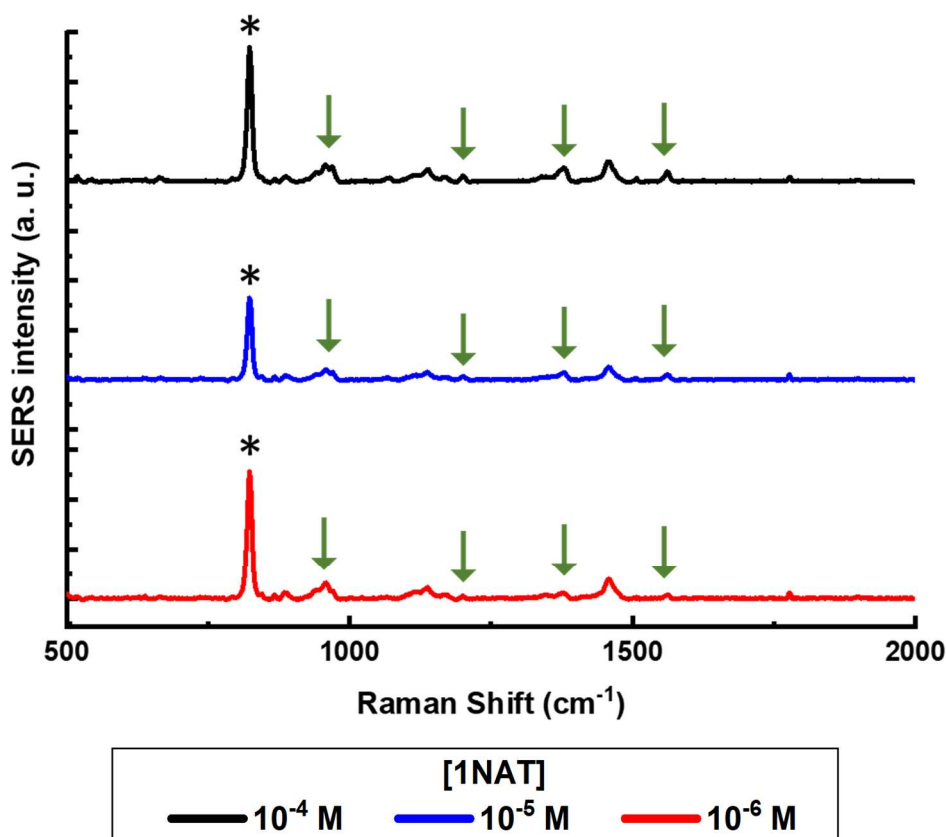


Figure 18 – SERS spectra of GNSs, with a R of 7.5, dispersed in isopropanol, and in presence 1NAT reporter at three different concentrations (10^{-4} M, 10^{-5} M, 10^{-6} M). The GNSs concentration is the same in every case. * marks the isopropanol reference peak, green arrows mark the location of the 4 Raman peaks chosen as reference for 1NAT and each corresponding 1NAT final concentration for each spectrum can be found in the box in the center bottom.

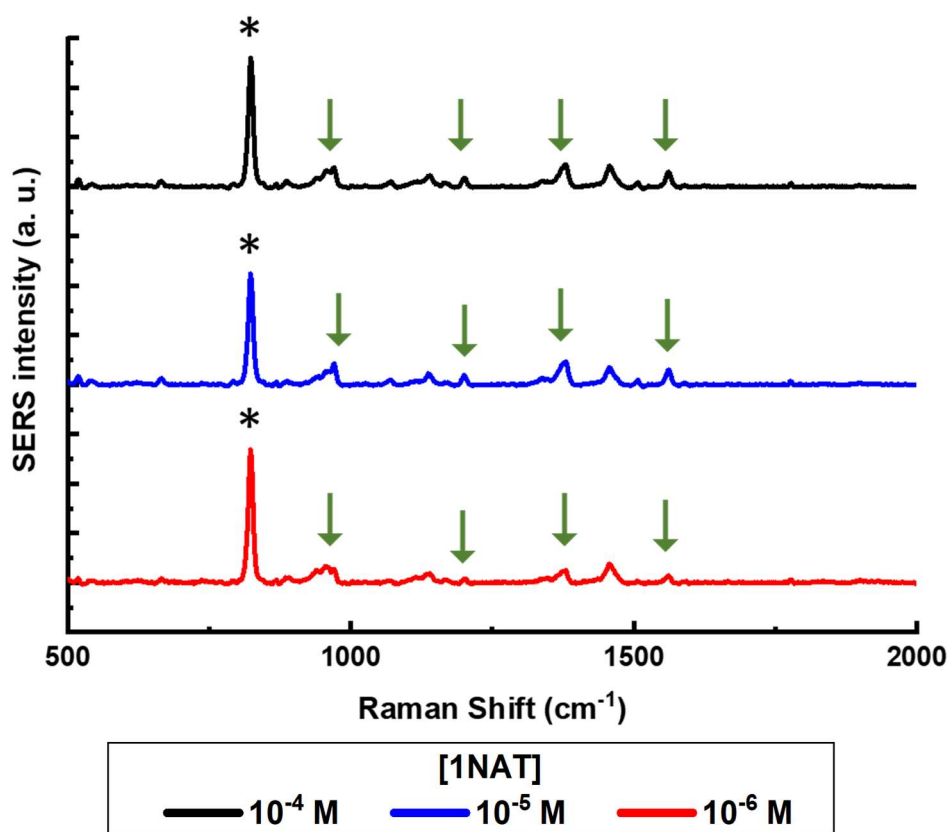


Figure 19 - SERS spectra of GNSs, with a R of 10, dispersed in isopropanol, and in presence 1NAT reporter at three different concentrations (10^{-4} M, 10^{-5} M, 10^{-6} M). The GNSs concentration is the same in every case. * marks the isopropanol reference peak, green arrows mark the location of the 4 Raman peaks chosen as reference for 1NAT and each corresponding 1NAT final concentration for each spectrum can be found in the box in the centre bottom.

By confirming that GNSs of R values of 7.5 and 10 offer good SERS signals with a limit of the detection at least as low as 10^{-6} M for the 1NAT reporter, it is now appropriate to consider to assemble these particles in a solid substrate which can potentially be used in the construction of the optofluidic device.

As mentioned earlier, transparent, and flexible SERS substrates are of considerable interest. By replacing rigid substrates with flexible and transparent ones, the applicability of SERS plasmonic substrate can be largely extended, not just for this project for many different future applications.

One material rather promising solid surface for flexible SERS is PDMS. Polydimethylsiloxane (PDMS) is widely used in biological detection and optical devices, due its stable chemical properties, nontoxicity, excellent mechanical flexibility, and low

cost. Its good plasticity, transparency and flexibility makes PDMS-based SERS sensors easy to integrate into surfaces of different shapes and sizes for *in situ* detection.[102,122] Additionally, as the most popular material in microfluidics at present,[60] a PDMS-based substrate is great to use in hybrid applications that require the combination of both SERS and microfluidics, such as the optofluidic platform intended to be developed in this project. Therefore, assembling plasmonic nanoparticles on PDMS, instead of a rigid substrate such as glass, or none-transparent such as paper, it can offer tremendous advantages. However, most works on this subject revolved around spherical particles, which are associated to lower SERS enhancements. To improve the current landscape and offer more innovation it would be interesting to assemble GNS on flexible PDMS substrate since these particles have remarkable plasmonic properties.[123]

As such, in this work, the protocol described by Wu *et al.* for glass functionalisation (by silanisation) and used in their original DNA biosensor was adapted for chemical assembly of the GNSs on a PDMS surface.

Silanisation is a process where reactive hydroxyl groups on the surface of the substrate react with methoxy- or ethoxy- residues of a silane molecule, leading to the formation of a covalent bond. The other end of the silane molecule consists of a carbon linker chain and a reactive residue which can be subsequently used for further functionalisation. Silanisation protocols typically consist of four main steps: cleaning and activation of the substrate to maximize the number of reactive hydroxyl groups on the substrate, the silanization itself performed in either vapour or liquid phase, and rinsing for the removal of unbound silane molecules, before a final (and sometimes optional) curing step.[124]

A similar procedure has been reported with a silane similar to MPTMS, (3-aminopropyl) triethoxysilane (APTES), for GNSs on a PDMS substrate.[123] However, APTES is more toxic and not as efficient for the immobilization of gold nanoparticles. Since, immobilization of gold NPs with APTES is based on electrostatic affinity of its amino group to colloidal gold, while MPTMS molecules have a mercapto- group which has a thiol end that forms a covalent (strong) bond between sulphur and gold.[125]

In the protocol from Wu *et al.*, the hydroxyl groups were introduced on glass by treating its surface with piranha solution for 1 hr, however this treatment is very aggressive, so plasma treatment for 2 min was chosen instead for PDMS.

The silanisation with MPTMS initially lasted for 1 hr, and acetic acid was added because it catalysis the silanisation process. However, increasing the time with MPTMS

and acetic acid solution for 2 hr improved results because holes on the bands of nanoparticles could be observed by naked-eye when the silanisation lasted one hour.

A curing step of 1 hr was implemented as well, similar to the original protocol, before adding the GNSs, which in turn were confined using a PDMS drilled piece.

At first, it was used a very high load of GNSs (10 mM [Au]). However, it was verified that GNSs when too densely assembled on a surface like PDMS, due to their photothermal effect, a material like PDMS can start to burn almost instantly from the heat generated from laser excitation, preventing measurements and lead to sample destruction. Even concentrations of 1 mM [Au] caused this problem as seen by the SEM images of Figure 20 and Figure A4 where very dense GNSs bands can be seen. Concentrations of 0.5 mM [Au] seem to offer the most appropriate spatial density of GNSs assembled, so it was the [Au] concentration of choice for future steps.

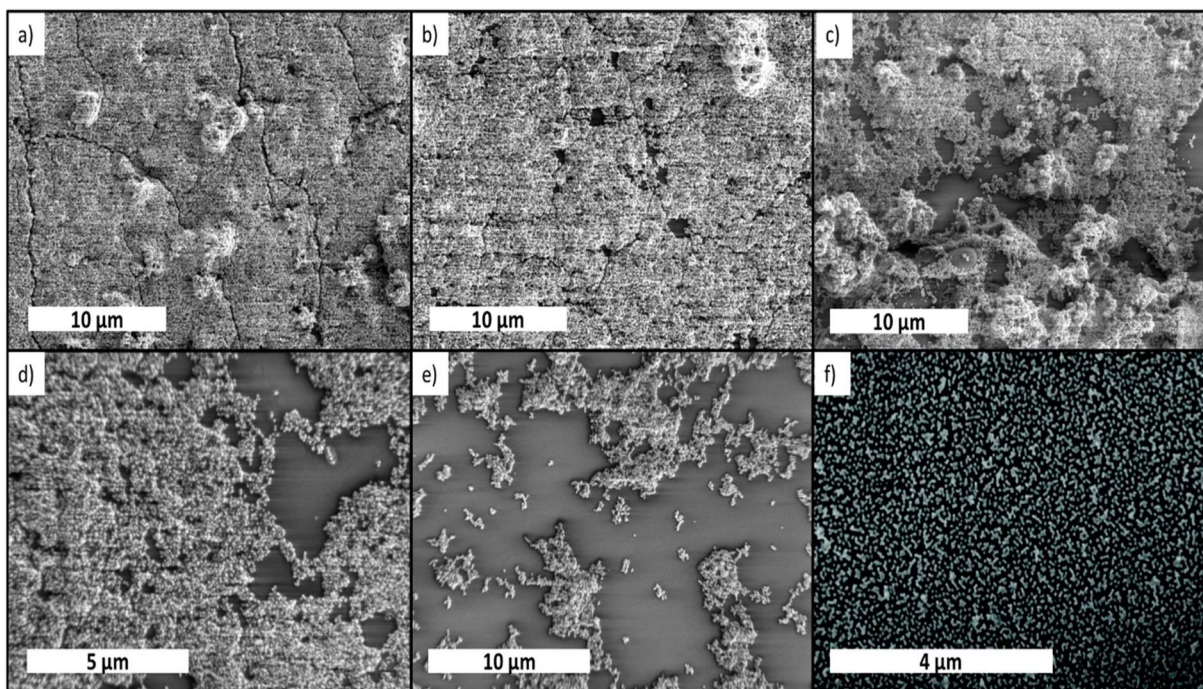


Figure 20 – SEM images of assemblies of GNSs in PDMS (a-e) at different conditions, and Ag@AgNRs on a glass surface (f); (a) 10 mM [Au] GNSs in 2mM NaCl solution; (b) 5mM [Au] GNSs in Milli-Q water; (c) 5 mM [Au] GNSs in 1 mM NaCl solution; (d) 1 mM [Au] GNSs R=20 in 1mM NaCl solution; (e) 0.5 mM [Au] GNSs PDMS in Milli-Q water; (f) Ag@AgNRs assembled of a glass surface, adapted from reference 7. Images (b-e) correspond to a MPTMS treatment of 2 hr, while for (a) an 1 hr MPTMS treatment was used. Images from (a-d) correspond to GNSs with R value of 20, while in image e), assemblies of GNSs with a R value of 10 are displayed. Molar concentration refers to the gold atoms in the dispersion.

Several solvents were tested as well, including isopropanol, Milli-Q water and NaCl aqueous solution of concentrations of 1 mM and 2 mM. Isopropanol was used to store the GNSs because these particles tend to be more stable in this organic solvent. NaCl is capable when added to reduce interparticle repulsion, which can in a small scale

improve assembly, however it is important to note that excessive salt concentration can lead to severe nanoparticle aggregation.[7]

Naked visual analysis alone was enough to determine that using isopropanol offered inferior results in terms of assembly homogeneity, when compared to using either Milli-Q or NaCl solution, and SEM analysis revealed that using NaCl even at a concentration of 1 mM drives aggregation leading to less homogeneous and reproducible assemblies when comparing with the assemblies that rely on Milli-Q water as solvent.

Based on the results, it was opted for MPTMS treatments of 2 hr, to utilise Milli-Q to redisperse the GNSs when placing them on the PDMS surface and for the concentration of GNSs to be around 0.5 mM [Au]. Assemblies in these conditions were produced with GNSs of 7.5 and 10 as R values, which can be found in Figure 21-a) and Figure 21-b) respectively.

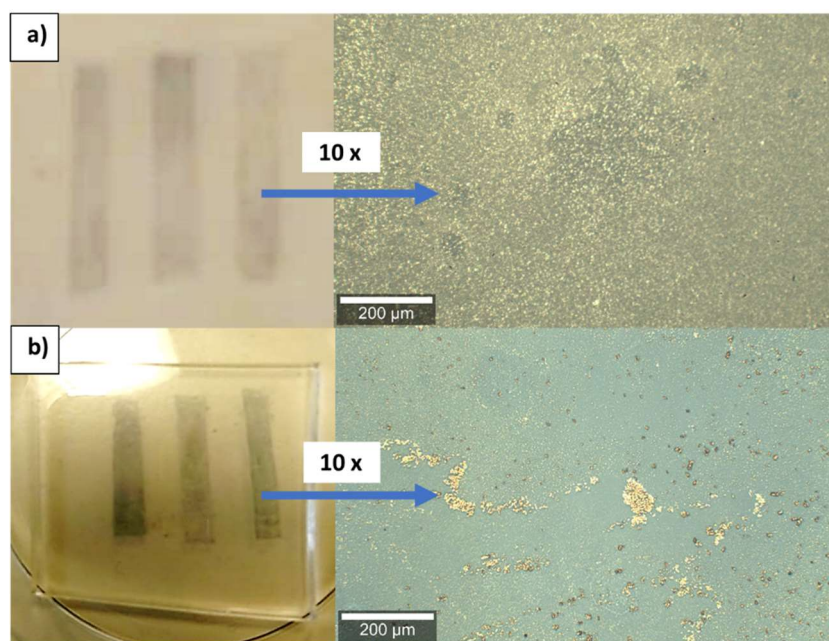


Figure 21 - Assemblies of GNSs with R values of 7.5 (a) and 10 (b). On the left side images can be seen of the GNSs bands on PDMS. On the right side, the corresponding bright field images taken with the 10x objective can be observed.

By trial and error, the laser power was chosen to be 50 mW, because at this intensity the PDMS did not burn in the time used for analysis, while still being relatively high power, since 100 mW was used for the GNSs in isopropanol. However, we were not able to detect any signal of the 1NAT reporter for concentrations as high as 10^{-4} M with any of the GNSs assemblies developed.

The lack of SERS signal might occur for several reasons, first GNSs assembled as they were might simply not be effective as plasmonic substrates, not being capable of

enhancing SERS signal to the level it would be required to detect 1NAT at 10^{-4} M at conditions used, since for example LSPR shifts occur when GNSs go from a dispersed state to a solid assembly.

Additionally, the GNSs produced might not have sufficient quality, for example in terms of size polydispersity, even if in solution these NPs were shown to offer good SERS enhancements and detection limits of at least 10^{-6} M.

The molecules of 1NAT might be degraded by the high temperatures caused from laser excitation even if the PDMS is not burnt when the GNSs are assembled. And even if PDMS is apparently not burning small, microscopic to sub microscopic changes might be happening such as local melting that is changing the substrate properties and preventing the detection of 1NAT.

Contaminants, for example, from the functionalisation process, might have also hidden the signal of the 1NAT, since SERS is an extremely sensitive technique.

To conclude the optical response of GNSs was optimised in solution, which made these particles when dispersed in isopropanol to offer good SERS performances when used as plasmonic substrate. On the other hand, the GNSs were successfully and efficiently assembled in a flexible PDMS substrate by a process of chemical self-assembly. However, the GNSs when assembled in PDMS did not offered better or even comparable enhancement levels when used in liquid phase SERS. The very poor SERS performance displayed makes these SERS substrates not appropriate for future applications, unless further optimisations are made that provide the necessary plasmonic properties.

B. Proof-of-concept: *BRAF* and *RdRp* gene detection

One of the core components of the optofluidic platform having more room to improvement is the plasmonic substrate associated, since it enhances Raman signalling allowing for sensitive detection at much lower concentrations. Unfortunately, GNSs PDMS-based substrates were not successful in producing satisfactory SERS signals, for the purpose of creating a functional optofluidic platform. Therefore, Au@AgNRs assembled glass slides, as described by Wu and co-workers were chosen instead as the plasmonic substrate.[7]

To show how simple is to adapt and how versatile Wu and co-workers' technology is, its optofluidic platform was adapted to very two different contexts, for the detection of *BRAF V600E* DNA and the RNA of SARS-CoV-2 *RdRp* gene. Detection of SARS-CoV-2 *RdRp* is particularly useful for the sensitive diagnosis and monitoring of COVID-19 patients, while *BRAF V600E* detection is clinically desirable in several types of cancer, especially in context of personalised therapy and patient stratification.

The changes in target is easily accomplished by switching the molecular probe sequence that recognises the target sequence. Therefore, at its core, in this project the detection of DNA associated to many types of cancerous cells (*BRAF V600E*) and an RNA sequence characteristic of a dangerous and potentially deadly viral infection (*RdRp* from SARS-CoV-2) was attempted.

As explained above in the introduction section, both these specific situations in a clinical setting can heavily benefit from this technology since it offers amplification-free, faster and ultra-sensitive detection at what is expected to be at a lower price when compared to the traditional technologies such as qPCR. Since it is a microfluidic device it is especially attractive as a POC platform since such devices offer precise control on quantities and the rate of flow of samples and reagents, enabling separation and detection of analytes with high accuracy and sensitivity.[126] Moreover, portable Raman microscopes make possible on-site detection.[95]

The usage and characterisation of Au@AgNRs, and their assembly on a glass substrate by silanisation, was previously discussed in detail by Wu and co-workers so it will not be object of review or discussion in this project.[7] An image of this substrate can be found on Figure A5, and a SEM picture of this substrate can be found in Figure 20 (above).

To test the ability of the plasmonic substrates in proving good SERS signalling the 1NAT molecule was used again, as Raman reporter to evaluate SERS performance.

A strong signal for the 1NAT molecule was detected at a concentration of 10^{-5} M (Figure 22– a), which was enough to build a SERS map (Figure 23-b).

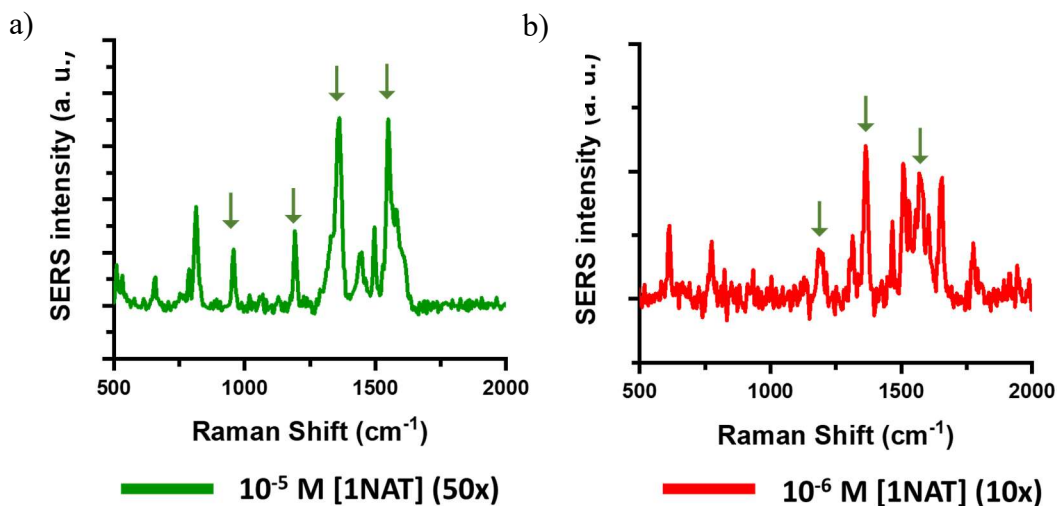


Figure 22 – 1NAT reporter is detected at concentrations of 10^{-5} M (green spectrum in a) and 10^{-6} M (red spectrum in b). Green arrows mark the characteristic Raman peaks detected of this reporter. The green spectrum (a) was acquired at a magnification of 50 $\times$ and the red spectrum (b) was acquired at a magnification of 10 $\times$, because it was at these magnifications the signal from 1NAT was the strongest for each respective case.

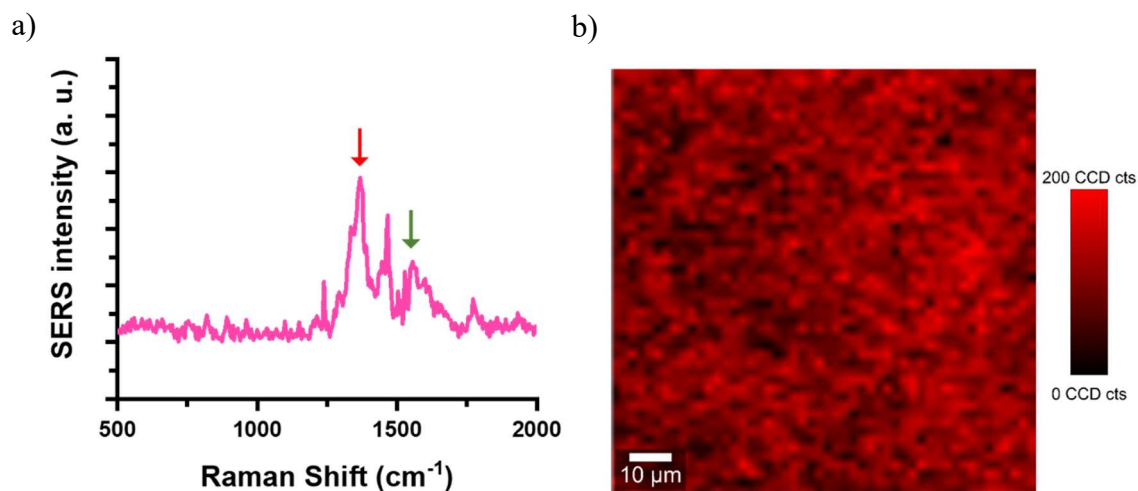


Figure 23 – SERS mapping of a plasmonic substrate which consists on Ag@AuNRs assembled on a glass surface, with the 1NAT reporter at a concentration of 10^{-5} M. The SERS spectrum (a) corresponds to the average of all 2500 individual spectra acquired by SERS mapping. Using these spectra, it was possible to create a SERS map (b) using the signal from the 1NAT reference peak (in the spectral area centered at 1360 cm^{-1}). Arrows on the Raman spectra point to the characteristic peaks of 1NAT that could be easily detected. The red arrow points to peak used to build the SERS map of the figure.

The SERS map of Figure 23-b) was built to assess the homogeneity of this substrate. It was created by measuring the SERS signal of 1NAT in area of $100 \times 100 \mu\text{m}^2$ in intervals of $2 \mu\text{m}$. The spectral area from 1320 cm^{-1} to 1400 cm^{-1} was considered in order to capture the signal of the characteristic peak of 1NAT at around 1370 cm^{-1} . Meanwhile, the pink coloured spectrum of Figure 23-a) corresponds to the average of all the spectra obtained from the SERS mapping.

From the observation on the Raman map in Figure 23– b) it is possible to conclude the homogeneity of the substrate is not the ideal however visually the dominant colour of the map is bright red (strong signal for the reporter). While there are some darker to black points it is not abnormal when dealing with SERS substrates. Particularly, because a large area ($100 \mu\text{m} \times 100 \mu\text{m}$) will be also be used to acquired and determine the signal given by the molecular components of the optofluidic assay, largely reducing the impact of the heterogeneity of the substrate on the SERS signal overall intensity.

Moreover, it was still possible to detect 1NAT reporter at a concentration of 10^{-6} M, as seen in Figure 22-b). And, since the molecular beacons used will be in the same order of concentration (micromolar), these substrates were considered acceptable for creating the optofluidic devices.

As seen in List A1, the extremities of the molecular beacons correspond to complementary C/G sequences, while the middle portions are the complementary sequences to the respective target that is intended to be identified. A thiol group at the 3' ends of the molecular beacons ensures their attachment of to the plasmonic silver/gold substrate.

All the molecular beacons utilised had attached, in its 5' ends, the Raman reporter 6-FAM, being FAM short for carboxyfluorescein, the source of the SERS signal that will be evaluated for the optofluidic molecular detections. When the target hybridises the linearisation of the molecular beacon leads to a huge decrease of the SERS signal given by the 6-FAM reporter (Figure 10), which is what allows to detect the presence of the target sequence. The SERS signature of 6-FAM has been described for this purpose by Wu and co-workers. The most prominent SERS peaks described correspond to 1128 cm^{-1} , 1325 cm^{-1} , 1548 cm^{-1} , 1630 cm^{-1} . [107] Nevertheless, in this work, the signal of the 6-FAM on the plasmonic substrate was also measured to ensure the feasibility of the reference information and such results can be found on Figure 24.

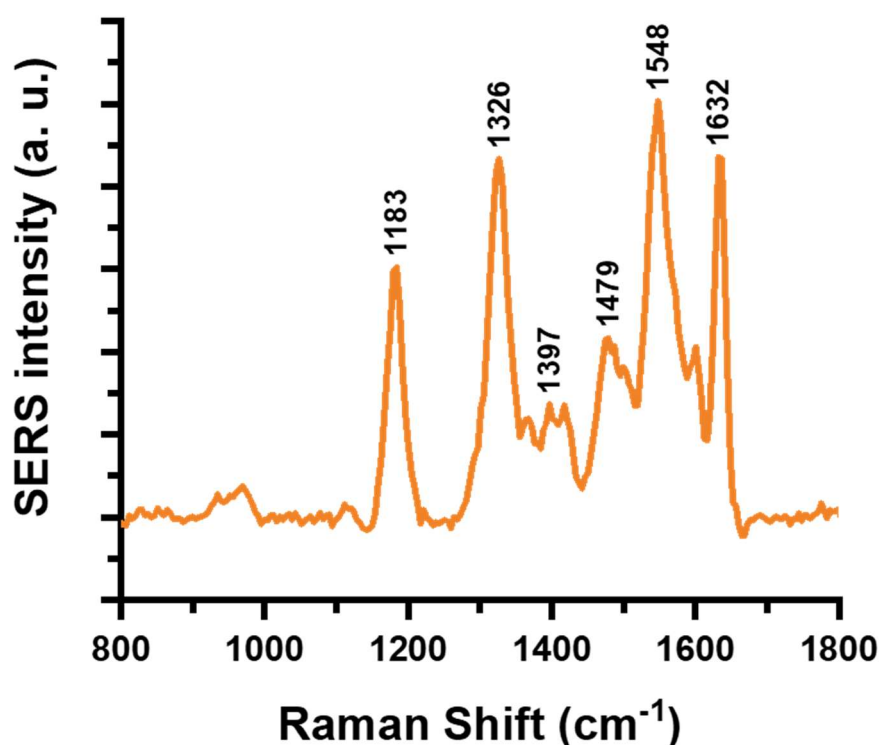


Figure 24 – SERS signal of the molecular beacons when in contact with the plasmonic substrate before adding the PDMS channels and target sequences. Most prominent Raman peaks which are characteristic of 6-FAM are marked with the value of the corresponding Raman shift (cm⁻¹).

From the analysis of Figure 24, it is possible to infer that the SERS peaks obtained for the molecular beacons used correspond very accurately to the ones previously published for the 6-FAM.[107]

Small synthetic nucleotides sequences, which can also be found on List A1, were used to test the detection capabilities of the optofluidic platform. After adding the microfluidic channels, it is possible to flow these sequences and after a 30 min incubation to measure the signal of the 6-FAM at the Raman microscope, to test the detection performance.

Essentially, in each optofluidic device a channel was filled with the buffer solution containing a known concentration of the synthetic target. While one other channel was filled with plain buffer solution without any nucleotide sequence to serve as reference for SERS intensity (blank). After incubation with the synthetic target, the channels were washed with PBS Tween 20 solution to remove any non-bound nucleotide sequences before measuring and comparing the SERS signal in the different cases.

For the SERS measurements, 625 spectra were taken for each condition in area of $100 \times 100 \mu\text{m}^2$ (step of $4 \mu\text{m}$), and the acquisition time was 1 s for each spectrum. This area was chosen because it offers relative short time of analysis (around 10 min) while providing enough signal strength and spatial resolution. Measuring this elevated number of points is meant to reduce the impact of the heterogeneity of the Raman signal in terms of quality and intensity and so to ensure reliable measurements, which is crucial for this technology to have practical application in the future. The average spectrum of all the spectra obtained from the mapping of the plasmonic substrates for the *RdRp* probe can be found in Figure 25, and refer to concentrations of *RdRp* synthetic target of 1 μM and 10 nM.

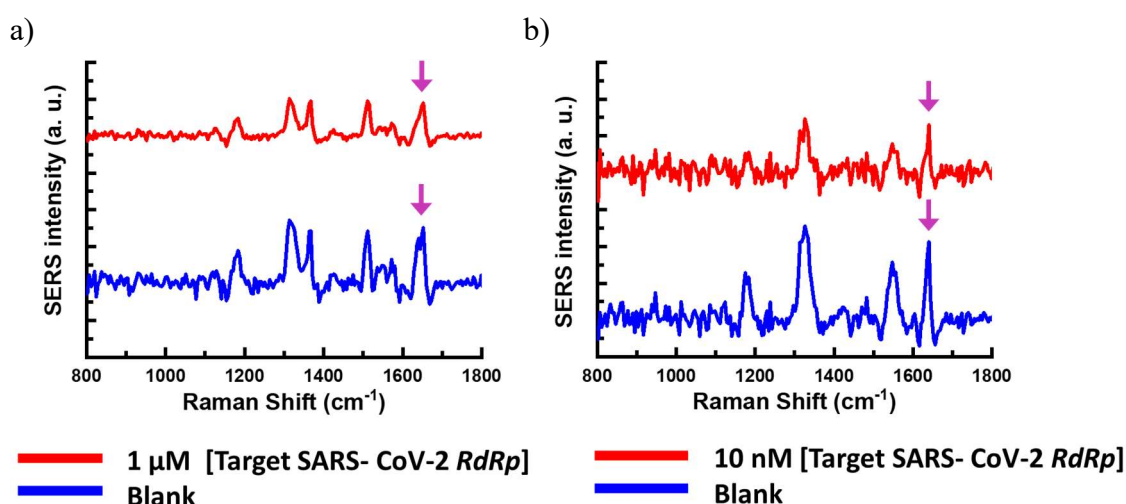


Figure 25 - SERS spectra obtained in the optofluidic assay for the *RdRp* gene at 2 different concentrations of synthetic target, 1 μM (a) and 10 nM (b). The spectra shown are the average of the 625 taken for each condition in an area of $100 \times 100 \mu\text{m}^2$. The top lines, in bright red, represent the average of the spectra for the target channels and the bottom spectra, in dark blue, are the respective blanks. Purple arrows indicate the characteristic peak of 6-FAM, at around 1640 cm^{-1} used to build the SERS maps of Figure 26.

First and foremost, it is possible to verify, in Figure 25, a decrease in SERS intensity in the target channel to the respective blank channel for both concentrations of target, which is what is expected since when the target is present the 6-FAM molecules are, in general, further away from the substrate so the Raman signal in result is weaker. However, and particularly for the *RdRp* gene at a target concentration of 1 μM , the Raman signal shape is visibly different from the reference signature of the reporter 6-FAM (Figure 24), and different from the signal when the *RdRp* target is at a concentration of 10 nM.

More specifically, in 1 μM of target for the *RdRp* gene new Raman peaks seem to have appeared and others disappeared for example at around 1300 cm^{-1} in both the target and blank channels.

A more in-depth analysis revealed the peaks present can also be found, but with very low intensity, in the reference for the Raman signature of 6-FAM. This is probably due to the change in the position of the reporter molecules relative to the plasmonic substrate, which causes different Raman shift peaks to be more intense than others. This is a problem because these different SERS responses can cause lack of reproducibility and it can make signals difficult to compare, since the most prominent vibration modes keep changing.

The SERS maps for the optofluidic channels were built considering the spectral area from 1600 cm^{-1} to 1680 cm^{-1} to capture the reference peak at 1640 cm^{-1} and compensate for eventual shifts. This peak was chosen because it was one of the peaks with highest relative SERS intensity, one of the narrowest and it was consistently present in all cases (even if small shifts were detected for this peak).

Figure 26 shows the SERS maps for the *RdRp*, and where a visible decrease in overall brightness, and so in signal strength is visible when the target is present.

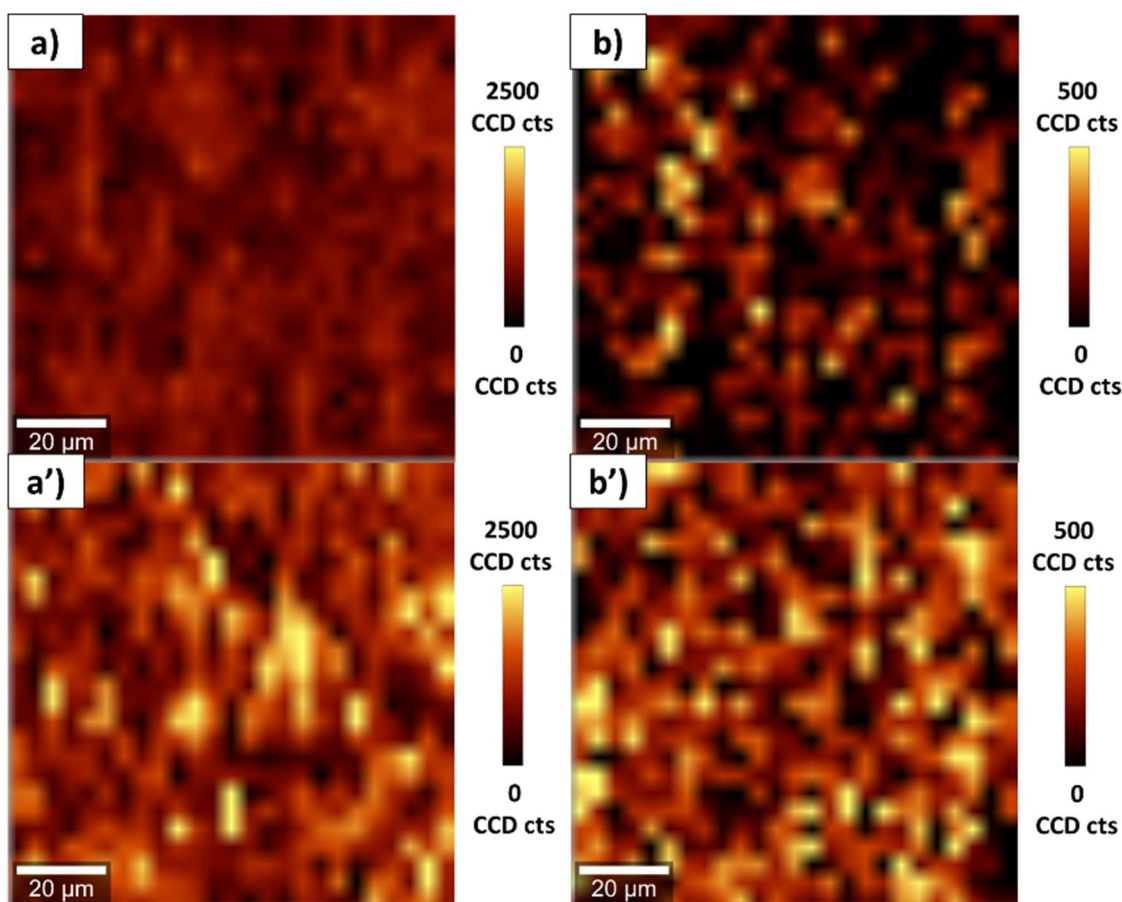


Figure 26 – SERS maps of the plasmonic substrate in the SARS-CoV-2 *RdRp* optofluidic assays. The maps in the left side, a) and a') correspond to the target channel and blank channel respectively for the detection of synthetic target at 1 μM . The maps on the left size, b) and b'), correspond to the target channel and blank channel respectively for the detection of the *RdRp* target sequence at a concentration of 10 nM.

The decrease of brightness verified in Figure 25 is to be expected and it is confirmed by the average spectra analysis shown in Figure 24.

The average SERS spectra obtained in the *BRAF V600E* detection studies can be found in Figure 27, while the corresponding SERS maps can be found in Figure 28.

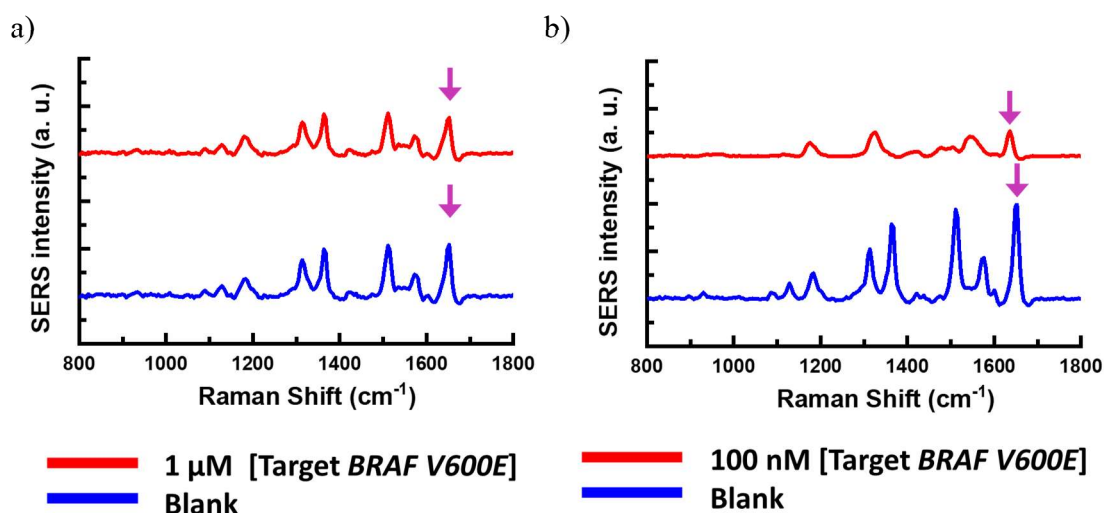


Figure 27 - SERS spectra obtained in the optofluidic assay for the human *BRAF* gene with the V600E mutation at 2 different concentration of synthetic target, 1 μM (a) and 100 nM (b). The spectra shown are the average of the 625 taken for each condition in area of $100 \times 100 \mu\text{m}^2$. The top lines, in bright red, represent the average of the spectra for the target channels and the bottom spectra, in dark blue, are the respective blanks. Purple arrows indicate the characteristic peak of 6-FAM, at around 1640 cm^{-1} used to build the Raman maps of Figure 28.

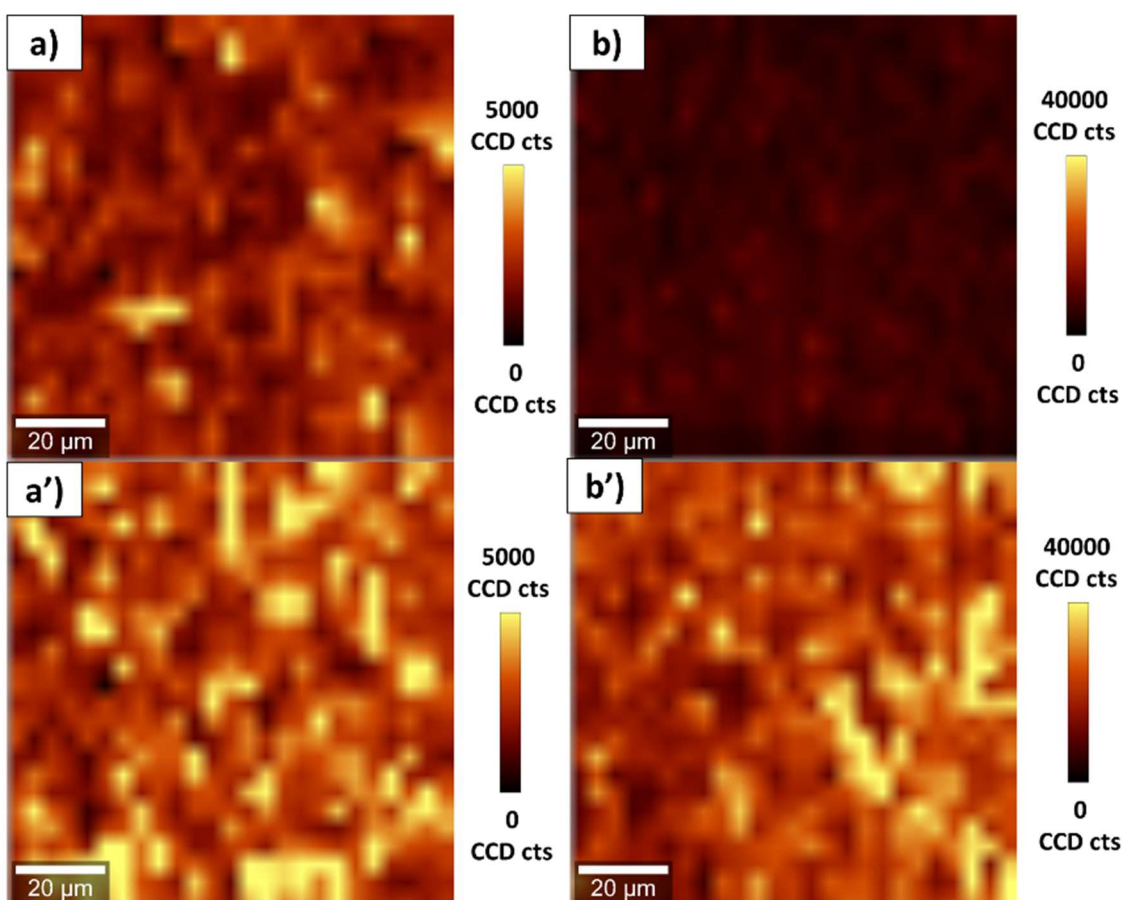


Figure 28- SERS map of the plasmonic substrate in the *BRAF V600E* optofluidic assays. The maps in the left side, a) and a') correspond to the target channel and blank channel respectively for the detection of target at 1 μM . The maps on the left size, b) and b'), correspond to the target channel and blank channel respectively for the detection of the *BRAF V600E* target at a concentration of 100 nM.

Once again, accordingly to what has been previously reported and experimentally verified for the *RdRp* gene, a noticeable decrease in SERS signal intensity is verified at the SERS maps of when the target sequences are present (Figure 28). Similarly, the overall intensity of the average Raman signal for the BRAF target channel decreases, particularly when looking at the reference peak of 6-FAM marked in purple (Figure 27).

The signal of the 6-FAM on the substrates is heterogeneous as seen in the SERS maps of both Figure 26, and Figure 28 for the *RdRp* and *BRAF* gene respectively. This is due to the inherent heterogeneity of the SERS seen above, in Figure 23, in relation to the 1NAT reporter, but it is also originated from the fact that there is target hybridization in some areas and not in not others unless the substrate is saturated with target, and it can also be cause by a non-uniform distribution of the molecules of molecular beacon on the SERS substrate.

Even though, the Raman maps, such as the ones in Figure 26 and in Figure 28 are a great way to graphically show a “turn-off” of the SERS signal with the addition of the target, it is also clear from the values of the colour scales in these images that the maximum relative intensities of signal vary in great deal. And, even different blanks which have the same Raman reporter (6-FAM) present different shapes in terms of Raman signature (spectra of Figure 25 and Figure 27) due to the motives explained above. These differences were in part to be expected and that is the reason why a blank channel was created for each individual assay.

In attempts to normalise the data, and to make it quantitative and comparable between different conditions the values of AUC in respect to the characteristic peak found at around 1640 cm^{-1} was computed for every spectra obtained in the optofluidic tests, similar to what was done for the Raman maps to capture the 6-FAM’s characteristic SERS peak. Then the average of the AUC values for each target concentration were divided by the AUC average value calculated for the respective blank. This was done to normalize and so make comparable the different AUC values obtained, since the levels of SERS intensity vary a lot between conditions. The calculations resulted in values of AUC_{norm} for each concentration of target tested, which were, then, plotted in the graph of Figure 29 for easy comparison. In turn, the numerical values involved can be found in the Table A2.

Nonetheless, it is important to note that the results from both genes cannot be compared with each other, because for one the length of the probe is different in both cases and the degree of probe hybridisation is also going to be different. Therefore,

comparisons should be extended only to the different concentrations within the same gene.

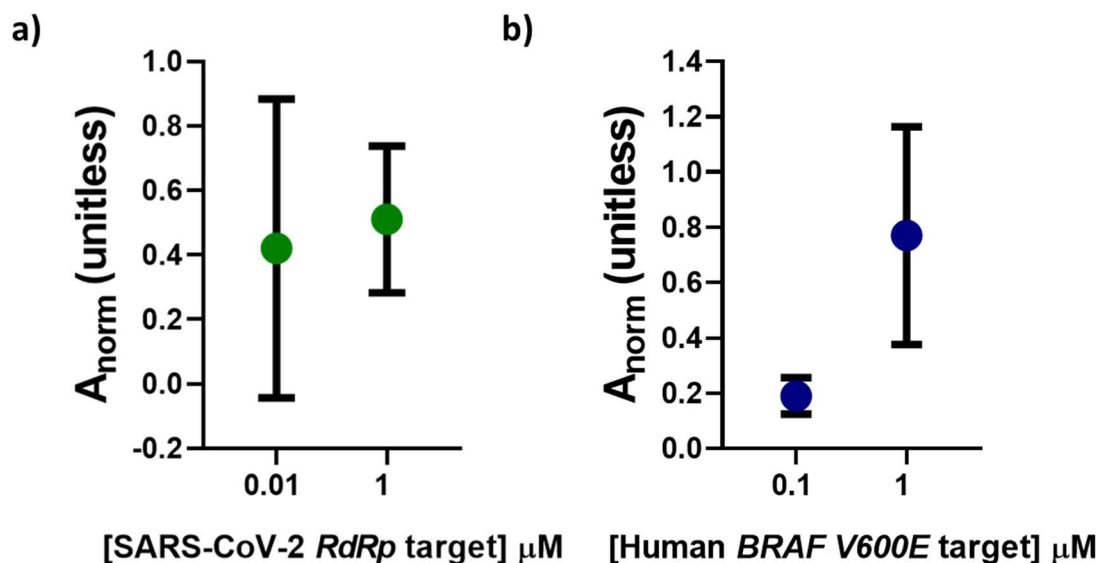


Figure 29 – Ratio between the average AUC of the SERS spectra of the target channels and the average AUC for the SERS spectra for the blank channels calculated in the optofluidic tests from 1620 cm^{-1} to 1670 cm^{-1} (A_{norm}). Green dots correspond to AUC_{norm} values related to SARS-CoV-2 *RdRp* testing (a) and the blue dots correspond to the *BRAF V600E* detection studies (b). All 625 spectra obtained by SERS mapping in area of $100\times 100\text{ }\mu\text{m}$ for each case were used for the determination of the average AUC values and respective standard deviation. Error bars correspond the standard deviation values of AUC_{norm} .

First, since the ratio between the average AUC for the target to the respective blank (AUC_{norm}) was always verified to be lower than 1, the signal given off by the Raman probe was verified to be consistently lower when the target sequence is present, as mentioned above. However, for higher target concentrations the AUC_{norm} , should have been lower because the higher the number of target sequences the lower is the Raman signal of the reporter according to previous reports by Wu and coworkers.[7] However, for the highest concentrations ($1\text{ }\mu\text{M}$) in both genes the AUC_{norm} was also higher, meaning the difference between the signal strength of the target and blank was lower. From this it is possible to conclude that the AUC_{norm} and so the SERS intensity of the target channel compared to the blank does not correlate well with concentration, at least for the conditions tested. This might be due to cross-hybridisation of the target molecules at high concentrations. The standard deviations of the values of AUC_{norm} , given by the error bars on the graph in Figure 29, are also usually quite high compared to average values of AUC_{norm} . However, this is to be expected due to inherent heterogeneity of the SERS substrate, spatial variation in the coverage of the molecular beacons attached and their

different conformations relative to the substrate. Such variations are the reason why so many spectra were taken for each condition.

In conclusion, the technology studied shows promise as a LB platform for both cancer therapy and viral detection. Specifically, in the context of COVID-19, as a POC platform that can help the grave situation the world currently faces. A solution such as this is urgently needed due to the high demand for quick and more cost-effective tests. It could also potential be useful to replace the swabs conventionally applied for sample collection by allowing detection in other fluids such as saliva.

In the case of *BRAF* gene, this system could be applied for the detection of either DNA provided from CTCs or possible cfDNA in blood. Making this optofluidic platform attractive for guiding clinical decisions.

The analysis itself is quite fast and it can be performed in less than one hour, which it is highly desirable in POC applications. However, despite promising, since the Raman signal is consistently lower when the target sequences are present, in the cases studied the target concentration did not seem to co-relate well with concentration, as seen with the AUC calculations. As such, it fails to provide accurate quantitative to semi-quantitative data, which is essential for clinical applications. On the other hand, a very limited number of concentrations of target were tested. In the future additional concentrations of target should be tested to obtain much more accurate, and reliable information.

The main downfall of this optofluidic technology is that it is based on the partial quenching of Raman signalling, signal which is always more or less present (never disappears form the spectra), and it can be affected by a number of factors. This method is particularly heavily user dependent, since for example just small changes in the focus positioning at the Raman microscope can be responsible for significant and drastic changes in signal. The signal intensity can also decrease just by bleaching of the probe over time, since 6-FAM is a fluorescent molecule and therefore subject to photodegradation, which in turn can lead to result variability. An alternative non-fluorescent Raman probe should be used instead in the future. The molecular elements, such as poorly stable RNA, can degrade quickly, as well, especially with laser exposure, leading to faulty results.

C. Droplet trapping device design and related CFD simulations

An interesting bottom-up approach recently applied by Gómez-Graña *et al.* to create SERS substrates is self-assembly by microfluidics-assisted microevaporation. The authors used a PDMS evaporation cell prepared by conventional soft lithography filled with a highly concentrated nanoparticle solution to obtain a large supercrystal of closely-packed Au nanooctahedra, with around 1 mm in length, 100 μm in width and 7 μm in depth. The driving force is the solvent evaporation which causes the nanoparticles to dislocate to and deposit on the end of the channel, where a supercrystal nucleates and then grows by continued nanoparticle deposition.[103] This work however was done in a continuous microfluidic fashion, so an interesting idea would be to switch this method to a droplet-based approach.

Droplet microfluidic assembly could be accomplished by trapping plasmonic NPs dispersions on microfluidic droplets, which would be spatially confined using hydrodynamic traps, so the droplets would gradually evaporate while confined. Droplet evaporation would cause the concentration of nanoparticles to reach a critical state, driving their assembly on the surface of the PDMS, due to their incompatibility with the oil phase (fluorinated). This shift to a droplet base approach could have a series of advantages. First, it is expected that this method may lead to the creation of very small but highly dense and organised assemblies (plasmonic super crystals), which are considered to offer high SERS enhancements and exceptional reproducibility.[103] Tuning the droplet size and the size the trapping areas would allow to easily tune supercrystal size, while the overall design can be adapted for each application. Additionally, this approach would reduce the amount of plasmonic NPs necessary, saving reagents. Finally, for multiple detection cross contamination could be avoided, since each detection area would be isolated and small even though located on the same chip.

However, if a traditional approach was followed to achieve a final microfluidic chip design for this application, it would probably require several rounds of microfluidic chip designing, fabrication and testing which are costly, time consuming and reagent consuming. So, in this section, CFD simulations were made in attempt to determine and test suitable conditions for effective droplet trapping, and so droplet-driven formation of supercrystals can be achieved in result.

An optimisation of the parameters such as flow rates and geometries for highly localised hydrodynamic droplet trapping and generation in a microfluidic chip was conducted. With such studies, it is intended to save both time and costs of prototyping and testing.

Among the different methods that can be deployed to simulate fluid flow, the interface capturing LS method is becoming increasingly popular because of its ability to accurately model flow problems involving sophisticated moving interfaces and complex morphologies.[86] The LS method has been quite successfully applied to 2D and to 3D multiphase flow problems such as bubble rising and growth in a stationary fluid, a droplet falling down, bubble adherence to a solid surface in a fluid and droplet pinching, collision and break up. In fact, numerical simulations based on the LS method are expected to play a pivotal role in the field of droplet-based microfluidics.[87] However, most studies with this method in the droplet microfluidic field were found to revolve mainly around droplet formation.[63,67,127]

Due to its accuracy, being well-described in literature and due to its importance in the droplet microfluidic field the LS model was used. To the best of our knowledge of the current literature, it was the first time this method was used to study passive hydrodynamic trapping in microfluidic systems. Therefore, it is not only useful to use this method as a practical CFD tool to save costs and time, as mentioned above, but it is also valuable for the scientific community in general. Since this tool can in this way be further explored for new circumstances in microfluidics other than droplet generation. It is expected that the simulation studies here undertaken provide the basis and pave the way for new and exciting developments not only in the field of droplet capture and confinement, but also in the field of multi-phase flow modelling in general.

Due to time and technical constraints, an extensive review of the parameters involved in droplet trapping and generation is not the goal of this chapter, however in the future this is a project very interesting and worthwhile to be explored and continued. One of the most important aspect for this application and respective CFD studies it is to select the appropriate fluids. Water droplets in oil spontaneously incline to attract each other and fuse together, this process is known as coalescence. Preventing this phenomenon is crucial for any droplet-based application, therefore the use of surfactants is required to ensure droplet stability. The degree to which the surfactant organises at the interface between the immiscible water and oil phases can be quantified by the reduction it causes on the interfacial tension at the oil/water interface. An efficient surfactant for

droplet microfluidics is expected to reduce surface tension to values below 20 mN/m.[104]

Novec™ HFE 7500 containing 2 % w/w Pico-Surf™ 1 fluorinated oil from Sphere Fluidics Pico-Surf™ was the main fluid considered for the continuous phase in the CFD simulations. This fluorinated oil-surfactant combination was shown to enable the generation of highly stable droplets and to allow droplet storage without coalescence.[105] Furthermore, fluorinated oils, such as this one, are both hydrophobic and lipophobic/oleophobic; in other words, they tend to reject non-fluorinated species whether they are hydrophobic or hydrophilic.[128] This allows to avoid cross contamination since diffusion of most compounds between droplets is prevented.[129] However, it also means that the NPs are incompatible with the oil phase, since fluorinated oils do not have affinity for non-fluorinated species. This aspect is useful since it will prevent the dispersion of the nanoparticles into the oil phase. The pure water was considered to be the discontinuous phase since it would be most likely the fluid of choice for dispersing the plasmonic NPs.

Mineral oil was used as well as the continuous phase for some simulations because it was what it was found to be most used in literature for microfluidic hydrodynamic traps.[4,74–77] Additionally, it was also useful to test the influence of the continuous phase in droplet behaviour. However, it was later switched back to HFE 7500 fluorinated oil because it is much more advantageous for droplet-based applications due to the reasons mentioned above.

One of the most important aspects of CFD is to define an appropriate computer mesh. A user-controlled mesh with free triangular geometry was created for every experiment. The main requirement of a good computer mesh is to be fine enough to resolve the physical effects occurring within the computational domain.[80] Initially to build the mesh maximum element size of 10 μm was considered, following the rule of thumb that the maximum element size should revolve around one-tenth of the droplet expected size.[115] However, a maximum element size of 5 μm showed visible better results in terms of visual quality for the plotting and animations of the results, while still allowing for reasonable simulation times.[115] Figure 30 is an illustrative example of this characteristic mesh applied to a microfluidic geometry.

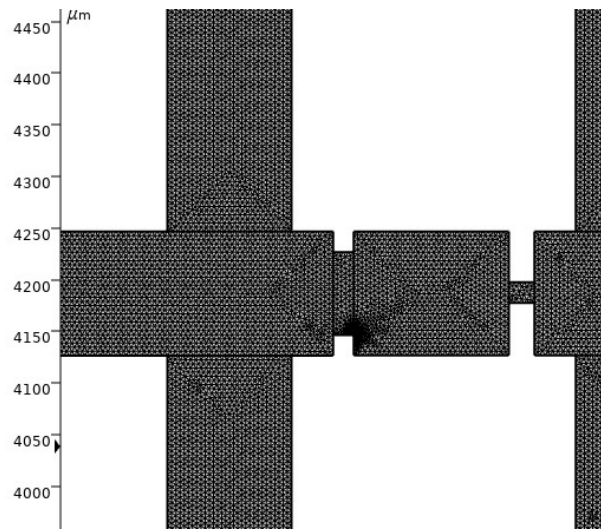


Figure 30 - To perform most CFD simulations a free triangular mesh with a maximum element size of 5 μm was created for the geometries under study.

For water-in-oil droplet applications the internal surface of the microfluidic channels is required to be highly hydrophobic. To achieve this with PDMS normally a material such as Aquapel® (PPG Industries, Inc., Pittsburgh, PA, USA), is used to make the internal channel surface highly hydrophobic. Therefore, all the experiments were performed with a wetted wall condition with a contact angle of π radians, to represent a purely hydrophobic interaction between the water droplets and the channel walls. However, this approximation can cause some difference between the simulation results and reality.

First, a design developed and tested previously in the lab for hydrodynamic trapping was simulated to attest for the capabilities of the simulation model. The traps were inspired in the design by Huebner *et al.*[73], found Figure 7. Figure 31 represents the main conclusions and results of the simulation for this design.

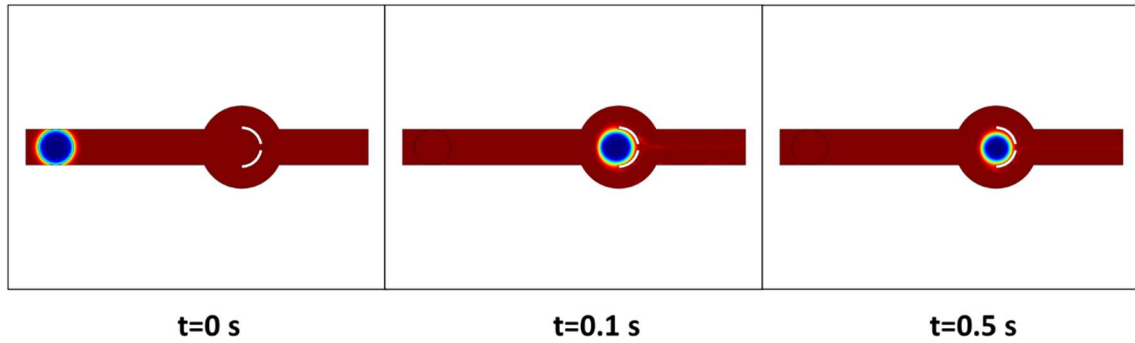


Figure 31 - CFD simulation for a hydrodynamic trap design previously created and tested in the laboratory. Three different time points, marked in the bottom, are represented. Dark reddish brown represents the continuous phase (HFE 7500 oil) and blue represent the dispersed phase (water). The respective time points can be found below each snapshot of the trapping region. A continuous phase flow was established from the left to right at a rate of 500 $\mu\text{L/hr}$. The trapping area is half of a circle with 120 μm of diameter. As seen in the picture, at 0.1 s the droplet is already confined and it remains so even after 0.4 s.

As seen in Figure 31, the trap is successful in confining the droplet. However, this design possesses a problem in experimental conditions, which is when the continuous phase flow is interrupted the droplets were found to displace themselves from the trapping areas, which impairs practical applications especially when long term storage is required. To solve this problem, other designs were explored. Namely, from Ma. *et al.*[4] and Dewan *et al.*[76] both based on Tan and Takeuchi traps shown in Figure 8.[74] These traps work by offering an alternative path to the trap that is longer to reach the outlet so it offers more resistance to the droplet passage, so the first droplet tends to enter the trap and it gets stuck since it cannot deform enough due to surface tension to leave through the small gap that allows the continuous phase passage. However, when this first droplet gets stuck it blocks the fluid flow through the small gap channel of the trap. Since the fluid flow through the traps is interrupted it now offers more resistance and new droplets that arrive are forced to take the alternative route.

Mineral oil was used for simulating these designs because it was the type of oil used by the authors of these traps to test them and apply them for its given applications. Nevertheless, as seen by the simulations in Figure 32 both trap designs failed to confine the droplet at the conditions of the simulations.

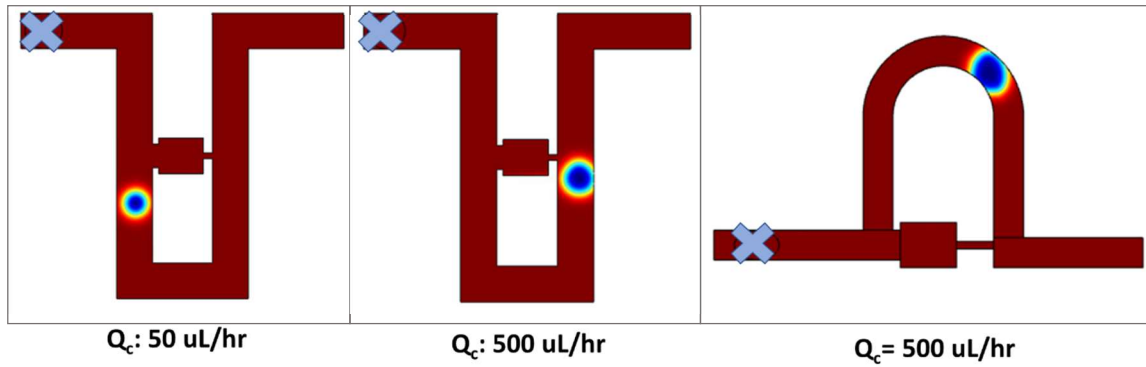


Figure 32 - CFD simulations for a hydrodynamic trap adapted from Ma *et al.* (two left ones) and Dewan *et al.* (right one). A continuous phase flow was established from the left to right. The respective flow rates (Q_c) used are marked in the bottom of the respective CFD result. Dark reddish brown represents the continuous phase (mineral oil) and blue represents the dispersed phase (water). A pale blue X-sign represents the original position of the droplet. The trapping area corresponds to a rectangular region with an area of $150\ \mu\text{m}$ (width) $\times$ $120\ \mu\text{m}$ (height). All traps at the conditions of the respective simulations failed to confine the droplet.

To achieve a better trapping efficiency so the droplet could enter the trap in the CFD simulations, two long symmetric channels were placed at opposite sides right before the trapping area both serving as alternative passages for the droplets. Using this design it was successfully confined a water droplet at a flow rate of $200\ \mu\text{L/hr}$ of mineral oil (continuous phase), as seen in Figure 33.

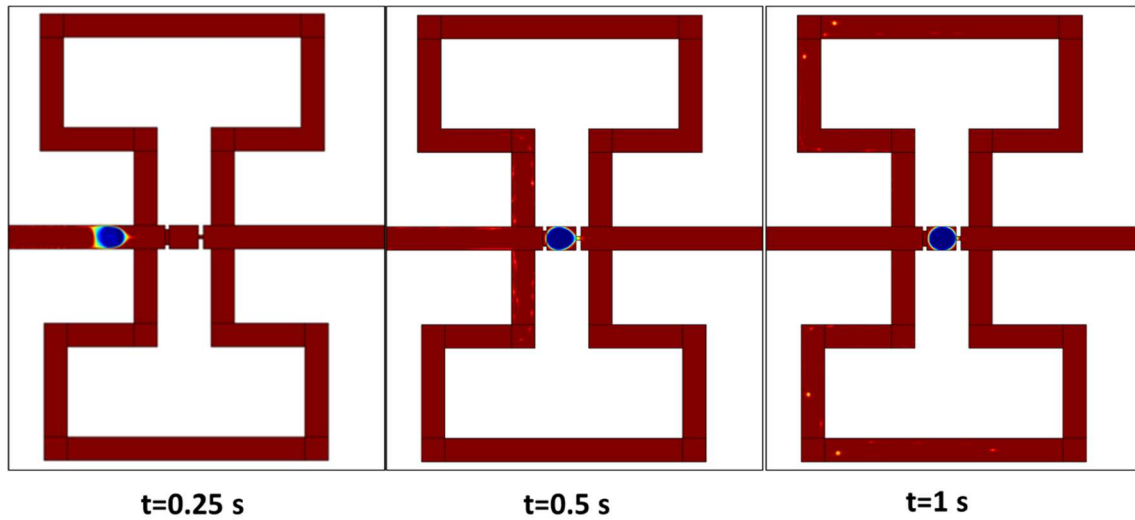


Figure 33 - CFD simulation of a novel design for droplet hydrodynamic trapping. Dark reddish brown represents the continuous phase (mineral oil) and blue represents the dispersed phase (water). The respective time points can be found below each snapshot of the trapping region. A continuous phase flow was established from the left to right, at a rate of $200\ \mu\text{L/hr}$. The trapping area corresponds to a rectangular region with an area of $150\ \mu\text{m}$ (width) $\times$ $120\ \mu\text{m}$ (height). At this flow rate the droplet was successfully confined in the trap.

The idea behind this is that when the droplet reaches the junction, the forces that propel the droplet to follow the alternative path are nullified since they are exerted in opposite directions. And long side channels are meant to create a great resistance for the

first droplet to go to the side channels, further stimulating its entrance in the hydrodynamic trap. In Figure 34, the trapping region is described in more detail and the rationale behind its design is graphically displayed.

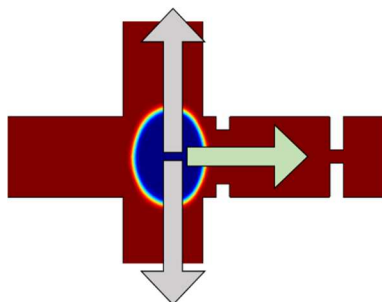


Figure 34 – Zoom in of the trapping region created. When the droplet meets at the cross junction the forces that propel the droplet to enter the alternative pathways cancel each other since they are exerted on the droplet in opposite directions. Additionally, for the droplet to enter the trap it is forced to deform since a small constriction of $80\ \mu\text{m}$ exists at trap entrance, while the main channels have a diameter of $120\ \mu\text{m}$. This was made to prevent the droplet from leaving the trap when the flow of oil is stopped. The small channel of $20\ \mu\text{m}$ at the exit of the trap is closed by the entrance of the first droplet, which is too small for a droplet of over $120\ \mu\text{m}$ to deform and go through, preventing other droplets from entering.

However, for the same structure when HFE-7500 oil is used at the same flow rate of $200\ \mu\text{L}$, instead of mineral oil as the continuous phase the droplet is not capable of entering the trap. This problem was solved by increasing the continuous phase flow rate to $1000\ \mu\text{L/hr}$ allowing for droplet capture even when using HFE 7500 oil as the continuous phase (results not shown).

This model can be further optimised by reducing the length of the side channels that constitute the two droplet alternative paths. Using smaller trapping units is preferable because it makes it easier to fit a larger number of traps in a small chip. Making it more practical for real word application where working, transportation and storage space is often limited. Figure 35 shows the novel design for a hydrodynamic trap fully optimised and CFD backed. A flow rate of $2000\ \mu\text{L/hr}$ for the continuous phase was applied when using this novel trap, the reason being that at flow rate of $1000\ \mu\text{L/hr}$ the droplet was not capable of deforming enough to enter the hydrodynamic trap. Moreover, the simulation was carried out with two droplets to test if only the first droplet entered the trap. As seen in Figure 35, after the first droplet fully enters the trap, a second droplet that arrives to the junction instead of also entering the trap it takes one of the sides channels as route and completely leaves the vicinity after a few instants, while the first initial droplet remains in confinement.

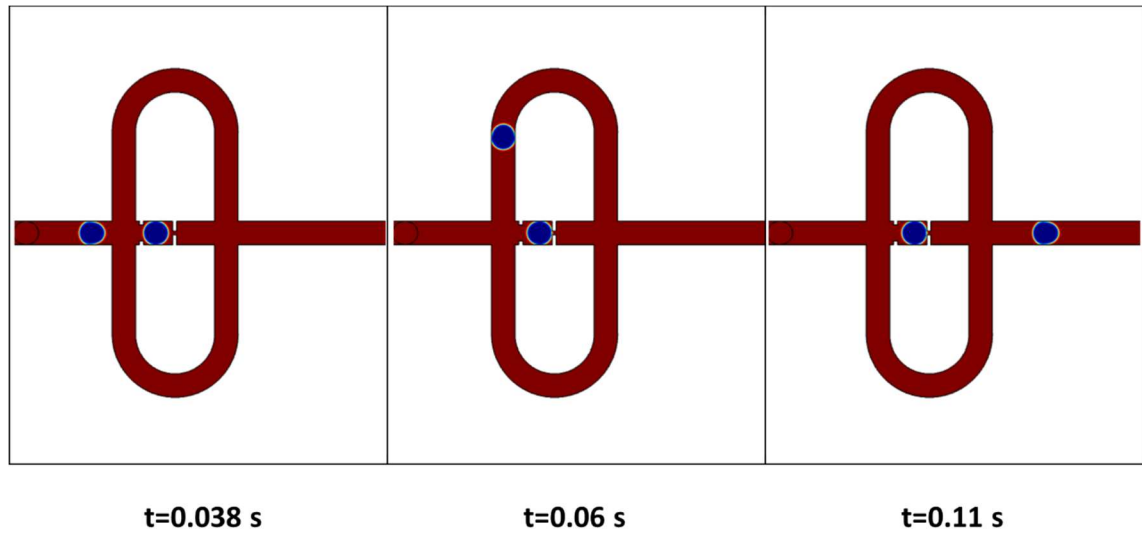


Figure 35 - CFD simulation of the final and optimised novel design for droplet hydrodynamic developed in this project. Dark reddish brown represents the continuous phase (7500 oil) and in blue is represented the dispersed phase (water). The respective time points can be found below each snapshot of the trapping region. A continuous phase flow was established from the left to right, at a rate of 2000 $\mu\text{L/hr}$. The trapping area corresponds to a rectangular region with an area of 150 μm (width)x 120 μm (height). At this flow rate the first droplet was successfully confined in the trap, and new droplets take one of the side channels (arc-shaped) as path, not entering the trap.

Despite the impressive results obtained thus far, it is also important to examine the conditions for droplet formation, even if briefly, since droplet trapping cannot be dissociated from droplet generation. As such, a flow-focusing MFDG geometry was studied at different capillary numbers (Equation 4). The results can be found in Figure 36. A flow-focusing MFDG was chosen because this type geometry was shown to offer a wide range of droplet sizes, enhanced generation rates and lower coefficients of variation (C.V.) in droplet size.[70]

Manipulating the capillary number (Ca) is essentially achieved by changing the flow rate of the continuous phase while maintaining the flow ratio between the continuous phase and the dispersed phase. Capillary number was considered because it was found in previous studies to be the main factor found to impact droplet size for a given flow-focusing MFDGs system.[130] Other factors can alter the droplet size such as the flow ratio between the dispersed and continuous phases, the interfacial tension between the two immiscible fluids, the wetting properties of the channel wall and the physical properties of the continuous phase. However, these parameters were kept constant and in accordance with the ones used in the CFD studies of hydrodynamic trapping.

The flow ratio being fixed at two means that the flow rate of the continuous phase (Q_c) at either side of the flow focusing junction was twice the flow rate of the dispersed phase (Q_d), which can be written as,

$$Q_c = 2 \cdot Q_d \quad (11)$$

While the total flow rate at the outlet channel of the flow-focusing junction, Q_t , is,

$$Q_t = Q_c + Q_c + Q_d \quad (12)$$

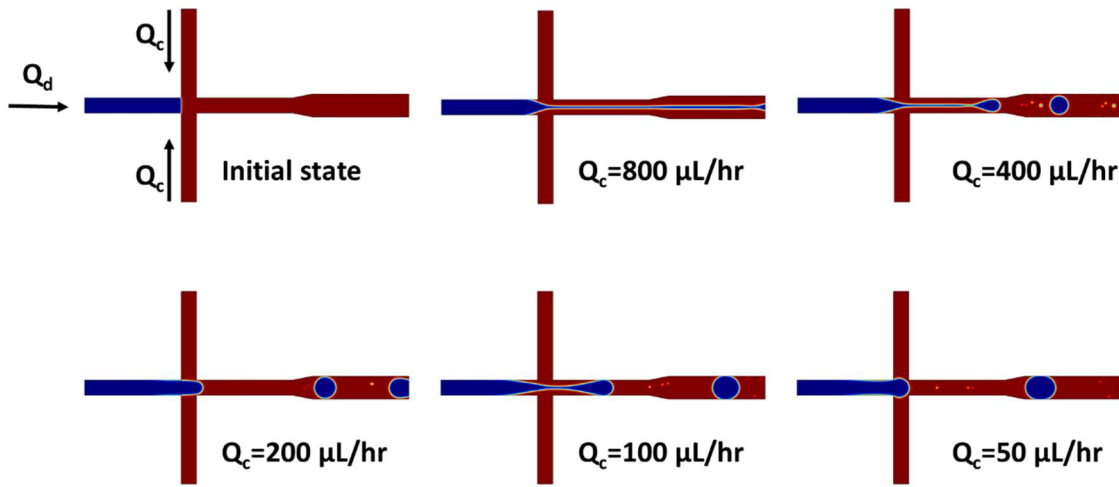


Figure 36 - CFD simulations for droplet generation in a MFDG flow focusing device at different capillary numbers. The respective Q_c values can be found below each snapshot. Dark reddish brown represents the continuous phase (HFE-7500 oil) and in blue is represented the dispersed phase (water). In the extremities of the two vertical channels, an oil inlet was added with the same Q_c . To the extremity of the left horizontal channel an inlet for water flow was added with a flow rate of Q_d . This is presented in the image of the initial conditions for every simulation which is marked as “Initial state”. A flow ratio of 2 between the continuous and dispersed phase was used for every simulation. At the junction all channels have a diameter of $80 \mu\text{m}$, the outlet channel eventually increases in diameter until it reaches $120 \mu\text{m}$, so it could be continued by the hydrodynamics displayed above in Figure 35.

The CFD results from Figure 36 indicate that Q_c values of 400 and 800 are associated to jetting modes of droplet formation and so are not desirable, since the droplet polydispersity would be quite high if produced in such high values of Ca .

In order that to have droplets with diameter around the width of the channels ($120\mu\text{m}$), it would require Q_c values of $200 \mu\text{L/hr}$ and $100 \mu\text{L/hr}$ with flow ratio of 2, so it would be in accordance to what was tested in Figure 35 for droplet size.. Furthermore, at this flow rates the droplet formation displays characteristics of dripping to squeezing regimes, which is desirable since these lead to monodisperse droplet formation, as previously mentioned. However, these Q_c values correspond to Q_t values of $500 \mu\text{L/hr}$

and 250 $\mu\text{L/hr}$, so the difference in flow rates between the MFDG and traps needs to be compensated so both size-appropriate droplets are generated and the entrance in the hydrodynamic traps is possible. As such, for the final design, it was added what it was decided to be called an oil accelerator. Basically, this part of the device would essentially consist of two channels, injected with continuous phase fluid that would meet the channel junction between the MFDG and the trapping area. The purpose is to increase the flow rate of the continuous phase after droplet generation. Injecting oil into the accelerator will increase the flow rate of oil for the traps, while maintaining the low flow rates at the MFDG device, since the flow direction is from the MFDG to the traps and then to the outlet.

The final design can be found in Figure 37. It consists of three key components: (i) a flow-focusing MFDG for droplet production as the ones shown in Figure 36, (ii) an oil accelerator so the droplets have enough speed to enter, and finally (iii) hydrodynamic traps (Figure 35) arranged in an array for multiple individual droplet capturing and storage.

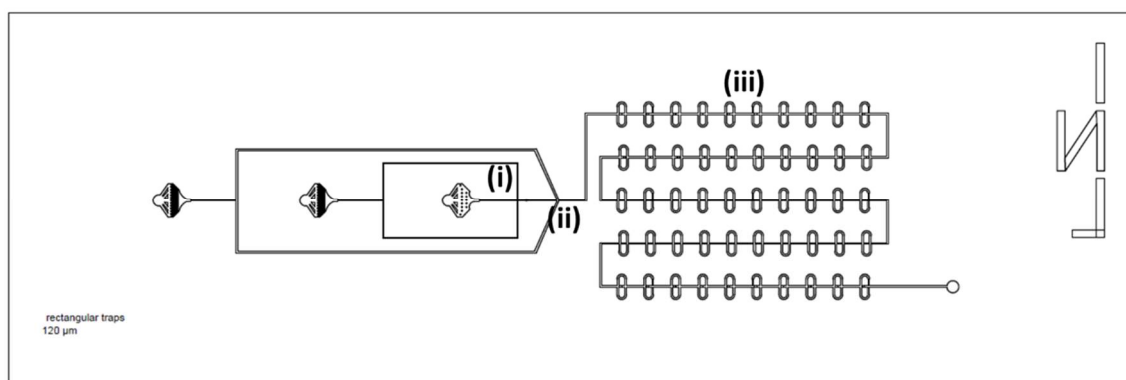


Figure 37 – Novel microfluidic device for droplet generation and hydrodynamic droplet trapping. The full design has the size of glass slide (rectangular box – 7.5 cm×2.5 cm). The main components are (i) a flow-focusing MFDG, (ii) an oil accelerator, (iii) an array of hydrodynamic traps. The channel dimensions in the MFDG segment have a width of 80 μm , while for the oil accelerator and trapping arrays the channels' width is 120 μm . Inlets and outlet have a diameter of 800 μm and each inlet has a filter system copulated.

Despite the success verified it is crucial to mention that experimental validation is fundamental to corroborate the simulation results. One interesting note is that direct trapping and droplet generations methods could have been explored as well, which for example resort to lasers or ultrasounds however, these methods are not as straightforward and such alternatives could alter the properties of the nanoparticles dispersed in the droplets affecting the final results. The molecular optofluidic analysis itself can also be performed in the future in trapped droplets for example to potentially reduce the amount of specimen required for analysis.

In terms of simulation quality, one of the limitations found was that some droplets seemed to shrink slowly over time in some cases, which might be due to numerical deterioration occurring at each step and other flaws of the model itself. One of the main down sides of this approach it is the long computation times at play due to the complexity of the mathematical and computational models involved. One full simulation could require a couple of days to fully run. Moreover, numerous iterations were required to achieve the results presented, which revealed to be quite time consuming. Fortunately, COMSOL allows to follow the solved results at real time so simulations can be stopped prematurely when the desired outcomes are not obtained, so it is not required to wait for each simulation to finish, when it is already known the desired outcomes are not going to be achieved. And finally, it is worth mentioning that the CFD work here presented was only possible because of the preventive lockdown measures enforced due to the COVID-19 pandemic which restricted all access to the INL laboratories for 3 months.

Conclusions

In this thesis an amplification-free optofluidic chip was studied, fabricated and applied for the detection of the *BRAF V600E* fragment and SARS-CoV-2 *RdRp* RNA. Since, the sensitive detection of these sequences is of great used in clinical practice to improve diagnosis and patient management in several cancer types and of COVID-19 disease, respectively.

The optofluidic platform combines both SERS and microfluidic to achieve accurate detection of nucleotide sequences, and so it potentially outperforms the molecular techniques currently used in clinical practice such as qPCR and NGS. This is seen in the previous version of this technology, developed by Wu and co-workers, which displayed detection limits and low as $50(\times 10^{-15})$ fM for the *KRAS* oncogene.[7]

In both cases of study, the SERS signal intensity of the reporter was lower when the target sequence was present, indicating the target hybridisation with the respective molecular beacons attached to the plasmonic substrate. The decrease in signal in turn can be used for detection of the target sequences as previously reported by Wu and co-workers.[7] However, the decrease in SERS intensity did not correlate well with concentration and a very limited range of concentrations could be tested. Therefore, the detection limits of the platform could not be determined. So even if the preliminary results seem promising, further testing and optimisation is required specially to achieve an optical response that can be used to obtain accurate semi-quantitative to quantitative results, which are most wanted in clinic. The genetic testing itself could be performed under an hour in optimal conditions, if a device is fully prepared for analysis that is. However, the application of this platform for POC testing is impaired since a Raman microscope and a trained user is required, and an initial step of DNA/RNA extraction is necessary when using clinical samples obtained from LB.

One attempt made in this work to further improve the technology, focused primarily on the plasmonic substrate used for SERS enhancement. Essentially, a flexible SERS substrate based of assemblies of GNSs, which have high SERS performances in solution, on a flexible PDMS surface was projected and tested. In order to do so, first GNSs were produced by the classic seed-mediated growth method, and its optical properties were successfully tuned for the application, as previously shown by Barbosa and colleagues.[120] Then these NPs were self-assembled on a PDMS surface by adapting a silanisation-based method described for glass.[7] So a flexible but efficient

SERS substrate could potentially be obtained. However, and unfortunately such approach did not provide satisfactory SERS performances, since the signal of the reporter (1NAT) was not detected in concentrations as high as 10^{-4} M. The lack of performance can be due to the low quality of the seeds and/or the nanostars produced from them, it can be caused by the presence of interfering molecules such as PVP and other contaminants, and finally the assemblies of the GNSs obtained in itself may not be appropriate to achieve high SERS enhancements. Therefore, quality control checks and further optimizations are necessary if attractive SERS performance are to be obtained with these substrates.

Finally, advanced CFD simulations were successfully applied for the study of droplet generation and droplet trapping. These simulations relied on the LS method, which to the best of our knowledge of the current literature has not been applied and described yet for this purpose. Furthermore, the CFD studies made allowed for the design of a novel microfluidic droplet trapping system, that allows for simultaneous droplet generation, acceleration, and entrapment. However, even though the device has the added advantage of being already backed by *in silico* studies, the conditions used were not experimentally validated in this context due to time constraints, so the real-word outcomes are expected to deviate even if slightly. However, it is safe to say that the design here presented, is rather promising for several future applications that can in turn benefit the aforementioned optofluidic platform, since it can be applied for testing a novel fabrication method of creating array-like SERS substrates and for droplet-based analysis of DNA/RNA samples.

Future perspectives

Even though the optofluidic platform shows promising results, further work is necessary to further develop and perfect this technology, so it can be used in the future as an *in vitro* diagnostic device. It is especially desirable later with enough optimisation, to achieve quantitative results with this approach.

The testing of the optofluidic platform is far from concluded. It is necessary first to test additional concentrations of target for example to determine the detection limits of this technology. After this initial step is concluded and shows promising results, then the platform should be further tested with cell-lines (for *BRAF*) and patient samples (for both COVID-19 and cancers such as colorectal). This was originally planned for this master thesis; however, this could not be pursued due to lockdown measures.

The possibility of multiplexing (detection of multiple nucleotide sequences in one test) should be considered since SERS is a great technique for doing so. One example where this would be relevant would be in SARS-CoV-2 detection where at least two molecular targets are required for molecular testing.

In the future, it would be invaluable to develop a software platform that can be used to process the spectra obtained, to allow easy interpretation of the results. Such solution would greatly increase the potential of this platform for POC testing.

One of the most logical steps to accomplish in the future is to fabricate the optimised microfluidic chip for droplet generation and trapping here presented. Then, if such system is confirmed, by experimentation, to be effective, it should be then applied for droplet microfluidic induced assembly of the GNSs or other plasmonic nanoparticles to create what it can turn out to be an outstanding flexible SERS substrate. Interestingly, the droplet trapping system here developed could also be used for many other applications, such as single cell isolation and analysis.

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Appendix

List A1 – List of the synthetic sequences used in this project, which includes the molecular beacons and respective synthetic targets.

Probe for *BRAF V600E* detection:

FAM CGCGCTAGCTACAGAGAAATCGCGCG (CH₂)₆ -SH

***BRAF V600E* target fragment:**

GATTTCTCTGTAGCTA

Wild-type *BRAF* allele (fragment equivalent to the target):

GATTTCACTGTAGCTA

Probe for *RdRp* detection of SARS-CoV-2:

FAM GACTAGTACCGGCAGCACAAGACATCTCTAGTC (CH₂)₆ -SH

SARS-CoV-2 *RdRp* target fragment (RNA):

AGAUGUCUUGUGCUGCCGGUA



Figure A1 – Au nanoseeds around 15 nm (left) and GNSs (right) color difference. The gold nanoseeds present a dark red wine-red color, while the nanostars derived from them present a dark grayish color with blue undertones.

Example of calculus A1 – List of the synthetic sequences used in this project, which includes the molecular beacons and respective synthetic targets.

For an Abs₄₀₀ value of 0.4 nm and a D_f of 3, the concentration of gold atoms in mM can be calculated as follows:

$$[\text{Au}] = \frac{\text{Abs}_{400}}{2.4} \cdot D_f \Leftrightarrow [\text{Au}] = \frac{0.4}{2.4} \cdot 3 = 0,5 \text{ mM}$$

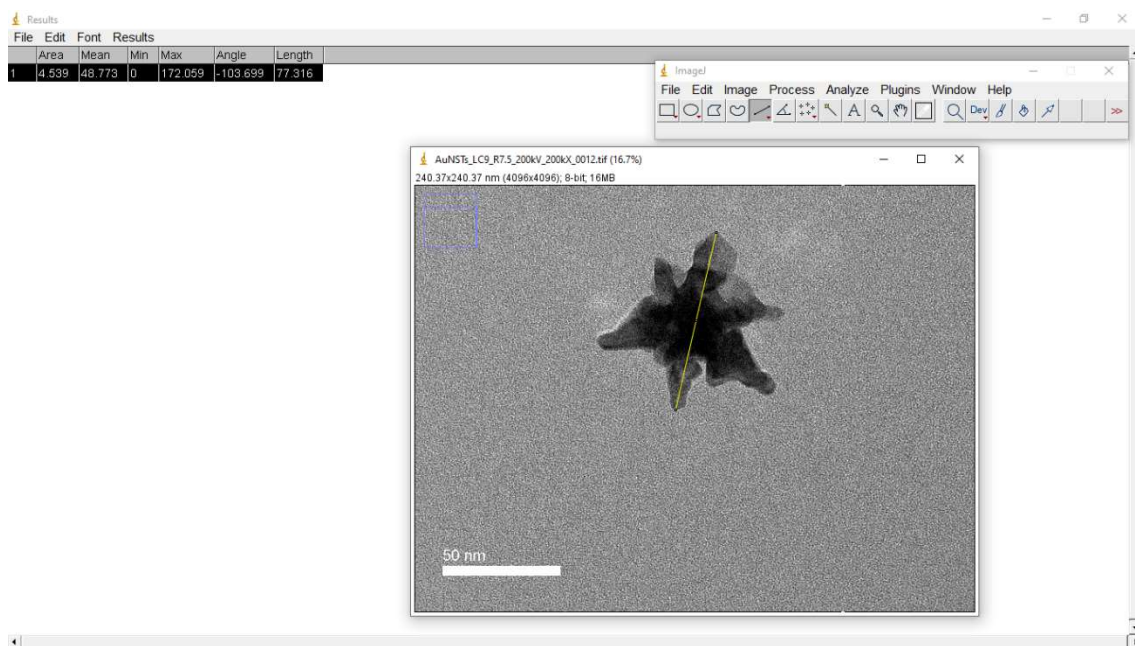


Figure A2 – ImageJ user- interface. The size of the GNSs corresponds to the length of the yellow segment that joins the two most distant opposite tips of the GNSs. This is possible due the white scale bar present in each TEM picture.

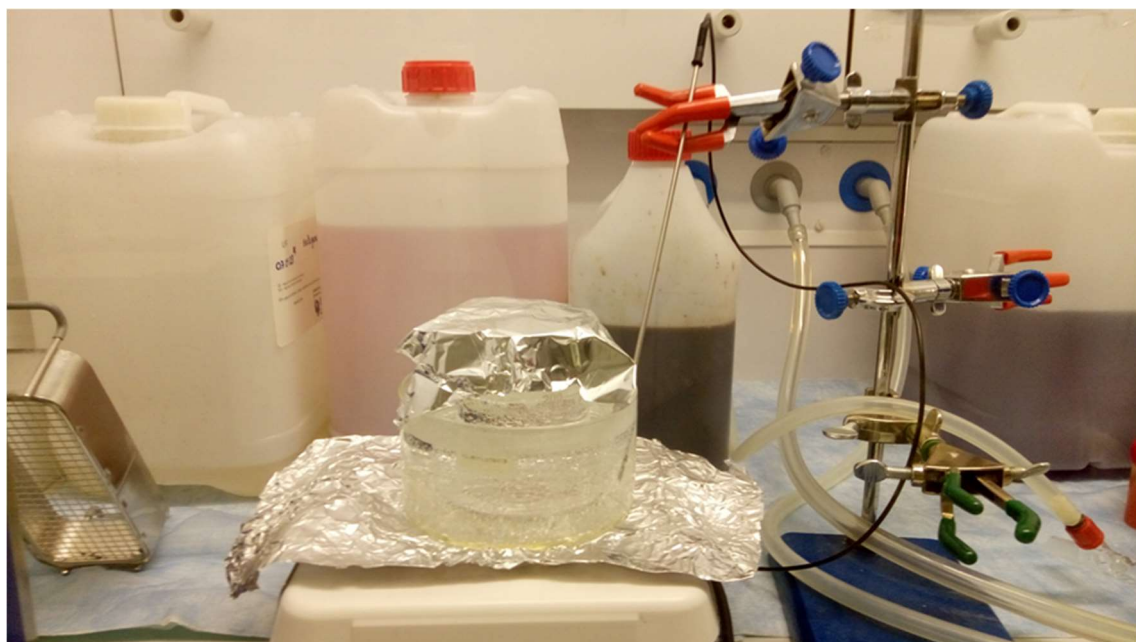


Figure A3 – Piranha treatment setup. Conventional optical microscopy glass slides were treated with fresh piranha, heated in a water bath at 80 °C for 1 hr under a chemical fume hood. Aluminum foil was used as cover to protect lab equipment and user from acid spills.

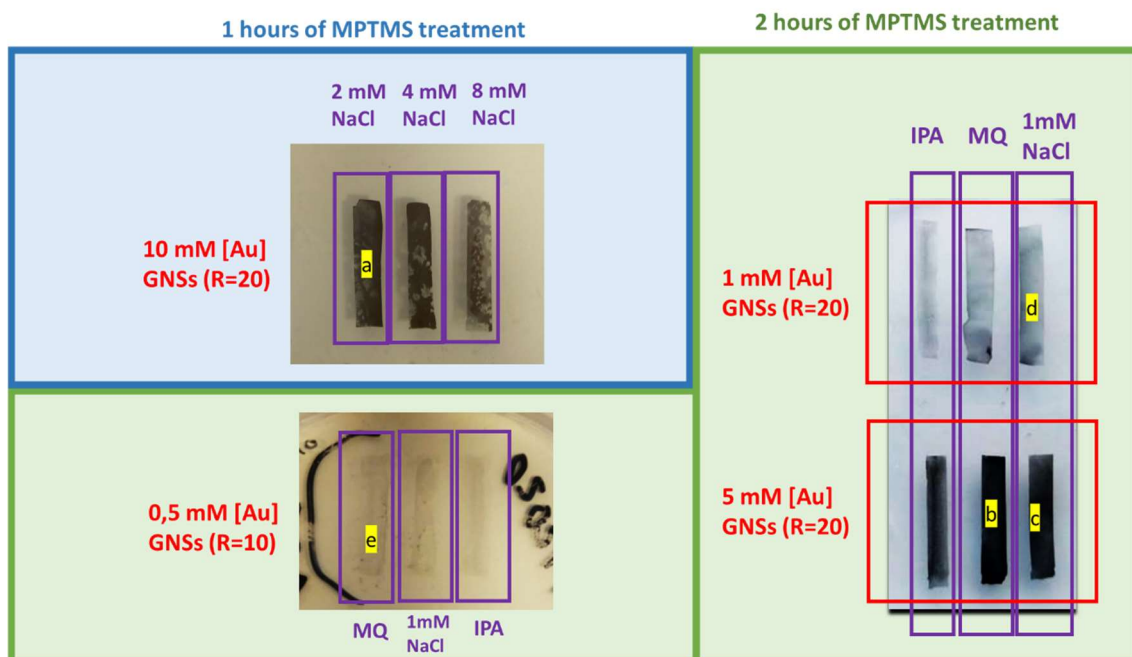


Figure A4 - GNSs assembled at different conditions on PDMS substrates. Blue background corresponds to 1 hr of silanisation, while green correspond to 2 hrs. In 1 hr, it is possible to see holes on the bands. In red are the different batches of GNSs used, including R values and [Au] concentrations. Solvents used during functionalization are marked with violet. The letters in the yellow background correspond to the respective SEM images of Figure 20.

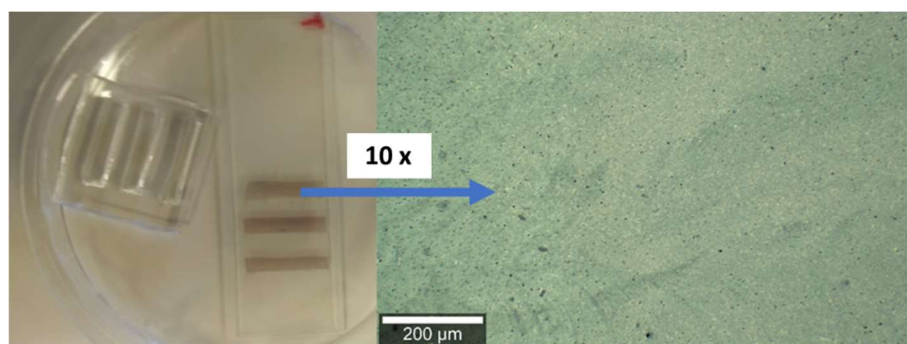


Figure A5 - Au@AgNRs assembled on glass slide and the respective PDMS grid used as mold to create the plasmonic bands (left side). On the right side an amplification (10x) of the surface of the plasmonic bands can be observed.

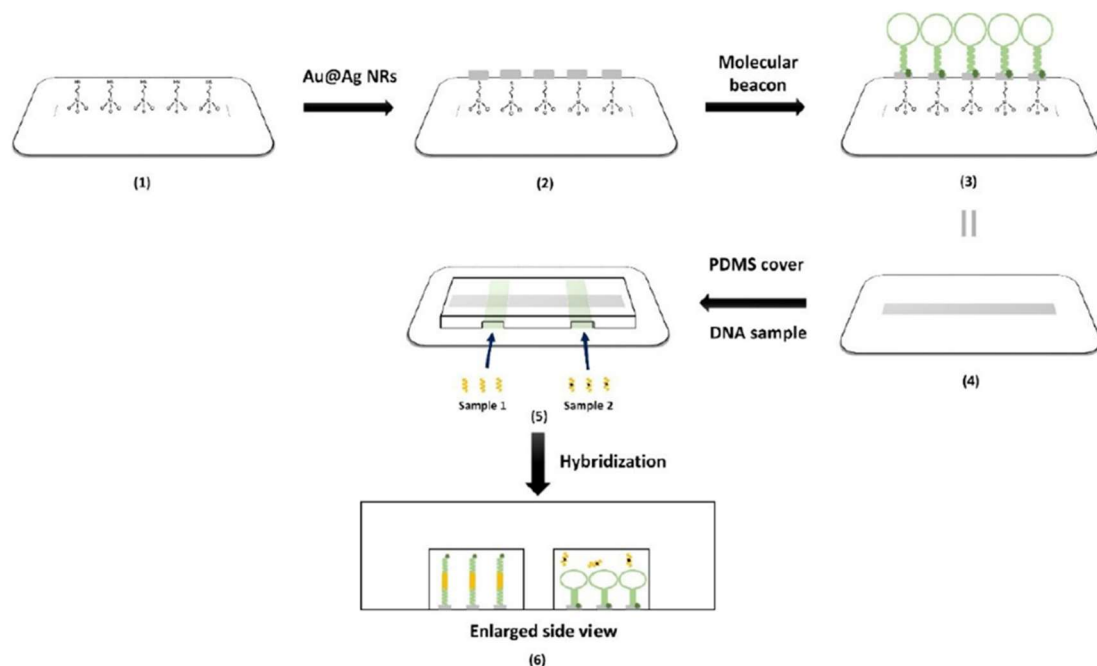


Figure A6 – Main steps taken to produce the optofluidic devices for molecular detection.

Step 1: The glass slides were modified with thiol groups through MPTMS-functionalization.

Step 2: To immobilize the nanoparticles onto the desired regions (rectangular shapes), a drilled PDMS piece (Figure A5) was used as a frame to confine the nanoparticle solutions. Essentially, for each functionalized glass slide a PDMS frame was reversibly bonded by simply pressing it against the glass surface. Then the Au@AgNRs colloid solution was added to the PDMS template, so that the Au@AgNRs were immobilized onto the glass slide in the confined rectangle region through the Ag-S bond.

Step 3-4: After washing, the molecular beacon probes were subsequently added to the template, so that the thiol-modified probes were attached to the nanorods through the Ag-S bond. The PDMS frame was then peeled off from the glass slide, while the molecular beacon-attached nanorods remained on the glass slide in a rectangle shape.

Step 5: The PDMS piece containing the microchannel was treated with oxygen plasma and then irreversibly bonded against the untreated glass slide previously modified with strips of nanorods and probes, in the orthogonal direction.

Step 6: DNA/RNA sequences were injected in the channels for specific hybridization in the microchannels.

Adapted from reference 7.

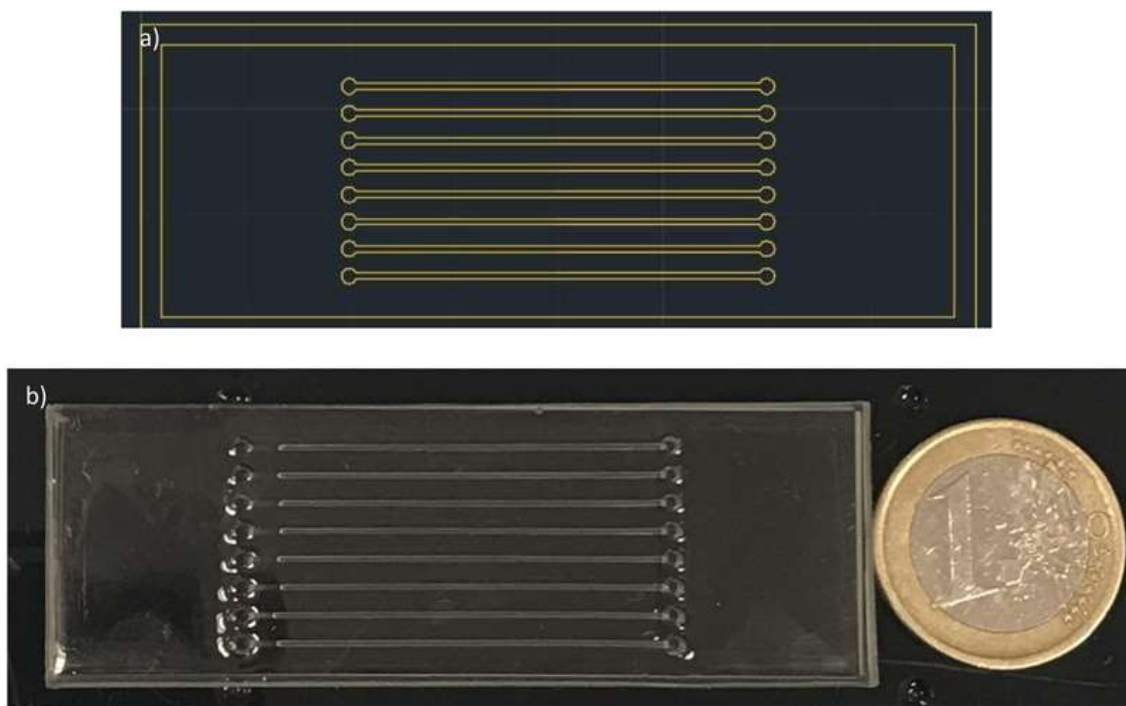


Figure A7 - Microfluidic chip computer design and replica. (a) Microfluidic chip design made with AutoCAD. (b) Final PDMS microfluidic device (PDMS replica bonded against a glass slide). The final dimensions of the microfluidic device were 4 mm (thickness) $\times$ 76 mm (length) $\times$ 26 mm (width) and it included a total of 8 capillarity-driven microchannels with a geometry of 40 mm (length) $\times$ 800 μ m (depth) $\times$ 600 μ m (width). Each microchannel had one inlet and one outlet, incorporated in the PMMA mold, and a volume capacity of 20 μ L. Adapted from reference 119.



Figure A8 – Setup for the injection of the different solutions in the optofluidic chip for analysis. A syringe connected to a pump by an EVA tube was used for each injection. Each injection was made at a flow rate of 5 μ L/min for 5 minutes, so the volume injected was 25 μ L for every case.

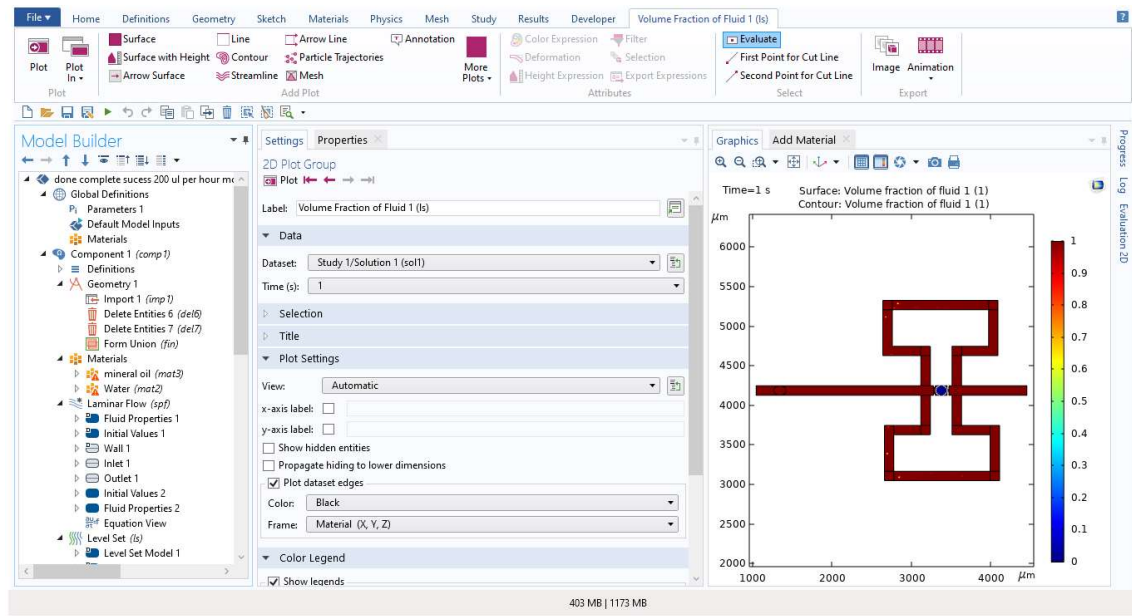


Figure A9 – User interface of COMSOL® Multiphysics v5.5.

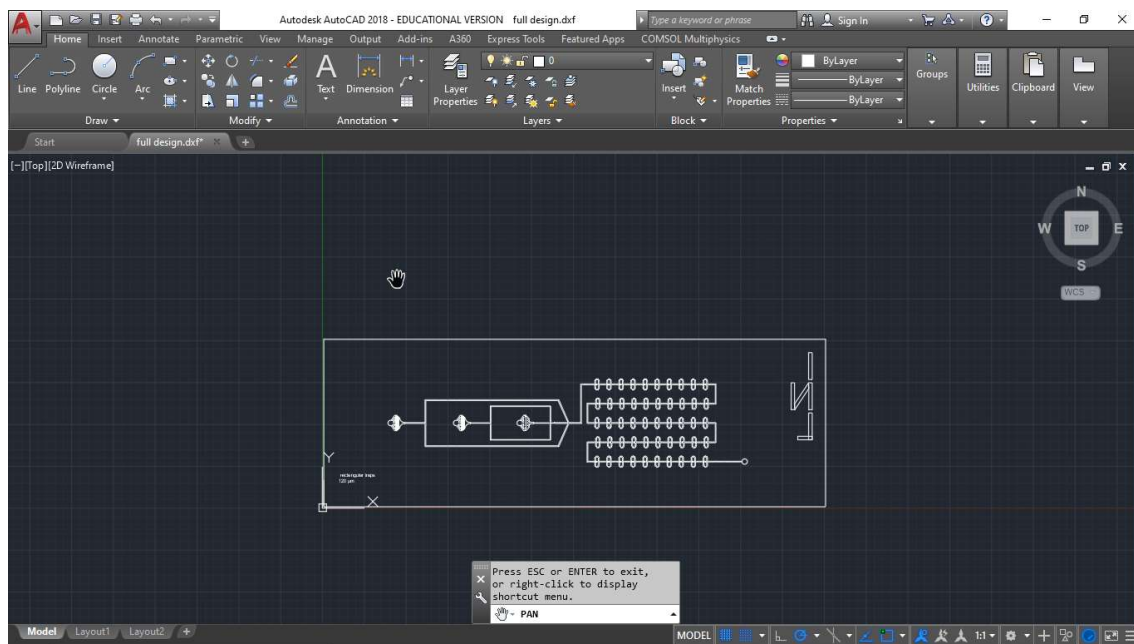


Figure A10 - User interface of AutoCAD® 2018 from Autodesk.

Table A1 – Fluid properties (absolute viscosity and density) of the continuous phases considered for CFD purposes.

Oil	Density (kg/m ³)	Dynamic Viscosity (Pa·s)
HFE 7500	1614	1.24e-3
Mineral oil	840	23.8e-3

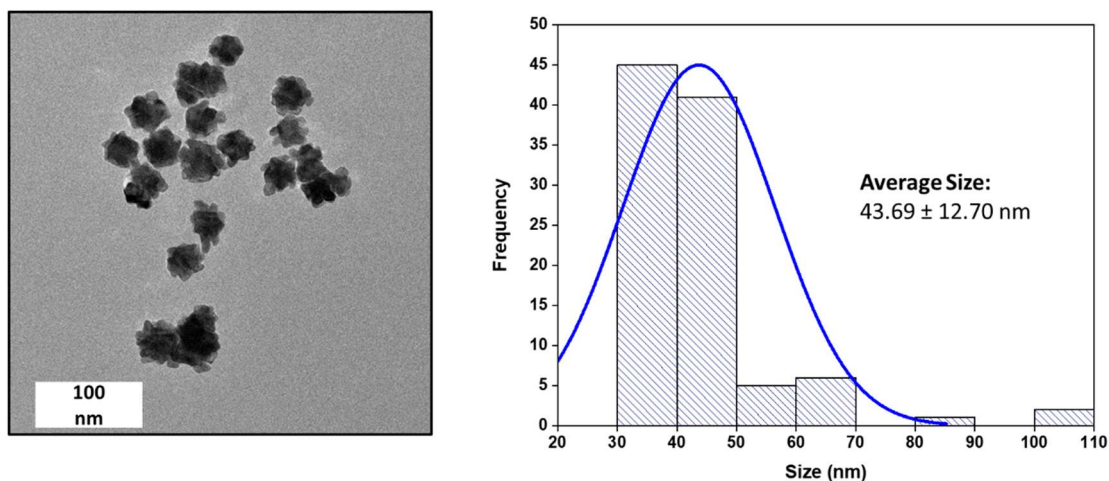


Figure A11 – Morphology and size distribution of the synthesized GNSs with an R value of 2.5. TEM image (on the left), shows a roundish morphology of the GNSs with small protruding lumps. The size distribution (on the right) shows that the average size is 43.69 nm, and the standard deviation is around 13 nm.

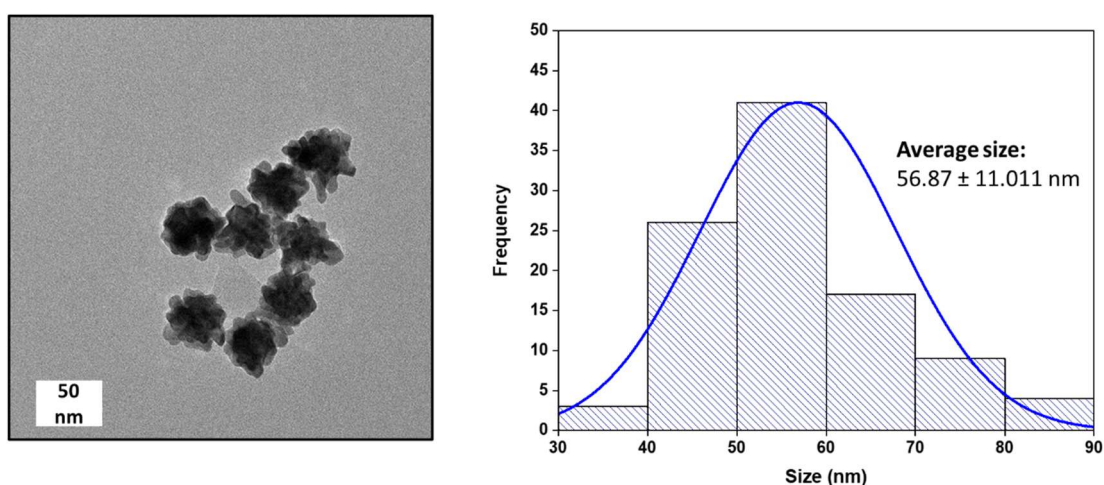


Figure A12 - Morphology and size distribution of the synthesized GNSs with an R value of 5. TEM image (on the left), shows a roundish morphology of the GNSs with small protruding lumps/tips. The size distribution (on the right) shows that the average size is 56.87 nm and the standard deviation is around 11 nm.

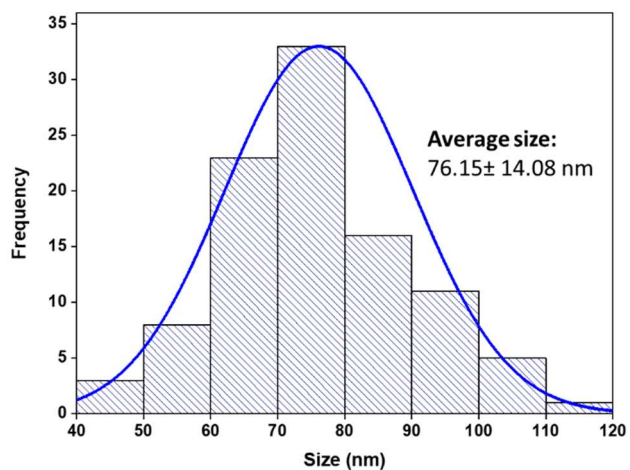
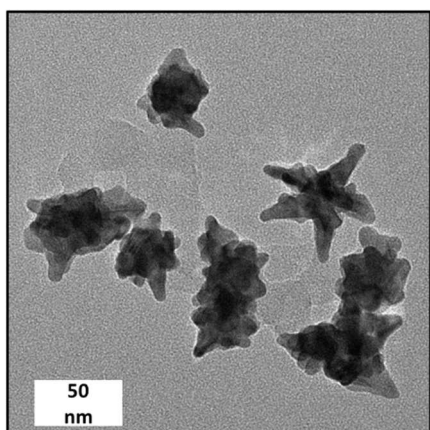


Figure A13- Morphology and size distribution of the synthesized GNSs with an R value of 7.5. TEM image (on the left), shows an irregular morphology with protruding lumps/tips for the GNSs. The size distribution (on the right) shows that the average size is 76.15 nm, and the standard deviation is around 14 nm.

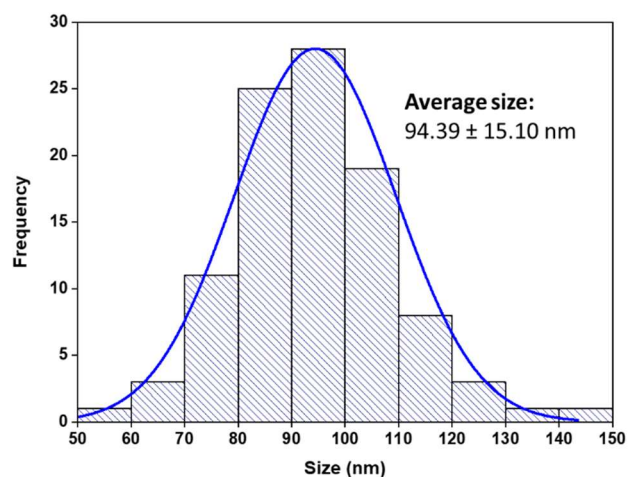
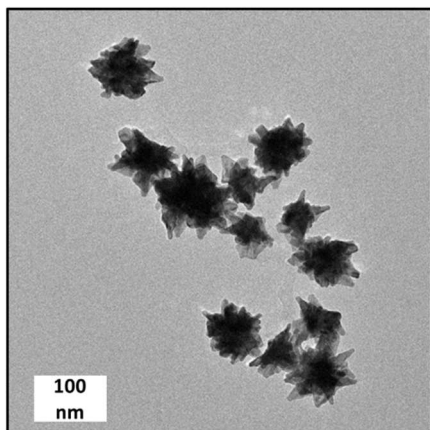


Figure A14 - Morphology and size distribution of the synthesized GNSs with an R value of 10. TEM image (on the left), shows GNSs with a central core with protruding tips. The size distribution (on the right) shows that the average size is 94.39 nm, and the standard deviation is around 15 nm.

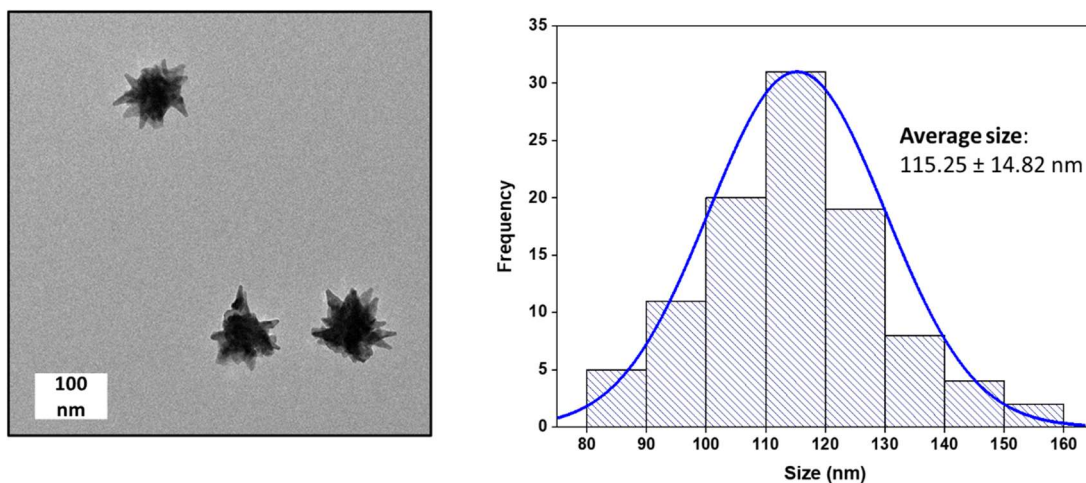


Figure A15 - Morphology and size distribution of the synthesized GNSs with an R value of 20. TEM image (on the left), shows GNSs with a central core with protruding tips. The size distribution (on the right) shows that the average size is 115.25 nm, and the standard deviation is around 15 nm.

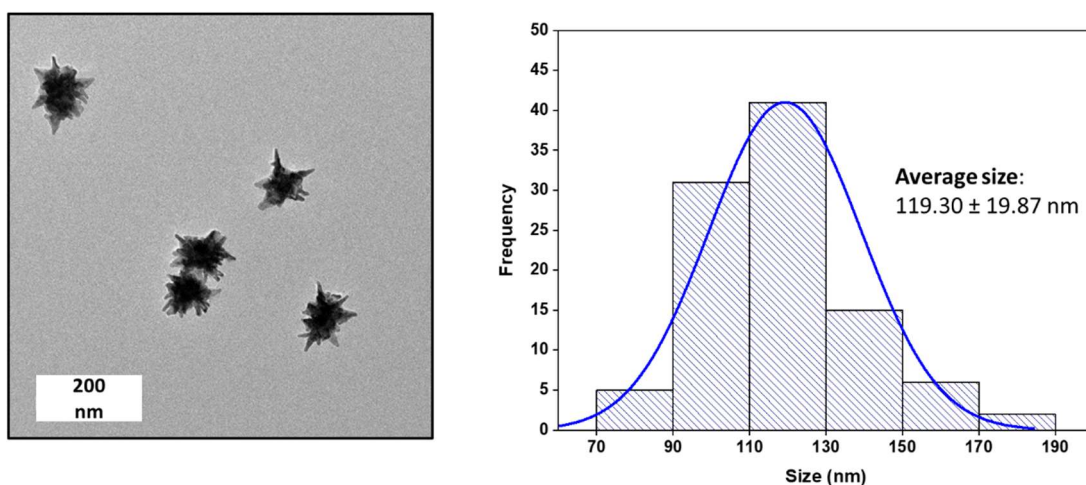


Figure A16 - Morphology and size distribution of the synthesized GNSs with an R value of 45. TEM image (on the left), shows GNSs with a central core with protruding tips. The size distribution (on the right) shows that the average size is 119.30 nm, and the standard deviation is around 20 nm.

Table A2 - Numerical results of the processing made for the spectra taken in the optofluidic studies for mutated BRAF and RdRp gene. The first column ($\overline{AUC}$) refers to the average AUC, and $\sigma(AUC)$ refers to the respective standard deviation. AUC_{norm} refers to the average AUC of the target divided by the respective blank and $\sigma(AUC_{norm})$ is the correspondent standard deviation values.

			$\overline{AUC}$	$\sigma(AUC)$	AUC_{norm}	$\sigma(AUC_{norm})$
<i>SARS-CoV-2 RdRp</i>	1 μ M	Target	4829	1176	0.508	0.228
		Blank	9498	3566		
	10 nM	Target	484	485	0.425	0.463
		Blank	1139	491		
<i>BRAF V600E</i>	1 μ M	Target	17424	5749	0.773	0.394
		Blank	22553	8779		
	100 nM	Target	27619	5639	0.190	0.066
		Blank	145704	41068		