

Electromagnetic sensing of cell suspensions in microfluidic systems

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DECLARATION

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Clare Jane Watts

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ABSTRACT

Cancer is one of the leading causes of death worldwide, and cancers which metastasise, the process of cancer spreading from one organ to another within the body, have the highest fatality rates. Although the mechanism of metastasis is not well understood, the study of cells in the circulatory system which are known to seed tumours is of great importance in cancer research. Current methods for detection of such cells, although specific and sensitive, result in their destruction, and have drawbacks associated with a bias towards specific cell markers.

This work seeks to assess the feasibility of using dielectric spectroscopy and the variation in dielectric properties of different cells at microwave frequencies to characterise and separate circulating tumour cells (CTCs) and other abnormal cells from peripheral circulating blood. This technique is non-destructive and label free, and opens the door to a flow-cytometer system based purely on the physical properties of different cells, capable of measuring them in their natural state.

A novel coupled-resonator sensor is introduced in this work, and used to make dielectric measurements of polystyrene microspheres as well as single, flowing, viable cells of three different types at ~ 10 GHz. The results have been compared to Raman spectroscopy analysis of the cultured cells, showing potential correlation between the water content of the cell types investigated, and the response of the novel system. This is of diagnostic value, with cell hydration linked to carcinogenesis.

PUBLICATIONS, CONFERENCES, AWARDS AND PATENT

JOURNAL PUBLICATIONS:

- S. M. Hanham, **C. Watts**, W. J. Otter, S. Lucyszyn, N. Klein. *"Dielectric measurements of nanoliter liquids with a photonic crystal resonator at terahertz frequencies"*. Applied Physics Letters, [Online], 21 July 2015.†
- **C. Watts**, S. M. Hanham, J.P.K. Armstrong, I. J. Pence, M. M. Ahmad, M. M. Stevens, N. Klein, *"Lab-on-chip microwave dielectric measurements on single flowing cells"*. Lab on a Chip, [In Peer Review], November 2017. ‡

CONFERENCE PRESENTATIONS AND WORKSHOPS:

- 17-18/03/2015 N. Klein*, **C. Watts**, S. M. Hanham, T. H. Basey-Fisher, E. N. Shaforost, W. J. Otter, B. Ng, J. McGovern, N. Guerra. *"Microwave-to-terahertz sensing in microfluidic systems"*, Lab-on-a-Chip & Microfluidics, Berlin, Germany.
- 25-27/05/2016 **C. Watts***, S. M. Hanham, N. Klein. *"Towards microwave sensing of single cells"*, Biosensors 2015, Gothenburg, Sweden. *Invited presentation*.
- 28-31/08/2016 N. Klein*, **C. Watts** et al. *"Microwave-to-terahertz cavities and waveguide structures for liquid sensing in microfluidic systems"*, SPIE Terahertz Emitters, Receivers, and Applications VII.
- 3-7/10/2016 **C. Watts***, S. M. Hanham, M. M. Ahmad, M. Adabi, N. Klein. *"Coupled Dielectric-Split Ring Microwave Resonator for Liquid Measurements in Microfluidic Channels at Nanoliter Volumes"*, European Microwave Week, London.
- 4-9/06/2017 S. M. Hanham*, **C. Watts**, M. M. Ahmad, S. Lucyszyn and N. Klein, *"Single Biological Cell Detection using Terahertz and Microwave Radiation"*, Emerging Applications of Terahertz Workshop, Int. Microwave Symposium, Honolulu.

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Contributions to publications:

- † Test solutions made up and biological solutions prepared and provided (these measurements were not reported on)
- ‡ All microwave measurements made and test solution/suspensions preparations done and paper written

GLOSSARY OF TERMS

DC	Direct current
RF	Radio Frequency
IR	Infrared
UV	Ultraviolet
CTC(s)	Circulating tumour cell(s)
EM	Electromagnetic
DNA	Deoxyribonucleic acid
VNA	Vector network analyser
CW	Continuous wave
PTFE	Polytetrafluoroethylene
TEM	Transverse electromagnetic
TE	Transverse electric
TM	Transverse magnetic
EH	Electric hybrid
HE	Magnetic hybrid
Q	Resonance quality factor
f_0	Resonance frequency
BZT	Barium zinc tantalate perovskite
SRR(s)	Split ring resonator(s)
CTMs	Circulating tumour microemboli
EMT	Epithelial-to-mesenchymal transition
ISET	Isolation by size of epithelial tumour cells
DEP	Dielectrophoresis/Dielectrophoretic
MRI	Magnetic resonance imaging
ROT	Electrorotation
DEP-FFF	Dielectrophoretic field-flow fractionation
CHO	Chinese hamster ovary
ACD	Acid citrate-dextrose
RBC(s)	Red blood cell(s)
WBC(s)	White blood cells(s)
PBS	Phosphate buffered saline
DI	Deionised

DR-DSRR	Dielectric resonator-double split ring resonator coupled system
DR	Dielectric resonator
DSRR	Double-split-ring resonator
PDMS	Polydimethylsiloxane
CNC	Computer numerical control
PM(s)	Polystyrene microsphere(s)
IFBW	Intermediate frequency bandwidth
EDTA?	
MSC	Microbiological safety cabinet
ΔQ	Shift in quality factor
ΔF_c	Shift in resonance frequency
CST MWS	CST MICROWAVE STUDIO
ECM	Equivalent circuit model
$\Delta(1/Q)$	Shift in inverse quality factor
F_{water}	Resonance frequency of water condition
Q_{water}	Resonance quality factor of water condition
HW	Resonant half-width
$\Delta\varphi$	Shift in phase (°)
ΔA	Shift in Amplitude (dB)
Q_0	Unperturbed resonance quality factor (DI water/PBS condition)
f_0	Unperturbed resonance frequency (DI water/PBS condition)
σ	Standard deviation
C2C12	Musculus muscle myoblast cell line cells (ATCC® CRL-1772™)
HMSC(s)	Human mesenchymal stem cell(s)

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1. INTRODUCTION

The interaction of electromagnetic (EM) waves with matter is well documented and of fundamental importance. Analysis of this interaction can yield information about a material's electrical properties, structure and composition.

Various properties of solids, liquids and gases can be probed from DC up to the very highest frequencies at gamma rays, and EM radiation of various frequencies/wavelengths has found applications in many diverse fields. Static electric fields can be used to detect cells using commercial devices such as the Coulter Counter by detecting differences in impedance. In the kHz to MHz regime (below 1 GHz), in conjunction with magnetic fields, information about the magnetic properties of atomic nuclei can be used to characterise the structure of molecules in NMR and the structure of tissues in the body in MRI. In the radio frequency (RF) range, the local heating effects of alternating electric fields are used in electrosurgery to cauterise, ablate and, more recently, characterise tissue during surgery.¹ In the field of analytical chemistry the frequency range of 10s to 100s of THz provides the basis of IR spectroscopy which gives characteristic information about the vibrational frequency of individual chemical bonds; UV-visible spectroscopy which gives quantitative determination of molecules based on electronic excitations; and Raman spectroscopy which shows fingerprint spectra of materials based on collective rovibrational modes. X-rays which range from 10 nm to 0.01 nm (30 PHz - 30 EHz) have a familiar application in medicine due to their penetrating ability, showing skeletal structure through soft tissues, and in security, scanning the inside of airplane luggage. Overlapping with X-rays are gamma rays which fall anywhere under 0.01 nm wavelength (more than 30 EHz). Despite low doses potentially causing cancerous mutations in cells, this highest frequency range is used in medicine, to kill cancer cells, and in radioactive tracers as well as in killing bacteria on surgical instruments and food.

This thesis focusses on the microwave frequency range, which occupies the 10 centimeter to 1 millimetre wavelength range, covering a frequency window of 3 to 300 GHz (10^9 Hz)². This band lies in between RF (radio waves) at lower frequencies and terahertz and far IR at higher frequencies.

Microwave frequencies have already revolutionised the world of wireless communications, and are also well known for applications such as radar, astronomical imaging and microwave heating. In the microwave frequency regime, however, information about the molecular structure of a material, the intermolecular bonds and the electrical conductivity can be gained from dielectric spectroscopy. The use of microwave frequencies to probe the composition and structure of biological materials has been explored since the 1950s,^{3,4} and there have been recent advancements in the application of

microwave frequencies to medical and biological fields, with the development of microwave-based imaging of cancerous tissues,⁵ therapeutic microwave-based heating for wound sterilisation and destruction of tumours.^{6,7}

In this thesis, the permittivity of single cells at microwave frequencies is investigated. A high frequency is chosen for measurements (~10 GHz) due to the strong interaction of electric fields with water in this range. It is postulated that at this frequency different cell types could be discriminated based on variations in their water content. At first, an existing simple dielectric resonator sensor is used, but in order to overcome its limitations in sensitivity and interaction volume a novel coupled resonator sensing system is developed. The sensitivity of the coupled system is tested using dilute ethanol solutions, the results of which have led to the development of an analytical model of the system which reveals a transducing property of the system as the basis of its enhanced sensitivity. Investigation of various experimental set-ups leads to the detection of individual, flowing polystyrene microspheres. Once successful sensing of cell-sized objects has been achieved the system is tested with three different types of biological cells, which despite their less significant permittivity contrast to the suspension medium, produce measureable shifts in the resonance properties of the system. Comparison of these results with those of the polystyrene microspheres confirms the system is sensitive to the composition of the flowing objects rather than their size. The biological cell results are compared to Raman spectroscopy measurements which show that the response of the sensor seems to be correlated to the water content of the cells, confirming the original hypothesis.

1.1. MOTIVATION

As will be discussed in Section 2.2, the strong interaction of microwaves with water makes biological tissues and cells a viable target for microwave measurement and sensing technologies, with most living things consisting predominantly of water. This is potentially particularly useful for the case of cancer, as malignant cells and tissues are widely cited as having an increased hydration compared to healthy cells and tissues.^{8,9} Strong contrasts in the microwave dielectric permittivity of tumour and healthy tissue have been measured,^{5,10} and for individual cell types the cancer aggressiveness grade of tumours can be distinguished using this technique.¹¹

Cancers count among the top causes of death worldwide, with metastatic cancers having the highest fatality rates.¹² As will be discussed in Chapter 4, metastasis is the process by which a primary tumour leads to secondary and maybe more new tumours in other organs and elsewhere in the body via “seed” cells which escape the site of the primary tumour into the circulatory system.

Metastatic cancer can occur in virtually all types of cancer, even cancer of the blood and lymphatic system,¹³ including the top four most common cancers (breast, lung, prostate and bowel) which in the UK in 2011 accounted for more than half of all reported cancer cases (53%).¹⁴ Cells known as circulating tumour cells (CTCs) are widely believed to be the culprits in metastasis, and studies show that patients with greater than 5 CTCs per 7.5mL blood face significantly lower survival rates than those with fewer.^{15,16} Current gold-standard histological characterisation and detection methods destroy or permanently physically alter the CTCs, and rely on very specific cell markers that may miss critical sub-populations.

Microwave dielectric spectroscopy potentially offers a complementary method of cell characterisation and detection which does not alter cells from their natural living state. This allows investigation of CTCs downstream from identification, an enormous advantage in helping to understand the mechanisms of disease progression as well as in developing and targeting new therapies for treatment. Along with this, basing sensing on physical characteristics such as water content may also allow detection of potentially threatening cells that do not conform to the standard phenotypes expected from traditional biology. The aim, therefore, is to test the viability of a new label-free method of detecting the cells responsible for metastasis, based on their electromagnetic properties at microwave frequencies.

1.2. THESIS OUTLINE

The work presented in this thesis covers the development of a sensor capable of detection of single flowing cells at microwave frequencies. The sensor is tested with different cell types to ascertain whether differences in the signals obtained can be correlated to physical differences between the cells, which may have diagnostic value.

Chapter 2 covers the theory of and mathematical basis for understanding dielectric spectroscopy. The dominating effects and the information that can be extracted at different frequencies are explored for solids, liquids, biological solutions and cells. Promising windows for investigation are identified.

Chapter 3 deals with the practical aspects of measuring dielectric properties across different frequency ranges, covering basic detection equipment such as the vector network analyser (VNA) as well as broadband and narrowband measurement techniques. The type of measurement approach used for different samples and experiments is explained and justified.

Chapter 4 briefly introduces the circulating tumour cell. The process of metastasis and the importance of CTCs and what they can be used to infer about disease state are explained. Shortfalls in current technology are examined and the advantages and challenges of a new method based on dielectric spectroscopy laid out.

Chapter 5 seeks to put the work in context by briefly summarising relevant current literature, starting with the early electrical measurements of biological solutions in the 1930s through to the latest work looking at scaling the process down to single cells, including work at a range of frequencies and other uses of electromagnetic waves such as dielectrophoresis and electrorotation, showing the broad spectrum of information that can be gleaned from cells.

Chapter 6 builds on Chapter 3, describing, in full, the experimental procedures carried out on all samples, including any sample preparation. In this chapter the sensor design is also outlined.

Chapter 7 presents and critically assesses the results gained from measurements, and introduces a theoretical model of the new sensor. Different versions of the sensor are tested with ethanol in water solutions, before being trialled with polystyrene microspheres and biological cells. The results are presented and discussed alongside the well-established measurement technique of Raman spectroscopy.

Chapter 8 summarises the results and conclusions that can be drawn from the results and outlines future work to improve the system and assess its viability as a CTC enumeration and detection device.

Appendix 1 will provide more detail of the equivalent circuit model, while **Appendix 2** will explain, in more detail, processing methods employed in Chapter 7 and **Appendix 3** will outline the method of cell size determination used.

2. MICROWAVE DIELECTRIC THEORY

2.1. INTERACTION OF MICROWAVE ELECTRIC FIELDS WITH MATTER

In dielectric materials an electric field can induce polarisation through the separation of charge on an atomic and/or molecular level. The polarisation opposes the applied field, and the degree of the polarisation depends on the material and its structure. Permittivity, ϵ , is an intrinsic value of a material which describes the degree to which it is polarised by an external electric field. It is normally expressed as a relative value which quantifies the permittivity of a medium as a ratio relative to that of free space:

$$\epsilon_r = \frac{\epsilon}{\epsilon_0} \quad \text{Equation 2.1.1}$$

ϵ_0 is a fundamental constant of nature that describes the strength of the Coulomb force. It has an exactly defined value which depends only on the unit system used. ϵ_r is the relative permittivity, also known as dielectric constant.

The separation of charge due to polarisation leads to the formation of electric dipole moments, p , as illustrated in Figure 2.1.1. The polarisation, P , of a material is the sum of these electric dipole moments per unit volume, and provides us with a general way of describing the response of the material to an electric field. The polarisation of a material decreases the electric field within the material with respect to the applied field, E . The resultant field is known as the displacement field, D , and is directly proportional to both the strength of the external field and the permittivity of the material:

$$\mathbf{D} = \epsilon_0 \mathbf{E} + \mathbf{P} = \epsilon_r \epsilon_0 \mathbf{E} \quad \text{Equation 2.1.2}$$

The relative permittivity of a material therefore describes the degree of polarisation of a material for a given applied field strength, due to the pushing and pulling effects the external field has on the distribution of charges within the material.

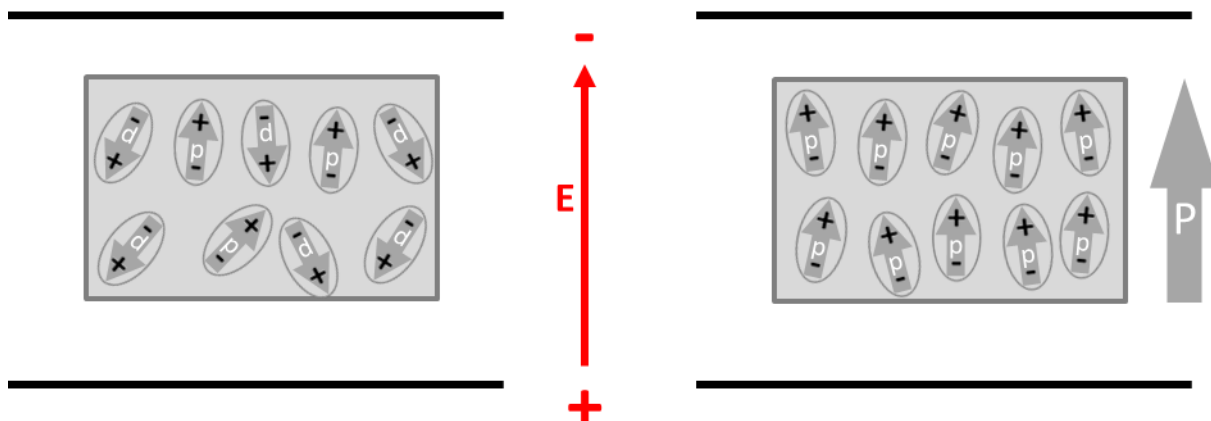


Figure 2.1.1: Schematic showing a polarisable material in a capacitor and the difference between the condition of no external field (left) and an external field (right). Polarisation in the dielectric results in a change in the field within its volume.

A perfect vacuum has no polarisation mechanisms and therefore its permittivity is, by definition, equal to 1. Since permittivity depends on the various inter/intra-molecular (or atomic) forces present, it is an intrinsic property, characteristic for different materials.

There are four general polarisation mechanisms in a dielectric:

- **Interfacial (space-charge)** – free charge carriers move with the field but are stopped by a boundary between different regions of the material.
- **Orientalional (dipolar)** – For free-moving molecules in liquids and gases, polar molecules can align themselves parallel to the external electric field, resulting in overall polarisation.
- **Ionic or molecular distortion polarisation** – Positive and negative ions in an ionic lattice or molecular solid can be displaced relative to each other via the stretching and bending of bonds, resulting in a net electric dipole moment.
- **Electronic or atomic** – The electron cloud around the nucleus of a neutral atom can be displaced relative to the nucleus, resulting in the formation of an electronic dipole moment within the atom.

The polarisation mechanisms interacting with electromagnetic fields in a given sample depend on the material and its structure, as well as the frequency of the applied electric field. Ionic and electronic polarisation have strong intrinsic restorative mechanisms, with electrostatic interactions and molecular bonds acting to pull back ions or charged molecular groups. This causes a resonance process, with the polarisation mechanisms modelled as dampened oscillators.

These resonances have high frequencies and form the basis of IR and UV/vis spectroscopy. Interfacial and orientational polarisation do not have such a restorative mechanism and therefore follow a relaxation from a polarised state back to a ground/equilibrium state when the external electric field decreases or is removed. This dielectric relaxation tends to be slower than the resonant

processes, and so these mechanisms are probed at lower frequencies, from DC to the microwave region.

For the purposes of this thesis, the focus will be the microwave dielectric properties of water-based solutions and biological liquids. At microwave frequencies, the dominant polarisation mechanism in water is the re-orientation of freely moving polar water molecules, though higher frequency (IR and UV) ionic and electronic polarisation mechanisms also contribute to the values of permittivity. For the case of more complex liquids such as biological materials, effects from free ions or charge carriers as well as biological macromolecules such as proteins must also be taken into account.

2.1.1. COMPLEX PERMITTIVITY

When the external field is removed, the energy used to create a displacement of charge is re-emitted as the polarisation dissipates and the material returns to its equilibrium state. This dissipates the net electric field across the material. A material with a permittivity greater than one can be thought of as storing an amount of energy proportional to its degree of polarisation, or its permittivity.

As well as being stored, some of the energy which induces the displacement field is lost to the material as heat in the process of polarisation due to, for example, collisions between atoms or molecules as they rotate or bend, the breaking of intermolecular bonds, overcoming electrostatic interactions during the stretching or bending of intramolecular bonds, etc. To encompass both the energy stored and the energy that is lost during polarisation, permittivity is represented as a complex value split into real and imaginary components:

$$\hat{\epsilon} = \epsilon' - i\epsilon'' \quad \text{Equation 2.1.3}$$

The real part of the permittivity, ϵ' , is the lossless permittivity and is a measure of the energy stored in the material, while the imaginary part, ϵ'' , is the out-of-phase loss factor and is directly related to the dielectric loss of the system due to bound charges and dipole relaxations¹⁷. i represents the imaginary number $i^2 = -1$. It is common to report losses using the loss tangent, $\tan\delta$, which is defined as the ratio of the imaginary to the real part of the permittivity:

$$\tan\delta = \frac{\epsilon''}{\epsilon'} \quad \text{Equation 2.1.4}$$

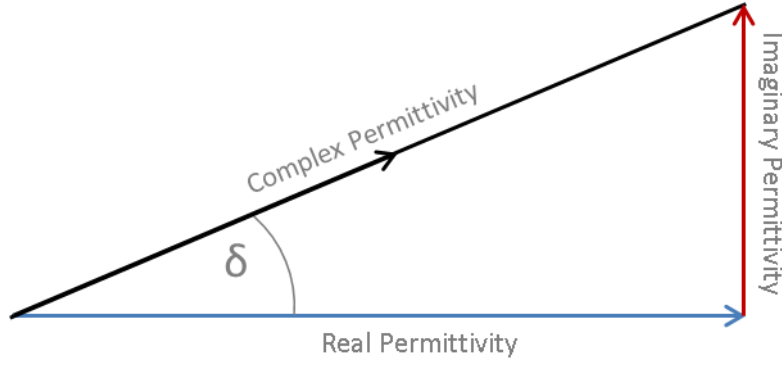


Figure 2.1.2: Visualisation of the loss tangent, showing the real and imaginary permittivity plotted on the complex plane.

2.1.2. FREQUENCY DEPENDENCE

The case is quite simple for static electric fields, but when dealing with microwave signals the fields are time-varying. The same polarisation mechanisms are present, but their effect on the dielectric behaviour of materials depends on the frequency of the oscillating fields. For this reason permittivity is a frequency-dependent property, meaning that its value for a particular material will change depending on the frequency (ω) of the applied electric field, so that Equation 2.1.1, Equation 2.1.3 and Equation 2.1.4 are better written as:

$$\varepsilon_r(\omega) = \frac{\varepsilon(\omega)}{\varepsilon_0} \quad \text{Equation 2.1.5}$$

$$\hat{\varepsilon}(\omega) = \varepsilon'(\omega) - j\varepsilon''(\omega) \quad \text{Equation 2.1.6}$$

$$\tan\delta = \frac{\varepsilon''(\omega)}{\varepsilon'(\omega)} \quad \text{Equation 2.1.7}$$

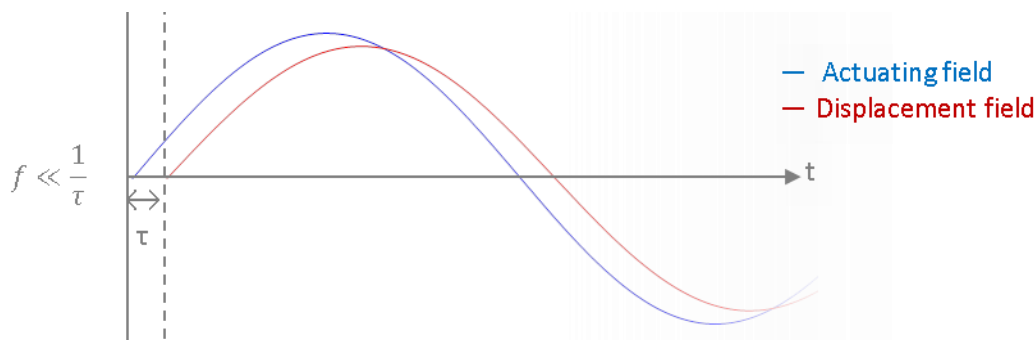
This means that both the energy stored and the energy lost from polarisation of a dielectric depend on the frequency of the actuating field. This frequency dependence arises from the fact that the response of polarisation mechanisms is never instantaneous; it is always a reaction to the external field. Different polarisation mechanisms have different characteristic response times, with time constants τ , which describe the speed of the displacement/stretch/bend/reorientation and its subsequent relaxation or resonance. The type of polarisation mechanism, the type of molecule or atom involved, and the surroundings of that molecule or atom, as well as the temperature determine the time constant.

When subjected to a time-varying electric field, the polarisation in a dielectric is forced to oscillate to align with the changing field. When the field is increasing in strength the polarisation increases, and when that field decreases to zero the polarisation returns to its equilibrium state, as governed by

electrostatic interactions with the surrounding molecules/atoms. The delay in the response of polarisation mechanisms results in a phase difference between the displacement field arising from the polarisation and the actuating external electric field. The relaxation of the displacement field re-emits the stored energy at the same frequency as the actuating field, but with this phase difference.

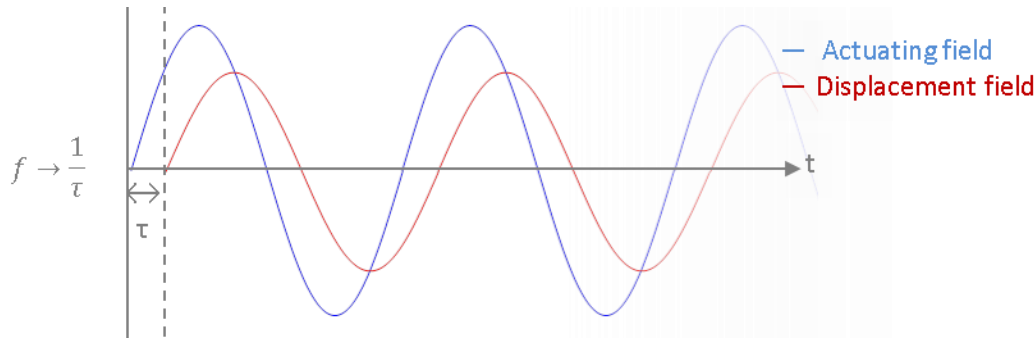
As the actuating frequency increases these different mechanisms will be unable to “keep up” with the changing field and so their contribution will drop off, leading to a characteristic dependence of the permittivity on frequency. Polarisation mechanisms that undergo dielectric relaxation exhibit a different frequency dependence to resonant mechanisms. Here relaxation mechanisms will be discussed, since these are the most significant contributors to the permittivity of aqueous liquids in the microwave regime.

At frequencies significantly lower than $1/\tau$, the response time is fast compared to the rate of change of the field, and polarisation seems to happen almost immediately. The phase difference between the actuating field and the displacement field is at a minimum:



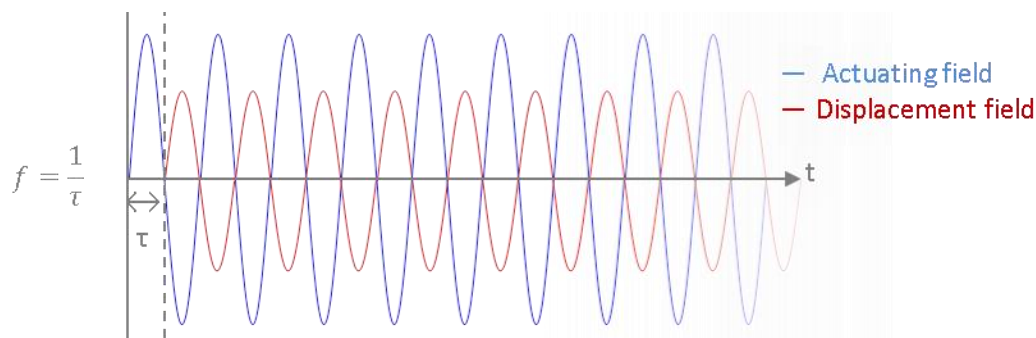
For non-resonant mechanisms, in this situation the degree of polarisation is at a maximum, as there is plenty of time per cycle for the mechanism to occur before relaxation. With both polarisation and relaxation happening almost in phase with the actuating field the energy stored is high, and the losses are low, leading to a maximum value of the real part of the permittivity (ϵ').

As the actuating frequency approaches $1/\tau$, the response time of the polarisation starts to become comparable to the rate of change of the field. The alignment no longer seems instantaneous; the lag in response, and therefore the phase difference between the actuating field and the displacement field, becomes more and more significant:



For non-resonant mechanisms, in this situation the degree of polarisation is limited by the response time. Before the polarisation mechanism has time to react fully to the maximum of the actuating field, the field has begun to change and oppose the polarisation, so the mechanism cannot fully align. Since the degree of polarisation achieved decreases with increasing phase difference, the energy stored begins to decrease, leading to a decrease in the value of the real part of the permittivity. With a steadily increasing phase difference between the displacement field and the actuating field, the polarisation of the field becomes less aligned with the external field, causing more energy to be lost in re-alignment. The re-emission of the energy stored in the displacement field upon relaxation is also becoming more and more out of phase with the external field, leading to destructive interference and increased losses. This results in a rapid increase in the imaginary part of the permittivity.

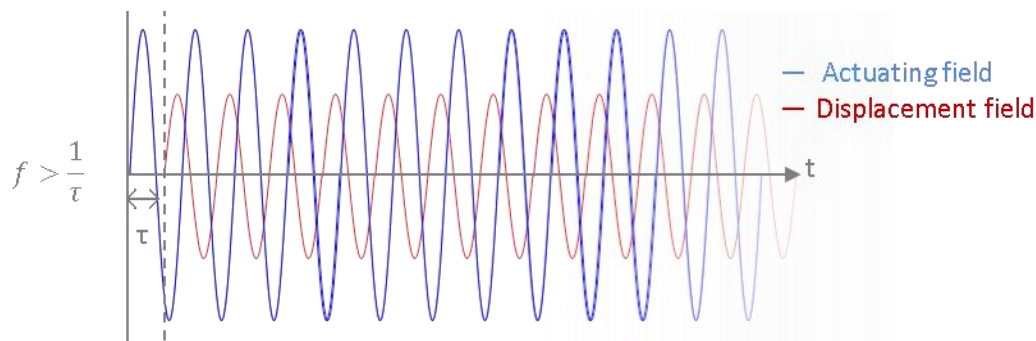
When the actuating frequency equals $1/\tau$ the rate of change of the field is equal to the time the polarisation takes to occur. Since the lag in response time is now equal to the time for the electric field to complete one cycle, the phase difference between the actuating field and the displacement field is exactly 180° :



In this situation the actuating field and the polarisation are exactly out of phase, with the maximum of the actuating field occurring as the polarisation is aligned exactly opposite to the field. For non-resonant mechanisms this results in the maximum amount of energy being lost in re-aligning the polarisation and through destructive interference. This causes a peak in the imaginary part of the

permittivity. The polarisation mechanism has even less time to react to the actuating field, so the degree of polarisation continues to decrease, causing the reduction in the real part of the permittivity to continue.

As the frequency exceeds $1/\tau$ the phase difference exceeds 180° and therefore starts to decrease with respect to the next cycle:



As before, the extent of polarisation continues to become more and more limited by the changing field, and in relaxation processes the real part of permittivity continues to decrease. With the phase difference now decreasing, the polarisation becomes more aligned with the field, and less energy is lost in re-alignment, causing the imaginary part of the permittivity to fall.

At frequencies much higher than $1/\tau$ the polarisation mechanism is no longer able to interact at all with the changing field, so the real part of the permittivity reaches a plateau. Since there is no interaction between the polarisation mechanism and the changing field, the energy loss due to that mechanism drops to zero and the imaginary part of the permittivity falls back to a minimum as well. The value of the real and imaginary permittivity at this point is determined by any other higher-frequency interacting polarisation mechanisms in the material.

Thus different polarisation mechanisms, with different characteristic time constants associated with them, cease to interact with electric fields at different frequencies, resulting in a unique dielectric spectrum for a particular material.

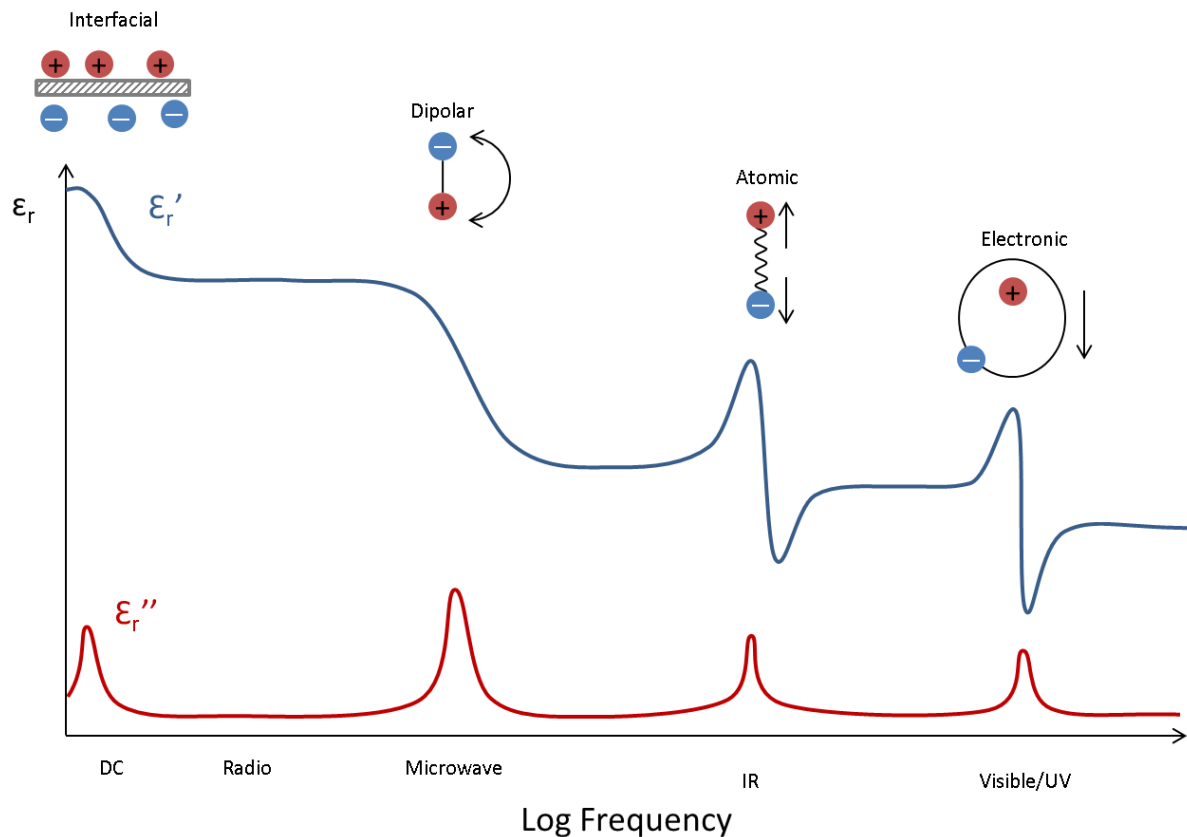


Figure 2.1.3: Schematic showing the frequency dependence of both the real and the imaginary permittivity arising from the four general dielectric polarisation mechanisms.

2.2. DIELECTRIC PROPERTIES OF WATER

Biological solutions consist mainly of water, so clearly when looking at the dielectric properties of biological solutions it is important to understand the dielectric response of water. As a liquid, water molecules have a degree of freedom which allows them to move and change their orientation.

Due to oxygen's two lone pairs of valence electrons, water molecules are non-linear in shape, with an angle between the hydrogen atoms of 104.5° . These lone pairs, along with the higher electronegativity of oxygen atoms, make the oxygen side of the molecule more negatively charged than the side with the hydrogen atoms. This permanent dipole in the water molecule results in an orientational polarisation mechanism, as shown in Figure 2.2.1.

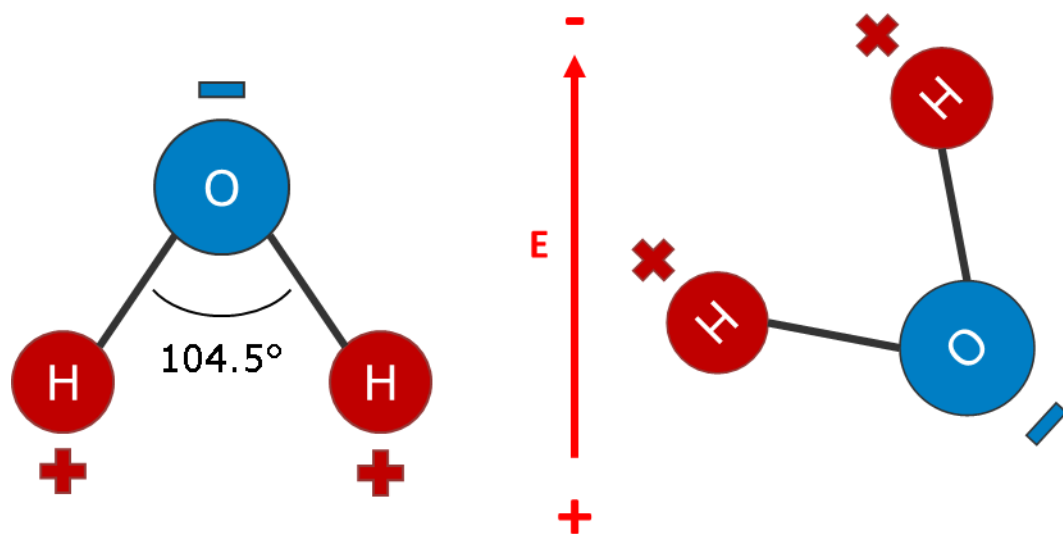


Figure 2.2.1: Schematic of a water molecule, showing on the left the distribution of charge across the hydrogen and oxygen atoms and on the right how this interacts with an electric field (red arrow) .

Under normal conditions the alignment of water molecules is governed by intramolecular electrostatic forces known as hydrogen bonds, and does not result in a net polarisation. The presence of an external electric field introduces anisotropy in the energy of different arrangements of molecules, causing the water molecules to rotate to align, on average, more parallel to the external field. Due to thermal motion, the alignment of dipoles with the field is not perfect, and the degree of alignment depends on the strength of the field, its alternating frequency and the temperature of the water.

In the microwave region, orientational polarisation dominates the dielectric response of water. The speed of the orientational polarisation response of water molecules is limited by inertia and the molecules' intermolecular interactions – the hydrogen bonds, dipole-dipole forces and Van der Waals interactions with its nearest neighbours. These processes result in a time constant of the relaxation of ~ 8 ps at room temperature, corresponding to a broad dipolar relaxation peak extending from around ~ 5 GHz to ~ 100 GHz at room temperature, as can be seen in Figure 2.2.2.¹⁸

At the low frequency limit (DC, $f=0$) the permittivity is known as the static permittivity ϵ_s , which represents the maximum polarisation achieved by all the polarisation mechanisms in that material. At frequencies above the relaxation of water the polarisation mechanism no longer significantly interacts with the oscillating field, and the permittivity is known as the infinite permittivity, ϵ_∞ .

The values of static permittivity, infinite permittivity, actuating frequency and relaxation time can be used to produce a mathematical model of the frequency-dependent complex permittivity of reorientational polarisation using the Debye formula:

$$\varepsilon^*(\omega) = \varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_\infty}{1 + i\omega\tau} \quad \text{Equation 2.2.1}$$

Multiple relaxation mechanisms and their characteristic permittivity and time values can be combined to yield a multiple term Debye equation that can be used to model complex materials with multiple polarisation mechanisms:

$$\varepsilon^*(\omega) = \varepsilon_\infty + \sum_{k=1}^N \frac{\varepsilon_k - \varepsilon_{k+1}}{1 + i\omega\tau_k} \quad \text{Equation 2.2.2}$$

where N is the number of relaxation processes.

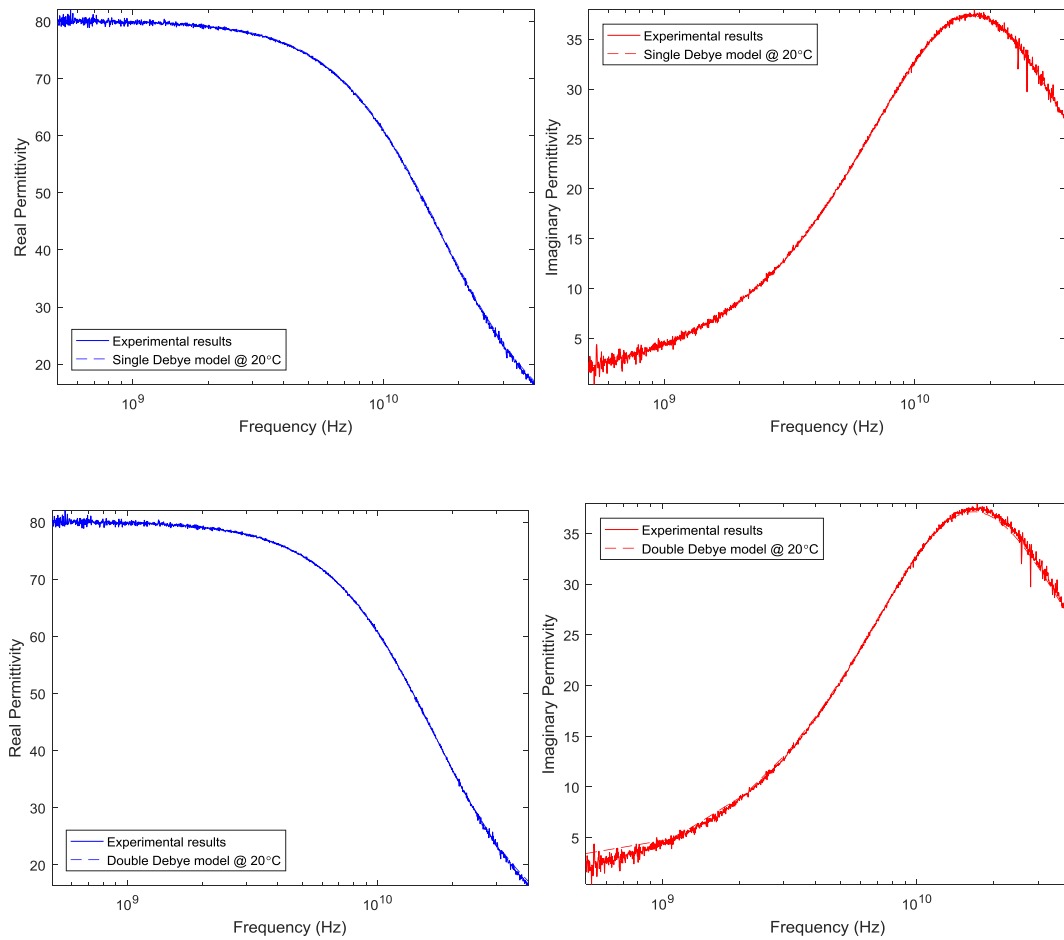


Figure 2.2.2: Comparison of both single and double Debye models of the complex permittivity of reorientational polarisation of water using values of permittivity and time constant from Kaatzé¹⁹ (single) and Buchner et al²⁰ (double) with experimental data. Real and imaginary parts are shown in blue and red, respectively, and experimental and debye model data shown in solid and dashed lines, respectively. The difference between the single and double parameter fit is significant only at higher frequencies at room temperature.

2.2.1. EFFECT OF TEMPERATURE

Higher temperatures mean that water molecules have greater kinetic energy. This random thermal motion opposes the alignment of water molecules with an electric field. The higher the temperature the more kinetic energy the water molecules have, causing them to be able to deviate more from alignment with the field. The more the molecules are able to resist aligning with the field, the less the polarisation and the less energy is re-emitted once that polarisation relaxes. Therefore, as the temperature of water is increased, the real part of its permittivity decreases. Although the infinite permittivity does not change significantly with temperature, the effect can be clearly seen in the real part of the dielectric spectrum of water as a reduction in the static permittivity, which is approximately inversely proportional to the temperature, as shown in Figure 2.2.3.

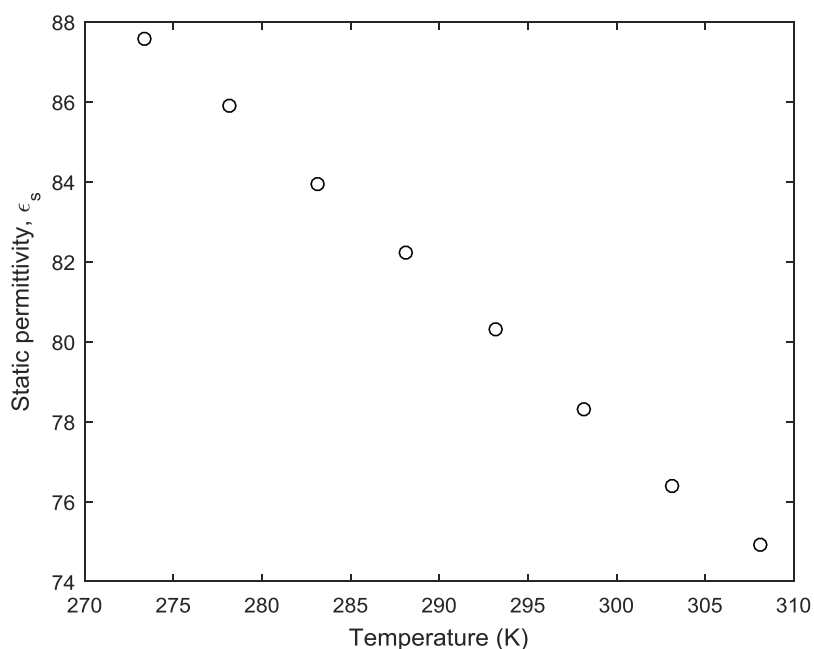


Figure 2.2.3: Values of static permittivity at a range of temperatures obtained via fitting dielectric parameters to the relaxation of pure water at a range of temperatures, from Buchner et al.²⁰

When individual water molecules have more kinetic energy they are more able to overcome intermolecular interactions. This means that less energy is lost to the breaking of intermolecular bonds when the water molecules rotate. Therefore, the imaginary part of the permittivity also decreases as temperature increases.

The time constant of the relaxation is strongly affected by the temperature of the water. Increased kinetic energy weakens intramolecular hydrogen bonds, increasing the speed of rotation of the

molecules and thus decreasing the time constant. The time constant is therefore dependent on the temperature-dependent Gibbs energy of activation of reorientation according to the Eyring relation:

$$\tau = \frac{h}{k_b T} \exp \left[\frac{\Delta G}{RT} \right] \quad \text{Equation 2.2.3}$$

where h = Planck's constant, k_B is Boltzmann's constant, R is the gas constant and T is the temperature.²⁰ In this way as temperature increases the relaxation of water moves to higher frequencies. It can be seen from Equation 2.2.3 that the time constant of the reorientational relaxation of water is inversely exponentially dependent on the temperature, resulting in large changes in the dielectric properties of water for small temperature changes.

As well as affecting the time constant through the disruption of intramolecular hydrogen bonds, the increase in kinetic energy at higher temperatures decreases the viscosity, η , of water, upon which the time constant is also dependent. A decrease in viscosity results in a decrease in the resistive force acting on the rotation of the molecules, which also decreases the time constant.

$$\tau = \frac{\pi \eta(T) r^3}{k_B T} \quad \text{Equation 2.2.4}$$

where r is the molecule's effective radius and η is the viscosity. The viscosity temperature dependence for common polar liquids is described using the Arrhenius relation:

$$\eta(T) = \eta_0 \exp \left[\frac{E_\eta}{k_b T} \right] \quad \text{Equation 2.2.5}$$

where E_η is the system activation energy. At low temperatures therefore, owing to high viscosity and low thermal energy of water molecules the relaxation time is high. As the temperature increases the relaxation time decreases due to both decreasing viscosity and increasing thermal motion of molecules. Close to freezing (0.2°C), the relaxation time of water molecules has been found to be 17.67 ps, while at 35 °C it decreases to less than 7 ps. Figure 2.2.4 illustrates these effects of temperature on the dielectric properties of water.

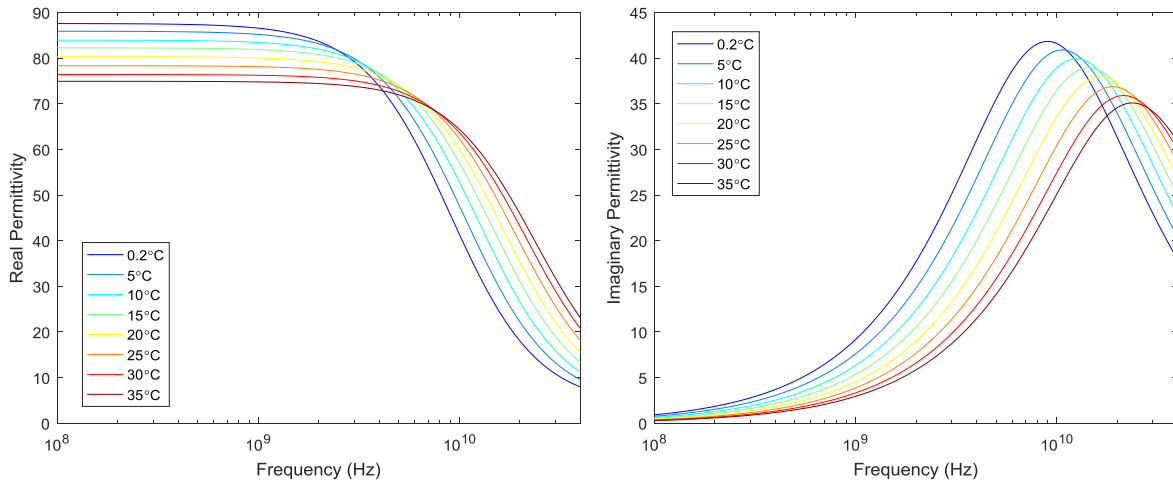


Figure 2.2.4: Debye modelling of the complex permittivity of pure water at temperatures varying from 0.2 to 35°C over a frequency range of 0 to 40 GHz using temperature dependent values of ϵ_s , ϵ_∞ and τ from Kaatz¹⁹.

2.3. INTERACTION OF MICROWAVE ELECTRIC FIELDS WITH BIOLOGICAL SOLUTIONS

2.3.1. IONIC CONDUCTIVITY/ELECTROLYTE DIFFUSION

When dealing with biological solutions, ionic interactions must be considered. Ions are commonly found in biological solutions such as blood, cell suspensions in medium and buffer solutions, as they stabilise proteins and are used by cells as molecular signals and to control osmosis. To ensure the survival of cells *ex-vivo* for any significant length of time, ions must be included in the suspension solution.

As charged particles, ions interact strongly with electric fields. EM radiation interacts with free-moving ions in solution via electrolyte conductivity, whereby the changing electric field drags the charged particles through the solution. The ions collide with other molecules as they move, dissipating their kinetic energy. The energy the electric field imparts to these charged particles by moving them around is lost as heat, and since there is no restoring force present, the ionic conductivity does not re-emit any energy and contributes only to losses in the system. The addition of ions to water therefore results in an increase in the imaginary part of the permittivity. In the real part of the permittivity an increase in ionic concentration causes a small decrease as the volume occupied by the added ions displaces water molecules. The ionic additions have a lower permittivity than water and therefore the static real permittivity is reduced to an extent proportional to the concentration. Due to the strong electrostatic fields surrounding ions, they attract and maintain a

shell of hydration water around them, which again results in an effective decrease in the volume of water interacting with the field, further decreasing the real part of the permittivity.

The contribution to the loss is frequency dependent. At lower frequencies ions travel longer distances and therefore undergo more collisions and dissipate more energy. At higher frequencies ions move shorter distances so collisions are less frequent and therefore the energy dissipated is less. This means that the ionic conductivity contribution to the permittivity of a biological solution drops off as the frequency is increased, falling with $1/\text{frequency}$. At high frequencies, as with other interactions, the changing field is fast compared to the amount of time needed for the ions to respond, and so the effect becomes negligible. At biological ionic concentrations ($\sim 8 \text{ g/L}$) this contribution to the losses becomes insignificant below 10 GHz, as can be seen in Figure 2.3.1, and is cited as negligible at frequencies over $\sim 40 \text{ GHz}$.²¹

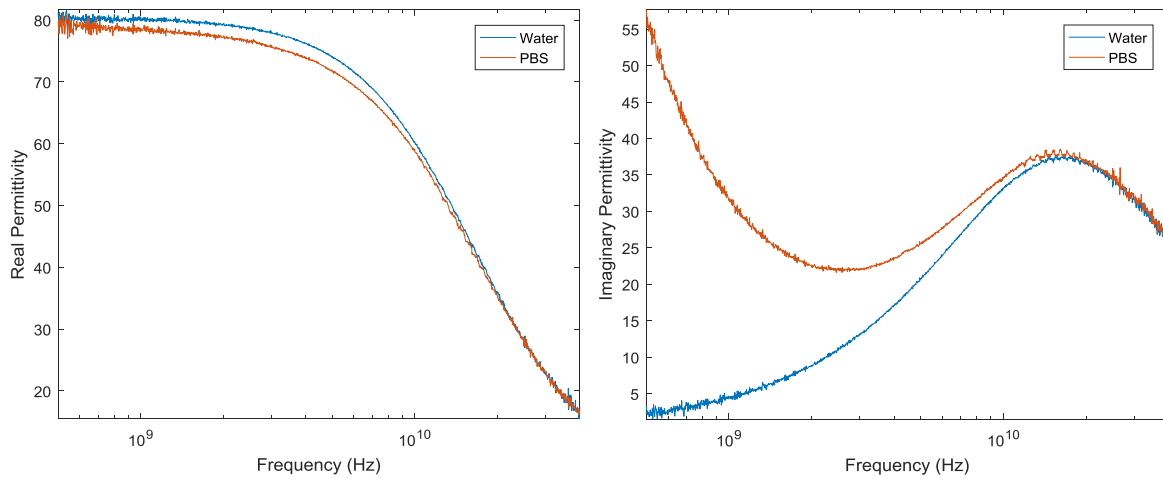


Figure 2.3.1: Comparison of dielectric spectrum of water and a salt solution (PBS). Experimental data measured using a broadband coaxial probe. PBS is a solution of various salts, mainly sodium chloride, in water at a total concentration of around 8g/L.

This effect of ions on imaginary permittivity can be modelled by adding a conductivity term to the Debye equation:

$$\varepsilon^*(\omega) = \varepsilon_{\infty} + \sum_{k=1}^N \frac{\varepsilon_k - \varepsilon_{k+1}}{1 + i\omega\tau_k} + \frac{i\sigma}{\omega\varepsilon_0} \quad \text{Equation 2.3.1}$$

where σ is the ionic conductivity in S/m and ε_0 is the permittivity of free space ($8.85 \times 10^{-12} \text{ F/m}$). The effect of temperature on the conductivity of a particular ion species is described by:

$$\sigma = \frac{qC}{6\pi r\eta} \quad \text{Equation 2.3.2}$$

where q is the charge of the ion, C is the concentration of the ions and r is the ionic radius. The viscosity, η , is temperature dependent, as in Equation 2.2.5. The effect of temperature on the conductivity depends on the hydrated ion radius and therefore on the type of ion present. As temperatures increase, the thermal motion of water molecules disrupts ion solvation shells, reducing the hydration radius of ions and therefore increasing their mobility through the solution. The decrease in viscosity at higher temperatures also increases conductivity by decreasing the resistance to movement of ions through solution. The increase in ionic conductivity causes an increase in ionic losses at low frequencies, as shown in Figure 2.3.2.

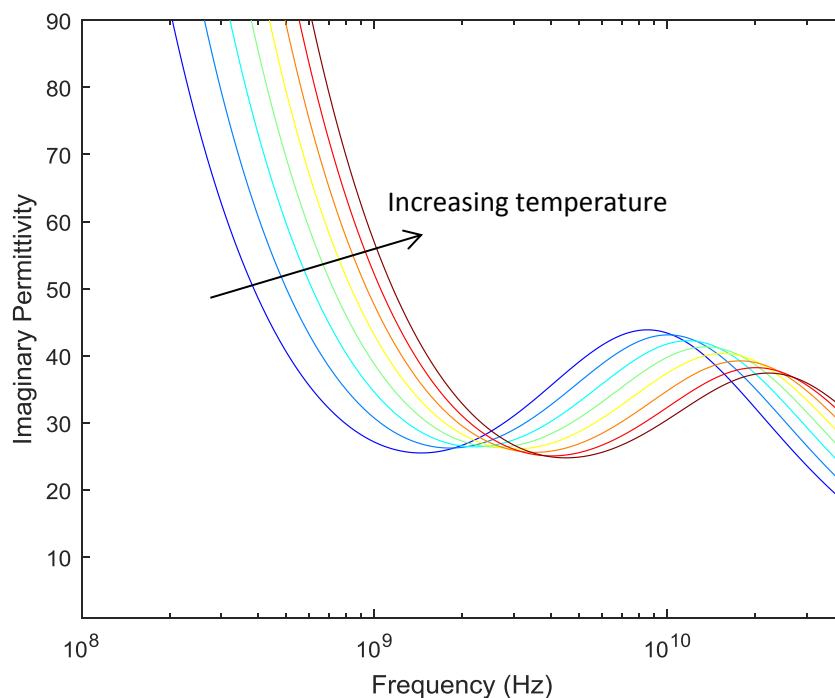


Figure 2.3.2: The effect of increasing temperature (blue to red, as indicated by the arrow) on the imaginary permittivity of an ionic solution, showing the increase in low frequency losses from ionic conductivity alongside the changes in relaxation time and permittivity. Values for conductivity in the model range from 1 to 3 Sm^{-1} as has been experimentally found to be the conductivity change for a 250 mM (14.5 g/L) NaCl in DI water solution over a temperature range of 6-66°C.²² A conductivity of around $1.4\text{-}1.8 \text{ Sm}^{-1}$ can be expected for phosphate buffered saline solution (generally 8 g/L NaCl with <2 g/L other salts including phosphates) according to the manufacturer.

2.3.2. EFFECT OF PROTEINS ON PERMITTIVITY

Proteins are common biological macromolecules consisting of peptide bonded amino acid chains folded into a particular three dimensional shape due to hydrogen bonding. They play important roles in many biological processes, carrying out a huge array of different functions, from metabolic reactions to DNA replication and transportation of smaller molecules. In biological systems such as cells, proteins exist in aqueous solution, so the effect of dissolved proteins on the permittivity of

water is important to understand when using dielectric spectroscopy as a characterisation technique.

The addition of proteins to water affects its dielectric spectrum. Proteins have a lower permittivity than water due to being large, organic, less (or non-) polar molecules. Proteins are made of amino acids, organic molecules which contain mainly carbon, hydrogen, nitrogen and oxygen. Their permittivity is low at high frequencies, generally less than 10, and is mainly due to electron polarizability and some flex in the structure of the protein, though their properties also depend on their environment (e.g. pH, hydration).²³ The low permittivity of organic molecules means that they do not strongly interact with GHz EM fields, and therefore the overall dielectric effect of increasing the concentration of proteins in water is to lower both the real and imaginary permittivity to a degree proportional to the volume of water they displace, due to an overall reduction in the interaction of the EM energy with the sample.

Adding constituents to water will also affect its viscosity, which will affect the characteristic relaxation time of the dipoles, as explained in Section 2.2.1. The relation between solute concentration and viscosity is given by:

$$\eta_r = 2.5k\Phi + 14.1k\Phi^2 \quad \text{Equation 2.3.3}$$

where Φ is the volume fraction of the solutes, k is a correctional term to account for shape of the molecules, and at low concentration ($\Phi < 0.02$) the relation is linear and the second term can be discounted.²⁴ As more salts and proteins are added to water the viscosity will increase, leading to an increase in the resistance to dipolar reorientation and a subsequent increase in the relaxation time. This shows on dielectric spectra as a shift of the relaxation towards lower frequencies, and the ratio of the change in static permittivity to the change in the relaxation time has been shown to be indicative of protein shape.²⁴

Globular proteins (approximately spherical) and disordered proteins (no fixed 3D structure), unlike membrane proteins or fibrous proteins, are free to move in solution and can contain polar groups and side chains. The reorientation relaxation of dipolar globular proteins occurs at low frequencies (a few MHz) since the size of these macromolecules hinders their movement. Smaller polar side chains of proteins (globular or disordered) can relax at higher frequencies and play a part in the microwave regime dielectric properties of a solution. Studies on protein solutions have shown a low frequency (< 100 MHz) dispersion caused by the reorientational motion of protein molecules commonly referred to as the β dispersion in protein solution dielectric spectroscopy.²⁵

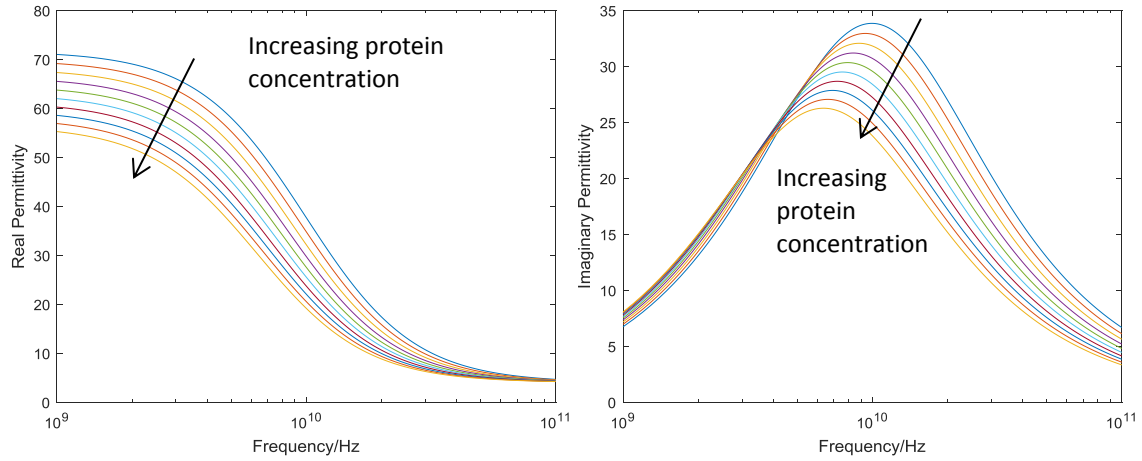


Figure 2.3.3: Schematic demonstrating the effect of increasing protein concentration on the permittivity of water.

2.3.2.1. MAXWELL-GARNETT THEORY

The effect protein concentration has on the permittivity of an aqueous solution can be modelled using a mixed media approach such as Maxwell-Garnett theory,²⁶ where the sample is assumed to be homogenous and a simple volume fraction occupation of the lower permittivity phase is used to predict the effect on the overall solution, known as the effective permittivity, ϵ_{eff} :

$$\epsilon_{eff} = \epsilon_m \frac{\epsilon_p(1 + 2\phi) + 2\epsilon_m(1 - \phi)}{\epsilon_p(1 - \phi) + \epsilon_m(2 + \phi)} \quad \text{Equation 2.3.4}$$

Where ϵ_m is the permittivity of the medium (in this case water), ϵ_p the permittivity of the other constituent dispersed in the medium and ϕ the volume fraction occupied by it. As the permittivity of water is well documented through large frequency and temperature ranges, modelling experimental data with this equation can be used to experimentally ascertain the permittivity of an inclusion (solute or suspended cells) in water even in low concentrations.²⁰

2.3.2.2. BOUND HYDRATION WATER

The surface of a protein molecule is complex, with hydrophobic areas, hydrophilic areas and unbound dipoles. These charges vary over the surface and interior of a protein, and can interact with water molecules due to their dipolar nature.

A large proportion of the surface of proteins is made up of amino acids with dangling dipole moments. These unbound charges aid in the process of folding to the correct conformation, but can also interact strongly with water molecules due to their dipolar nature. This is known as protein

hydration and is important in stabilising the 3D structure of proteins in solution. This interaction has a large effect on the mobility of water molecules interacting directly with the surface of the protein as well as the further surrounding water. Studies have shown that water up to 2nm away from the surface of a protein is affected by its charges.²⁷ The interaction depends on the position of the water molecule, as the charges vary widely over the surface and the interior of a protein

The water in a protein solution may therefore be split into two populations: bound (or hydration) water and bulk water. The interaction of polar water molecules with charged or polar functional groups, either on proteins inside cells or on the surface of cell membranes, leads to a modification of their orientational polarisation and a change in its relaxation time. Bound water exhibits a slightly higher orientational relaxation time (100s of MHz to <2.5 GHz),^{8,25,28,29} and the effects of bound water on the permittivity of protein solutions can be modelled by adding additional relaxation terms to the Debye equation, according to Equation 2.2.2. At high frequencies, bound water has a pronounced effect on the THz absorption of protein solutions, contributing to additional dispersions over 40 GHz.^{30,31}

2.3.3. PERMITTIVITY OF CELL SUSPENSIONS

The effect of cells on the permittivity and dielectric spectrum of water is frequency dependent. As well as electrolyte diffusion, protein effects, orientational polarisation of water molecules and the effect of water displacement by organic compounds, additional relaxation mechanisms arise from the complex physical structure of cells.

2.3.3.1. CELL MEMBRANE

All animal cells have a cell membrane constructed from a selectively permeable phospholipid bilayer (plasma membrane) with various organic biomolecules, such as proteins, carbohydrates and cholesterol embedded within it. The cell membrane contains many pores and ion channels which connect the extracellular liquid with the interior of the cell and control the concentration of ions and macromolecules through selective transport. The cell membrane also supports extracellular structures such as cell surface antigens. Some membrane inclusions or surface-bound molecules hold a charge. For instance sialic acid, which creates a negative cell surface charge. The bilayer is held together entirely by non-covalent interactions of the hydrophobic tails of the amphipathic lipid molecules and exists in a liquid crystalline state, whereby molecules can move almost freely across the membrane.

2.3.3.1.1. COUNTERION DIFFUSION/POLARISATION (α -DISPERSION)

Under normal biological conditions the charges present on a cell's surface are bound and stabilised by counterions in the surrounding solution. As well as charges due to negatively charged surface-bound molecules (e.g. sialic acid) the membrane potential of most animal cells contributes to strongly associated positive ions on the surface of a cell membrane.

Cells control their ion content via specific ion channels in order to stop uncontrolled osmosis into the cell due to the relatively high macromolecular content of the intracellular fluid. Cells expel sodium and chloride ions and take up potassium ions from the extracellular solution. The concentration control of different ion species inside and outside a cell leads to a membrane potential, with the intracellular side of the membrane slightly negatively charged. Positively charged free ions in the extracellular solution balance this charge and keep the voltage confined to the cell membrane.

The counterions present on the surface of a cell can be moved around by an external time-varying electric field. Upon removal of this field, counterion charges that have accumulated in particular areas on the surface of the cell membrane repel each other, resulting in a restoring force of the polarisation mechanism. This leads to an additional relaxation in the dielectric spectrum of cell suspensions, which is generally referred to as counterion diffusion, or α in the literature.^{28,32,33} As with all other polarisation mechanisms, there is a time constant associated with the movement of these ions. As they are strongly electrostatically bound to the surface of the cell membrane they are not completely unhindered, and as the frequency of the changing field increases above ~ 10 kHz they are unable to keep up. The speed of counterion diffusion can give information about the physical structure of the cell membrane, such as roughness, which can be characteristic of different cell types.³⁴

2.3.3.1.2. INTERFACIAL/MAXWELL-WAGNER/LOOYENGA POLARISATION (β -DISPERSION)

The membrane of a cell is an interface that restricts the free movement of ions. It also controls the movement of charged species and proteins, maintaining a specific chemical composition inside the cell that differs from the extracellular medium. This results in a system where two liquids with different permittivities and conductivities are separated by a non-conducting interface, causing an additional relaxation similar to space-charge polarisation described in Section 2.1, known as Maxwell-Wagner, Looyenga or interfacial polarisation.

Maxwell-Wagner polarisation occurs in inhomogeneous or layered dielectric materials and arises due to a separation of charge across a poorly conducting interface separating areas of different permittivity.^{28,35,36} In the case of cells, cell membranes separate the extracellular solution from the cytoplasm inside; these have different ion concentrations and viscosity and therefore different dielectric properties. In the presence of an electric field, at frequencies in excess of 10 kHz, free charged particles both inside and outside the cell are pulled through solution. When they come into contact with the cell membrane, the otherwise free moving particles are obstructed and accumulate at these boundaries, causing a strong capacitance to build across the cell membrane and a high degree of polarisation across the cell. This effectively blocks the EM radiation from interacting with the cell interior. The high polarisation results in a high real permittivity and an increase in the imaginary permittivity due to the movement of ions through the solution. The charging of the membranes has a time constant associated with it and therefore results in an additional relaxation in the real permittivity and a slight increase in the losses, the magnitude of which decreases with 1/frequency, as in electrolyte conductivity. For ion concentrations typical in biological solutions this dominates in the single MHz frequency range, becoming negligible at frequencies higher than ~2GHz.²¹ In the literature this effect is referred to as the β dispersion and tends to be significant at around 0.1 – 10MHz.²⁸ This interface polarisation can be a useful property, as with a suitable dielectric model and measurements dielectric parameters of cell structural parts can be determined at low frequency, such as membrane capacitance and the conductivity and permittivity of inter- and extracellular solutions.^{34,37,38} This polarisation can be modelled as an intrinsic relaxation using the Debye equation discussed previously.³⁶

The exact frequency of the interfacial polarisation relaxation depends on the electrolyte concentrations, and at the levels found in blood commonly occurs in the single MHz frequencies, becoming negligible at ~2 GHz.²¹

2.1.1.1.1. SUMMARY

The cell membrane causes two important relaxation mechanisms below 2 GHz known in the literature as α and β dispersions. Both counterion diffusion (α -dispersion) and interfacial polarisation (β -dispersion) result in screening of the cell interior from the external electric field. The polarisation resulting from trapped charges either at the interface of the cell membrane or within the cell membrane opposes the external field and screens the interior of the cell from the EM energy. This means that in this frequency regime the information gained from dielectric spectroscopy relates only to the extracellular contents and the cell membrane. Measurements in this frequency regime can give information about extracellular medium conductivity and permittivity, cell membrane

capacitance and physical characteristics such as membrane inclusions and texture, but little interaction with cell contents occurs. These parameters have been explored using lower frequency microwave dielectric techniques, and shown to be of diagnostic value, and can be indicative of cell type,³⁴ as well as disease state, which will be discussed in Chapter 5.

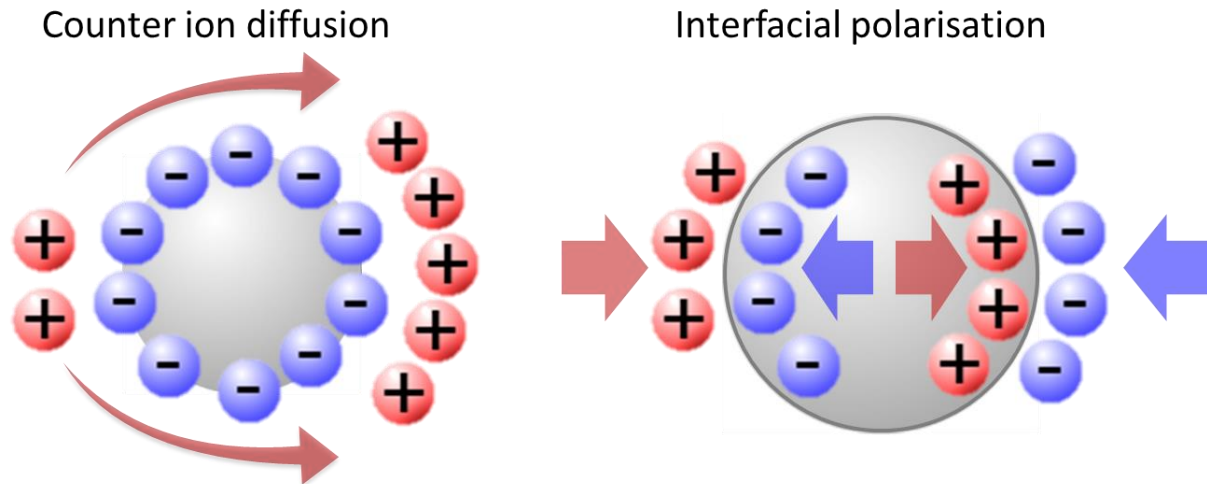


Figure 2.3.4: Schematic of the counterion diffusion and interfacial polarisation that cause the α and β dispersions, respectively.

2.3.3.2. CELL INTERIOR

At frequencies higher than 3 GHz the dielectric contributions from counterion diffusion and interfacial polarisation have dropped off significantly. The polarisation mechanisms cannot keep up with the changing field and the screening field does not form across the cell. EM energy is able to penetrate inside the cell and interact with its contents. The main constituent of cells is water, so at frequencies between 3 and 20 GHz the main contributing mechanism to the dielectric constant of cell suspensions is the orientational polarisation of water molecules, as discussed in section 2.2.

2.3.3.2.1. RED BLOOD CELLS (RBCs)

Red blood cells differ from most other cells in the human body, in that they do not have a nucleus. A mammalian RBC consists of a cell membrane surrounding densely packed haemoglobin, to ensure the maximum amount of oxygen can be transported by each cell. The water content of red blood cells is in general lower than the water content of other cells, around 65%.³⁹ Haemoglobin is a large protein with a low permittivity (*ca.* 10)⁴⁰, therefore it contributes mainly to lowering the real part of permittivity via displacement of water, as shown in Figure 2.3.5.

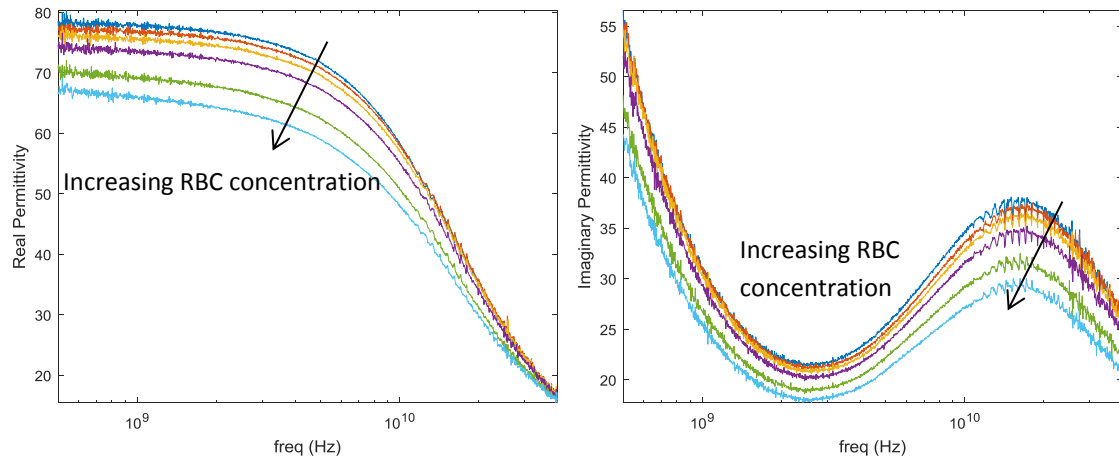


Figure 2.3.5: Dielectric probe measurements of blood cell solutions ranging in concentration from approximately 500 MHz to 40 GHz. The region under 500 MHz is not measured by the permittivity probe used, so that the beta dispersion (significant <10 MHz) is not seen.

The cell membrane of RBCs contributes to interfacial polarisation, though whole blood and RBC suspensions do not exhibit an alpha dispersion.⁸ This could be due to the non-spherical shape of RBCs, or the fact that the membrane does not carry such a strong charge (there are no ion channels for osmosis), or it could simply be weak and drowned out by the larger effect of electrode polarisation at low frequencies.⁴¹

2.3.3.2.2. NUCLEATED CELLS (WHITE BLOOD CELLS (WBCs)/CIRCULATING CELLS/TUMOUR CELLS)

Most cells in the human body have a more complex composition and contain a membrane-enclosed nucleus as well as other complex intracellular structures such as granules, vesicles, endoplasmic reticulum, mitochondria and Golgi apparatus. Even within cell populations there is a large degree of heterogeneity and different cell types have significantly different physical properties as well as contents. Different cell types are considered in this thesis so the general properties of nucleated cells will be discussed here.

The material inside the cell, excluding the nucleus, is known as the cytoplasm. As well as the protein and ion packed solution known as the cytosol, the cytoplasm also encompasses the organelles.

The cytosol is densely packed with macromolecules and certain ions, with protein concentrations of up to 30 g/100mL in the cytoplasm.⁴² The ionic concentration of the cytosol is heavily controlled by ion channels in the cell membrane, with a much higher concentration of potassium and charged macromolecules (amino acids), but lower concentrations of sodium, chloride and potassium than in plasma. Proteins carry out important functions inside a cell, from RNA transcription to molecular

transport. Most proteins are freely moving within the cytosol, whereas some proteins are not fully solvated and are bound to or weakly associated with the cell membrane or the intracellular membranes of organelles. The high concentration of large molecules in the cytosol leads to macromolecular crowding, an important phenomenon that enables the chemical processes of living cells.

Anchored to the cell membrane is the intracellular cytoskeleton, which gives the cell its physical structure and mechanical properties. It is made of filaments of fibrous actin proteins which hold the cell up like scaffolding. The cytoskeleton is a complex and diverse structure and is involved in a wide variety of cell processes, but for the purposes of this work it can be understood simply as a cell's rigid protein skeleton, contributing to the amount of organic, non-polar cell contents.

Organelles are specialised sub-cellular units, usually enclosed within their own separate lipid bilayer, which carry out a specific function. Most animal cells contain mitochondria, vacuoles and of course a nucleus. In addition some circulating white blood cells contain granules and special vesicles for phagocytosis. It has been suggested that at lower frequencies (10-100 MHz) interfacial polarisation of various organelles and nuclear membrane mechanisms may contribute to a cytoplasm permittivity higher than that of pure water.⁴³

The nucleus of the cell is the largest structure within animal cells, occupying approximately 10% of the cell volume on average, with the nuclei of some leukocytes making up to 65% of the cell volume.⁴⁴ The nucleus consists of a membrane encasing the nucleoplasm, which is similar in composition to the cytosol and consists of the liquid nucleosol along with sub-nuclear bodies such as DNA and the nucleolus.

The nuclear membrane is similar to the cell membrane, consisting of a lipid bilayer, with proteins embedded within it and small holes known as nuclear pores for the transport of particles in and out of the nucleus. The outer membrane of the nucleus also contains proteins connected to the cytoskeleton, which gives the nucleus structure, maintains its position and provides the basis for the cells mechanosensory function. Unlike the cell membrane, the nuclear membrane is studded with ribosomes, the molecular machines that read RNA and synthesise proteins accordingly. The membrane of a nucleus gives rise to interfacial polarisation and for this reason the permittivity of nucleated cells is best modelled using a double shell model to take into account the nucleoplasm and nuclear membrane.⁴⁵ The double shell model approximates to an additional Debye-type Maxwell-Wagner dispersion to the dielectric spectrum of a nucleated cell compared to that of a non-nucleated cell. These differences are only apparent at lower frequencies (<1 GHz).^{46,47}

The most well-known constituent of the nucleus is the cell's DNA. If the DNA in a mammalian cell was stretched out and made linear it would be almost 2 meters long, yet it is only ~ 20 Å wide, leaving plenty of room for water to ensure it is highly hydrated.⁴⁸ Most of the time, the DNA of a cell is stored as chromatin, a structure made up of building blocks known as nucleosomes in which segments of DNA are wrapped around histone proteins, (though there is recent evidence to contradict the higher order chromatin structure).⁴⁹ DNA has been demonstrated to exhibit a dispersion below 1 GHz due to various rotational and vibrational motions when in solution,⁵⁰ but when in a complexed state the electrostatic binding between DNA and histones results in a low permittivity, meaning that DNA mainly contributes to the amount of "non-water" present in our samples.⁵¹ The highly negatively charged nature of the phosphate backbone of DNA results in screening by counterions, and a high degree of solvation and therefore bound water, which may contribute to additional relaxations in the low frequency regime as well as absorptions in the THz regime, similarly to protein-bound water. The nucleolus is the largest sub-nuclear body, and forms around specific regions of the chromosome for the purpose of ribosome biogenesis. This structure therefore consists of DNA, RNA and proteins and has no membrane. The nucleolus does not experience interfacial polarisation and the contribution to the higher frequency permittivity will also essentially be the exclusion of water.

There is not much in the literature on the relative water content of the nucleus and cytoplasm of mammalian cells, but for amphibian oocytes it has been shown by desiccation and NMR that the nuclei of such cells contain more water, on average than the cytoplasm.^{52–54} The higher percentage of water in the nuclei compared to the cytoplasm may be due to the hydration of DNA and its tight packing. As will be discussed in Chapter 4, the increase in hydration of malignant cells is seen alongside an increase in overall cell size as well as nuclear size due to an increase in the amount of redundant DNA and an increased rate of cell division. The nuclear to cytoplasmic ratio of cells is therefore used as a diagnostic marker and is indicative of pathological state.⁵⁵

Although the nucleus and sub-nuclear bodies may have low frequency dielectric dispersion mechanisms, these will be small in comparison to other low frequency effects, and are not well understood as yet. At the higher frequencies employed during the work in this thesis, it is the water content of the cell and its nucleus that will be the most significant determinant of cell permittivity.

2.4. SUMMARY

The dielectric spectrum of cell suspensions is affected by dissolved inclusions such as ions and proteins, as well as cellular structures such as the cell membrane and nuclear membrane. In

summary, when salt and proteins are added to pure water three effects are observed: an increase in ϵ'' with a $1/f$ dependence; a decrease in the real static permittivity; and a decrease in the characteristic relaxation time of dipolar reorientation.

When dealing with complex systems such as cell suspensions in water the characteristic frequency dependence of the dielectric spectrum can give us a lot of information about the composition and physical structure of the cells as well as their environment.

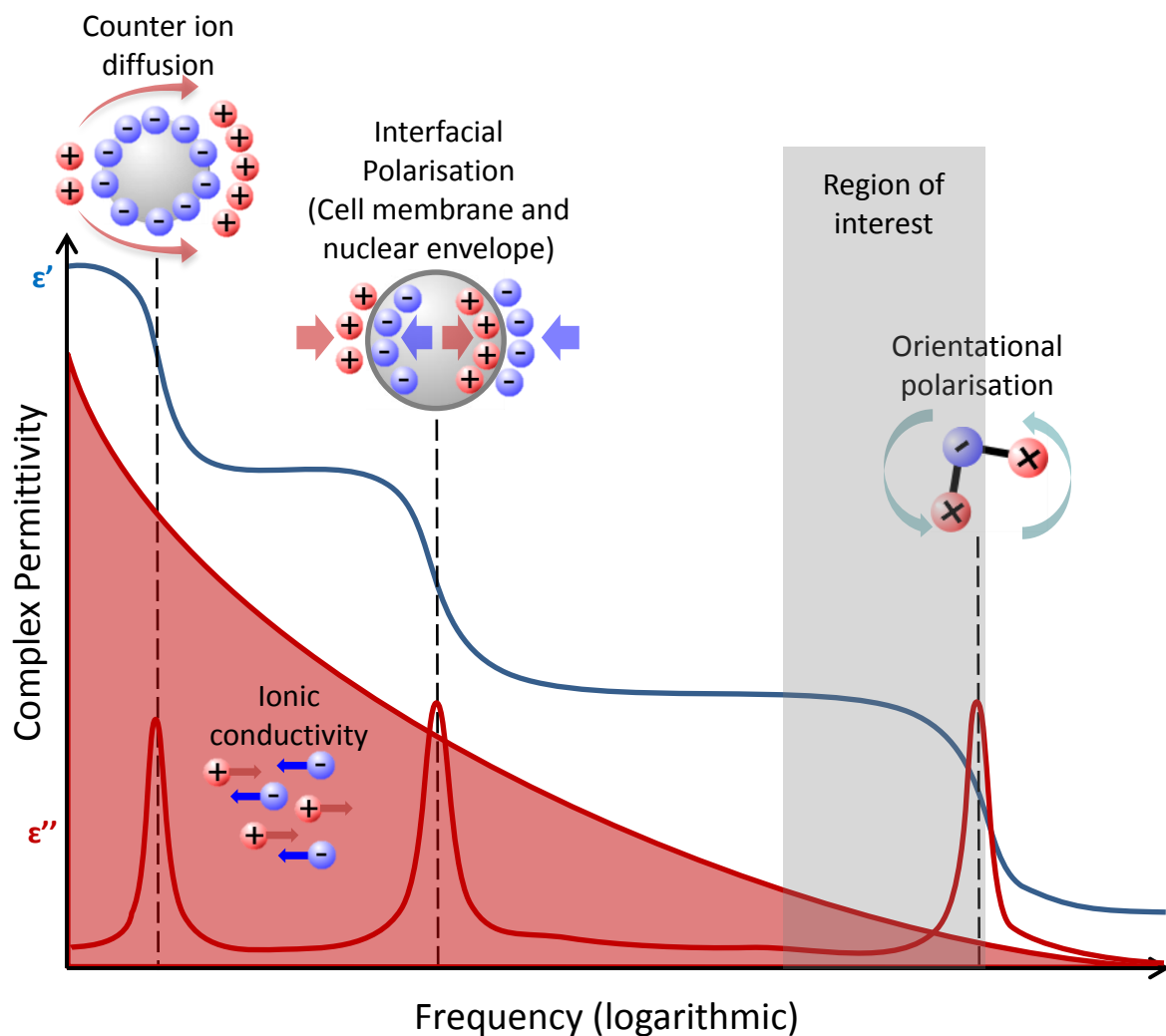


Figure 2.4.1: Schematic (not to scale) showing the frequency dependence of both the real (ϵ' , in blue) and imaginary (ϵ'' , in red) permittivity of a cell suspension, showing the contributions from counter ion diffusion (α), interfacial polarisation (β) and water orientational polarisation (γ) as well as the contribution to the imaginary part from ionic conductivity (red area). In reality the plateau from interfacial polarisation is orders of magnitude larger than the reorientational polarisation.

In the RF region of the dielectric spectrum the response of cell suspensions is largely due to effects of ions and cell membranes, with high permittivities due to counterion diffusion and interfacial

polarisation, as well as high losses owing to ion conductivity. Probing cells at in this frequency range provides information about the structure of the membrane, such as its capacitance, conductivity and permittivity, which can be related to physical properties such as cell membrane area, thickness and degree of folding, as will be discussed in Chapter 5. Analytical modelling of such dispersions is best done with a double-shell model that takes into account the deviations from the single shell model arising from the small influence of the nuclear envelope.⁵⁶ Proteins, DNA and bound water may contribute to dispersions at frequencies less than 3 GHz but these are small in comparison to the membrane effects of cells.

However, at frequencies above 3 GHz the contribution from the cell and nuclear membranes to the dielectric properties becomes less and less significant and the response of a cell suspension system is therefore dominated by the interaction between the EM energy and water molecules. By probing samples at this frequency we can determine physical differences between different cell types based on water content, since different cellular constituents have different chemical compositions. At frequencies around 10 GHz, as discussed in this thesis, the lipids of cell membranes and the hydrocarbons of proteins and DNA contribute to the permittivity of cells via the exclusion of water molecules, decreasing both the real and imaginary parts of the permittivity.

Above 15 GHz, due to the frequency dependence of the relaxation mechanism, the permittivity contrast between water and organic molecules decreases, making the frequency window between 3 GHz and 15 GHz ideal for studying the water content of cell suspensions, due to minimal electrolyte losses and membrane effects as well as maximum permittivity contrast.

3. MICROWAVE DIELECTRIC MEASUREMENT TECHNIQUES

Experiments conducted in this thesis rely on accurate broadband and narrowband dielectric measurements using advanced microwave equipment and techniques. In this chapter, the basic operation principles and theory behind some of these are described.

3.1. VECTOR NETWORK ANALYSER (VNA)

The standard instrument used for microwave characterisation is the Vector Network Analyser (VNA). This acts as a source, detector, processor and display, giving signal phase and amplitude information over a wide frequency range. The VNA is able to monitor both amplitude and phase information of signals generated and received. Working in a two-port configuration it is able to measure the reflected or transmitted signals, with either port able to act as an output or detector. Both experimental set-ups are employed in this thesis. Figure 3.1.1 shows a schematic explaining the measurement configurations that are possible using a two-port set-up.

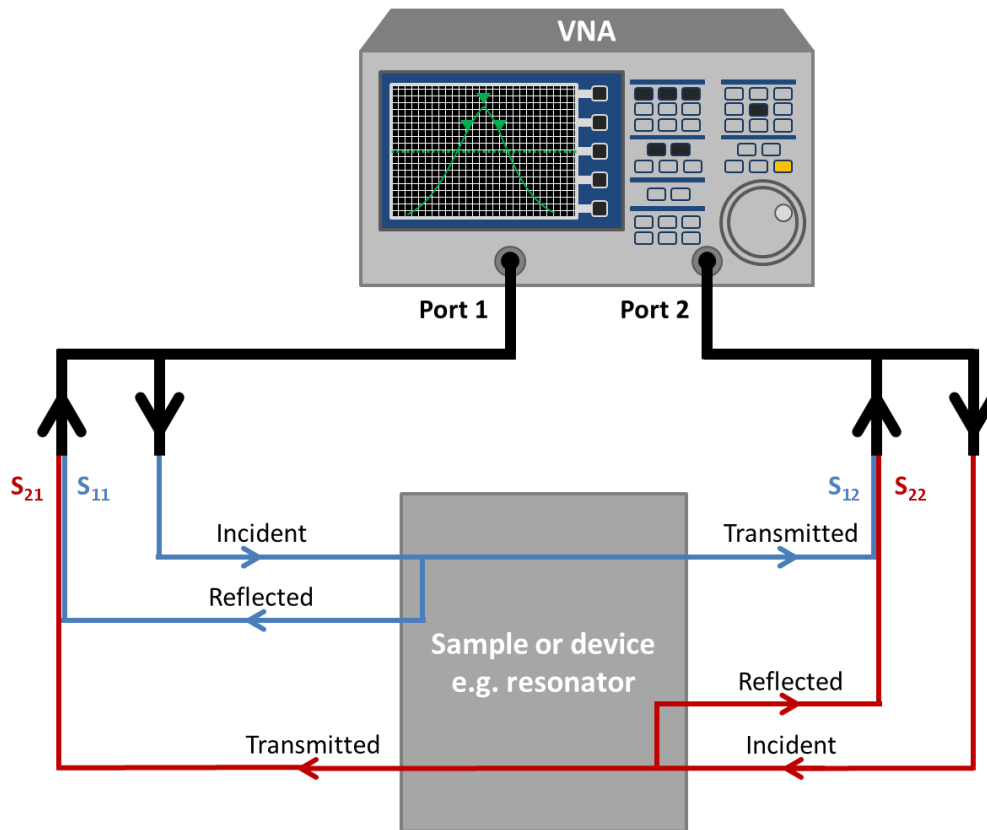


Figure 3.1.1: Schematic of the two-port VNA measurement set-up showing the S parameters that can be measured. Blue lines and arrows indicate port one being used as the signal source and port two as the detector, while red indicates the opposite. The S parameters are shown in the same colour and positioned next to the lines representing them.

The VNA measures the S-parameters, where S_{11} and S_{21} are defined as a function of the voltage of the incident and reflected or transmitted signals:

$$S_{11} = \frac{\textit{Reflected}}{\textit{Incident}} \quad \text{Equation 3.1.1}$$

$$S_{21} = \frac{\textit{Transmitted}}{\textit{Incident}} \quad \text{Equation 3.1.2}$$

The S-parameters are complex values, with the real part directly related to the magnitude and the imaginary to the phase of the incident and reflected or transmitted signals. The values of S_{11} or S_{21} are therefore used to calculate the values of amplitude and phase. This can be done by the VNA as measurements are taken or the VNA can be connected to a computer to collect the raw S parameters directly to speed up data acquisition.

A VNA provides a continuous wave (CW) signal, and can be used either at a single frequency or in sweep mode where it steps through a range of frequencies making measurements at each frequency point. Continuous wave measurements generate outputs of amplitude or phase as a function of time, whereas swept measurements produce spectra of amplitude or phase as a function of applied frequency.

In this thesis two VNAs are used, an HP 8510 VNA with a frequency range of 50 MHz to 40 GHz for reflection measurements using a coaxial probe (see Section 3.2), and a Rohde & Schwarz ZVA 24 VNA with a frequency range of 10 MHz to 24 GHz for transmission measurements using a dielectric resonator.

3.2. COAXIAL PROBE

A coaxial cable is a common type of radio - microwave frequency transmission line. Above a few tens of kHz the length of cabling can exceed the wavelength of the signal frequency and ordinary electrical wires used to carry low frequency mains power become excessively inefficient for transmission, due to radiation of energy and reflection at connectors due to impedance mismatching.

A coaxial cable consists of a cylindrical inner conductor separated from a hollow cylindrical outer conductor (usually both copper) by an insulating dielectric material (e.g. PTFE). Signals propagate via an alternating electromagnetic field that is confined to the dielectric between the two conductors. The precise spacing of the inner and outer conductors, as well as the dielectric constant of the insulator determines the impedance of the coaxial cable.

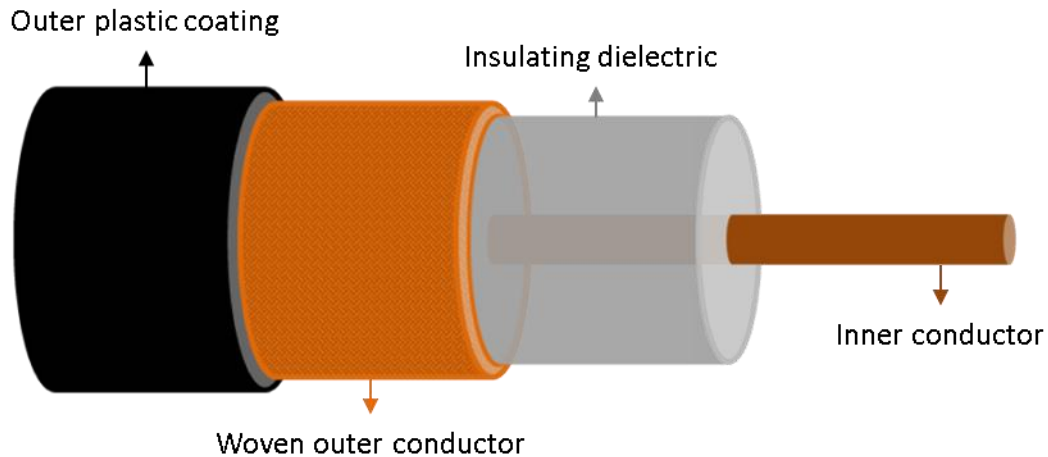


Figure 3.2.1: Schematic of a coaxial cable showing each layer.

A coaxial probe is a measurement device that uses coaxial transmission line theory to bring electromagnetic fields into contact with samples. The probe consists of a cut-off coaxial cable, with the termination surface exposing concentric circles of outer conductor, dielectric and inner conductor. At the end of the probe, the electromagnetic field between the inner and outer conductor leaks into the surroundings and is known as a fringing field. This fringing field is very sensitive to the dielectric properties of the material it occupies, and therefore bringing the tip of the probe into close contact with samples causes it to perturb significantly. This perturbation has a knock-on effect on the amplitude and phase of the signal reflecting back through the coaxial cable. These changes are measured using a VNA and, with the correct calibration procedure, can be used to calculate the complex permittivity of the material being measured.

The TEM (transverse electromagnetic) mode, consisting of a radial electric field distribution between the inner and outer conductor, can be excited at a range of frequencies. Higher order modes are also possible, so in order to prevent unwanted interference a coaxial cable of certain dimensions and dielectric material must be chosen to ensure that the onset of the second mode will not be excited at the frequencies used.

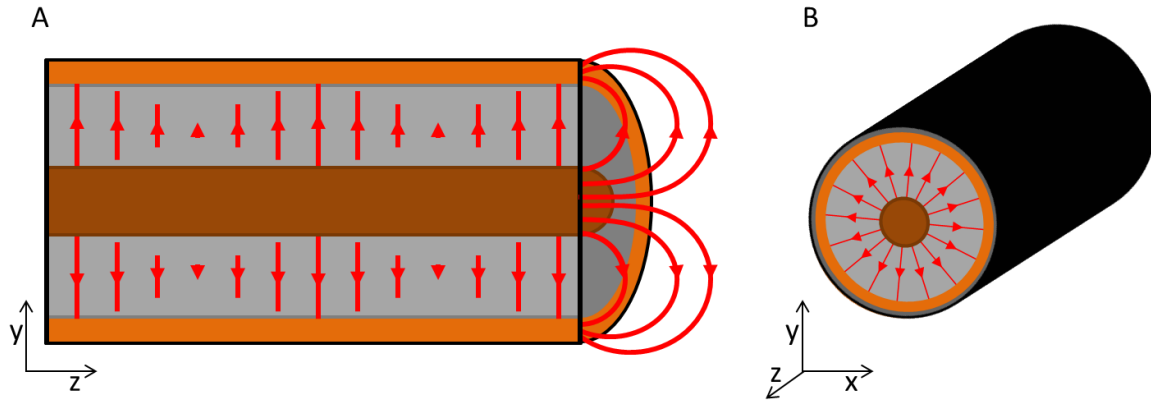


Figure 3.2.2: Schematic showing the TEM mode of a coaxial probe. A cut plane in the x-direction showing the propagation of the electric field between the inner and outer conductor, and the fringing field at the termination of the probe (A). A cut through in the z-direction showing the radial distribution of the electric field between the inner and outer conductor (B).

In order to be able to map changes in the signal due to the interaction of the fringing field with the sample back to absolute values of complex permittivity of the material under test, the coaxial probe needs to be calibrated. This process also allows the software to account for the length of the cable the probe is attached to and removes effects from any additional reflections from this connection or any defects.

The calibration standard used for measurements in this thesis is the air-short-water calibration. This is an appropriate calibration technique given that in this work the probe is used to measure polar liquids (water-based cell suspensions). First the coaxial probe is left open to air and the reflection parameters are measured over the frequency range being used. Since air represents a sample with negligible permittivity and high impedance, the reflected wave should have zero phase change with respect to the incident wave. Second, a short circuit is created between the outer and inner conductors at the probe surface, using either metal foil or a shorting block provided with the probe. In this condition the reflected wave should leave the interface of the probe and the short with a 180° phase shift. Lastly the probe tip is immersed in deionised water ($18 \text{ M}\Omega$) of a known temperature. Since the complex permittivity of water is well known over a large frequency range, differences in the measured and expected values can be corrected for. The software supplied with the dielectric probe corrects measurements using the differences in the measured and expected values for these three conditions.

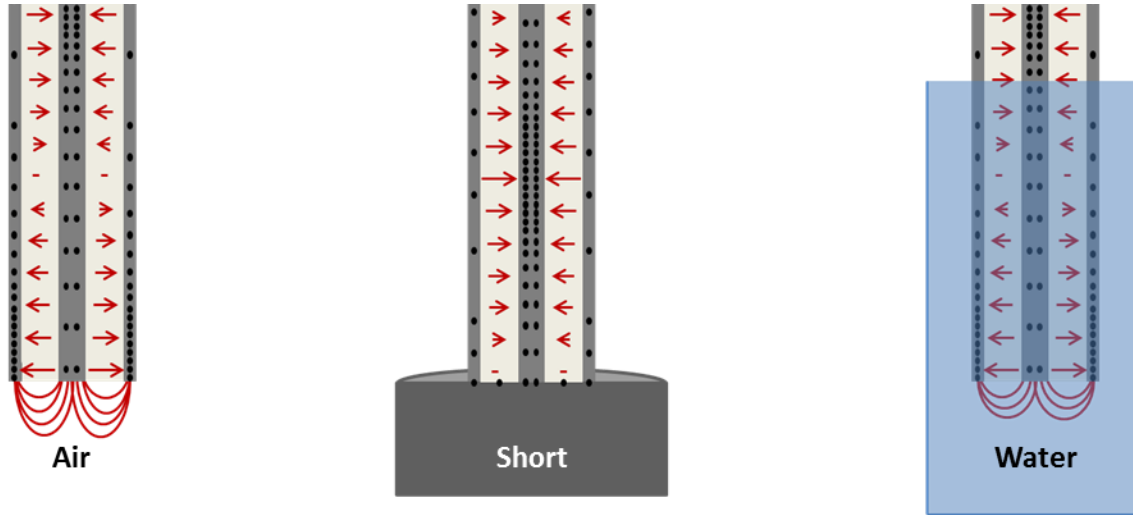


Figure 3.2.3: Schematic of the air-short-water calibration technique for a coaxial dielectric probe.

In this thesis, broadband measurements made by coaxial probe are conducted using the Agilent performance probe kit. This probe has a quoted accuracy of $\Delta\epsilon' = \Delta\epsilon'' = 0.05|\epsilon^*|$, which can be considered valid for polar and semi polar liquids.

3.3. DIELECTRIC RESONATORS

The boundary between a dielectric and air can form the basis of an electromagnetic resonator. The large difference in impedance between a high permittivity dielectric and a low permittivity environment results in efficient reflection of the fields, which along with the low losses of certain dielectrics, enables high field confinement. Resonant modes occur at frequencies where the confined fields are able to constructively interfere and cause synchronised polarisation across the material. The shape and size of the dielectric therefore govern the properties of the resonator, determining the possible resonant modes and their frequencies.

Although dielectrics are able to confine EM fields within them in this way, at the boundary between the dielectric and air the fields are non-zero. At the surface of the dielectric resonator the induced dipoles result in an electric charge on the surface of the material. This charged surface sets up an electric field as shown in Figure 3.3.1. This field is known as an evanescent field and its strength decays exponentially with distance from the surface of the dielectric, making it highly spatially confined, resulting in the surface of a resonator being very sensitive to its environment. Introducing a sample into the volume close to the surface of a resonator alters the permittivity of the material at the surface, which affects the reflection of EM fields at the boundary. This has the knock-on effect of changing the resonance properties of the dielectric resonator. Monitoring the changes in the resonance can give information about the permittivity of the material, and can form the basis of

sensing and analysis techniques.^{21,22,24,57,58} Different areas of the resonator will be more or less sensitive depending on the mode, with high field regions being the most sensitive and the distribution of high field regions changing between different modes. Some energy of a resonant mode is also lost to a radiative component of the mode. Different modes have different field distributions, and consequently different resonance properties, with different ratios of confined, radiative and evanescent fields.

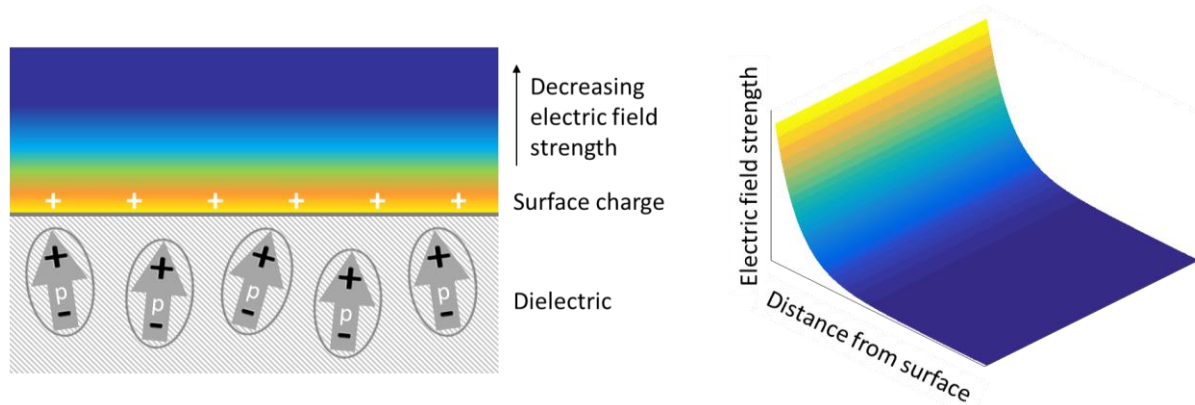


Figure 3.3.1: Left: schematic of the surface charge in a polarised dielectric and the resultant field outside the dielectric that decays with distance away from the surface. Right: surface plot of the strength of the field as a function of distance from the surface of the resonator.

3.3.1. MODES OF CYLINDRICAL RESONATORS

A resonator of a given geometry and material will exhibit an infinite number of constructive field interference patterns within the resonator and therefore multiple resonance modes at discrete, stepped frequencies, each with a characteristic field distribution. Modes are named in terms of the distribution of the electric and magnetic fields along certain axes. Cylindrical dielectric resonators can support a family of modes:

- **Transverse electric** (TE_{mnp}), in which the electric field has no component travelling in the vertical plane. This is analogous to the coaxial transmission line case, where the electric field in the TE mode is perpendicular to the direction of propagation in the cable.
- **Transverse magnetic** (TM_{mnp}) which are analogous to transverse electric modes, but in which there is no component of the magnetic field along the vertical plane
- **Hybrid** modes (EH_{mnp} or HE_{mnp}) in which there are components of both magnetic and electric fields in all planes. These are named either EH_{mnp} or HE_{mnp} depending on whether the electric or magnetic field, respectively, is dominant in the direction of propagation.

The subscript numbers m , n and p denote the azimuthal, radial, and axial mode numbers respectively, i.e. the number of nodes contained within the resonator in the radial, azimuthal and axial directions. In the case of a dielectric resonator in a cavity, boundary conditions dictate that only TE modes with azimuthal symmetry ($m = 0$, i.e. $TE_{0,n,p+\delta}$) and hybrid modes exist.

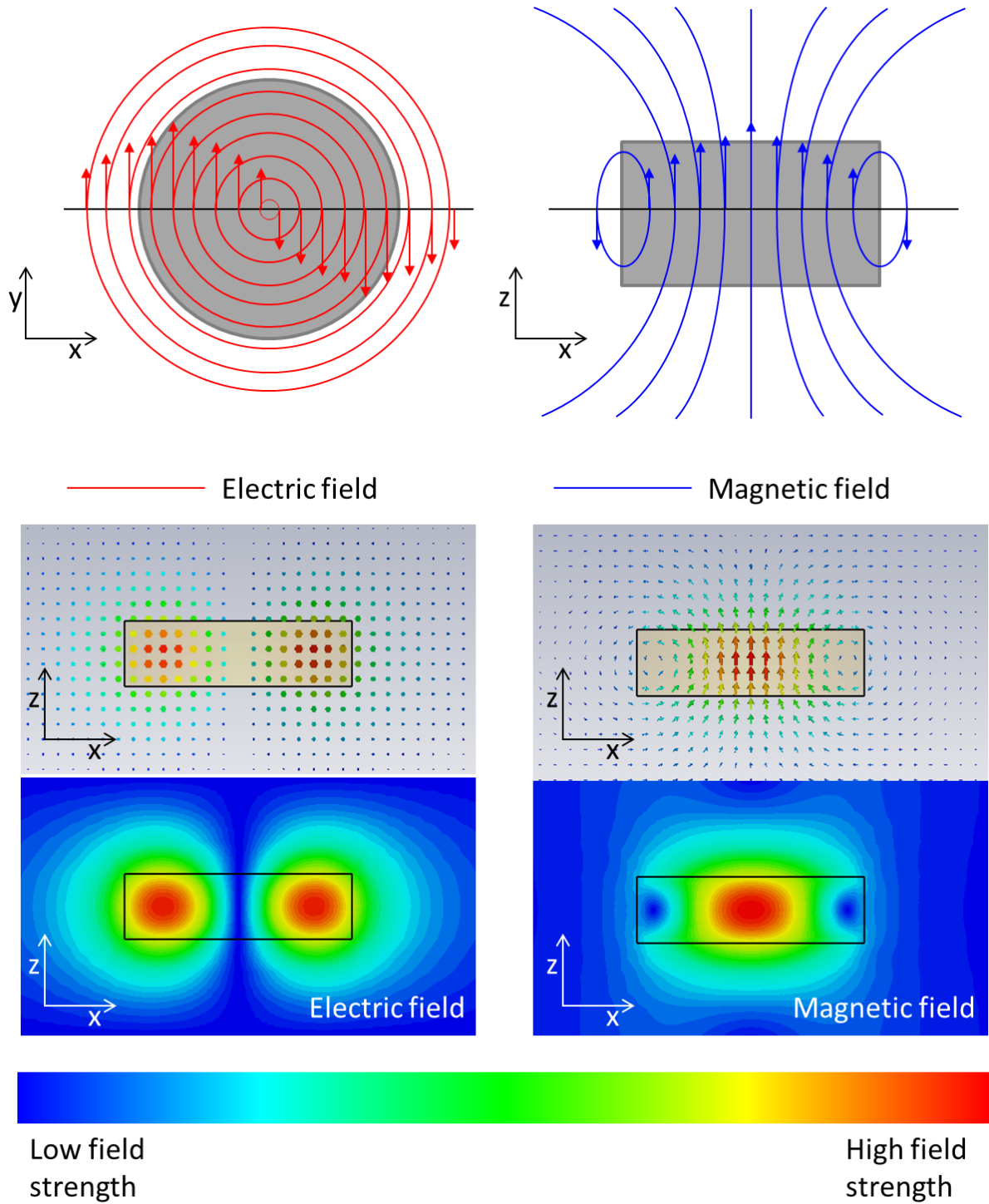


Figure 3.3.2: Left: schematics of the distribution of electric and magnetic fields in the TE_{016} resonant mode of a cylindrical dielectric resonator. The electric field (left) is shown as viewed from above the resonator, while the magnetic field (right) is viewed at a cross section down the middle of the resonator. Arrows denote field lines, with stronger fields illustrated using longer arrows. Right: CST Microwave Studio simulations of the TE_{016} mode of a resonator used for measurements, showing the electric (left) and magnetic (right) field distributions at a cross section down the middle of the resonator. Arrows denote field lines, with stronger fields illustrated with larger arrows, while colour plots denote the absolute strength of the field.

For all measurements in this thesis resonators are operated in the fundamental mode for cylindrical dielectric resonators: the TE_{016} mode. The TE_{016} mode consists of a circularly symmetric electric and

magnetic field, with the magnetic field forming circular loops which pass through the dielectric and a circularly propagating electric field, as shown in Figure 3.3.2. The $TE_{01\delta}$ mode therefore goes through no variations in phase along the radial direction and one change in the azimuthal direction. The symbol δ is used to denote the fact that the axial length of the resonator is smaller than a half-wavelength of a propagating EM wave of the same frequency so that the fields are non-zero at the vertical boundaries. For higher order modes the axial wavenumber is quoted as integers + δ .

Although the electric field is predominantly confined to the resonator in this mode, there are significant evanescent and radiative components. In order to remove the radiative component of the resonant mode, resonators used in this thesis are surrounded with a copper housing. This maximises the energy stored in the resonator, by reflecting radiated energy back to the resonator, and produces higher quality factor resonances. As can be seen from the simulations in Figure 3.3.2, the $TE_{01\delta}$ mode has a significant field component in the air around the resonator. Higher order modes, such as whispering gallery modes, have greater confinement of the electric field within the resonator and lesser radiative components, resulting in higher quality factors. The quality factor of the resonance therefore depends on the material of the resonator and its surroundings as well as the mode excited. The resonance frequency depends on the geometry of the resonator and the mode.

3.3.2. QUALITY FACTOR

An important metric for evaluating a resonator or particular resonant mode is the quality factor, Q , which is a dimensionless value representing the ratio of energy stored in the system to energy lost per cycle. A higher value of Q therefore denotes a resonator with lower losses. When viewing a resonant curve of amplitude as a function of frequency, a higher Q resonance appears as a thinner, sharper peak. For resonances in which the width of the resonance curve at the half-peak value (full-width-half-maximum or FWHM, shown in Figure 3.3.3) is small compared to the resonance frequency, f_0 , Q can be approximated as:

$$Q = \frac{\omega W}{P} = \frac{f_0}{FWHM} \quad \text{Equation 3.3.1}$$

where ω is the frequency, W is the energy stored in the resonator, and P is the total power loss. A lower quality factor therefore denotes higher losses and resonators with low quality factors have higher FWHM values, producing flattened, more spread out resonance peaks. Low quality factor resonators make measurement of the resonance frequency less precise.

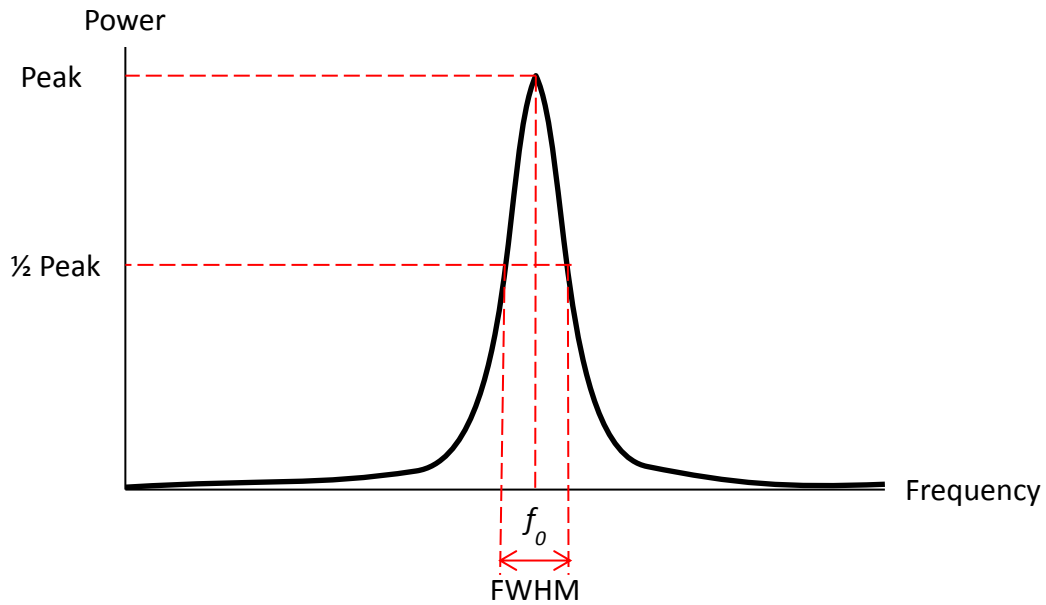


Figure 3.3.3: Schematic of the resonance parameter as measured by the VNA as a function of frequency showing a resonance curve with the peak frequency, f_o , and full width at half maximum (FWHM) values displayed.

There are three contributions to the losses of a dielectric resonator: intrinsic dielectric losses, radiation losses and ohmic losses. Intrinsic dielectric loss is the energy lost as heat through polarisation of the dielectric material. High permittivity materials are used for dielectric materials in order to minimise dielectric losses. In this thesis cylindrical dielectric resonators used are made from barium zinc tantalate (BZT), a perovskite with a permittivity of 28 and a Q_f (quality factor due to dielectric losses multiplied by frequency) value of 168 000 GHz.⁵⁹ Radiation losses occur as a result of the propagation of standing electromagnetic waves away from the resonator and are dependent on the shape of the resonator and the mode excited. As mentioned previously, these losses can be removed by housing the resonator in a metallic cavity, which reflects radiated fields back to the resonator. This, however, results in ohmic losses in the conductive cavity resulting from the movement of electrons through a resistive material. The use of high conductivity materials for microwave cavities, such as copper, can minimise these losses.

Intrinsic dielectric, radiative and ohmic losses contribute to the unloaded quality factor, Q_{UL} , while losses induced via coupling contribute to the loaded quality factor. The strength of the coupling to the dielectric resonator determines the loaded quality factor, while the material and environment of

the resonator determine the unloaded quality factor. In this thesis coupling loops are used to both excite the DR and measure the transmitted signal. Coupling loops connect the inner and outer conductor of a coaxial cable in a loop shape, allowing current to flow between them, which sets up orthogonal alternating magnetic fields. The orientation of the coupling loops determines the orientation of the resulting magnetic fields and therefore the mode induced in the dielectric resonator. To excite the $TE_{01\delta}$ mode the coupling loops are oriented perpendicular to the axis of the dielectric resonator as in Figure 3.3.4, so that the vertically aligned magnetic fields induce a transverse electric field in the resonator. In the case of coupling loops, the distance between the loops and the resonator determines the strength of the coupling. In order to ensure optimum coupling, so that maximum energy is retained within the resonator, the position of each coupling loop is adjusted so that the reflection measurements of each are approximately the same, and the quality factor and amplitude of the resonance measured in transmission mode is approximately at a maximum.

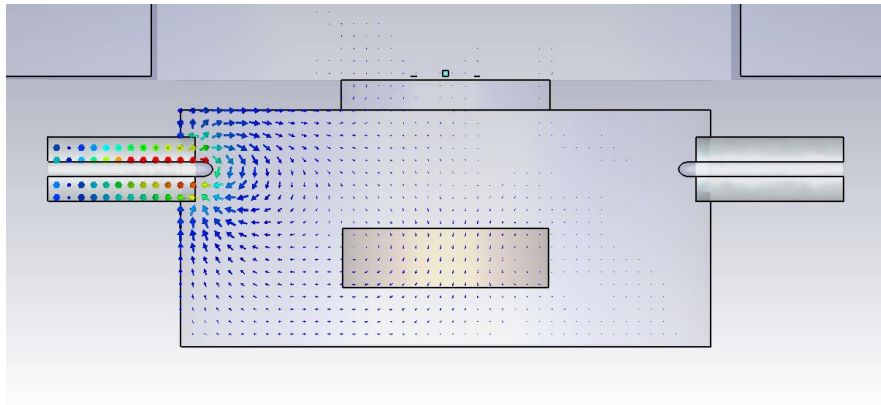


Figure 3.3.4: Schematic of the shape of the magnetic field distribution from one coupling loop, showing the circular, vertical shape of the field that would interact with the dielectric resonator.

The total quality factor can be calculated from the quality factor contributions from all the losses according to:

$$\frac{1}{Q_{total}} = \frac{1}{Q_{dielectric}} + \frac{1}{Q_{radiative}} + \frac{1}{Q_{ohmic}} + \frac{1}{Q_{coupling}} \quad \text{Equation 3.3.2}$$

since the inverse quality factor is proportional to the losses of the system, as in Equation 3.3.1, which are additive.

3.4. SPLIT RING RESONATORS (SRRs)

Metal structures can be used to confine alternating electric fields to small volumes. A ring structure can support a resonant mode at a frequency dependent on its geometry. Introducing a gap into a ring structure produces a split ring resonator (SRR) which can be operated at modes that set up alternating electric fields in the gap via capacitive charging of each side of the gap. In this thesis a circular split ring resonator with two gaps is used for measurements and referred to as a double split ring resonator (DSRR), as shown in Figure 3.4.1.

A split ring resonator operates by a synchronised movement of electrons through a metal structure. It is most commonly excited using a microstrip.^{60–65} In this thesis a DSRR is excited in the mode illustrated in Figure 3.4.1, which consists of electric currents running around each half of the DSRR in the same direction, alternating between clockwise and anticlockwise. This results in charge accumulating on one side of each gap each time the direction changes and sets up capacitive electric fields in the gap region.

Several modes with different resonant frequencies can be excited in a split ring with a given geometry. The resonances at which these modes exist depend on the geometry of the rings, with degeneracy sometimes occurring when small changes are made to the shape of the ring. This can be problematic, since for the sensing application discussed in this thesis it is important to set up strong alternating electric fields in the gaps, and as can be seen in Figure 3.4.1 there are plenty of modes that can be excited which result in no field in the gaps whatsoever.

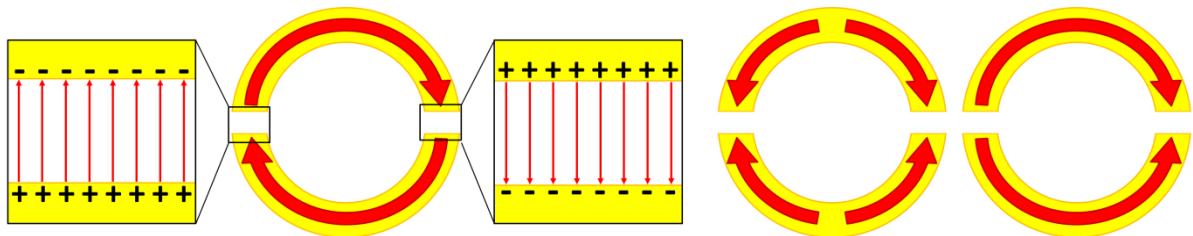


Figure 3.4.1: Left: Schematic of a resonant mode in a circular DSRR showing the (conventional) current moving in the ring (large red arrows) as well as the electric fields set up in each gap (zoomed in sections, small red arrows) when a dipole-dipole mode is excited. Right: Schematic showing two other resonant modes that exist in a split ring which do not result in electric fields in the gaps. For the rings used in this thesis these resonant modes can be close together in frequency.

The frequency of a split ring resonator therefore depends on its geometry and its shape. The quality factor of metal split ring resonators is low compared to dielectric resonators due to conductivity losses. Energy is also lost from SRR due to radiation, but since the size of the DSRR is small (>6 mm) compared to the free standing wavelength of the operation frequency (3 cm at 10 GHz), radiation losses are negligible compared to ohmic losses.

For the purposes of this thesis the mode excited exhibits two opposing dipoles which result in electric fields in the gaps of the DSRR, as shown in Figure 3.4.1. This mode is excited via inductive coupling with the DR, which is explained in Chapter 7.

Changes in the resonant parameters of a SRR can be related back to the permittivity of samples by representing the SRR as a resonant LCR circuit, with values of inductance representing the two half rings, capacitance representing the gaps and a resistance to represent radiative and conductive losses. This is discussed further in Chapter 7.

4. CIRCULATING TUMOUR CELLS (CTCs) AND THEIR DETECTION

As outlined in Chapter 1, the motivation for this project is to develop a label-free cell sensor capable of differentiating cell types based on physical properties, with the ultimate aim of detecting rare circulating tumour cells (CTCs) in blood. As will be discussed, non-destructive, physiological-based methods of CTC detection have been investigated and proposed already. Such a system could provide advantages over current techniques such as quick measurements, easy-to-interpret results, and most importantly undamaged CTCs that can be processed and studied after detection. Alternative methods, however, generally struggle with selectivity, sensitivity and ambiguity, falling short of the current gold standard techniques. Dielectric spectroscopy (the investigation of permittivity as a function of applied frequency) offers an interesting alternative to other characterisation techniques, as it offers potential specificity and sensitivity not seen in other approaches based on physiological properties.⁶⁶ The case of circulating tumour cells is potentially particularly well suited to microwave dielectric measurements due to evidence of significant differences in water content of malignant cells.⁹ This section briefly builds the case for a microwave dielectric measurement technique for CTC detection and enumeration.

4.1. METASTASIS

In metastatic cancer, cells escape the site of the primary tumour into the circulatory system and find their way to new organs or other places in the body, where they proliferate and form new tumours,⁶⁷ although the exact nature of the cells responsible for metastasis is currently debated.^{68,69} Associated with over 90% of cancer fatalities, metastatic cancer is one of the leading causes of death in the world today,^{70,71} yet the mechanism of tumour metastasis remains unclear.⁷²

The current interpretation is that metastasis occurs via a series of thus far poorly understood steps, starting with the escape of cells from the primary tumour into the circulatory system (most commonly the vasculature route⁷³). These cells survive the circulatory system, travelling to small vessels in tissues downstream of the original tumour site and extravasate (move from the blood vessel into the surrounding tissue and organ) to proliferate and seed secondary tumours in the new microenvironment.⁶⁷ It is this spread of tumours around the body that proves fatal for so many millions of cancer patients each year, as the treatment for multiple tumours becomes difficult and

the cancer may spread to more vital organs, for example breast cancer is prone to spread to the lungs, liver, bones and brain.¹³

In accordance with this “seed and soil” theory, cells found circulating in peripheral blood which bear some specific hallmarks of malignant tissue (tumour) cells are defined as circulating tumour cells (CTCs).^{74,75}

4.2. CLINICAL SIGNIFICANCE OF CTCs

If metastasis could be inhibited or stopped, the fatality rate of cancer sufferers would be significantly reduced. Elucidation of the complex process of metastasis and the nature of the cells responsible is of the utmost importance if metastatic cancer is to be tackled.

The detection, separation and enumeration of these particular cells has already proven useful as a diagnostic tool to monitor disease progression, quantify efficacy of treatment and therefore prognosis, inform treatment choices, and even aid in diagnosis, as CTCs retain markers characteristic of the site of their parent tumour.^{72,73,76–78} In this way, collection and subsequent analysis of CTCs from a patient's peripheral blood offers a minimally invasive way of probing the tumour by way of a pseudo-biopsy, and if cultured, CTCs could also be used as a test population for possible treatments, or aid in the identification of new therapeutic targets.⁷⁹

Another advantage of sampling circulating tumour cells over carrying out invasive tumour biopsies is the ease of sampling over time, as the innate heterogeneity shown in tumours makes biopsies often unrepresentative of the disease. CTCs can, in theory, be shed from all different parts of a tumour, potentially leading to a more complete picture of the overall genomic content of the tumour if sampled many times.⁷⁴

Further investigation into the specific cell-surface antigens expressed by these cells may also help developments in treatment of cancers, for example using a patient's CTCs to create personal medicine tailored to their specific cancer, or to guide the body's own immune system towards fighting tumours (cancer immunotherapy, e.g. Sipuleucel-T, and other antigen-specific therapies).^{80,81} Targeted therapies have already shown themselves to be beneficial, with two methods gaining FDA (Food and Drug Administration, the US federal agency responsible for regulation of, among other things, medical treatments) approval.⁸¹

Owing to the constant evolution of cancer at the molecular level, resistance to drugs is often acquired so CTCs could prove invaluable as a source of easily available, continual monitoring of this

evolution, and may help in keeping track of or even anticipating changes that will affect the response of tumours to medicines.⁷⁴

4.3. CTC CHARACTERISATION

Clearly the process of metastasis cannot be directly observed and so there is no unambiguous proof of which cells are the culprits, and there is in fact doubt over whether the cells that are detected in peripheral blood can possibly be responsible for metastases, given their persistence in the blood stream and continued motility through narrow capillaries.⁷² However, tumour cells found circulating in the blood have been shown to initiate metastases in otherwise healthy individuals following xenograft procedures.¹⁵ This proves that the cells currently defined as CTCs have at least the potential to seed new tumours and are therefore worthy of investigation. It has long been known that circulating tumour microemboli (CTMs, large clusters of tumour cells sometimes embedded in blood clots or with other cell types) have a higher capability of metastasis initiation compared to single cells,⁸² and recently the presence of such clusters in patient samples has been linked to poorer prognoses.^{83,84}

The current understanding of CTCs relies on cell surface markers for unambiguous identification. A cell is characterised as a circulating tumour cell if it fulfils all the following criteria:

- found in circulating peripheral blood
- has a nucleus
- greater than 5 micrometres in diameter
- tests positive for an epithelial cell surface antigen (usually EpCAM), which indicates the cell is of an epithelial phenotype and therefore of epithelial origin (accounts for 85% of cancers)
- tests positive for cytoplasmic expression of tumour specific cytokeratins (CK 8, 18 and/or 19), which indicates the cell is malignant
- shows negative for leukocyte specific markers (CD45, which occurs on all leukocytes) to rule out identification as a circulating white blood cell

In this way, cells that show all the common hallmarks of “normal” tumour cells that can be found in the blood stream are identified.⁷⁴

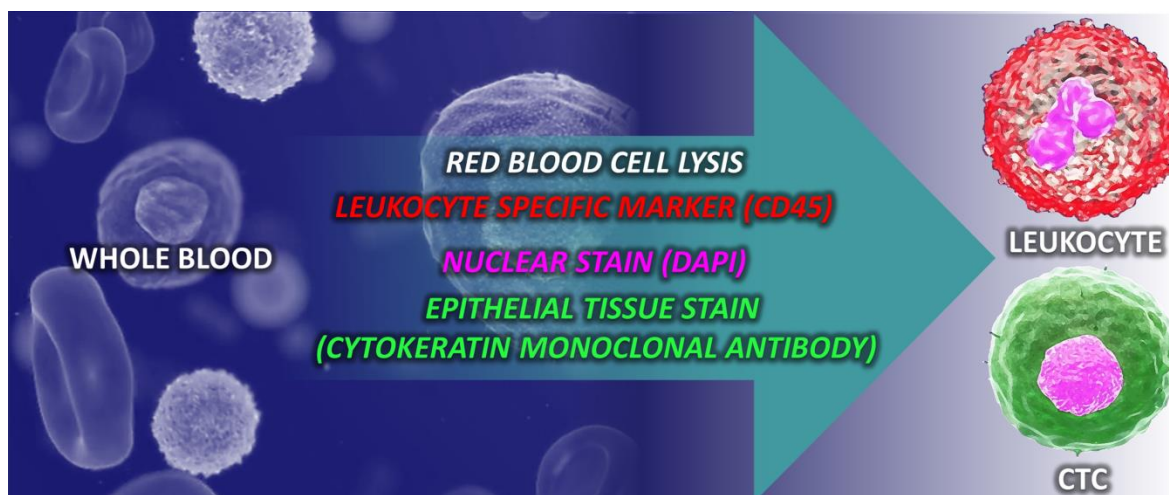


Figure 4.3.1: Schematic of the immunocytochemical approach to detecting CTCs, showing the process of taking whole blood through the steps of RBC lysis and staining for differentiation between CTCs and leukocytes, adapted from CellSearch®.⁸⁵

4.3.1. IMMUNOCYTOCHEMICAL METHODS

There are many antibody-based CTC capture assays relying on tumour cell-specific traits. These technologies are generally split into two camps for enrichment of CTCs, one which uses antibody coated magnetic beads or wire to physically pull relevant cells together and another chip-based methodology which uses microfluidics to carry cells over antibody coated surfaces for immobilisation.⁸⁶ Both then go on to downstream focussing and detection based on further immunocytochemistry or simple microscopy. Initial microfluidic approaches used arrays of antibody-coated micropillars to trap cells,⁸⁷ and more recent developments include the HB-Chip,⁸⁸ and the *in-vivo* CellCollector CANCER01 wire-based trapping technology.⁸⁹ More commonly used is the magnetic enrichment technique, with methods including MagSweeper,⁹⁰ CTC iChip,⁹¹ Ephesia CTC-chip,⁹² IsoFlux⁹³ and the only FDA approved method for CTC detection and enumeration, CellSearch®.^{69,85} CellSearch® first enriches for CTCs using ferromagnetic iron nanoparticles with a coating of antibodies specific to the epithelial cell surface antigen EpCAM. Fluorescently labelled cytokeratin and CD45 antibodies are then used to confirm malignant epithelial cells and to rule out any possible white blood cell contaminants, respectively. Cells are then permeabilised and a nuclear stain is added (DAPI) to confirm the presence of a nucleus. A magnet is used to pull captured cells together for collection. Visual examination by a trained pathologist is then required to check cells that test positive for epithelial and cytokeratin markers and nucleic stains, but negative for the leukocyte-specific marker CD45.⁸⁵

This immunocytochemical approach is not only expensive (high cost of conjugating antibodies to magnetic nanoparticles/surfaces/fluorescent labels), but also relies on a narrowly defined

combination of cell surface markers that can occur on circulating cells caused by other conditions. For example, patients with some benign inflammatory colon diseases will test positive for CTCs when no cancer is present.⁷² Cell surface antigen-based detection can also cause other problems, such as the surface of CTCs becoming obscured by an aggregation of platelets around it,⁷³ physical trapping of other cell populations among beads and enrichment of some leukocytes due to nonspecific binding.⁷⁴ Due to innate heterogeneity in cancer cell populations, the expression of surface markers also varies widely, leading to a large range of yields for these immunocytochemical methods.⁹⁴ Current techniques currently also discount circulating tumour microemboli, despite their proven metastasability.

Another potential problem with this method stems from the use of epithelial cell markers, when the assumption of an epithelial phenotype may not be correct. There is still much discussion as to whether the tumour cells that actually cause metastases of carcinomas go through epithelial-mesenchymal transition (EMT) upon dissemination from the primary tumour,^{73,95,96} but there is evidence to suggest significant portions of CTCs in patient samples are of the mesenchymal phenotype.⁷⁵ EMT is the process by which a cell changes its phenotype from epithelial (an adherent, solid tissue cell with strong physical connections to its surroundings) to mesenchymal (a cell that is only loosely bound to its surroundings and therefore migrates from the primary tumour easily). If this is the case, CellSearch® and other immunocytochemical approaches which rely on epithelial cell markers may only be detecting a portion of CTCs that have not yet undergone EMT, or undergone only partial EMT and could potentially be insensitive to a critically important subpopulation.⁷⁴ There are also varying levels of EpCAM expression between different cancer cells, and basing detection on the presence of EpCAM markers of course omits any non-epithelial cancers, e.g. melanoma or leukemia.⁹⁷

All immunocytochemical methods also rely on at least permanently altering the CTCs with antibody conjugation, and often completely destroy the cells due to nuclear staining or immobilise the cells by conjugating them to magnetic nanoparticles, nanowires or surfaces. Although enrichment techniques that allow captured cells to be released have been reported,⁸⁹ these methods still generally prevent additional analysis or culturing of the cells after detection and collection. This prohibits CTCs being studied in detail to determine their role in metastasis or to develop a therapy specific to these cell types.

4.3.2. OTHER METHODS

Not all methods for detecting and collecting CTCs are biological but, due to innate cell heterogeneity, for most separations there is some degree of immunocytochemical screening involved to unambiguously identify a cell as a CTC.

Malignant cells, on the whole, tend to be larger and have a greater nucleus to cytoplasm ratio than normal, healthy cells.^{86,98,99} This has led to the development of methods for enrichment of CTCs from whole blood which rely on their physical characteristics, such as their size or density; such methods include density gradient centrifugation (Ficoll, OncoQuick, Accucyte),^{86,94} size selection by filtration (isolation by size of epithelial tumour cells - ISET, for example the ScreenCell® technology, the cluster chip),^{97,100,101} as well as electromagnetic approaches such as dielectrophoresis (DEP), which will be discussed further in Chapter 5, and approaches based on the deformability of cells.⁸⁷ Other approaches based on photoacoustic methods are capable of detection and even destruction of CTCs *in vivo*,^{102,103} while simple hydrodynamic flow focussing approaches have demonstrated high throughput separation of larger CTCs from mixed samples.¹⁰⁴

Using these physical characteristics to separate cell types removes many of the problems associated with the immunocytochemical approaches, but does not give a definitive characterisation of a cell as a CTC, due to the heterogeneity in tumour cells, in CTCs even from the same patient and other cell populations with similar physical characteristics such as monocytes.⁸⁷ To do this requires further analysis based on antibody methods such as those outlined above or other gold-standard approaches such as flow cytometry

Although it is generally agreed that CTCs and other cells from solid tumours are larger than blood cells there is some evidence that CTCs undergo cytoplasmic loss during migration, causing a spectrum of CTC morphologies in the blood stream.¹⁰⁵ This may render these techniques overly simplistic, potentially missing CTCs that do not adhere to the traditional tumour cell model. Indeed, size-based methods alone have proven relatively insensitive,¹⁰⁶ possibly due to the wide variety of sizes of CTCs as identified using the CellSearch® system.⁷⁵ Centrifugation techniques also suffer from inherent problems of purity due to leukocyte contamination, while there is contradictory evidence on the relative deformability of CTCs.⁸⁶ The vast variation in morphological features of CTCs presents problems for physiological-based approaches, but may lend insight into CTC populations, potentially indicating degrees of aggressiveness or invasiveness.

Analysis of the DNA can also be used to characterise cancerous cells, but despite the theoretical high sensitivity and selectivity of this approach, expression of markers from healthy cells produces a

strong background, making false positives an issue.¹⁰⁷ This also suffers from problems of tumour heterogeneity, and of course destroys the cells.

More recently, an approach based on dielectrophoresis (DEP) has been proposed which separates viable CTCs from whole blood using low-frequency dielectrophoretic properties of cells, combined with their size.¹⁰⁸ This is discussed further in Section 5.3, and has led to molecular biology research into separated CTCs.¹⁰⁹

4.4. CHALLENGES AND OPPORTUNITIES

There is potential for a new, simple and fast, label-free method of detection, characterisation and possible separation, based purely on physiological differences between normal and malignant cells; a method that unambiguously identifies cell types while preserving them in their natural *in-vivo* state.

As discussed in Chapter 2, in the frequency range 5-20 GHz EM radiation interacts strongly with the dipole of a water molecule and this makes microwave frequencies particularly sensitive to the amount of water present in a sample. Microwave dielectric spectroscopy, and the microwave dielectric properties of different cells, can be thought of as measuring the amount of water and non-water in a sample. For biological samples this is a useful metric for characterisation, as the amount of water in a cell can give information about its size, membrane permeability, the presence of granules and nuclear to cytoplasmic ratio.

Malignancy of cells is associated with higher water content than found in normal cells and a corresponding larger size,⁹⁹ and higher nuclear to cytoplasmic ratio.^{55,98} An increase in the level of cell hydration has been linked to carcinogenesis, due to the ability of cell hydration to inhibit cell differentiation and apoptosis as well as encourage cell division, with the degree of malignancy increasing with the degree of cell hydration.⁹ The ability of dielectric measurements to differentiate cancerous and healthy cells is discussed in more detail in Chapter 5. The differences in tissue water in malignant and normal tissue are routinely used in clinical medicine to define the extent of tumours using MRI,¹¹⁰ and there is evidence to suggest inhibiting cell hydration may be an effective treatment for colon cancer.^{111,112}

Microwave dielectric measurements therefore offer a method that is:

- Contactless

- Based on physiological properties – does not require any cell modification and is not subject to antibody bias
- Non-invasive – can sample small amounts of peripheral blood
- Non-destructive – allows investigation of living cells, potentially in real time, as well as analysis downstream
- Scalable to cell dimensions and low cell concentrations – possibility of single cell analysis
- Possible to conduct in a biological medium environment - keeping cells in their natural *in-vivo* state.
- Capable of achieving high-throughput with sub millisecond measurement times
- Continuous rather than a batch process, for real time monitoring
- Capable of being integrated with microfluidic systems for high-throughput lab-on-chip applications - possibility of automated separation.

Circulating tumour cells do, however, present some considerable challenges to microwave detection. The difference in water content between different cell types is expected to be small and therefore a highly sensitive system will be required, and efforts will have to be made to ensure that any measured effects are greater than those induced by noise or other external factors such as fluctuating temperature, which dielectric spectroscopy of water is particularly susceptible to. It would also be helpful to miniaturise the measurement to single cell scales so as to avoid measuring the overall properties of the cell and its surroundings, thus enhancing the contrast measurable between different types of cells. Tumour heterogeneity and the physical similarity between CTCs and different cell populations may also be a problem with microwave dielectric approaches, but potential issues of specificity may lead to detection of populations of malignant cells currently missed by gold standard techniques, therefore offering complementary information.

5. ELECTROMAGNETIC METHODS FOR CELL DETECTION

The field of electromagnetic biosensing has been around almost as long as electrical technology itself, with the 20th century bringing the development of the Coulter counter,¹¹³ electromagnetic heating technologies,¹¹⁴ and theoretical and mathematical frameworks for the dielectric properties of biological materials,³ as well as reports of differences in the low frequency electrical properties of cancerous and benign tumours as early as the 1920s.¹¹⁵ Advances in microwave sources and techniques have allowed higher frequency properties of cells and other biological materials to be probed, leading to the characterisation of their broadband dielectric properties and discrimination of different tissue types based on their water and protein content,¹¹⁶ as well as the discovery of a high contrast in permittivities of healthy and malignant tissues.^{5,10} Models to account for the dispersions seen in cell suspensions and tissues were put forward, identifying the α , β and γ dispersions discussed in Chapter 2,^{28,32,33} and various human and animal tissues were characterised over wide frequency ranges.^{117–119} Clinical and therapeutic uses of microwave frequencies, such as diathermy, wound sterilisation,⁶ the destruction of malignant cells,⁷ and tissue imaging,^{5,120} as well as the safety of these applications have been widely studied.¹²¹

This chapter will briefly summarise some of the literature relevant to the work in this thesis, focussing on the progress made in cancer cell and single cell analysis in the microwave regime, as well as relevant approaches such as dielectric and split ring resonators.

5.1. TISSUES AND BIOLOGICAL SOLUTIONS

Simple biologically-relevant solutions have been widely studied at a range of frequencies, for example the dielectric response of simple protein solutions has been determined and found to be a promising means of identifying different proteins and their concentrations with signature microwave responses.^{22,24} Resonator experiments on protein solutions have also shown remarkably high sensitivity to small volumes, down to nanolitres.⁵⁸ Higher frequencies have also been used to probe the interaction of proteins and water, with the THz regime providing insight into the thickness of proteins' water shells and the physical extent of the electrostatic interactions involved.³⁰ Microwave glucose sensors have been proposed by a number of researchers for the medical field for the purposes of mediator-free diabetes monitoring, using both whole blood and serum only from <4 GHz to >9 GHz.^{122–125} Alcohol solutions have also been widely studied due to their similarity to biological molecules,¹²⁶ as well as for the purposes of fermentation monitoring¹²⁷ and food identification.¹²⁸ Investigation of the dielectric properties of DNA solutions has shown both low frequency dispersions

from DNA molecules as well as high frequency differences assumed to be due to water molecules electrostatically bound to polar groups⁵⁰

Whole tissues have been investigated for years, and it has been shown that over 30 types of biological tissues can be identified, including skin, muscles, heart, lungs and even different types of bone, brain and fat.^{4,17} It is well known that the anomalously high water content of cancer cells allows microwave technology to be used for both detection and treatment,⁹ and the ability to differentiate between tumorous and non-tumorous tissue was demonstrated as early as 1926 by Frick and Morse, who showed that malignant breast tumour tissues have a higher permittivity than both normal breast and non-malignant tumour tissues.¹¹⁵ This has already led to the development of microwave technologies for detection of skin cancers,¹²⁹ imaging of breast and liver tumours^{5,66,120,130} and thermal treatment of various cancers.¹³¹

High levels of sensitivity and selectivity have been achieved via immobilisation of biomolecules such as DNA (via biotin-streptavidin binding or DNA hybridisation) directly onto microwave resonator sensors at < 3 GHz, showing the ability to detect down to 1 ng/mL of DNA fragments with selectivity to fragment size.¹³² Surface-modified split-ring resonators operating at higher frequencies (around 10 GHz) have shown large frequency shifts for 1 μ M streptavidin *via* single-stranded DNA-linked biotin,¹³³ a sensing limit of 100 pg/mL to the binding of prostate-specific antigen,⁶³ and a limit of 10 ng/mL complementary single-stranded DNA for both DNA and proteins in low concentrations,^{64,133} due to the sensitivity of the area around the surface of the metal.

5.2. BLOOD AND CELL SUSPENSIONS

The dielectric properties of blood have been investigated for many decades, and some of the earliest higher frequency measurements on the resistivity of whole blood served as experimental evidence for the membrane hypothesis of cells.³ Early broadband investigations on blood led to the description of the α , β and γ dispersion mechanisms seen in cell suspensions,³⁶ while a more recent extensive investigation has extended into the higher frequency regime, outlining the dielectric properties of whole blood in the frequency regime 1 Hz to 40 GHz and further investigating the main dispersions.⁴¹

As well as broadband techniques, resonator measurements at single frequencies have also been carried out on blood, showing high sensitivity in the microwave window to levels of protein in blood cells, as well as cell concentration.²¹

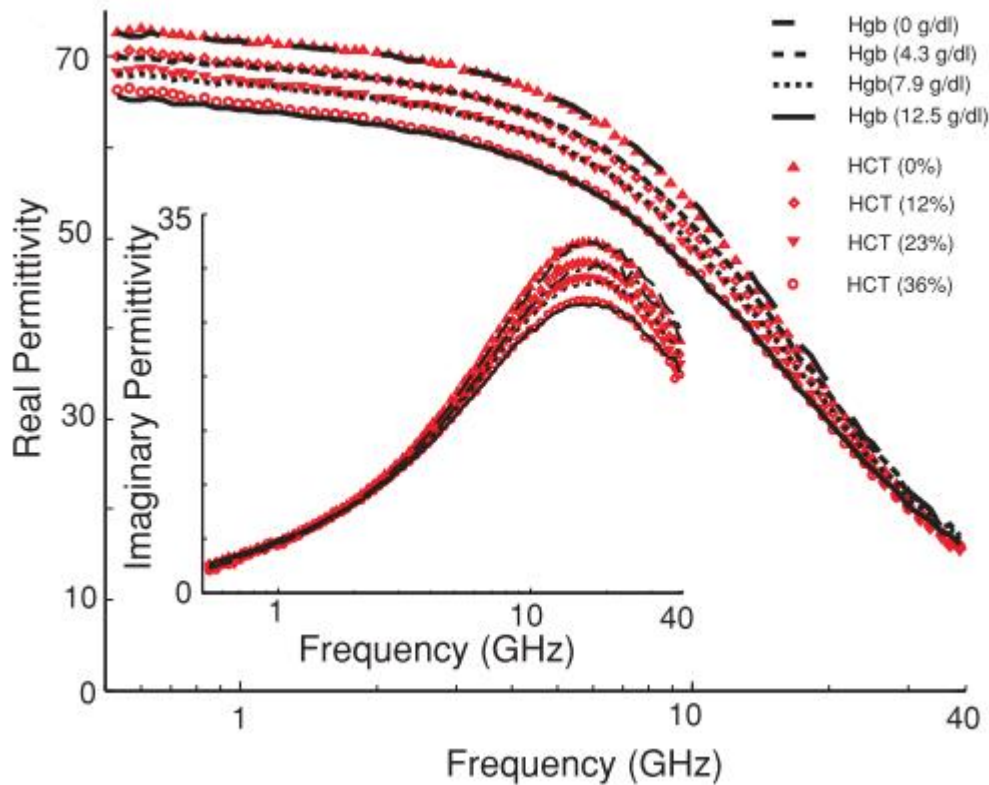


Figure 5.2.1 Broadband permittivity measurements showing the dependence on increasing concentration of haemoglobin (Hgb) and red blood cell (HCT) concentration. Reproduced from Basey-Fisher et al.²¹

Dielectric sensing is attractive for blood tests due to the possibility of using small sample volumes for non-invasive tests on peripheral blood. Given the sensitivity of the microwave frequency window to changes in water and ion content it has been shown to be possible to sensitively detect lymphatic disease even with small sample volumes of peripheral blood, an improvement on current techniques.¹³⁴ In non-uniform fields, dielectrophoresis (DEP) forces have been shown to be able to differentiate between sickle and normal red blood cells,¹³⁵ and radio frequency measurements are capable of determining the haematocrit of whole blood samples.³⁵

At low frequency (<1MHz) the difference in response of white and red blood cells has been used to differentiate between them,¹³⁶ and some discrimination based on size can be achieved at this frequency between white blood cell subpopulations.³⁷ For intermediate frequencies (up to 100s of MHz) the response of white blood cells is governed by cell membrane capacitance, which is proportional to cell membrane surface area and therefore to both size and morphology. Different white blood cell subpopulations have different cell surface morphology due to having different functionality, and thus one type can be distinguished from another on the basis of cell membrane roughness, thickness, composition, number of microvilli, ruffles or other projections such as surface immunoglobulin molecules.³⁴ It has been shown that suspensions of B and T lymphocytes can be

distinguished in this way.¹³⁷ This difference in specific cell membrane capacitance can also allow normal and malignant cell populations to be differentiated at up to 3 GHz.⁵⁶

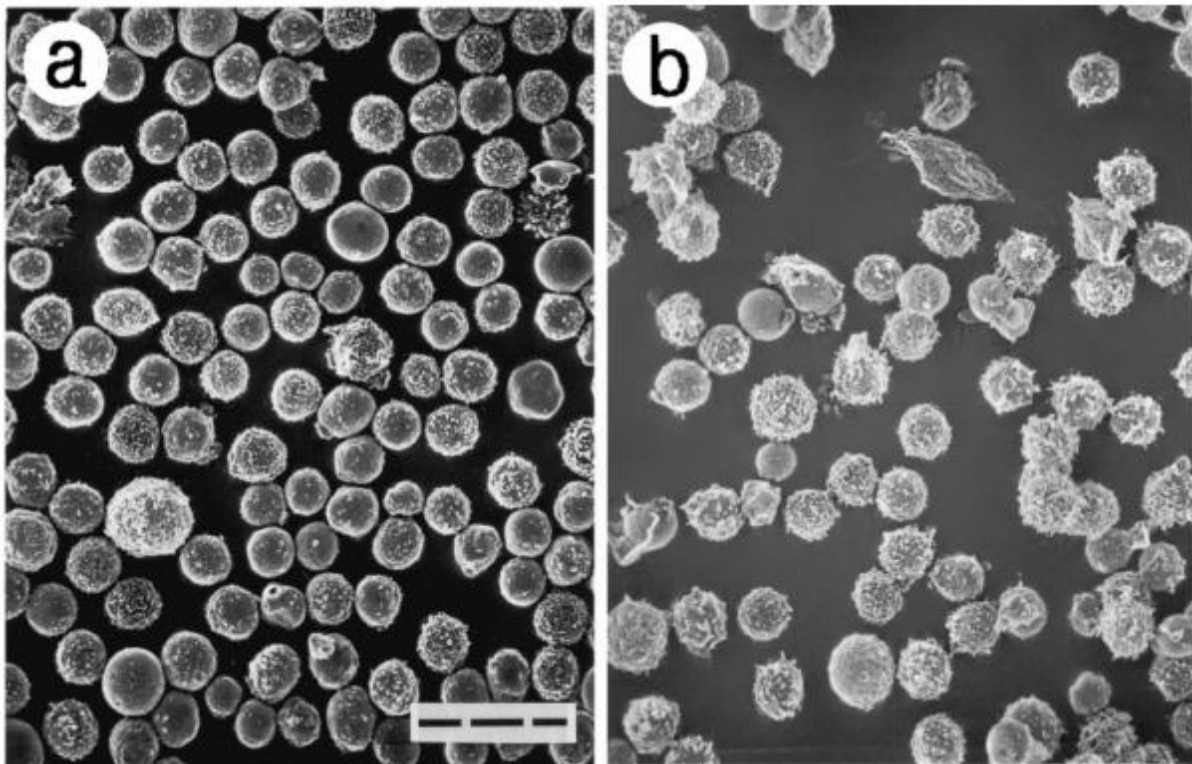


Figure 5.2.2: Scanning electron micrographs of different types of human leukocytes : a) T-lymphocytes, b) B-lymphocytes, reproduced from Yang et al,³⁴ showing the variety of membrane morphologies between different cell types which provides contrast to electromagnetic characterisation techniques at low frequencies. Bar length denotes 20 μm for both micrographs.

At higher frequencies the inside of cells can be probed, giving information about the conductivity and permittivity of the cytoplasm and nucleoplasm and the amount of water vs proteins. Broadband measurements of the effects of suspensions of live and dead cells on interdigitated capacitors have shown contrast in differences of conductivity and capacitance over a range of 0.04-40 GHz.¹³⁸ Coplanar waveguide approaches from 0.85-40 GHz have shown a decrease in both the real and imaginary permittivity of suspensions of dead cells compared to live cells of the same type,¹³⁹ while from 1-32 GHz a dependence of measured permittivity of cell suspensions on their progressively deteriorating viability has been shown,¹⁴⁰ possibly due to a combination of membrane electrical potential effects at low frequency and the breakdown of cell membranes causing the local release of ions and proteins at higher frequencies. A dependence of the real and imaginary permittivity of suspensions on the concentration of cells has been shown up to 40 GHz, also demonstrating the decreasing contribution from salts in biological media with increasing frequency.¹⁴¹ Whole blood and its constituents has been probed up to 2 THz, showing different effects for protein solutions and plasma, but a lower contrast between water and whole blood than at lower microwave

frequencies.³¹ Suspensions of *E. coli* and T-cells have been successfully measured up to 0.5 THz using low frequency DEP focussing, showing sensitivity to the concentration of *E. coli* cells.¹⁴²

Single-frequency approaches to cells suspended in biological media have also shown sensitivity and selectivity, with a coplanar approach able to distinguish live from dead cells suspended in medium at 6 GHz,¹⁴³ and an interdigitated capacitor approach able to correlate measured capacitance changes at 3 GHz to the number of cells settled on the sensor, down to 20 individual cells.¹⁴⁴ Differences in permittivity and conductivity of whole blood at 2-3 GHz were able to distinguish between samples from normal volunteers and those from patients suffering from a parasitic lymphatic disease using a resonant cavity perturbation technique.¹³⁴

5.3. CANCER CELLS AND TISSUE

At low frequencies cancer cells have shown characteristic differences from normal cells, due to their membrane capacitance, intracellular conductivity, as well as possibly the difference in their cell surface charges, with aggressive metastatic tumour cells often exhibiting an increase in cell surface conjugates with negative charges, promoting cell detachment via charge repulsion.¹⁴⁵

So far much work has been done on dielectrophoretic separation techniques, focussing on the low to mid frequency regime where separation is achieved on the basis of cell membrane capacitance or intracellular conductivity. DEP as a separation technique for cancer cells was first demonstrated in 1992,¹⁴⁶ and since then has been investigated extensively. AC electrokinetic methods DEP and electrorotation (ROT) have been widely used to ascertain the dielectric properties of cells at low frequency (below 1 GHz).^{43,147} DEP uses an inhomogeneous electric field to polarise cells which are then drawn either towards or away from areas of high field concentration, depending on the polarisation relative to the medium and the orientation of polarisation. Different cells experience different degrees of polarisation due to varying dielectric and membrane properties and therefore this can be used as a separation technique in methods such as dielectrophoretic field-flow fractionation (DEP-FFF) and electroaffinity columns. The DEP response of cells is also frequency dependent, causing a repulsive force at frequencies lower than the particle's characteristic crossover frequency (the frequency at which the particle experiences no net force from the field) and an attractive force at frequencies much higher. This allows elucidation of the properties of cells by fitting DEP spectra with appropriate cell models.^{43,148,149} The same can be done for ROT measurements, whereby a rotating electric field is used to pull particles/cells around in circular paths and their ability to keep up with the field and direction of rotation depends on the specific

dielectric properties of each cell and the frequency applied.⁴³ ROT is a single cell approach but is time consuming, low throughput and complex, both in measurement and analysis of results.¹⁵⁰

Once the dielectric properties of the different cells are known, it is possible to predict how they will react under DEP forces. In general, for epithelial (solid) tumour cells it has been shown that the first DEP crossover frequencies are significantly lower than those for other blood cells, due to higher cell membrane surface area and size leading to increased membrane capacitance, while for circulating malignant cells, the distinction is less significant.^{151,152} This has been used to separate oral squamous cell carcinoma cells from normal epithelial tissue cells.¹⁵³ Separation can be achieved in different ways, for example: DEP-FFF, where the height of a cell or a particle in a flow with a parabolic velocity profile is controlled by cells' varying responses to a particular frequency, such that different cells elute at different times,^{148,154} or dielectric affinity columns, in which the frequency is gradually changed to preferentially release certain cells to elute, but retain a particular cell type.^{38,105,148,155} Techniques that employ targeted DEP forces on specific cell types have proven successful at concentrating human cervical cancer cells at 1 MHz in a handheld device,¹⁵⁶ and separating mixtures of polystyrene microspheres, cultured human kidney cells and mouse neuroblastoma cells,¹⁵⁷ as well as cultured human breast cancer cells from colorectal cells at 1-5 MHz.¹⁵⁸ The same breast cancer cell line (MCF-7) has been separated from normal epithelial breast tissue cells using differences in DEP forces felt at 40 MHz,¹⁵⁹ while a combination of hydrodynamic flow focussing based on cell size and targeted DEP forces has achieved effective separation of the breast cancer cells from red and white blood cells, using a 900 kHz electric field with recovery of cancer cells up to ~71%.¹⁶⁰

The human breast carcinoma cell line (MCF-7) is used as a model for CTCs to demonstrate the potential of DEP forces in their separation, but more recently CTCs themselves have also been separated. Membrane morphology depends on the cell type, with cells originating in tissues tending to be larger (due to stacking), and malignant cells tending to have a high degree of folding in their membranes as a result of increasing disorganisation. Therefore cells that originate in tumours and invade the blood stream will have very folded cell membranes as a result of balling-up for circulation. Although cell size and membrane area is reduced due to shedding of vesicles of cancer cells in suspension (and therefore can be expected for CTCs in the blood stream), it is still generally significantly higher than healthy circulating cells, which tend to have smooth membranes and minimal surface area due to their nutrient-rich environment and ease of metabolic exchange, resulting in substantially lower DEP crossover frequencies for malignant cells. The same trend is seen, to a lesser extent, for leukaemias, where the onset of malignancy causes increased disorganisation of surface morphology.¹⁵²

The DEP-FFF approach is being commercialised for the separation of viable CTCs from whole blood.¹⁶¹ In the ApoStream® technology, when a signal less than 100 kHz is applied to electrodes at the base of a flow chamber, CTCs experience a positive DEP force, attracting them to the bottom of the channel, where they pass into a collection tube. Healthy circulating cells experience higher crossover frequencies and therefore experience a negative DEP force, resulting in repulsion away from the bottom of the channel and subsequent elution to waste. This technology shows high throughput as well as high recovery rates, showing the promise of dielectric-based separation and characterisation techniques.¹⁰⁸

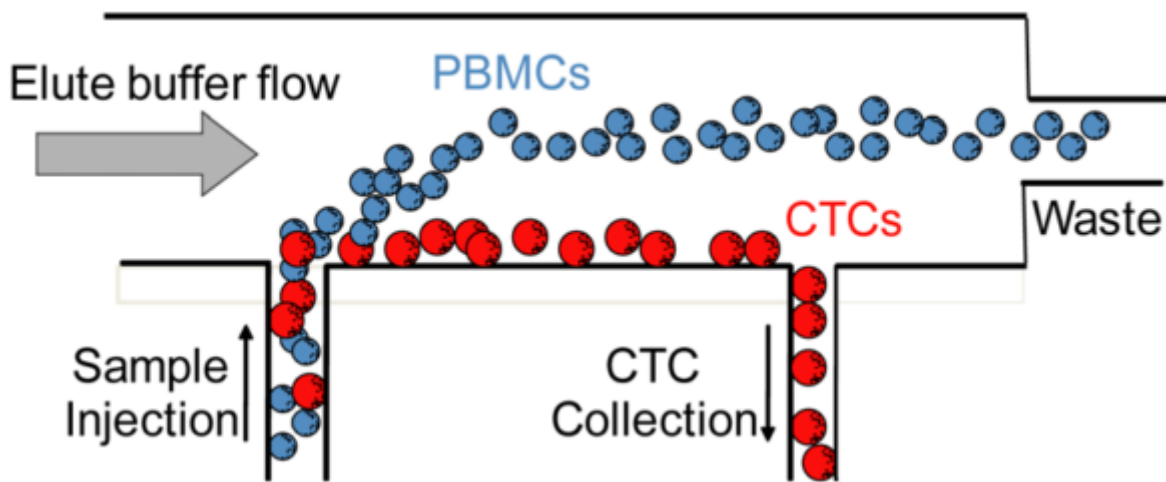


Figure 5.3.1 Schematic of the operation of the ApoStream technology showing the direction of flow and the DEP-induced movement of peripheral blood mononuclear cells (PBMCs) and circulating tumour cells (CTCs). Adapted from Gupta et al¹⁰⁸

The conductivity of the cell medium is required to be lower than that of the intercellular conductivity for DEP separation to be achieved. For most approaches this requires re-suspension of cells in a low conductivity medium, which can result in leakage of the cytoplasm through the membrane and reduce cell viability.^{152,162} However, one study achieved low conductivity by a hydrodynamic focussing of cells to high densities combined with ion diffusion to achieve separation of CTCs from clinical samples of separated peripheral blood mononuclear cells in a physiological medium.¹⁶²

A wideband investigation of living cultured cancer cells has shown the expected dependence on frequency as seen with other cell types and tissues, with the dominating response between 10-20 GHz that of the reorientation of water molecules.¹⁶³ At higher frequencies differences between normal and malignant cells continue to be seen, and may be of particular diagnostic value. Higher frequency measurements are less adversely affected by physiological salt concentrations, and so detection and enumeration of live cells in biological medium can be achieved.¹⁴¹

The strong permittivity contrast between healthy and malignant tissue in the microwave regime has led to the development of imaging and diagnostic technologies for a range of cancers.^{66,120} An *in-vivo* open-ended coaxial probe approach for the characterisation of skin lesions up to 6 GHz demonstrates the ability to discriminate normal, malignant and benign tissue based on differences in water and salt content.¹²⁹ Another wideband open-ended coaxial probe approach up to 20 GHz identifies breast cancer as a particularly promising target for microwave dielectric detection due to the high permittivity contrast between normal and malignant tissue,¹³⁰ explained by the higher water content of malignant breast tissue,¹⁶⁴ and several approaches for *in-vivo* diagnostic imaging based on this high contrast have been proposed.^{5,120,165–167}

5.4. SINGLE CELL ANALYSIS

Impedance-based single cell detection at DC has been demonstrated since the 1950s, with characterisation based on cell size.¹¹³ Low frequency alternating current techniques have shown sensitivity to size as well as to the capacitance of cell membranes, with approaches at <1MHz - 15 MHz able to count erythrocytes and latex beads,¹⁶⁸ as well as discriminate leukocytes in a flow cytometer-like system based on a combination of size and cell membrane differences.³⁷ At higher frequencies (<3 MHz), measurements of the impedance of trapped single breast cancer cells can be used to discriminate between different cell lines, due to increasing cell membrane permeability with pathological progression of cancer cells.¹⁶⁹

At higher frequencies, approaches that use planar metallic resonator structures to confine microwave frequency fields to small interaction volumes are often used for the spatial resolution necessary for single cell detection. However, traditional approaches employing planar metallic microwave structures suffer from low quality factors, which are only lowered further by the introduction of aqueous cell media. The low quality factors of these approaches limit sensitivity,¹⁷⁰ making single cell analysis difficult due to the low permittivity contrast between biological cells and the surrounding media. Sensors following this design often rely on immobilisation of cells directly onto sensing elements, only then achieving single-to-few cell analysis.^{60,66,170,171} This is often achieved by fixation and dehydration of cells, either on the sensor or prior to physical deposition, in order to facilitate measurements. Although this approach retains sensitivity and selectivity, with one approach able to differentiate between colorectal cancer cell lines on the basis of aggressiveness,^{11,172} it is a slow and destructive batch process, unable to monitor cells in real time in their natural state.

Very high spatial field confinement has been proposed using conducting/dielectric near-field probe techniques with sub-wavelength resolution in the microwave regime,¹⁷³ and the terahertz regime,¹⁷⁴ and the ability to map the dielectric properties of individual bacteria has been achieved at 19 GHz.¹⁷⁵ These approaches do not, however, lend themselves to high throughput cell identification, and have not achieved measurement of cells in medium. Resonators in the terahertz regime have also been developed with the potential interaction volumes necessary for single cell measurements,^{176–179} but in this high frequency regime the contrast between water and organic cellular contents is at a minimum.

5.5. FLOWING, LIVING SINGLE CELL ANALYSIS

The ability to measure single cells in their living condition is clearly advantageous compared to approaches in which cells are destroyed; keeping cells in aqueous media does, however, present a problem for microwave-based sensing approaches, due to high dielectric losses of water in the microwave regime. The detection and measurement of cells one-by-one may give more reproducible results than deposition or culturing of cells on-sensor as the number of cells probed is well defined and the position of the cell can be controlled.

The DEP approaches discussed in Section 5.3 offer single cell sensitivity as well as high throughput analysis, but rely on cell membrane effects and low conductivity suspension media as well as previous knowledge of the dielectrophoretic properties of different cell types to achieve separation. However, DEP-based approaches have shown single cell sensitivity, capable of monitoring cytoplasm conductivity changes in single Chinese hamster ovary (CHO) cells,¹⁸⁰ analysing the DEP response of flowing CHO cells as well as 10 μm polystyrene microspheres over a frequency range of 1-400 MHz,¹⁸¹ and continuous cell sorting of healthy and parasite-infected red blood cells based on DEP responses from 50-150 kHz.¹⁸² At frequencies under 30 MHz a flow cytometer-like system has been developed, with the ability to measure individual leukocytes at two discrete frequencies and differentiate different types based on their size and membrane capacitance.³⁷ This approach, however, cannot discriminate phenotypes of cells, and the use of phenotype-specific marker conjugated beads has been proposed in conjunction with low frequency impedance measurements to increase specificity.¹⁸³ Another flow cytometer-like approach shows that under 15 MHz, impedance measurements of erythrocytes and ghost erythrocytes at two different frequencies can distinguish between them based on internal conductivity differences.¹⁶⁸ At frequencies of <2 GHz, where dielectric losses are low and effects of electrode polarisation are insignificant, capacitance-based sensors have showed detection of free flowing live yeast cells and similarly sized polystyrene

spheres,¹⁵⁰ as well as Chinese hamster ovary cells, with dependence of signal amplitudes on the size of the particles,¹⁸⁴ and their viability-dependent DEP properties.¹⁸⁵

Polystyrene microspheres have been used as a higher permittivity contrast target for microwave single-particle analysis for some time; to demonstrate the spatial resolution of sensors and for proof of concept of cell detection devices. Discrimination of single small (4.3 and 5.14 μm) polystyrene spheres at low frequency (~ 1 MHz) based on size has been achieved with impedance measurements,¹⁸⁶ while a similar approach up to 15 MHz shows discrimination between latex microsphere sizes based on signal amplitude as well as speed.¹⁶⁸ Higher frequency approaches have proven capable of detecting stationary single polystyrene spheres between *ca* 5 and 6 GHz,¹⁸⁷ as well as flowing polystyrene spheres in an aqueous environment in the few-GHz range,^{60,184} and even of measuring their dielectric properties at 2-3 GHz with a simple split-ring resonator approach.⁶⁰

However, there has been little reported in the field of higher frequency (>3 GHz) measurement of live, flowing single cells, owing to the difficulty of low contrast between cells and cell medium once the effects of the cell membrane become insignificant. A successful approach at around 5 GHz was able to measure the dielectric properties of single yeast cells, discriminating between viable and non-viable individual cells, however this approach required a DC field for cell immobilisation in the sensing region and suspended cells in DI water rather than cell medium.¹⁸⁸

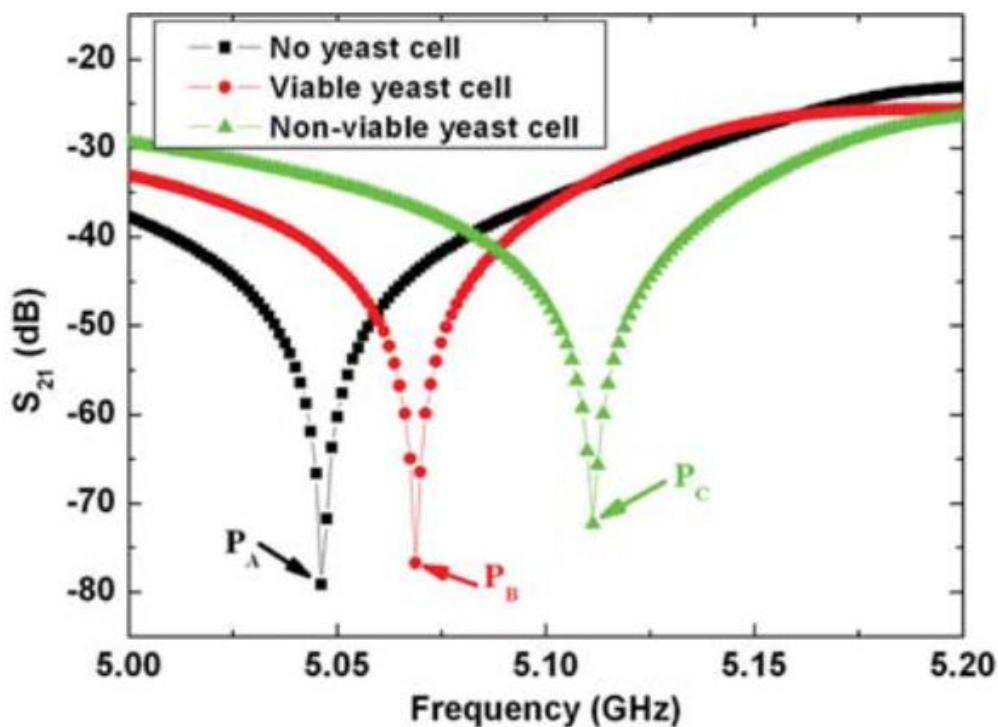


Figure 5.5.1 Measurements of transmission parameters from Yang et al¹⁸⁸ showing the ability of a microwave frequency interferometer system to distinguish between single viable and non-viable yeast cells.

For living cells in their natural environment, high frequency, high throughput, flowing detection remains a challenge. Although single cell analysis must deal with complications arising from heterogeneity within populations or tissues, the ability to characterise on a cell-by-cell basis has clear advantages, with the gold standard technique of flow cytometry yielding information about complex mixtures of cells as well as the ability to separate cells of interest from heterogeneous samples. The application of a flow cytometer-like system based on microwave dielectric properties could offer information different and complementary to that currently used for cell identification. Considering work done thus far it seems reasonable to assume that such a system could offer unambiguous cell identification as well as diagnostically relevant information, and due to the non-destructive nature of the measurements could provide important opportunities for ongoing cell biology research.

5.6. SPLIT RING RESONATORS

SRR-based devices, like other planar metallic structures discussed, effectively confine electric fields to arbitrarily small interaction volumes, and allow potentially high sensitivity due to resonance properties which depend strongly on the surrounding environment. Various SRR geometries have been proposed for sensing applications, ranging from crack detection,¹⁸⁹ to dielectric characterisation of solids.¹⁹⁰

In the field of liquid sensing, various split ring structures have been used for dielectric characterisation of aqueous solutions using microstrip line coupling at a range of frequencies, owing to the high sensitivity of the area around a SRR to changes in dielectric properties,^{191–193} but these approaches suffer problems of low quality factor due to high losses incurred when the liquid samples are placed in high field strength areas. Coupling to a split ring resonator via a dielectric resonator has been shown to dramatically increase the quality factor of a SRR system (~40 fold), enabling accurate measurements of resonance frequency and quality factor but rendering the resonance frequency nearly insensitive to changes in the sample.¹⁹²

For split ring resonators specifically, the sensitivity achievable by confining fields using metallisation has demonstrated single flowing polystyrene microsphere detection and complex permittivity characterisation at ~3 GHz,⁶⁰ and as discussed previously, conjugation of biomolecules directly onto the surface of SRRs gives high sensitivity and selectivity to DNA and proteins,^{64,133} even when high quality factors are maintained through insulation of the metal structure from biological fluids.¹⁹⁴ However, to my knowledge, at present a split ring resonator has not been used for the detection of single viable biological cells.

6. EXPERIMENTAL

6.1. PREPARATION AND MEASUREMENT OF RED BLOOD CELLS AND PLASMA

6.1.1. SEPARATION OF WHOLE BLOOD

Whole blood was taken from volunteers in the morning and processed immediately. Four filled 5 mL acid citrate-dextrose (ACD) coated vacutainers of whole blood were combined into a 20 mL falcon tube and centrifuged at 2500 rpm in an Eppendorf 5804R centrifuge for 10 minutes at 4°C with a low brake. This separates the blood into three layers: the plasma at the top is removed and discarded, the thin buffy layer in the middle is carefully collected using a pipette and decanted into a new falcon tube, and the bottom layer consists of red blood cells (RBCs) which are re-suspended in phosphate buffered saline solution (PBS, VWR). The RBCs are spun down then re-suspended twice more to remove as much of the remaining plasma and anti-coagulant as possible. The collected buffy layer consists of white blood cells (WBCs), platelets, plasma and RBC impurities. In order to get rid of the RBCs the buffy layer fraction is suspended in 3mL ice-cold lysis buffer (155mM NH_4Cl , 10 mM KHCO_3) and left on ice for 10 minutes and the tubes inverted regularly. After 10 minutes the suspension is centrifuged for 5 minutes at 1500 rpm at 4°C in order to stop the cell lysis. The supernatant buffer solution is gently removed using a pipette and the WBC pellet left in the tube is re-suspended in PBS and washed twice more to remove as much as possible of the lysis buffer.

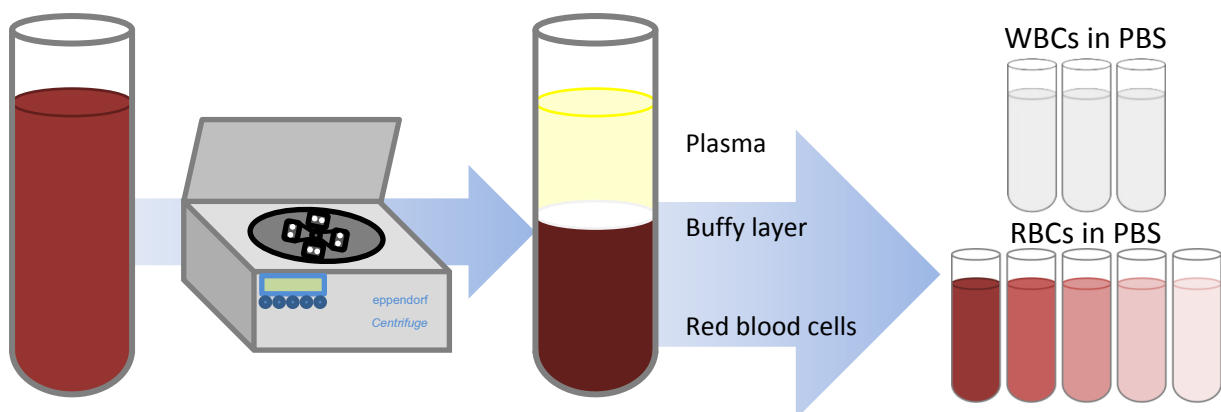


Figure 6.1.1: Schematic showing the process of separation of whole blood into fractions and preparation of suspension of variable WBC and RBC concentrations.

RBC suspensions were made up to ~40% in PBS by eye, to mimic the concentration in whole blood, then fractions were taken out to make up the lower concentrations measured. WBC suspensions were made as concentrated as possible, with the volume of PBS added depending on the size of the pellet. Generally, WBC suspensions were on the order of 50 - 100,000 cells/mL. Lower concentrations of WBC suspensions were then made up by further dilution of fractions. The concentration of cell suspensions in cells/mL was determined using a haemocytometer (C-Chip disposable Haemocytometer, Neubauer improved, labtech.com), with trypan blue added to WBC samples for visualisation and counting. Concentration by volume was calculated using values of cell volume taken from the literature: $9.1\text{e-}14\text{L}$ (90fL) for RBCs and $4.13\text{e-}13\text{L}$ for WBCs.^{195,196} Only the original RBC and WBC suspensions were analysed using the haemocytometer, with the concentration of other suspensions calculated according to their dilution factors.

The use of haemocytometers for ascertaining cell concentration is common practice in the field of life sciences, due to its ubiquity and versatility. However, the method suffers from many sources of error, and the total error can be as high as 30-40% depending on the technique employed. For example, errors can arise from:

- Human error in defining cells
- Volume and dilution errors (usually from pipettes)
- Small measured volume not representative of the overall sample (statistical error) due to non-uniform cell suspensions or very low or high concentrations
- Cells not distributing evenly in the counter
- Suspensions not filling the chamber correctly due to debris or bubbles

The overall error is subject to how the counter is used, the experience level of the user and the concentration of cells. Although a single use, fixed-volume improved Neubauer grid cytometer is used, given the fact that cell and polystyrene sphere solutions are normally measured at relatively low concentrations by a relatively inexperienced user and without cell staining, in this thesis an estimation of the error of average cell count value is estimated from a study as between $\pm 30\%$ and $\pm 50\%$ depending on concentration.¹⁹⁷ This is a very large error, and contributes significantly to uncertainty in measurements carried out on cell suspensions.

Two sizes of variable volume mechanical pipettes available from VWR were used for all liquid volume measurements. A version with a 2-20 μL range and one with a range of 100-1000 μL were used. Both the accuracy and precision of the pipettes is provided by the manufacturer. The accuracy varies from $\pm 0.8\text{-}3\%$, and $\pm 0.6\text{-}1.6\%$ for the smaller and larger pipettes respectively, while the precision of the pipettes is quoted as between $\leq 0.3\text{-}1.5\%$ for the smaller pipette, and $\leq 0.15\text{-}0.4\%$ for the larger pipette. These values depend on the volume measured, with higher values of accuracy

and precision achieved with measurement of larger volumes. To ascertain the total possible systematic error that arises from use of the pipettes, if used correctly and at room temperature, the relevant values of accuracy and precision are added together. In efforts to minimise this error, the largest volume increment possible for dilutions was used to make up the total volume required.

Prepared blood cell samples were measured the same day as collection and disposed of directly afterwards. The maximum time between collection of samples and measurement was on the order of a few hours, ensuring that cells had the maximum viability possible in a stripped down medium. However, the viability of cells was not quantified. For bulk measurements above ~2 GHz the viability of cells is not important, as the overall properties of the suspension will be the same whether or not the cell membranes have lysed. For individual flowing cell measurements a lysed cell will have a different measured response, however, due to the real-time video footage, cells that have lysed can be fairly easily spotted and excluded.

6.1.2. MICROWAVE BROADBAND COAXIAL PROBE TECHNIQUE

The first approach taken to measuring blood cells was a broadband technique using a microwave coaxial probe. For these measurements an Agilent performance probe 85070 (as described in Chapter 3) is used with a HP 8722ET VNA for large-scale liquid measurements over the frequency range of 200 MHz to 40 GHz. The VNA is set up in reflection mode and the probe connected using a coaxial cable with 2.92 mm K-type connectors, specified for use up to 40 GHz. The probe is mounted on a clamp stand so as to hold the whole set-up as still as possible, reducing the effect of mechanical wobbles on the measured results. In order to account for differences in room temperature, connection to and length of the flexible coaxial cables a calibration is done before each measurement, as described in Chapter 3.

Liquid samples to be measured are placed in a small plastic vial with a volume of approximately 5 mL. The probe is then submerged in the liquid under test so that the end of the probe has around 2 cm of liquid underneath the coaxial aperture, to ensure that the evanescent field is distributed only in the sample, and not spreading out of the liquid and interacting with the container or surrounding air, as shown in Figure 6.1.2. Care is taken in all measurements to ensure that the bottom surface of the probe is free of any air bubbles, which would significantly affect measurements. If any bubbles are present, a plastic pipette is used to dislodge them using a stream of bubbles from below.

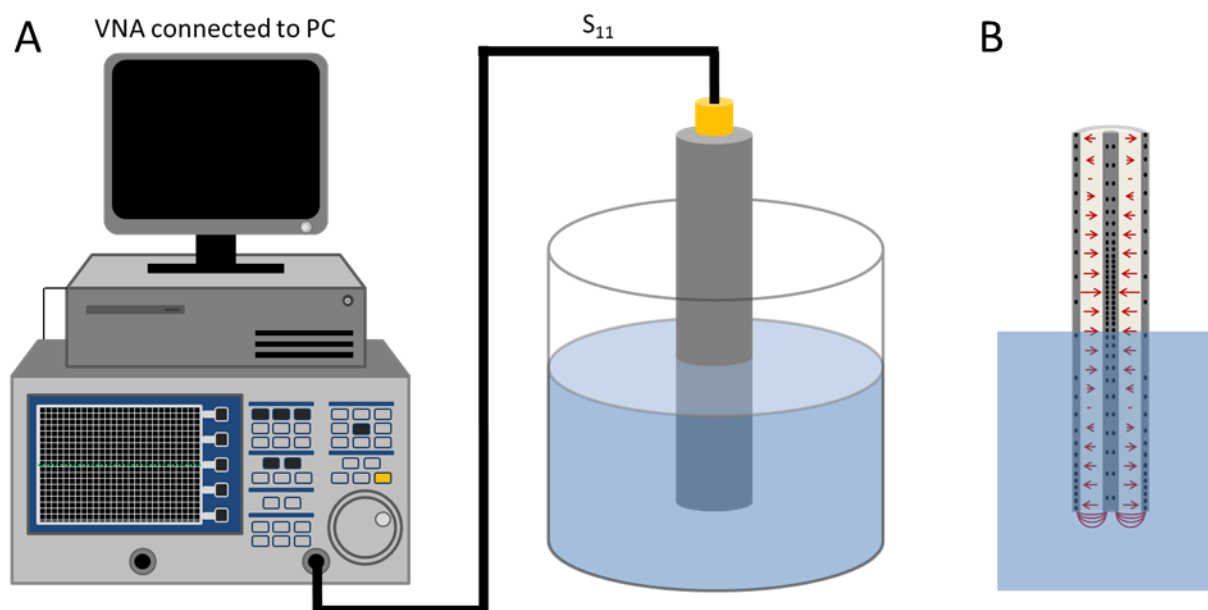


Figure 6.1.2: Experimental set-up using the broadband coaxial probe showing the VNA and coaxial probe immersed in a liquid sample (A). Schematic showing the distribution of the electric field in the coaxial probe in the TEM mode as well as the fringing field in the liquid sample (B).

Measurements of complex permittivity are taken from 500 MHz to 40 GHz on all liquid samples, with a calibration step being performed in between each measurement. Each measurement is performed three times and an average taken of each measurement for the final results. The probe is carefully rinsed with water, and, for biological measurements, sterilised using Virkon, at the end of each experiment.

6.1.3. NARROWBAND DIELECTRIC RESONATOR TECHNIQUE

Single frequency measurements were initially taken using a large dielectric housed resonator inside a microwave cavity. The resonator consists of a 13 mm x 9 mm barium zinc tantalate (BZT, permittivity ~29) cylindrical puck mounted on a Teflon stand encased in a hollow cylindrical copper housing with a lid, fabricated using standard CNC machining .

A holder for a Microfluidic ChipShopTM chip is integrated underneath the lid of the cavity, positioning the channels so that one of them passes directly on top of the resonator at the highest field region of the TE₀₁₆ mode. A ZeonorTM straight channel microfluidic chip consisting of four 200 µm diameter channels in a low permittivity material sealed with a 40 µm film is used in order to bring the sample as close as possible to the evanescent field of the resonator.

The lid of the copper cavity encloses the channel chip, trapping most of the electromagnetic energy within.

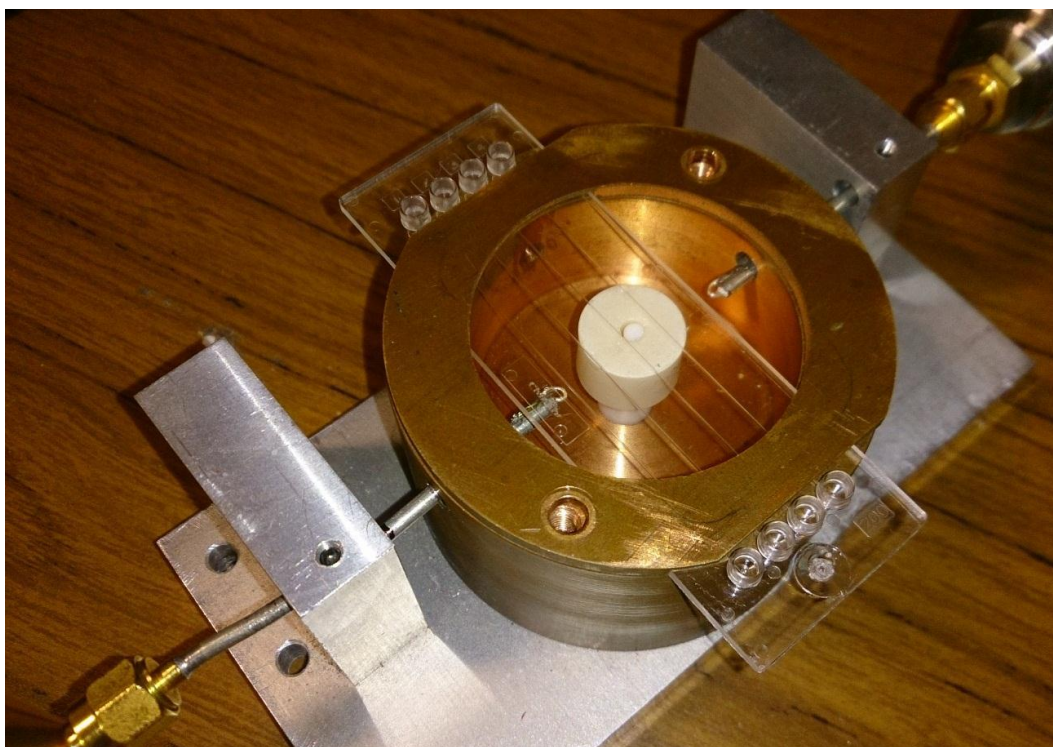


Figure 6.1.3: Initial resonator, shown without lid. The microfluidic chip is held in place when the lid is screwed on through the copper holder into the cavity body, with the microfluidic channels passing directly over the dielectric resonator. Coupling loops can be seen in the sides of the cavity, with supports outside to minimise movement.

The resonator is set up with the HP 8722ET VNA in transmission mode and operated at the resonance frequency of the TE_{016} mode at 4.19 GHz. Large flexible coaxial cables (GORE VNA Microwave/TF Test Assemblies) attached at one end to semi-rigid cables terminated with coupling loops are used to couple the signal in and out of the microwave resonator from the VNA. Coupling loops were made by cutting off the outer conductor and the isolation tube from one end of the semi-rigid cable and subsequent bending and soldering of the inner conductor to the edge of the outer conductor. Coupling loops are inserted into the copper cavity through small holes in the sides and holders for the coupling loops are included in the resonator housing design to avoid mechanical drift effects during measurements.

This resonator was designed for use with Zeonor[®] straight channel microfluidic chips purchased from Microfluidic ChipShop[™]. Chips consisting of four 200 μm diameter channels in a low permittivity material (dielectric constant = 2.3 and loss tangent = 0.0002 @1 MHz¹⁹⁸) sealed with a 40 μm film were used. The resonator includes a holder for the chips mounted between the body of the cavity and the lid. This ensures the microfluidic channels remain still and have a repeatable position in relation to the dielectric resonator.

The commercially purchased microfluidic chips are supplied with tubing and connectors, allowing test liquids to easily be introduced into the channel. Liquids to be measured are pumped into the microfluidic channel using a peristaltic pump and left to flow for a few seconds to ensure the channel is full and there are no air bubbles. In between samples the channel is flushed for several seconds with a blank solution, either phosphate buffered saline (PBS) when cell suspensions are being measured, or DI water when solvent solutions are being looked at. This blank solution is also measured in order to cancel out any mechanical or temperature drift of the system over time. Cell suspensions are measured in concentration order, (more concentrated samples measured after less concentrated ones) so that the lower concentrations are not contaminated by residues left by the more concentrated samples. Measurements are taken in triplicate, with the liquid moved along in the channel in between each. The average of these measurements is used as the final result, to make sure that the measurement is representative of the whole, despite small sample sizes.

During measurements the pump is turned off and the liquid is stationary when measured so that there are no pressure variations affecting the results. The microfluidic channel used with this resonator measures 200 by 200 μm , so only a few microliters of sample are required for each measurement.

For these experiments the quality factor and frequency of resonance are measured using a custom algorithm that fits both the phase and amplitude information of the complex resonant curve.¹⁹⁹

6.2. INDUCTIVELY COUPLED DIELECTRIC RESONATOR - DOUBLE SPLIT RING RESONATOR (DR-DSRR)

The new resonator system consists of a cylindrical dielectric resonator (DR) in a copper cavity aligned coaxially beneath a circular aperture in the lid. A second resonator, a metal double-split-ring resonator (DSRR), is deposited onto a thin glass coverslip and aligned directly over the aperture. A polydimethylsiloxane (PDMS) microfluidic channel is arranged and sealed over the gaps of the DSRR, as shown in Figure 6.2.1.

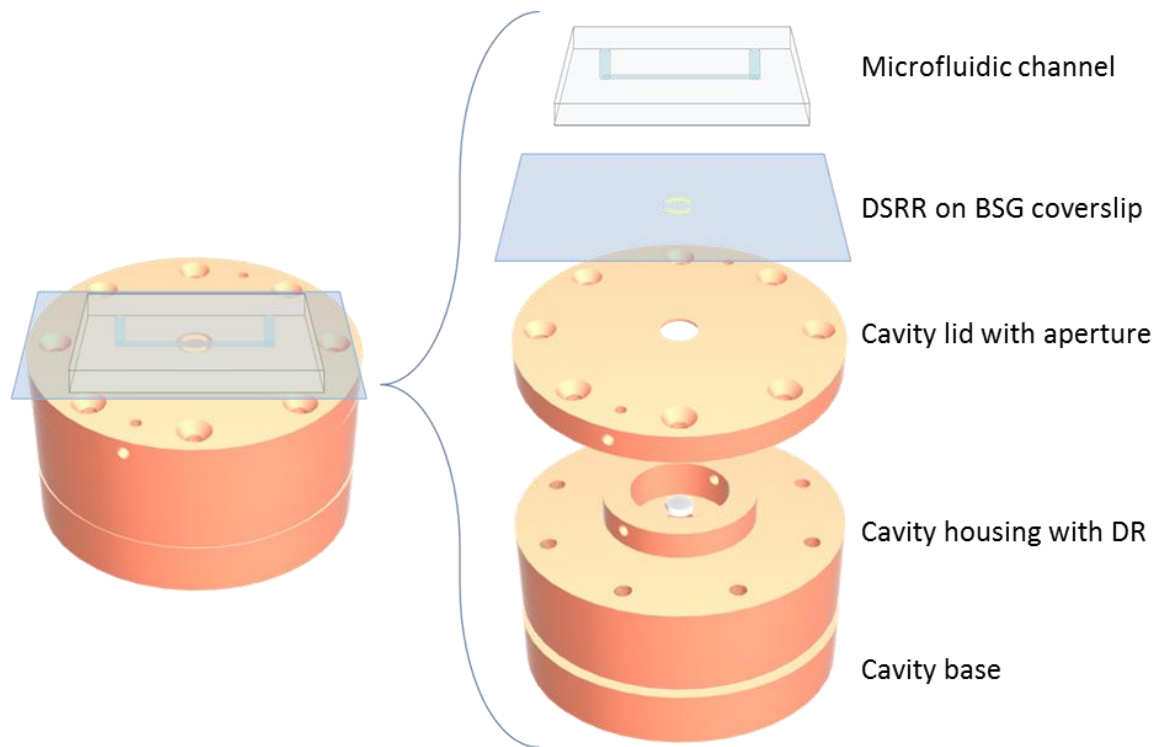


Figure 6.2.1: Schematic of new system showing the assembled sensor on the left, with each component separated and labelled on the right.

6.2.1. FABRICATION AND ASSEMBLY OF RESONATORS

A cylindrical BZT dielectric resonator with a diameter of 7 mm and a height of 2 mm with the TE_{016} resonance at ~ 9.81 GHz was used for the new coupled-resonator system. A fused quartz cylinder acted as a stand for the DR, which was mounted onto one end using superglue.

The copper microwave cavity was first designed using SolidWorks, and the housing then fabricated using CNC machining. The cavity was designed with two main differences to the previous system. The lid of the cavity was tight fitting and did not accommodate a microfluidic chip inside, it had a lip around the edge to keep as much of the EM energy inside the cavity as possible. The lid was made as thin as possible (1 mm) and also had an 8 mm diameter aperture drilled into the centre, to align exactly with the dielectric resonator sitting inside. The bottom section of the resonator was designed in two separate parts: the lower section of the cavity housing and a separate cavity base. Both parts had threaded holes through their centres, to accommodate a screw-mounted quartz stand which would support the dielectric resonator. This allowed the resonator to be moved up and down incrementally inside the cavity by turning the screw. The two-part base formed a jam nut which helped keep the screw-held resonator secure and also extended past the end of the screw, providing a flush surface on the bottom of the system so it could sit level. A hole was drilled into one end of a

cut M6 screw, and a line sawed through the other to be used with a screwdriver. The quartz cylinder was glued into the drilled hole in one end of the screw and inserted into the cavity before the dielectric resonator was glued to the top. This assembly is shown in Figure 6.2.2.

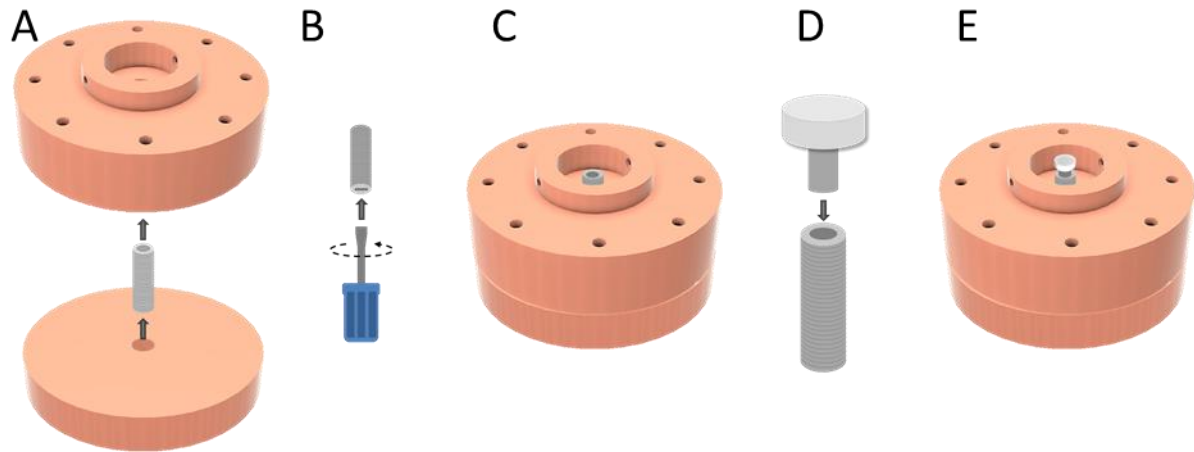


Figure 6.2.2: Schematic showing the alignment of the copper microwave cavity, the screw and the base (A). Bottom view of the screw showing how it is manoeuvred up and down (B) to form the assembled cavity and base (C). Dielectric resonator on a quartz stand inserting into the drilled hole in the screw (D), and how this fits into the assembled cavity and base (E).

The threaded hole in the cavity base allowed the screw to be reached from underneath without having to move the whole system, as shown in Figure 6.2.3. Placing the set-up on a raised platform with a hole in the centre allowed a screwdriver to be used to make small changes to the height of the resonator (and therefore the distance between the resonator and the aperture/DSRR) independently of other variables such as the position of coupling loops or twisting and moving of flexible coaxial cables.

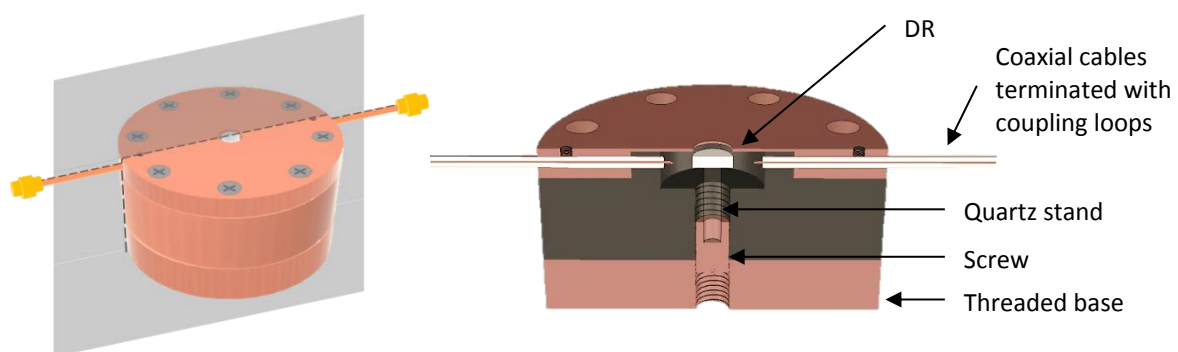


Figure 6.2.3: Schematic of a cross section through the middle of the cavity as shown on the left, showing the DR in the cavity, the screw and quartz stand that can be used to adjust the height of the DR in the cavity, as well as the coupling loops used to excite the DR. In the cross section on the right hand side the body of the cavity is shown in a contrasting colour for clarity.

The walls of the cavity were designed to be quite wide (42 mm), and the holes for coupling loops only slightly wider than the coaxial cables, so that the loops were held securely and would not have

room to move around once put into place. To increase stability of the coaxial cables, large holders were made which held the connectors in place just outside the cavity. The lengths of flexible coaxial cables were held down using tape to minimise mechanical drift. These mechanical control measures meant that once set up, the system could be left between experiments and not move or change significantly, and helped to ensure that results could be comparable even when taken months apart.

The metallic DSRR were fabricated using standard microfabrication techniques. 130 μm thick 22 x 64 mm borosilicate glass (BSG) coverslips are sputter coated with a few nm of chromium as an adhesion layer, followed by copper or gold. For rings a few hundred nm thick the metal is sputtered only, whereas for thicker rings the metal is electroplated after sputtering for thicknesses up to 7 micrometres. After metal deposition the substrates are spin coated with positive photoresist S1813 and exposed to UV through a photomask designed with multiple DSRR geometries. After removal of the uncured photoresist, unwanted areas of metal are etched off by wet etching, followed by removal of the remaining cured photoresist in an organic solvent (acetone and 1165 resist stripper) to produce a range of DSRRs on a substrate.

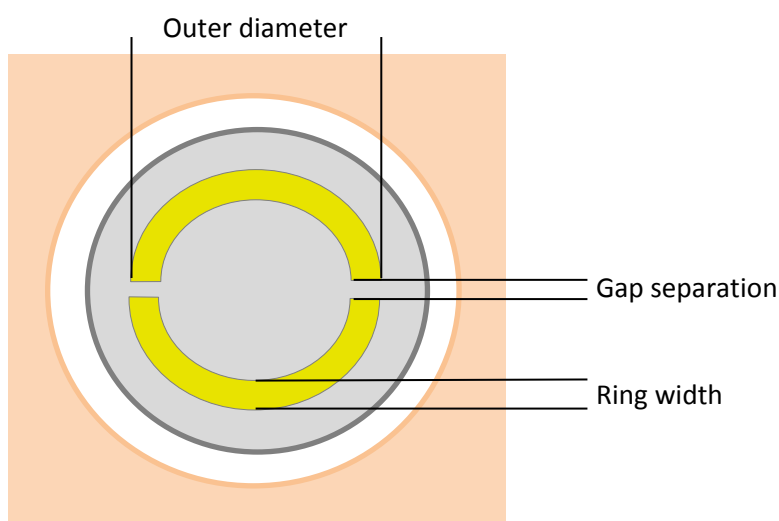


Figure 6.2.4: Schematic of the DSRR (yellow) aligned over the aperture (white circle) and DR (grey circle below), showing the dimensions of the rings which are varied.

Rings with outer diameters ranging from 4-6 mm, gap separations of 0.02-0.2 mm and ring widths of 0.05-1 mm were made (Figure 6.2.4 defines these values). Both gold and copper rings were fabricated, in thicknesses from 200 nm-7 μm . These were to be systematically investigated to find the optimum dimensions of the ring for sensitivity. The outer diameter of the DSRR was constrained by the size of the DR and aperture, while the size of the gaps had to take into account the size of the available channels (the photolithography mask made for ring fabrication was designed with the commercially available microfluidic channels in mind, because a protocol for creating microfluidic

channels in-house had not yet been developed), as well as aim to keep the interaction volume small enough to achieve single cell resolution and large enough to allow cells to pass and significantly load the coupled resonator system. The thickness of the rings was increased once PDMS open channels were used in order to increase the interaction volume between the electric field and the liquid; however, this had to be balanced with the ability of the PDMS to conform around the DSRR and seal. When in-house production of PDMS channels became possible, DSRRs and channels were paired such that channels could cover completely the gap area and encompass the edge of the metal, allowing the electric field to penetrate the liquid. The alignment of the DSRR on the BSG coverslip over the DR and aperture is shown in Figure 6.2.5.

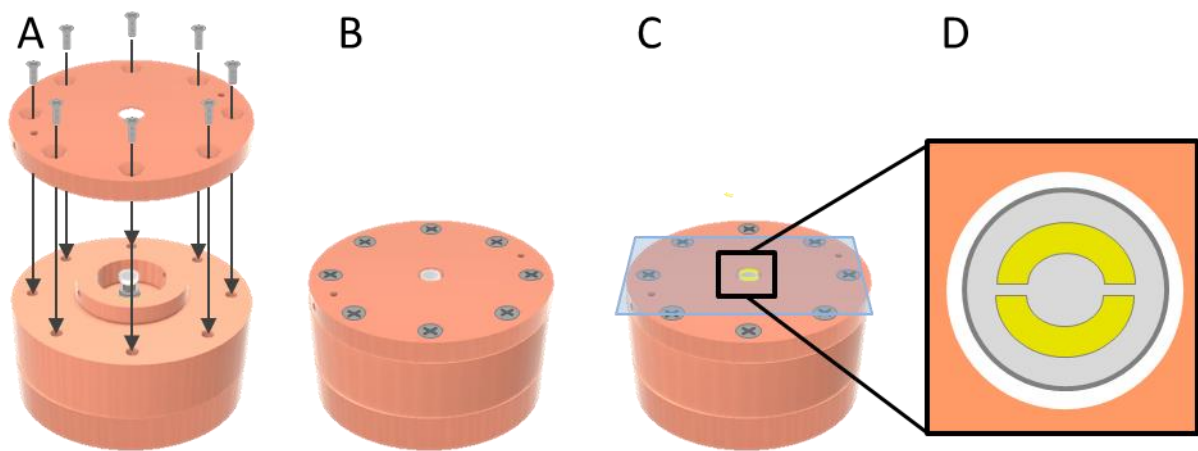


Figure 6.2.5: Schematic showing the attachment of the lid to the copper microwave cavity (A and B), as well as the alignment of the borosilicate glass substrate-mounted DSRR with the aperture in the lid and DR below (C and D).

The DSRR was designed with two gaps due to the results of early simulations which showed that with only one gap, the symmetry of the DR TE_{016} mode was significantly disturbed when loaded by the asymmetric ring, and simulations predicted a higher sensitivity to small permittivity perturbations for the SRR with two gaps. The presence of two gaps rather than one has also allowed for the loaded resonance frequency of one of the DSRRs fabricated to be close enough in frequency to that of the DR, resulting in the high sensitivity to be reported later, as explained in Section 7.2.2.

6.2.2. OVERVIEW OF THE SYSTEM

6.2.2.1. INTER-RESONATOR COUPLING

The alignment of the circular DSRR coaxially above the DR over an aperture allows inductive coupling of the two resonators. As in the previous resonator the TE_{016} mode of the DR is excited via coupling

loops carrying the signal to and from a VNA. As discussed in Chapter 3, this mode consists of a circularly symmetric electric and magnetic field distribution, as shown in Figure 6.2.6. Due to the circular shape of the aperture and the DSRR, when the DR is placed directly underneath the DSRR, the circularly symmetric magnetic field is able to protrude through the aperture in the 1mm thick cavity lid and induce currents in the two halves of the DSRR. The new system uses a smaller dielectric resonator and therefore operates at a higher frequency, around 9.8 GHz.

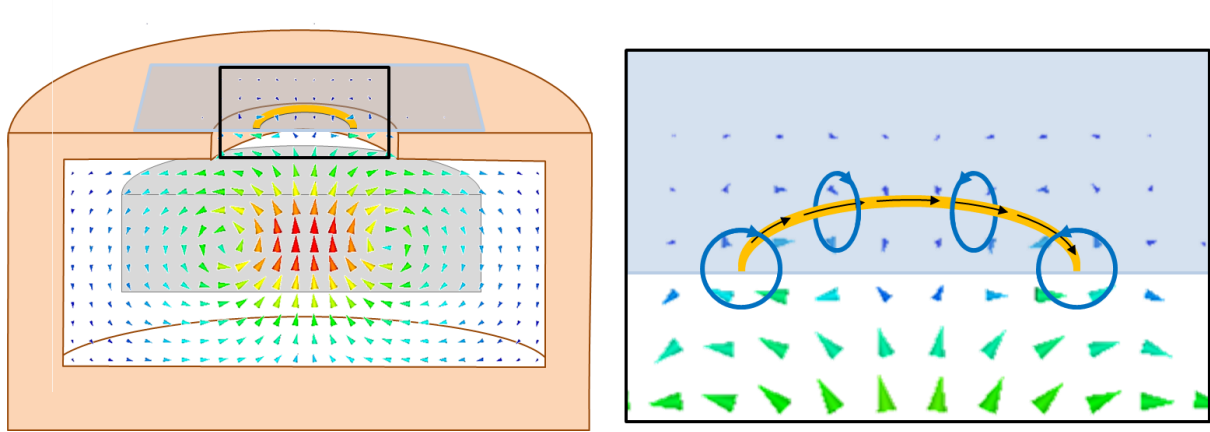


Figure 6.2.6: Schematic of a cross section through the cavity and DR, showing the magnetic field of the DR protruding out of the aperture and inducing a current in the metal of the DSRR.

The lid of the cavity is only 1 mm thick, allowing the DR to be placed very close to the DSRR. The screw-mounted DR allows the distance between the two resonators, and therefore the strength of the coupling between them, to be varied and controlled.

With the right configuration, the currents set up in the DSRR move in the same direction, resulting in a build-up of charge across the gaps and producing an electric field, as shown in Figure 6.2.7. The spatial confinement of the field can be controlled via the design and fabrication of DSRRs with different geometries.

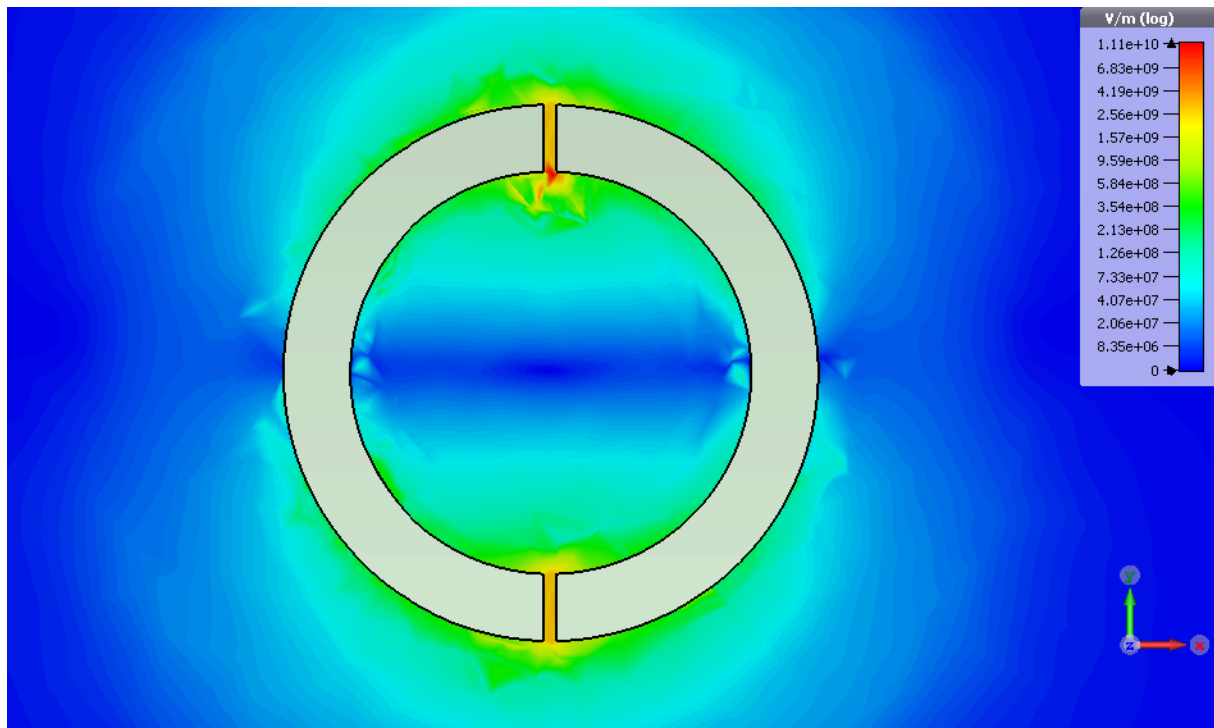
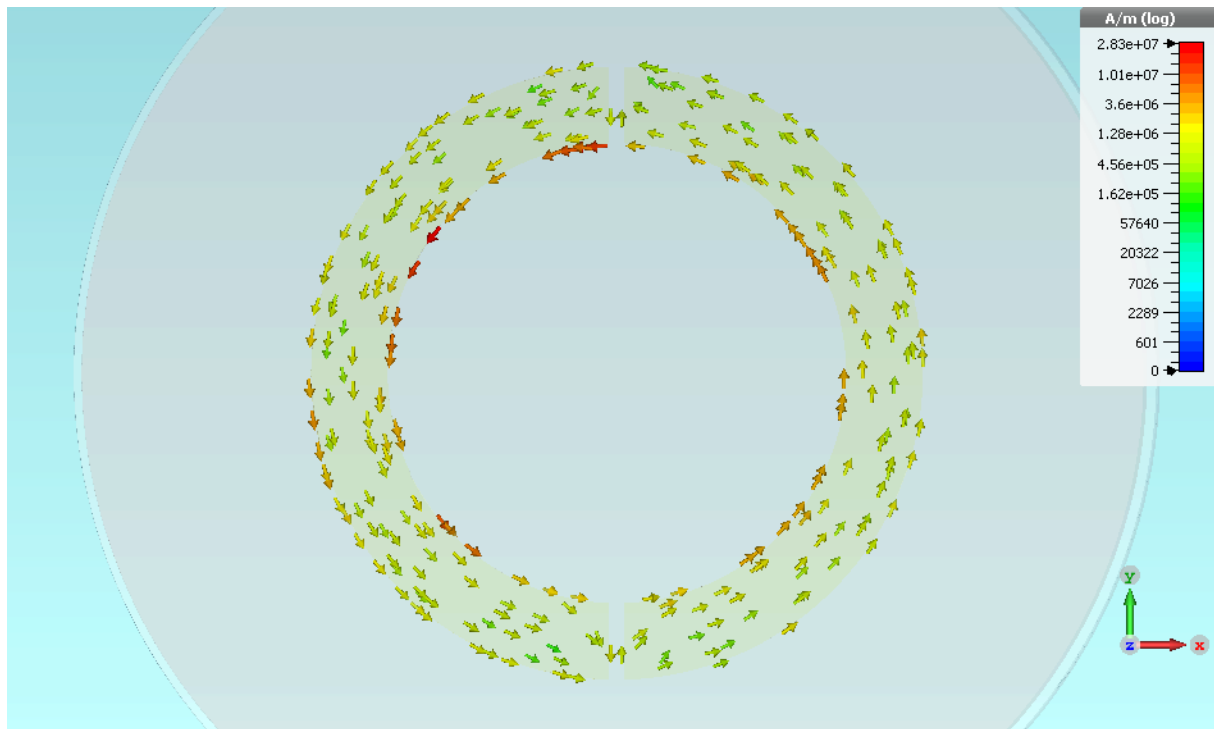


Figure 6.2.7: Simulations of the DSRR. Left: the surface current of the DSRR induced by the DR, showing the propagation of current clockwise in both halves of the ring. Right: the electric field the currents result in, showing the strongest field in the gaps between the two halves of the ring. The strength of the electric field is plotted as a colour map. Both cross sections are taken at the upper surface of the DSRR.

The system of indirect coupling to the DSRR sensing element using the DR, rather than direct excitation via e.g. a microstrip, also offers other unique advantages. By utilising existing DR

technology and expertise in the group, the difficulty and expense of on-chip microwave frequency connectors and circuitry was avoided, and the microwave components could be separated entirely from any biological or other liquid samples interacting with the DSRR, opening the possibility of cheap, disposable chips. The DR offered temperature stability and high quality factor operation at high frequencies and with high loss liquids in the sensing regions, giving the new system a relatively high quality factor even for water-based samples (water-loaded quality factor = ~ 1600). In this way, high quality factor measurements could be carried out at a sufficiently high frequency for the electric fields to overcome Maxwell-Wagner screening effects and interact with the contents of biological cells, producing a sensor dependent on cell content rather than size.

6.2.2.2. INTEGRATION OF MICROFLUIDICS

With a highly confined electric field, the sample needs to be brought as close as possible to the sensing region. In order to achieve this, custom microfluidics are made in-house as described in Section 6.2.3. The PDMS channels are specially aligned and sealed directly on top of the capacitive gaps, so that the channel passes directly over the edges of the metal in the gaps, as shown in Figure 6.2.8. In this way the liquid sample can be flowed directly through the high field region of the sensor, for maximum sensitivity.

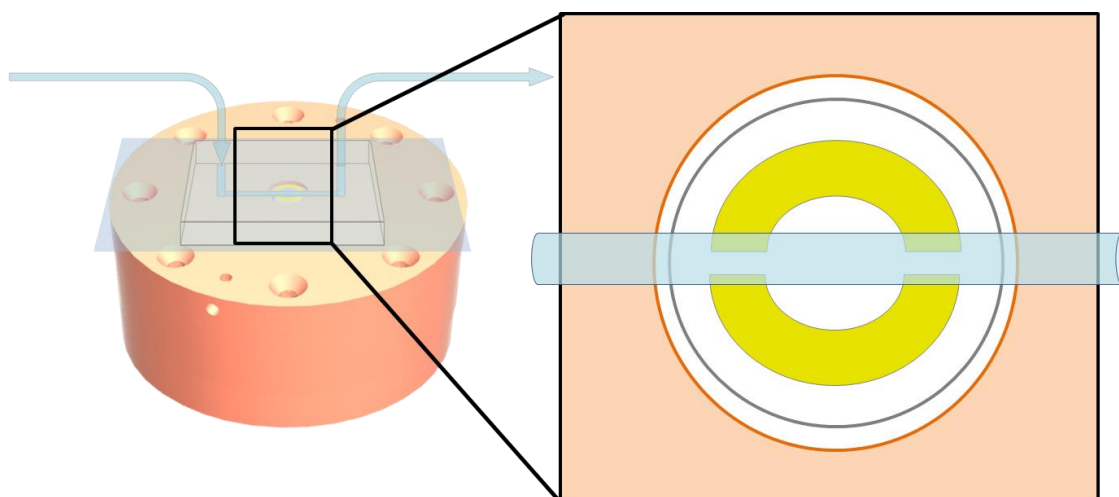


Figure 6.2.8: Schematic of the assembled coupled resonator system (left) and expanded view of the alignment between the DR (white circle with grey edge), DSRR (yellow split ring) and aperture (orange edged circle), as well as the alignment of the microfluidic channel (light blue cylinder) across the two gaps in the DSRR.

Although microfluidic channels with particularly thin coverslips ($50\ \mu\text{m}$) had been used with the previous resonator system, simulations showed that the sensitivity of the new system could be increased by bringing the liquid samples into direct contact with the DSRR and the gap, by increasing the strength of the electric field interacting with the sample, as can be seen in Figure 6.2.9. By using

open channels and sealing them directly onto the coverslip and DSRR, the electric fields were able to penetrate more fully the volume of liquid, resulting in a ~600-fold increase of maximum electric field strength in the sample according to CST MICROWAVE STUDIO® (CST® MWS®). This drastic increase may also be due to the change in loading of the DSRR resulting in a significant shift of its resonance frequency, as will be discussed in Section 7.2.2.6, which would have the knock-on effect of changing the distribution of energy between the two resonators. The distribution of the field in the gap can be seen to be approximately the same in both channel conditions in Figure 6.2.9, showing that moving the channel further away from the gap will still result in a decrease of field strength. In-house production of microfluidic channels also provided the flexibility to arbitrarily vary the channel dimensions to optimise the sensitivity while avoiding blockages.

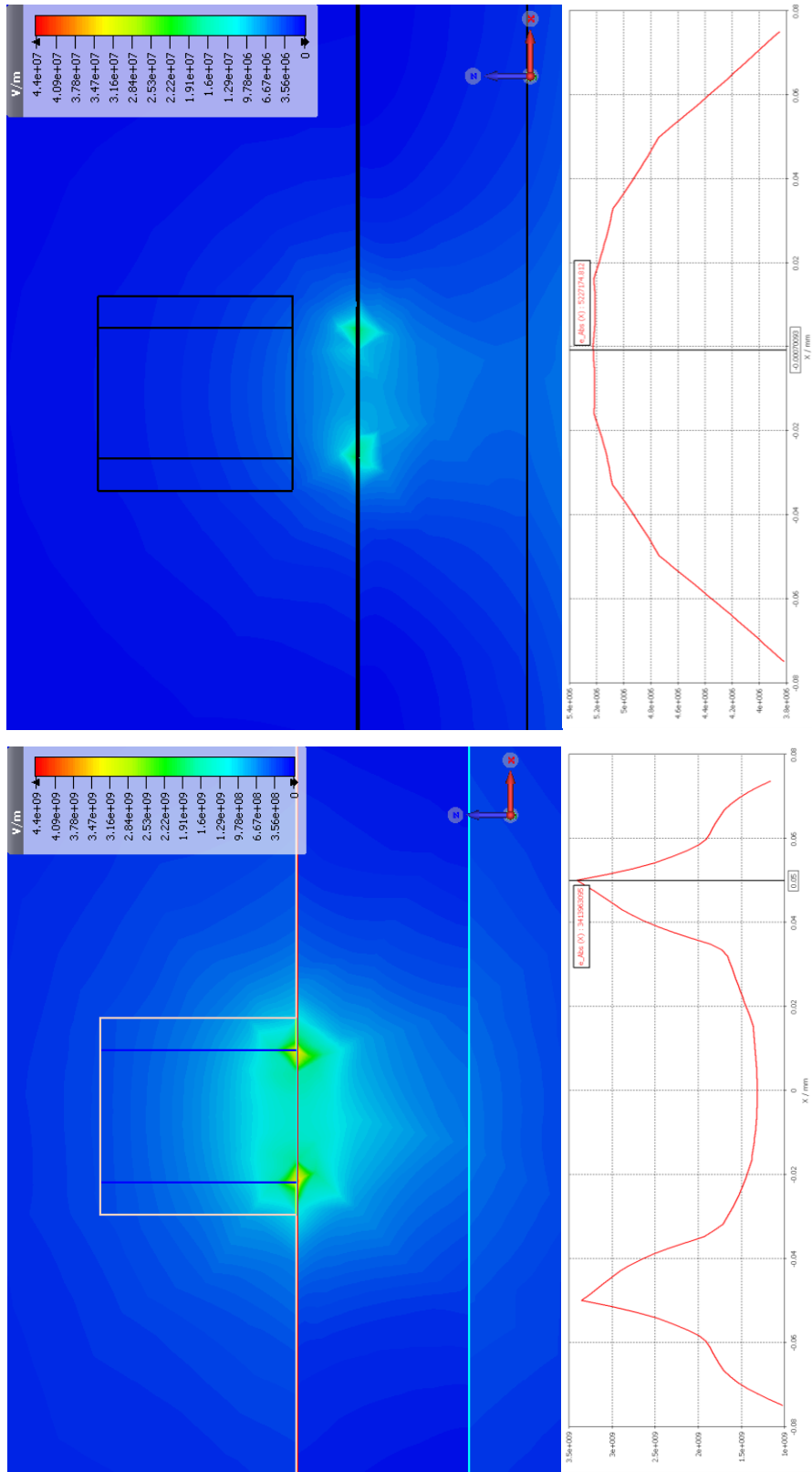


Figure 6.2.9: CST MWS simulation results of two experimental configurations: on the left the channel in direct contact with the metal of the DSRR, and on the right the channel separated from the DSRR by a polymer seal. In all other respects the simulations are identical. The top left shows a cross section through the middle of one of the capacitive gaps of the custom PDMS channel configuration, showing the rectangular cross-section of the channel directly over the edges of the DSRR. The strength of the electric field in the channel is plotted as a colour map. The top right shows the same cross section of the other configuration; the channel separated from the edges of the DSRR by a 50 μm coverlid. The strength of the electric field is again plotted as a colour map, but with a scale more than 100x lower. The bottom two graphs show the variation in field strength across the bottom of the channel from one side to the other (left to right in the top cross sections), crossing the capacitive gap, at the mid-point of one of the gaps, for both configurations. Units for electric field strength in all plots are V/m.

6.2.3. IN-HOUSE PRODUCTION OF POLYDIMETHYLSILOXANE (PDMS) MICROFLUIDICS

Since the commercial channel chips originally used separated the DSRR from the liquid with a plastic coverslip, and simulations showed this resulted in a significant decrease in field strength within the liquid, it was important to ensure that the liquid under test was brought as close as possible to the surface of the metal in order to optimise the sensitivity of the device. In order to achieve the maximum perturbation of the capacitance by the liquid and therefore the DSRR resonance, custom microfluidic channels were made in-house and sealed directly on to the BSG coverslips so that the channel was in direct contact with the metal surface.

Microfluidic chips were made using RTV 615 A + B PDMS, a two-part curing soft siloxane polymer commonly used as a potting agent and for microfluidic applications, purchased from Circuit Specialists Europe Ltd. This consists mostly of long chain vinyl-containing polydimethylsiloxane, vinyl-containing silicone resin, methyl hydrogen-siloxane, a small amount of solvent (benzene/toluene) and a catalyst (chloroplatinic acid). In order to make a hollow channel a negative was used to cast the PDMS around. Some channels were moulded using micrometre-scale glass capillaries before 3D printed negatives were made, which offered a more well-defined geometry.

RTV 615 requires a ratio of 10:1 of parts A:B, with part A being the vinyl-containing resin, and part B the hydrosiloxane copolymer cure. Experiments were done varying this ratio to obtain the best conformation and sealing to a glass slide. It was found that a ratio of 12:1 gave a fully cured but very elastic polymer which conformed very well over even thick rings and sealed quickly to be watertight.

The moulding process is shown in Figure 6.2.10. Channel negatives are placed inside a mould and coated in mould release spray, before the liquid PDMS A+B pre-polymer mixture is poured on top and left to cure over a couple of days (Figure 6.2.10, **A**). Once solid, the PDMS is removed from the mould and the section of the PDMS with the channel imprint is cut out and the rest is discarded (Figure 6.2.10, **B**). The resulting PDMS channel is a solid rectangle with the imprint of the negative forming a straight and hollow channel down its centre (Figure 6.2.10, **C**). A biopsy punch is then used to punch out small vertical cylinders of PDMS at the beginning and end of the channel to allow tubing carrying liquid samples to be connected to the microfluidic chip (Figure 6.2.10, **D**). The cylinders of PDMS excised are the same outer diameter as the tubing used with the pumping system, and therefore no special interface is required, standard microfluidic tubing can simply be inserted into the holes in the PDMS chip. The completed PDMS channel is then rinsed in acetone or isopropyl alcohol then water and sealed onto the DSRR glass coverslip and aligned over the gap. A droplet of water is pipetted onto the glass before the PDMS is lowered on top and forms a thin film between

the surface of the glass and the PDMS. This thin film allows the PDMS to slide easily over the surface of the glass and using an optical microscope it can be manipulated so that the channel passes over both gaps in the DSRR. The assembly is then left to dry, and as the water evaporates the surface of the PDMS is pulled into close contact with the glass and strong hydrogen bonds are formed at the interface, sealing the channel (Figure 6.2.10, E).

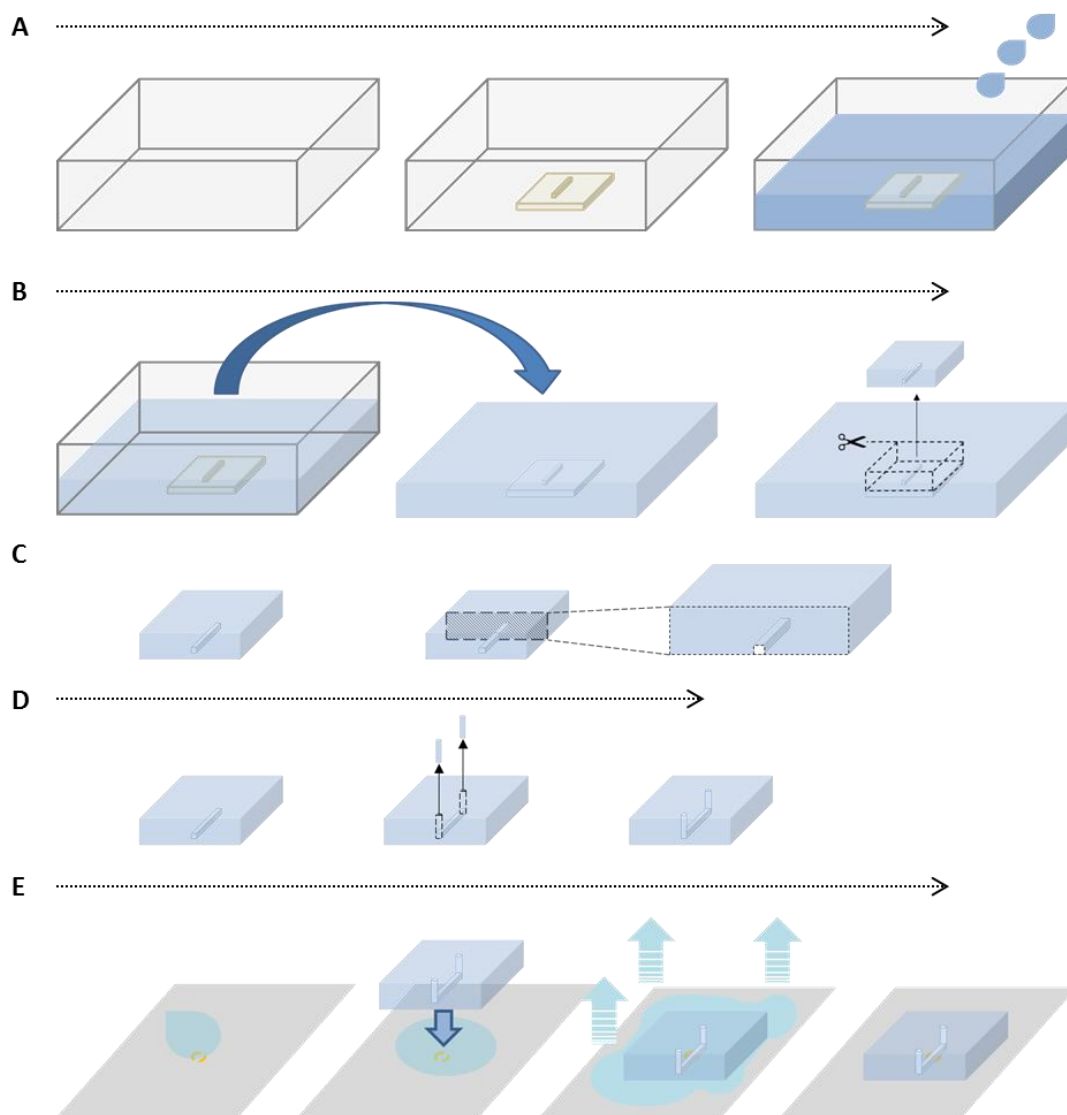


Figure 6.2.10: Schematic showing the process of fabricating PDMS microfluidic channels. The negative of the channel (in this case a 3D printed mould) is placed in a clean, shallow container and mixed RTV A and B PDMS pre-polymer is poured over the mould (A). This is left to cure for several days before being removed from the container and the excess PDMS removed (B). This leaves a PDMS block with an open channel in the bottom (C). A biopsy punch is then used to excise vertical cylinders of PDMS at each end of the channel to act as inlet and outlet holes (D). The cleaned PDMS channel is then aligned over a DSRR using a drop of water as lubricant under a microscope, then left to dry, temporarily sealing the PDMS to the glass substrate of the DSRR and creating a watertight channel passing through the gaps (E).

The microfluidic channel was designed to be wider than the gaps in the DSRRs, to ensure that the liquid was not screened from the electric field by excess PDMS covering the metal. The overall size of

the PDMS block was made to be large enough to produce a good seal with the glass, but not so large as to significantly hang over the edge of the DSRR substrate. PDMS chips therefore were mostly around 2-3 x 3-4 cm in size, with channels of around 2-3 cm long and around 200-400 μm wide.

6.2.4. SOLUTION MEASUREMENT SET-UP

Before real time-measurements of flowing cells and particles were conducted, measurements similar to those done with the previous resonator sensor were carried out, in order to characterise and systematically investigate the new system. Solutions of ethanol in deionised water were made up in concentrations varying from 0-100%, with more low concentration solutions than high concentration, in order to allow better resolution data in the high permittivity regime, in which the system will have to have high sensitivity for single cell measurements to be possible. These solutions were used to ascertain the sensitivity of the system to changes in permittivity of the material in the capacitive gaps. The permittivity values of these solutions at 10 GHz were taken from the literature.^{126,200} All solutions were made up using the two variable volume mechanical pipettes.

The system is set up as in Figure 6.2.11. Coupling loops were made from semi-rigid cables as described in Section 6.1.3. As before, these cables were attached to one end of the large, flexible coaxial cables (GORE VNA Microwave/RF Test Assemblies) via SMA adaptors, while the other ends of the flexible cables were connected to the VNA ports. The coupling loops were inserted into the holes in the sides of the assembled resonator system and adjusted for coupling to the DR in transmission mode. The plane of each coupling loop was adjusted to be oriented perpendicular to the DR axis in order to achieve optimum coupling to the $\text{TE}_{01\delta}$ mode, as described in Chapter 3. The heavy VNA cables were fed through holes in large, steel support blocks specially made for the purpose, with screws to hold the cables steady.

Microfluidic tubing is inserted into the inlet hole in the PDMS channel sealed over the DSRR. Hand-held syringes are then connected to the tubing using standard microfluidic connectors and solution pumped into the channel by hand. Measurements of frequency and quality factor, or amplitude and phase are taken for each solution and repeated three times, with the solution pumped through and left for the pressure to equilibrate between each repeat. Measurements of blank solutions of DI water are made in between every ethanol solution in order to cancel out effects of temperature or mechanical drift. Again the quality factor and frequency of resonance are measured using a custom algorithm that fits both the phase and amplitude information of the complex resonant curve.¹⁹⁹

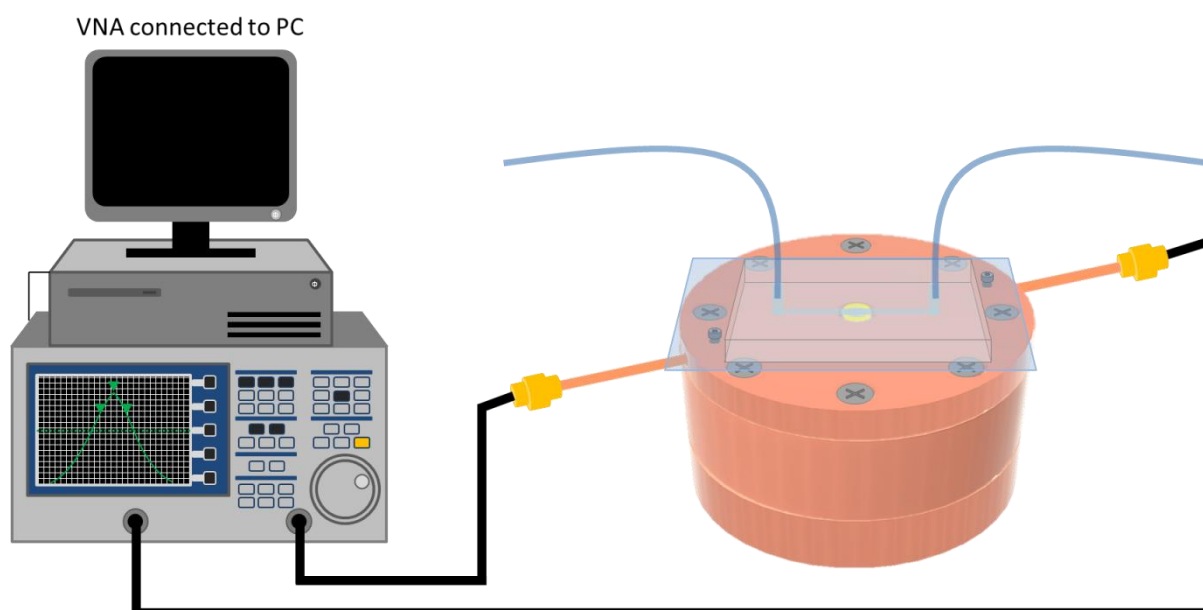


Figure 6.2.11: Schematic of measurement set-up showing the VNA connected to the coupling loops via coaxial cables (black), and the microfluidic tubing connecting (blue) with the PDMS microfluidic channel. The holders for the flexible coaxial cables are not shown.

For experiments done on several different rings, the same channel was used each time and re-sealed over each ring being investigated. Efforts were made to keep the channel positioning as consistent as possible, but it is not possible to ensure this to any great degree of accuracy.

6.2.5. REAL-TIME SINGLE PARTICLE/CELL MEASUREMENT SET-UP

Measurements of single flowing cells and polystyrene spheres were carried out using a real-time monitoring set-up. This set-up, as described below, allows for visual recording of cells or particles whilst simultaneously collecting either resonance frequency and quality factor or phase and amplitude data in transmission mode at resonance from the VNA.

Equipment for real-time experiments is shown in Figure 6.2.12 and consisted of:

- The DR in the microwave cavity on a custom stand with the DSRR/PDMS microfluidic channel assembly aligned over the aperture in the lid.
- A Rohde & Schwarz ZVA 24 VNA with flexible coaxial cables connected to semi-rigid coaxial cables terminating in coupling loops inserted into the microwave cavity so as to excite the TE_{016} resonant mode of the dielectric resonator.
- An Elveflow OB1 pressure pump connected to a nitrogen gas cylinder providing the driving force for fluids contained in small reservoirs. These vessels were connected directly using standard microfluidic 1/16" OD PTFE (polytetrafluoroethylene) tubing to the inlet of the PDMS microfluidic channel.
- An optical microscope set-up with a low-magnification, high working distance microscope objective (from Dolomite Microfluidics) focussed on one of the capacitive gaps, with the images picked up using a high speed CCD camera capable of capturing up to 150 fps.

Control of the pumps, VNA and camera feed is all done using a computer. A MATLAB script automates simultaneous triggering of image capture from the camera and signal sampling from the VNA.

The signal and video feed are displayed in real time. This means that the positions of cells can be manipulated through adjustment of the pressure pump controls in order to obtain multiple measurements of the same cell, as well as to ensure that all measurements are of single cells rather than multiple cells. Collecting the data this way also allows later analysis to reveal information about the relationship between the signal recorded and the size of the cell or the path it takes through the sensing region. Data such as this could be used to normalise signals to cancel out these effects and extract differences in responses which arise directly from differences in internal composition between cells.

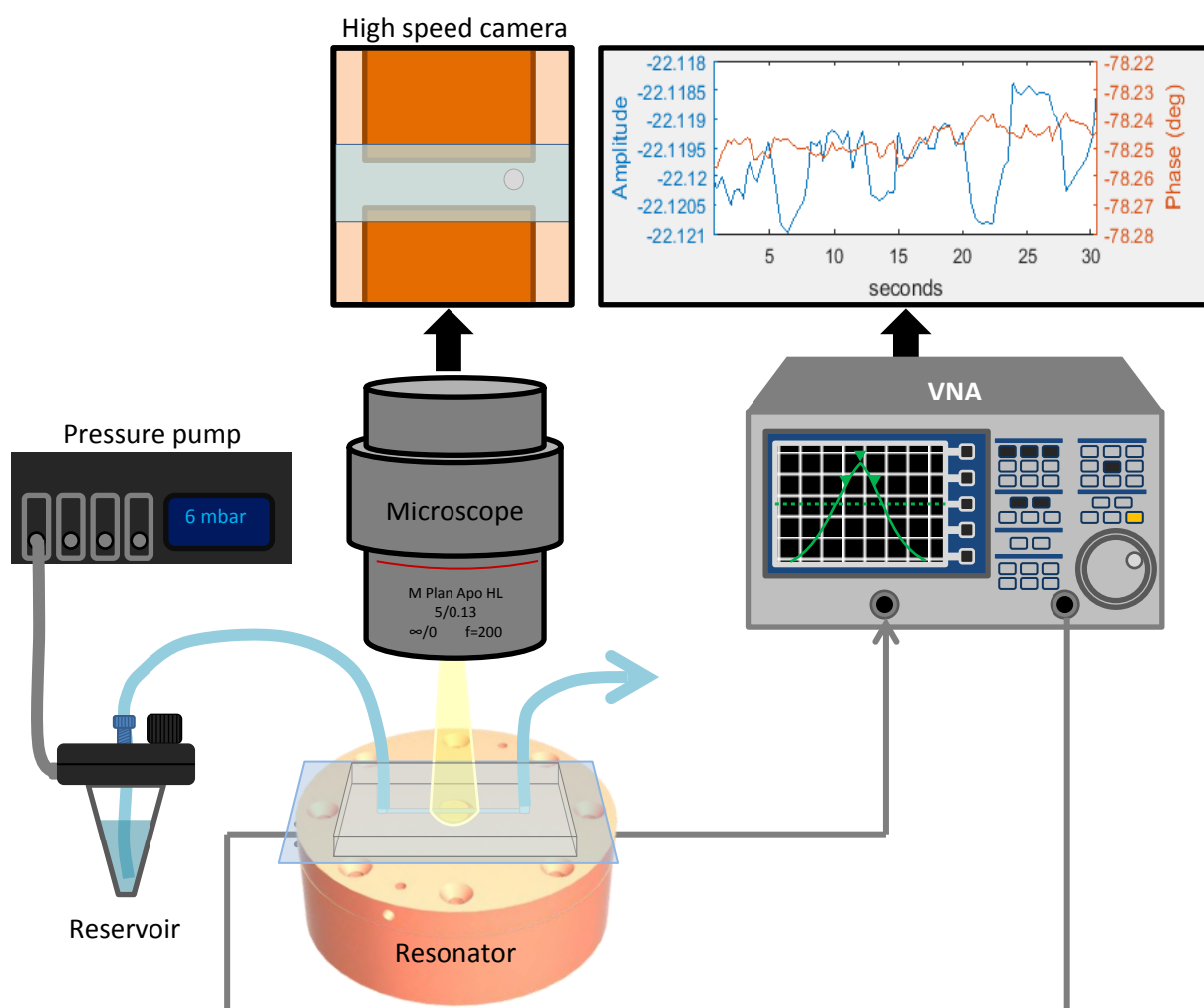


Figure 6.2.12: Schematic of the real-time experimental set-up, showing the pressure pump connected to the microfluidic channel. The microscope and camera set-up is trained on a gap of the DSRR, while the VNA is locked into the resonant frequency and records amplitude and phase as a function of time.

For all measurements, the samples and blank background solutions (either DI water in the case of polystyrene microspheres (PMs) or PBS in the case of cells) were kept at room temperature beforehand to ensure that all measurements were at approximately the same temperature. PBS had to be removed from the fridge a few hours before an experiment to ensure that its temperature fully equalised with the environment.

Some time before measurements are to begin the system is set up and allowed to settle for a while, to ensure that any mechanical movements, air bubbles in the channel, or temperature effects are given time to equilibrate or dissipate. Blank background solution is pumped through the channel for several minutes and the resonance parameters monitored until no significant change (<0.01 dB and $<0.1^\circ$ per few tens of seconds in amplitude and phase) is seen over time.

For the most accurate results the VNA is operated in CW mode at the resonant frequency of the liquid-loaded resonator system. For all measurements the difference between the suspended particles and the solution they are suspended in is measured. Therefore the system's unperturbed state is defined as that in which the background suspending solution (either water for ethanol solutions, or PM measurements, or PBS for cell suspension measurements) is loading the DSRR. To ensure maximum contrast in signal upon the passage of a particle across the gaps, the sensor needs to be operating at the exact frequency of the peak of the TE_{016} mode resonance in this unperturbed state. Consequently, before (and several times at intervals during) measurements, a high resolution frequency sweep of the sensor with only the blank background solution filling the gaps was carried out. The peak resonance was recorded and used to set the frequency of the VNA when put into CW mode for measurements. The deviation of the transmitted signal around the resonance peak is then measured as changes in amplitude and phase.

Once the system has been set up and calibrated with the blank solution, the samples are introduced to the sensor. The camera is used to track samples through the channel without recording, until a suitable cell is found for measurement. Once a cell is found that can be clearly seen and controlled using the pumps, the MATLAB script is employed to trigger simultaneous optical recording of the cell passage and digital recording of the signal displayed by the VNA. These are recorded once every 0.2 seconds at (approximately) the same time, and a video file of the cell in the gap as well as a text file of the amplitude and phase of the signal along with a time stamp are generated.

Cells under control are passed multiple times over the sensing region by manipulation of the pressure. The pressure pumps are controlled with a computer interface which allows incremental

changes of the pressure to be made. This measurement process is repeated on as many individual cells as possible to build up as accurate a picture of that cell population as possible.

Although the technical specifications of the VNA list the absolute accuracy of the VNA to 0.01 dB, for this type of relative measurement the accuracy is not quoted by the manufacturer. The stability of the trace is quoted in the technical datasheet of the VNA as typically 0.001 dB at a 0 dBm source power and an IFBW (intermediate frequency bandwidth, which corresponds to the inverse of the integration time for single frequency measurements) of 1 kHz, as measured over the course of one hour. For continuous wave measurements taken of PMs and cells, an IFBW of 10 Hz was used, with measurements of background and target taken a few seconds apart with the collection of data for each individual target taking generally less than a minute, up to a couple of minutes maximum. Therefore, a much higher stability for these measurements than the quoted 0.001 dB is assumed, and indeed the standard deviation for background measurements across biological cells does not exceed 0.0004 dB. In the absence of a known absolute precision for these measurements, all average amplitude and phase values are quoted to a significance level of no more than their standard deviation.

6.2.6. PRESSURE PUMPS

Initial experiments used syringe pumps and peristaltic pumps; however, the continuous flow and quick response time offered by pressure pumps made control of individual particles and cells much easier.

In order to achieve a smooth flow profile so that particles could be accurately controlled in the sensor, a pressure pump was purchased from Elveflow (OB1 MK3, 0-200 mbar). Pressure pumps use very accurate piezoelectric valves to finely control pressure from a source (e.g. compressor, gas cylinder) and use it to steadily push fluids from a reservoir, as in Figure 6.2.12. This results in a highly steady flow, with none of the bumps or steps associated with peristaltic or syringe pumps. Since water is not compressible, the lead time for changes in pressure is small, and the flow is easily varied by changing the pressure. This steady and easily manipulated flow of liquid allows a high degree of control over the positions of suspended particles, even on the micro scale.

The pressure pump is connected to a small, air-tight sample vessel which is partially filled with the liquid to be tested. The vessel is sealed with a lid which keeps it air tight and also holds PTFE microfluidic tubing and the connection to the pump. The pressure from the pump forces nitrogen into the vessel, pushing the liquid up and out of the vessel and through the tubing sealed into the lid of the vessel. PTFE tubing with an inner diameter of 500 μm and a standard outer diameter of 1/16"

was used to connect the sample vessel with the microfluidic channel via the holes punched through the PDMS to the channel. Clamps were used to hold the tubes still during experiments to avoid any vibrations or sudden movements due to pressure changes or the movement of liquid in the tube. Tubing was initially connected in the same way to the outlet of the PDMS chip but this set-up caused problems: the large flow resistance caused by the outlet tubing required high pressure and associated high flow rate to push the fluid out of the outlet. In order to flow the samples slowly enough to witness particle or cell passages, the outlet tube had to be removed. In this set-up, sample liquid pooled at the top of the punched hole in the PDMS and was absorbed with Kimtech wipes and disposed of. For fine control of cells and particles in small microfluidic channels, low flow rates are required, so pressure pumps operating from 0 to 200 mbar were chosen to provide the highest pressure resolution at low absolute pressures.

6.3. PREPARATION AND MEASUREMENT OF POLYSTYRENE MICROSPHERES

Polystyrene microspheres (PMs) were purchased from Polysciences Inc in 25 μm and 11 μm average diameters, suspended in DI water at a concentration of 2.5 % by weight ($\sim 2.91\text{e}6$ particles/mL for 25 μm spheres and $\sim 4.55\text{e}7$ particles/mL for 11 μm spheres). The mean and standard deviation of the diameters of the spheres, as measured by the manufacturers, is quoted as $24.9\text{ }\mu\text{m} \pm 0.77\text{ }\mu\text{m}$ and $11\text{ }\mu\text{m} \pm 0.25\text{ }\mu\text{m}$. For measurements, small amounts of the stock solution are diluted with further DI water, using mechanical variable volume pipettes to achieve a concentration between 50-100,000 particles/mL. The concentration was determined using a haemocytometer (C-Chip, Cronus Technologies), to ensure that the particle solutions were neither too dilute nor too concentrated for individual flowing measurements. The preferred concentration range was determined via trial and error, with overly dilute suspensions resulting in long waiting times between particle passages or excessive particle speeds, and very concentrated suspensions either causing blockages or rendering single particle measurements impossible due to the number of particles in the capacitive gap at any one time.

Suspensions of polystyrene spheres were loaded into the pressure pump vessels in the same way as calibration solutions, but care was taken to ensure they were regularly agitated to avoid the spheres settling in the bottom of the tube and clustering together.

6.4. PREPARATION AND MEASUREMENT OF CULTURED CELL LINES

Mouse myoblast cells (C2C12) purchased from ATCC (ATCC® CRL-1772™), human mesenchymal stem cells (hMSCs) derived from bone marrow purchased from Lonza and human monocytes (THP-1) purchased from ATCC (ATCC® TIB-202™) were provided by a collaborator. All cells were cultured at 37°C under sterile conditions and a 5% CO₂ atmosphere using standard protocols. Cultured cells were harvested using trypsin-EDTA and spun down to a pellet and rinsed with PBS before being suspended in PBS at concentrations of around 100,000 cells/mL. As with the PMs, the concentration was ascertained using a disposable haemocytometer and any necessary dilutions done with PBS in order to allow single cell measurements. Before measurements, cell suspensions were kept on an orbital shaker in a microbiological safety cabinet (MSC), and only transferred to the pressure pump vessel immediately before use, to ensure minimal clumping or adherence, and experiments were carried out as quickly as possible after cell harvesting. As in the case of PMs, the vessel was regularly agitated to avoid cell clusters and adherence.

Waste cell solution from the outlet of the sensor was absorbed into Kimtech wipes and disposed of immediately into biological waste bins. Cells were harvested the morning of each experiment and disposed of immediately afterwards.

7. RESULTS AND DISCUSSION

7.1. INITIAL BLOOD CELL MEASUREMENTS

Broadband measurements were made using the Agilent performance probe 85070 on suspensions of red blood cells (RBCs) in phosphate buffered saline solution (PBS) as described in Section 6.1.2. The results, shown in Figure 7.1.1 show, as expected, that as the concentration of RBCs increases both the low frequency real and imaginary parts of the measured permittivity decrease.

From these measurements it was possible to ascertain the permittivity of a single red blood cell. The permittivity of each RBC suspension at a single frequency (4.19 GHz) was plotted against concentration and the results fitted with a Maxwell-Garnett (M-G) mixed media formula:

$$\epsilon_{eff} = \epsilon_m \frac{\epsilon_i(1 + 2\Phi) + 2\epsilon_m(1 - \Phi)}{\epsilon_i(1 - \Phi) + \epsilon_m(2 + \Phi)} \quad \text{Equation 7.1.1}$$

Using this formula a first estimation of an overall RBC permittivity was calculated by assuming the permittivity of haemoglobin (Hg) to be 3 (from previous related work²¹) and the volume occupation of Hg to be ~30%. This first estimation was then fed into a second M-G formula to work out the overall permittivity of a suspension of RBCs, ϵ_{eff} . A value of 73.5 was taken from measurements and used as the permittivity of the PBS medium, ϵ_m , the calculated permittivity of the RBCs was used as the inclusion permittivity, ϵ_i , and the volume inclusion of RBCs, Φ , was varied from 0 to 100% (0-1) to create a curve, as shown in Figure 7.1.2. The initial values for Hg permittivity and volume inclusion of Hg in RBCs were then systematically varied to achieve the best fit with the permittivity probe measurements via an iterative least squares approach.

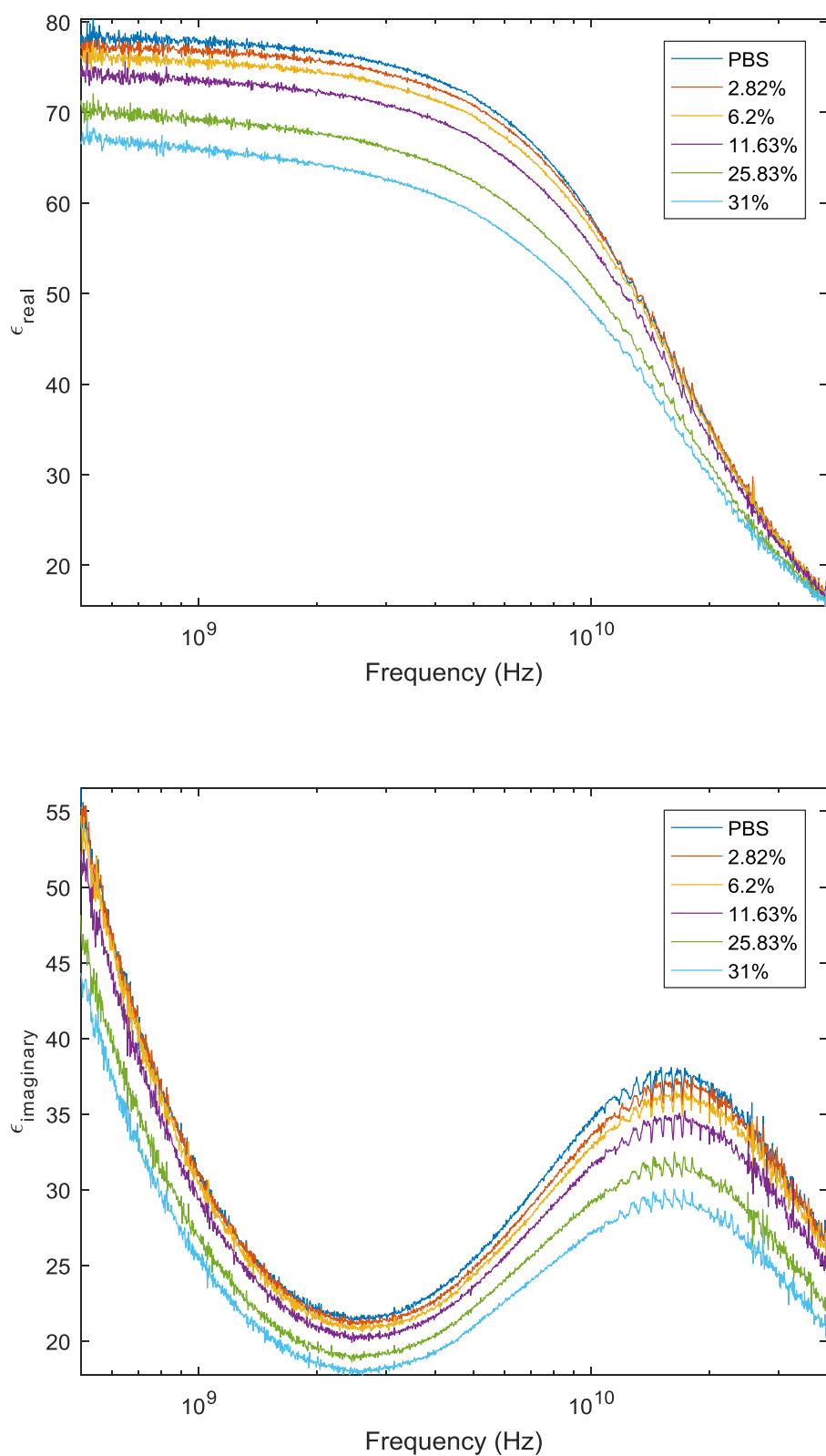


Figure 7.1.1: Permittivity probe results of suspensions of RBCs in PBS taken over a frequency range of 500 MHz – 40 GHz. Concentrations are quoted as volume %, calculated using a haemocytometer and an assumed RBC volume of 91fL ($91\mu\text{m}^3$).¹⁹⁵

$$\epsilon_{eff} = \epsilon_m \frac{\epsilon_i(1 + 2\Phi) + 2\epsilon_m(1 - \Phi)}{\epsilon_i(1 - \Phi) + \epsilon_m(2 + \Phi)}$$

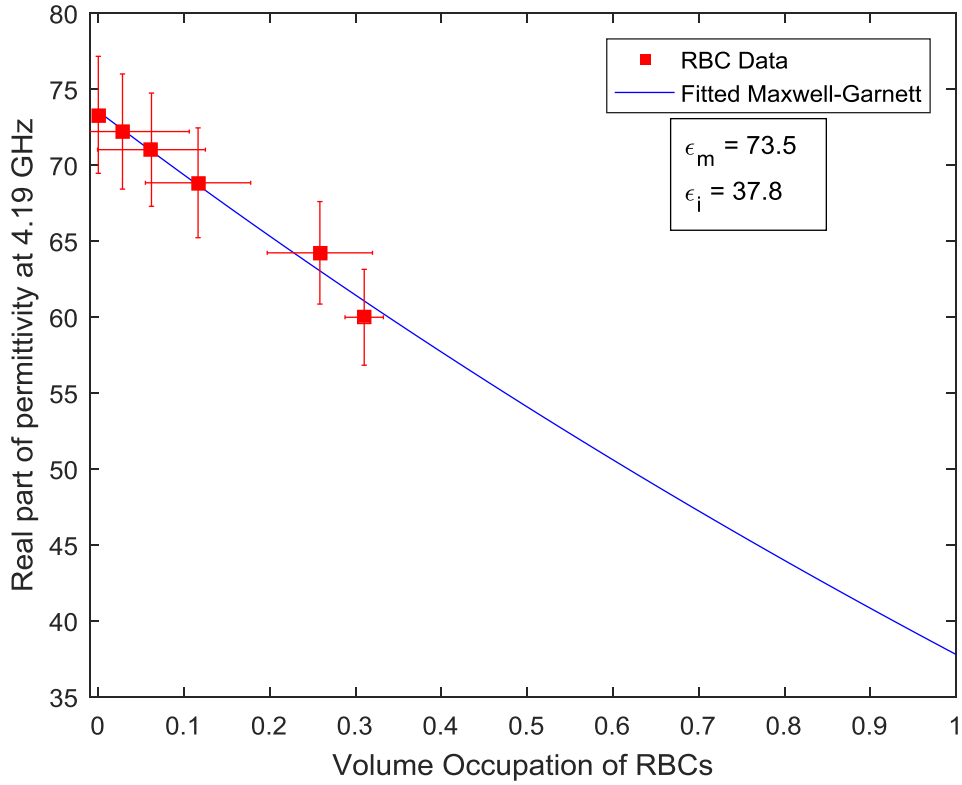


Figure 7.1.2: Plot of permittivity vs volume occupation of RBCs for RBC in PBS solutions, showing the fit obtained using the Maxwell-Garnett mixed media formula. Vertical error bars denote the stated accuracy of the probe permittivity measurements from the manufacturer, which is much larger than the standard deviation of repeat measurements. Horizontal error bars denote the accuracy of the concentration of RBCs estimate, based on errors from the cell counter for the highest concentration, and a combination of the errors from the cell counter and the errors from the micromechanical pipettes for the others.

Extrapolating this best fit to a volume occupation of 1, the overall permittivity of RBCs was therefore found to be approximately 37.8 at 4.19 GHz.

Unfortunately, due to the large volume and relatively high concentrations necessary to measure solutions with this broadband method, suitable white blood cell (WBC) suspensions could not be made up from the blood samples taken in order to carry out this process for WBCs. An impractical amount of blood would need to be collected and processed to produce the number of WBCs necessary for detection, and suspensions with such high concentrations tend to result in clumps of cells, making the probe technique unsuitable. Thus, in order to measure WBCs, a lower volume and higher sensitivity approach was needed, leading to the use of a microwave dielectric resonator. Originally it was thought that by using red blood cells suspensions as calibration solutions, the permittivity of a single “white blood cell” could be extrapolated from measurements made using the

resonator. If this worked, different cell types could then be investigated to see if the microwave resonator approach was able to differentiate them.

Attempts were made at measurement of white blood cells using an existing dielectric resonator (DR) sensor set-up, but despite the field confinement offered by the DR, problems with producing large enough volumes of high enough concentrations of WBC suspensions and the very small shifts induced by them meant that WBC measurements were mostly within the error of repeat background PBS measurements, as shown in Figure 7.1.3. Blank measurements were not taken in between every WBC sample, leading to considerable drift of the system in between measurements and quality factor shifts in the opposite direction to what was expected according to perturbation theory. Ascertaining the exact concentration of solutions was also difficult, with large errors inherent in haemocytometers and multiple dilutions.

Even with more frequent blank measurements, this resonator would not be sufficiently sensitive for the single-cell measurements required for the proposed application. In order to maximise the contrast in response from PBS and nucleated cells, a more sensitive system is required. The sensitivity depends, among other things, on the confinement of the field and the extent to which it is affected by the sample under test. For this reason a new resonator-based sensor with greater field confinement and a smaller interaction volume was developed, as described in Chapter 6. In the next section the operation principles of the new resonator-based system are presented.

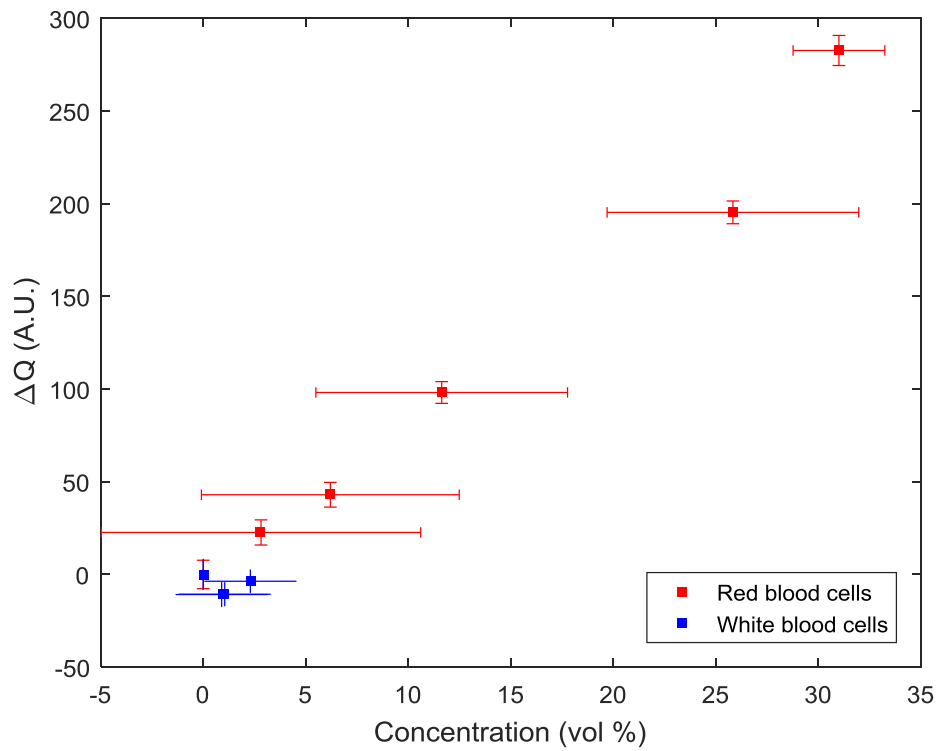
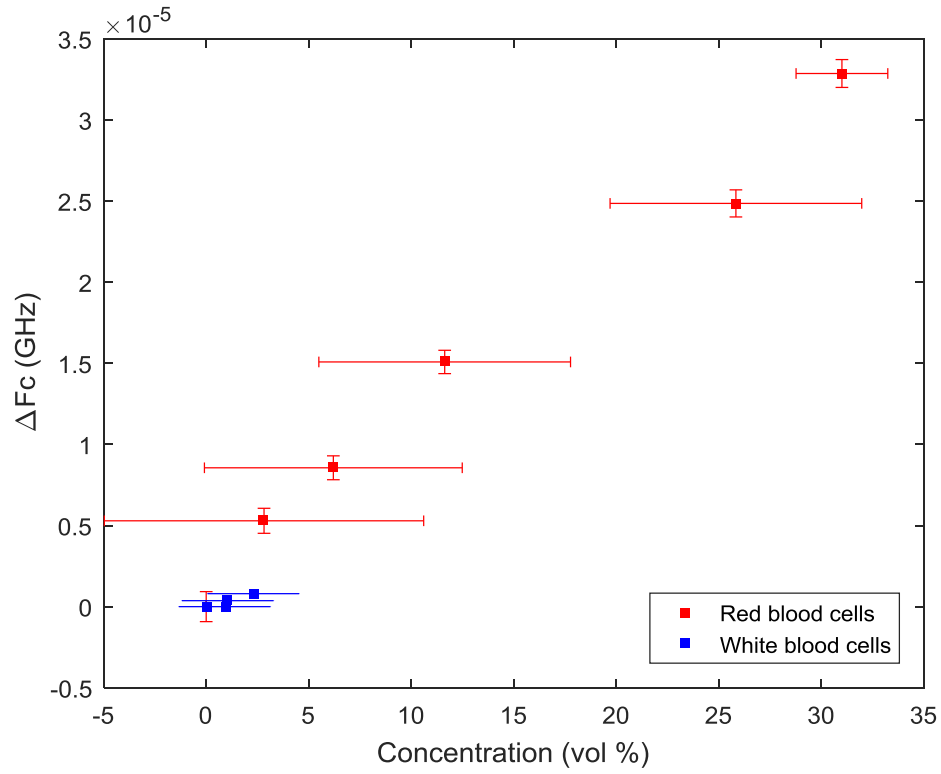


Figure 7.1.3: Results from the existing resonator sensor showing white blood cell suspension measurements alongside red blood cell measurements of change in frequency and quality factor. The measurements for frequency shift are all well inside ± 1 standard deviation of repeat measurements of the PBS background, while the quality factor measurements show significant drift within measurements. Total length of error bars denotes two standard deviations of the data.

7.2. COUPLED DIELECTRIC RESONATOR – SPLIT RING RESONATOR (DR-DSRR)

In order to move to the detection and measurements of single nucleated cells, a new system with improved sensitivity and increased spatial resolution was built. The new system was designed to further confine the microwave field to a volume comparable to that of the cells to be measured. A metal capacitive structure is used to achieve this; by shrinking down the size of the gap, the volume of the electric field can be arbitrarily controlled. Due to the circular shape of the DR, the fields of the DR are circularly symmetric. In order to excite a metallic structure at microwave frequencies using the DR it should also be circularly symmetric, and so a double split ring resonator (DSRR) was used. The coupled DR-DSRR as described in Section 6.2 was able to confine a microwave electric field to a volume on the same order of the cells to be measured.

7.2.1. CST MICROWAVE STUDIO® MODELLING

Attempts to understand the system from a theoretical perspective were made using CST MICROWAVE STUDIO® (CST® MWS®), using both eigenmode and frequency domain solvers. It was hoped that simulations could provide a means of optimising the system without trial and error experimentation. Initial eigenmode simulations of the two coupled resonators showed that there are other DR resonant modes with different magnetic field distributions resulting in different current distributions in the DSRR occurring around the $TE_{01\delta}$ mode. Most of these modes resulted in no electric field in the capacitive gaps, so it was useful to confirm that the frequency of operation of the system was resulting in a mode of the DSRR which gave rise to a strong electric field in the gaps. Simulations of the DSRR only showed that small changes to the shape of the DSRR brought some of these different, non-sensitive modes closer to the frequency of the sensitive modes, making it a useful tool for checking potential changes to the system for optimisation. eigenmode simulations were useful for ascertaining the unloaded resonance frequencies of various DSRRs which were not straightforward to measure experimentally, due to the measurement set-up as well as the inherently low quality factor of the loaded DSRRs. Comparisons between simulations could still be drawn in terms of electric field strength, and as discussed in Sections 6.2.2 and 7.4.1.5, eigenmode simulations were used to compare the difference between using the system with home-made PDMS channels and that using commercially available channel chips, as well as the differences due to channels of different shapes.

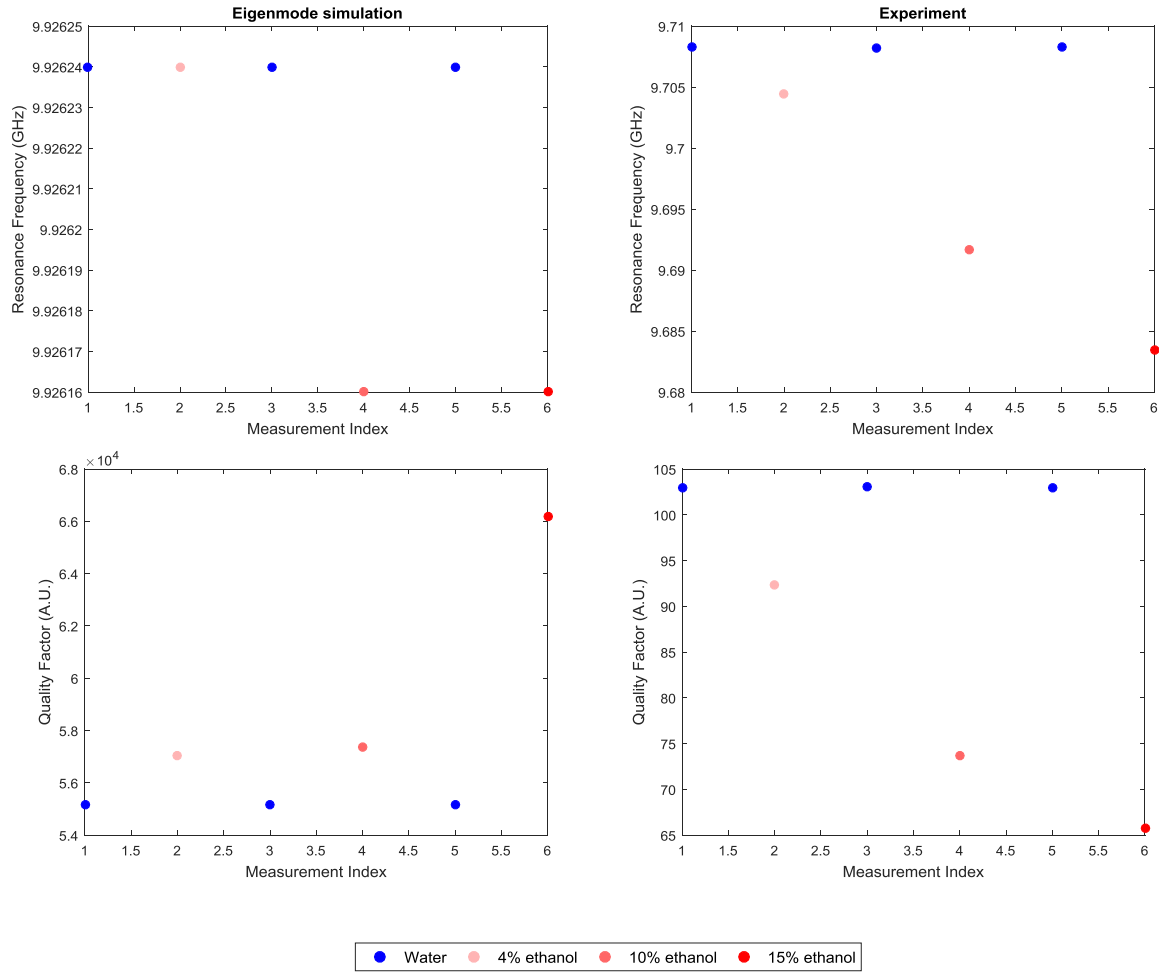


Figure 7.2.1: Comparison of simulated and experimental results. The left hand plots show the Eigenmode simulation results for a particular configuration with water as well as 4, 10 and 15% ethanol in DI water solutions filling the channel. The right hand plots show experimental results from the equivalent configuration. Both the average resonance frequency and quality factor measurements are shown. The value of measurement index denotes the order in which measurements were taken, i.e. 1 = first blank (water) measurement taken, 2 = first sample measurement taken, 3 = second blank measurement taken etc. Both simulation and experimental results are plotted in the same order for ease of comparison.

However, eigenmode simulations do not take into account losses in the system until post-processing, meaning that significant changes in field distribution or resonant half-width arising from the introduction of liquids like water are not modelled. For this reason, despite the ease and speed of simulations, this method was not ideal. When tested, the eigenmode simulations were not able to reproduce experimental results, as shown in Figure 7.2.1, meaning they could not be used to understand the system or predict sensitivity for different configurations.

Frequency domain simulations, which do take into account materials with high losses, were tried, but did not show good agreement with experimental results, being unable to reproduce the trends seen from an equivalent experimental system as shown in Figure 7.2.2.

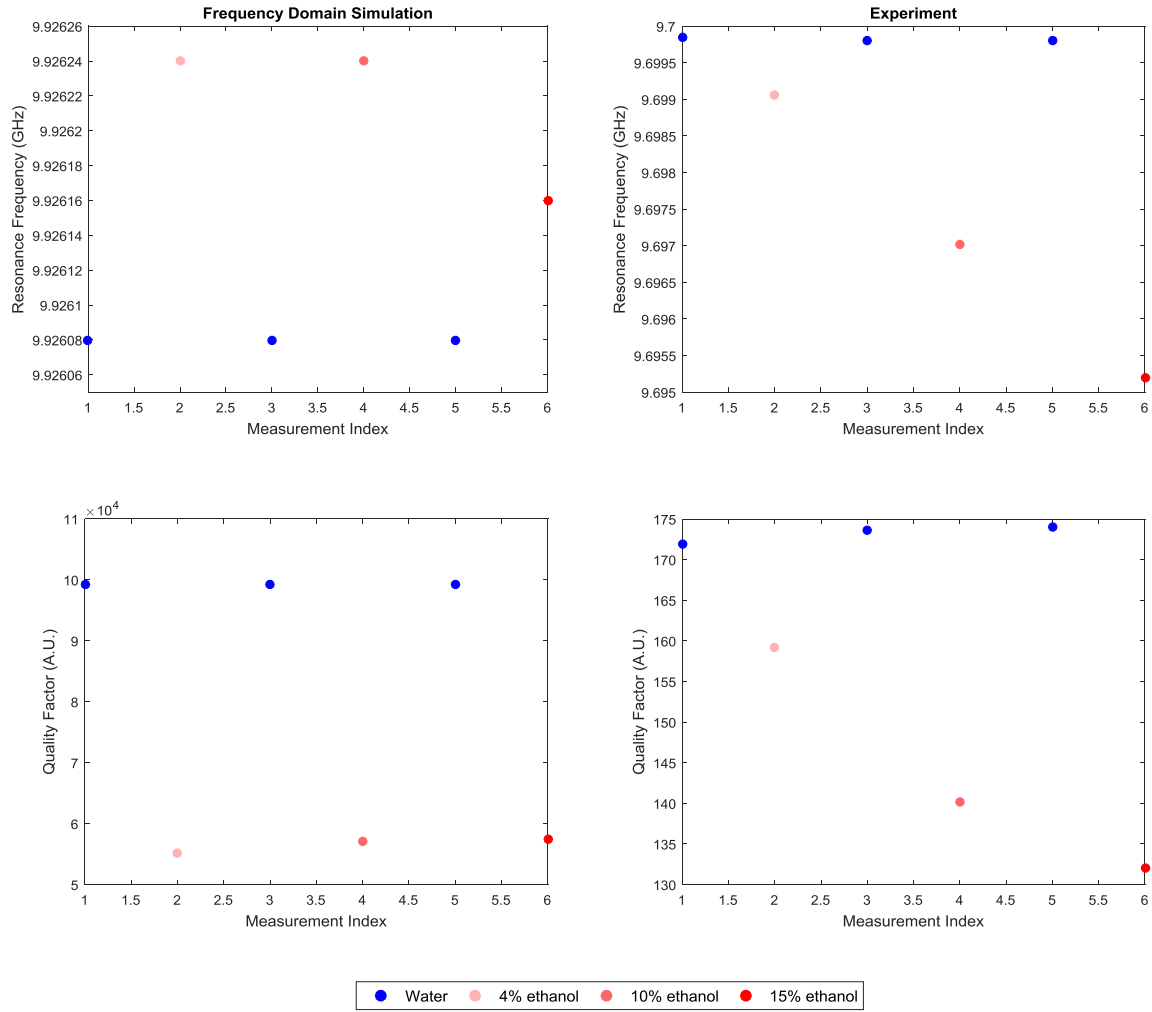


Figure 7.2.2: Plotted results of simulated and experimental data of the same system showing the significant differences in trends from the frequency domain simulation. On the left the results of resonance frequency and quality factor from the simulation are plotted for water, 4, 10 and 15 % ethanol in DI water solutions. On the right average resonance frequency and quality factor of the equivalent experimental configuration are plotted for the same samples. The value of measurement index denotes the order in which measurements were taken, i.e. 1 = first blank (water) measurement taken, 2 = first sample measurement taken, 3 = second blank measurement taken etc. Both simulation and experimental results are plotted in the same order for ease of comparison.

The problems with both CST MWS modelling approaches may be due to the difficulty in characterising the system accurately, making it impossible to reproduce the geometry of the system in simulations. For example, the puck height and the dimensions of channels cast using glass capillaries were difficult to ascertain and the alignment of the gaps and the channel by hand (and therefore the degree of overlap between the liquid and the capacitive electric field) was not easy to control or determine by eye. These discrepancies may lead to significant differences in outputs for equivalent experiments, given the sensitivity of the system to changes in the geometry of the capacitive sensing region, as discussed in Section 7.2.2.6.

7.2.2. EQUIVALENT CIRCUIT MODEL (ECM)

A new approach to modelling the system was adopted in order to better account for unknown quantities by taking into account empirical data. An equivalent circuit model (ECM) has been developed by Norbert Klein,²⁰¹ and is the subject of a future publication. The ECM mathematically approximates the system as two LC resonant circuits inductively coupled in parallel. The ECM includes an analytical model for DSRR, which consists of a partially liquid filled capacitor with a parallel resistor to account for the loss tangent of the liquid. As two main fitting parameters, the model contains a “filling factor” which describes the fraction of the electromagnetic energy of the capacitive electric field which is stored in the sample, and a coefficient which describes the strength of the DR-DSRR mutual coupling. The resonant parameters of the unloaded DR and the empty DSRR were taken from experiments and CST MWS eigenmode simulations, respectively, while values of the complex permittivity of the liquid under test can be taken from literature. The values of the filling factor and coupling coefficient cannot be measured or known from the physical set-up and so are obtained via a least squares fit of the ECM to experimental data. Due to this combination of theory, empirical data and literature values, the ECM produced results that were much closer to experiment than simulations.

7.2.2.1. GENERAL BEHAVIOUR

A full derivation of the analytical model is provided in Appendix 1. For the purpose of understanding the limits of this model and its application in this thesis it is important to understand its basis. The model first calculates, in terms of normalised complex impedance, the properties of each unloaded resonator individually, approximating them as LCR circuits. The sample-loaded properties of the DSRR are then calculated by approximating the two gaps as a single capacitance and a parallel shunt resistance which is dependent on the permittivity of the solution and a “filling factor” to represent how much the DSRR is affected by the liquid sample. The effect this liquid-loaded DSRR has on the DR in the coupled system is calculated by assuming a mutual inductance between the two circuits.

For comparison to experimental results, the loaded frequency and quality factor of the coupled system can be taken from this calculated impedance. Plotting the normalised admittance of the system produces the expected resonance curves for the system under different conditions, as shown in Figure 7.2.3.

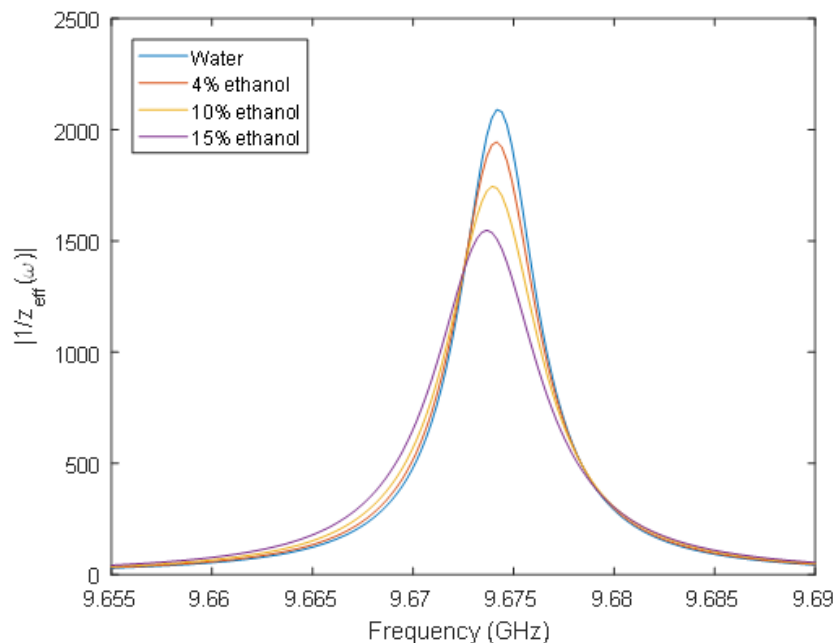


Figure 7.2.3: Example of outputs of ECM, showing the plotted results of the ECM for the coupled system with water and ethanol solutions in the capacitive gaps. Permittivity values for the ethanol solutions are used as inputs, and are taken from literature.¹²⁷ The normalised admittance, $|Y|=|1/z_{\text{eff}}(\omega)|$, is plotted against the resonant frequency of the system. In this set-up, increasing concentrations of ethanol result in a decrease in the resonance frequency and quality factor of the coupled resonator system.

7.2.2.2. POSSIBLE LIMITATIONS OF THE ECM

The ECM provides a good means of understanding the system but due to the difficulty in ascertaining some of the input values it cannot be used to predict the sensitivity of alternative set-ups (e.g. the use of different channel geometries). In addition to this, the ECM relies on a perturbative approach and therefore does not take into account significant local deformations of the electric field due to the sample. This approximation is only valid for small changes in permittivity,² and for the situation in which the material filling the capacitive gaps is homogenous and isotropic. This means that although the ECM may be theoretically able to give good agreement with the effects of samples consisting of low concentrations of ethanol in water, it is unable to encompass the effects of particles smaller than the size of the capacitors, particularly if they are of a significantly different permittivity to the surrounding medium, and therefore may not fare so well when modelling measurements of polystyrene spheres suspended in water.

The modelling of the two sensing gaps of the DSRR as a single capacitance leads to another shortcoming of the ECM when dealing with small particles. Modelling using the total capacitance assumes that the same medium fills both capacitive gaps at all times, whereas for measurements of single particles significantly smaller than the dimensions of the gaps, the effects are localised to one

gap only. The ECM is unable to model each gap separately, and therefore cannot encompass situations in which the capacitance of the two gaps is unequal. This asymmetry may have additional effects on the resonance properties of the DSRR which are not accounted for by the current model.

7.2.2.3. OUTCOME OF THE ECM: UNDERSTANDING THE SENSOR

The ECM allowed a better understanding of the coupling and provided insight into how this affected the sensitivity. It showed that for the experimental set-up used, the shifts in frequency and quality factor can be understood as the changing overlap between the resonances of the DR and the DSRR loaded with different samples.

With an empty channel (i.e. air, $\epsilon_r = 1$, in the gaps of the DSRR), the resonant frequencies of the DSRRs is higher (from eigenmode simulations, around ~14 GHz) than the DR (~9.8 GHz from experiment). The resonance of the empty DSRR, according to simulations, has a quality factor of around 400 in this situation, lower than that of the DR (several 1000), which is to be expected due to the higher losses in gold.

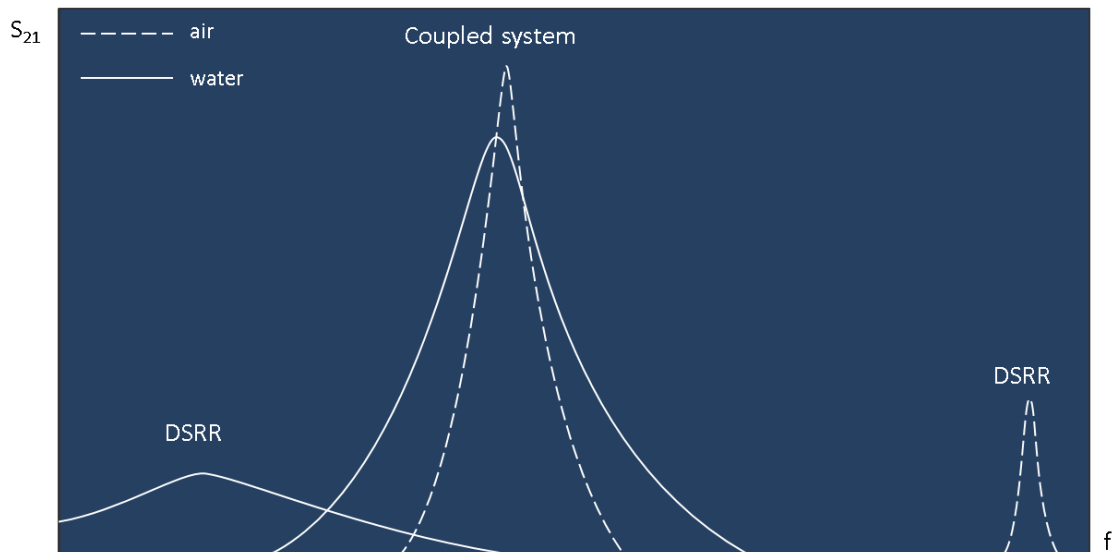


Figure 7.2.4: Schematic of the DR (coupled system) and DSRR resonances showing the change to both the DSRR and the coupled system resulting from filling the empty capacitive gaps with water, with the dashed line traces representing the air-filled system and solid line traces the water-filled system.

When the capacitive gaps of the DSRR are filled with water, $\epsilon_r \approx 63 + 29i$ at ~10GHz,¹²⁶ the capacitance of the DSRR resonance increases as:

$$C = \frac{\epsilon_r \epsilon_0 A}{d} \quad \text{Equation 7.2.1}$$

Which results in a decrease in the resonance frequency of the DSRR:

$$\omega_0 = \frac{1}{\sqrt{LC}}$$

Equation 7.2.2

This decrease in frequency brings the resonance of the DSRR closer to that of the DR. At the resonance frequency of the DR, the contribution to the losses from the DSRR increases. The resultant resonance is therefore much more influenced by the DSRR. Given the strong interaction between the alternating electric field and water at the DR resonance frequency, the presence of water in the capacitive gaps causes an increase in the losses of the DSRR, and therefore a drop in its quality factor. The combination of the decrease in frequency and the increased spread of the resonance (decrease in Q) of the DSRR results in an increased overlap between the two resonances. This increased overlap affects the resonance properties of the DR, decreasing both the frequency and quality factor of the resonance, as illustrated in Figure 7.2.4.

The resonance frequency and quality factor of the DSRR are very sensitive to changes in the permittivity of the material in the capacitive gaps. If the water in the gaps is replaced by a solution of ethanol in water or a suspended cell passes through the gap, the effective permittivity of the liquid decreases. This results in an increase in both the frequency and the quality factor of the DSRR resonance, and therefore an increase in the overlap between the two resonances. This is measured as a decrease in the quality factor (or amplitude) and frequency (or phase) of the DR, as illustrated in Figure 7.2.5.

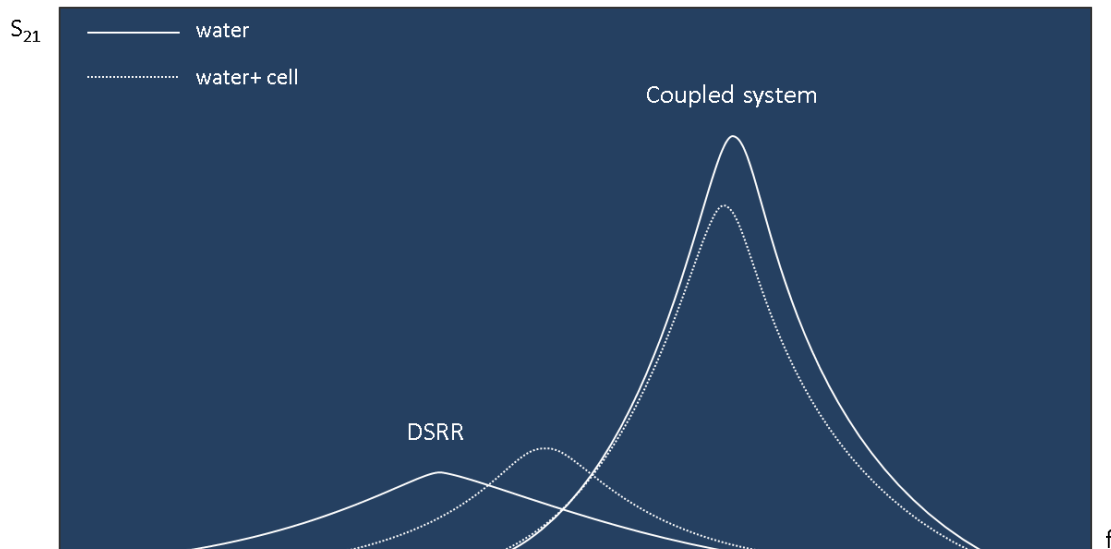


Figure 7.2.5: Schematic of the DR and DSRR resonances showing the change to both the DSRR and the coupled system resulting from replacing the water in the capacitive gaps with a liquid of lower permittivity, such as an ethanol solution or a cell suspension, with the solid line traces representing the water-filled system and dotted line traces the cell suspension-filled system.

Like other simple resonant circuits operating at microwave frequencies with high loss liquid materials, the quality factor of the water-loaded resonance of the DSRR is very low; around 5 from simulations and the ECM. Although the sensitivity of the DSRR to small changes in permittivity of liquids under test is high, measurements of low Q resonances are more difficult and inherently less precise than measurements at higher quality factors. Use of the DR to indirectly measure changes in the resonance properties of the DSRR allows for high Q and therefore precise frequency and quality factor (or amplitude and phase at resonance peak) measurements and therefore a higher sensitivity to small changes. Conversely, use of the DR on its own would yield highly precise measurements due to a high quality factor, but low sensitivity due to the relatively high stability of the resonance to changes in permittivity of liquids under test, as well as the difficulty in confining the fields and bringing them in such close proximity to the liquids.

The novel coupling system is therefore operating as a transducer, relaying information about the shift in resonance position of the DSRR due to different permittivity liquids in the capacitive gaps via readout of the resonance properties of the DR (as shown in Figure 7.2.6). This keeps measurements sensitive while maintaining a high frequency and quality factor resolution.

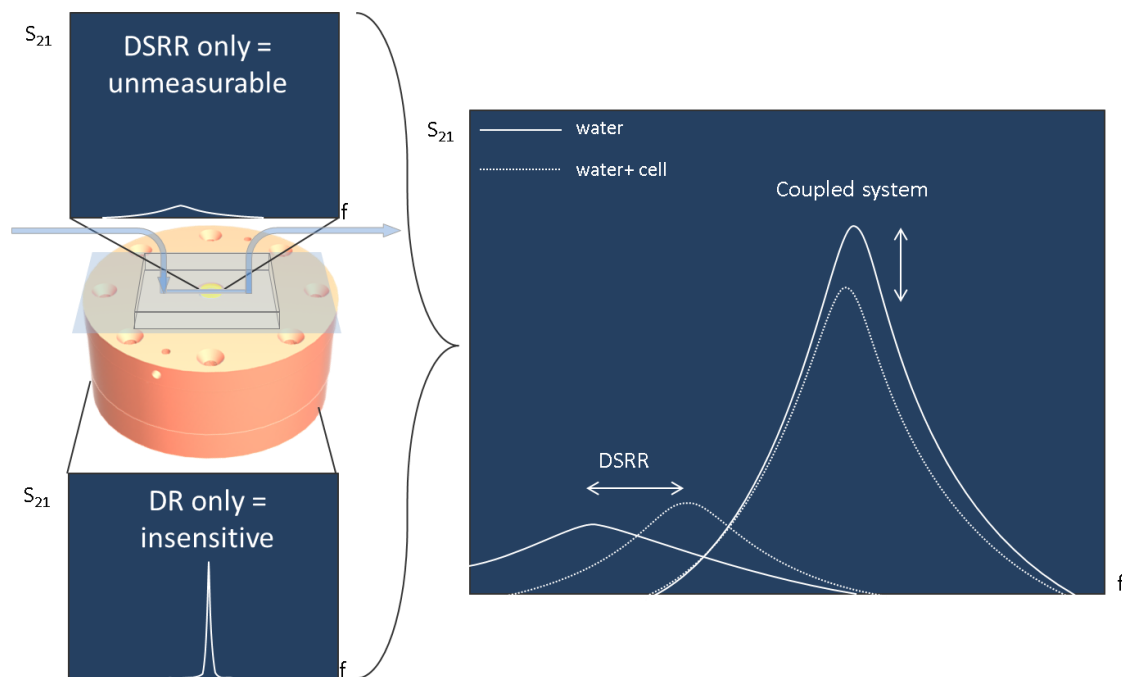


Figure 7.2.6: Schematic of the system showing the resonance properties of the DR and DSRR individually (left) and the properties of the coupled system (right). Neither the DR nor DSRR alone can produce the precise and sensitive response exhibited by the coupled system.

In order to achieve this sensitivity the system needs to be “tuned” so that small changes of permittivity in the high permittivity regime result in a significant change in overlap. If the loaded resonance frequency of the DSRR moves out of this window, for example to frequencies much lower

than that of the DR, this effect will not be seen and the system loses sensitivity. The effects of changes in the loaded resonance frequency of the DSRR relative to that of the DR are discussed in more detail in Section 7.2.2.6.

7.2.2.4. FITTING TO EXPERIMENTAL DATA

The results of the ECM fitted to experimental data (discussed in Section 7.3.1) are shown in Figure 7.2.7, showing good correlation for shifts in both frequency and quality factor of the coupled system. Both quality factor and resonance frequency measurements are quoted as shifts relative to that of the reference solution (water) to avoid problems of drift of the system due to temperature or mechanical movement. From the least squares fit, the values of coupling coefficient and filling factor for this configuration are 0.020 and 0.046, respectively.

Values of frequency shift and quality factor shift are expressed as values relative to the reference such that:

$$\frac{\Delta F_c}{F_{c_{water}}} = \frac{F_{c_{sample}} - F_{c_{water}}}{F_{c_{water}}} \quad \text{Equation 7.2.3}$$

$$\Delta\left(\frac{1}{Q}\right) = \frac{1}{Q_{sample}} - \frac{1}{Q_{water}} \quad \text{Equation 7.2.4}$$

These values represent the relative changes in the real and imaginary parts of the complex resonance frequency of the system normalised to the unperturbed resonance state, where $1/Q$ is proportional to the total losses in the system. This also helps to calculate and visualise the relative sensitivity of the two measured values, according to Equation 7.2.5 and Equation 7.2.6.

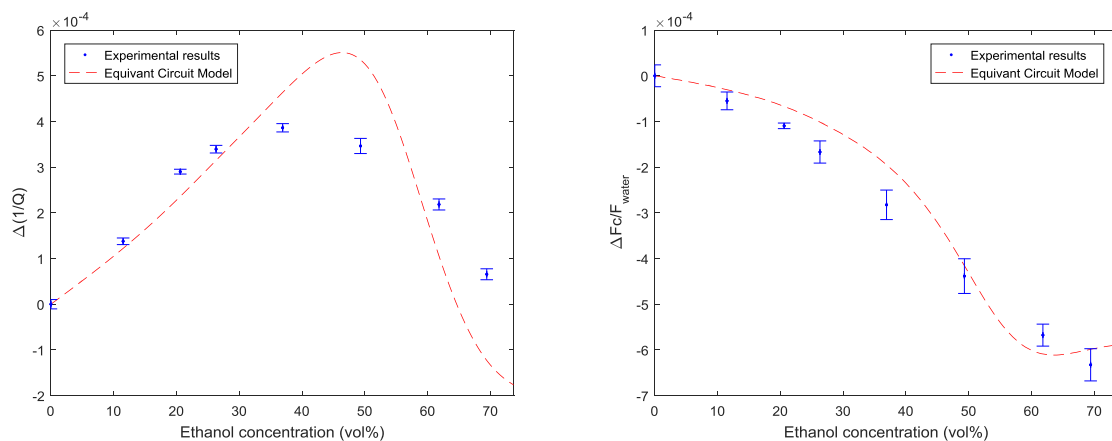


Figure 7.2.7: The frequency and quality factor shifts from experiment (blue) and the ECM (red) plotted against the concentration of ethanol. The total length of all error bars denotes two standard deviations of the data. Horizontal error bars are too small to see.

It can be seen that the ECM is able to roughly reproduce the trends observed experimentally, especially at low concentrations, however there are differences in both the magnitude of the shifts and the point at which quality factor shifts cross over from negative to positive (the concentration at which the frequency of the DSRR exceeds that of the DR). It is possible that, by making the unloaded DSRR resonance frequency and/or quality factor a fitting parameter, a better resemblance could be achieved, but errors in concentration as well as permittivity (temperature) of the solutions measured may be contributing to the discrepancies as well.

An estimation of the shifts predicted by the ECM for the case of a single biological cell perturbing the capacitive gap can be made by calculating the shifts induced for a theoretical “99% water” solution, in which the real and imaginary parts of the permittivity are both reduced by 1%, as could be expected for a very low concentration of proteins in water. At ~10 GHz this corresponds to a change in the real part of the permittivity of water of ~0.6, and in the imaginary of ~0.3. This small change in permittivity results in a predicted shift in resonance frequency of ~12 kHz, which corresponds to a shift of $\Delta F/F_{\text{water}}$ of -1.3×10^{-6} , and a change in quality factor of ~20, which corresponds to a shift in $\Delta(1/Q)$ of 4.1×10^{-6} . These shifts can be normalised to the resonant half-width (HW) to ascertain their significance via the following figures of merit:

$$\text{Sensitivity in } \Delta Q \text{ figure of merit} = \frac{\Delta HW}{HW_{\text{water}}} \approx Q_{\text{water}} \times \Delta \frac{1}{Q} \quad \text{Equation 7.2.5}$$

$$\text{Sensitivity in } \Delta F \text{ figure of merit} = \frac{\Delta F}{HW_{\text{water}}} = Q_{\text{water}} \times \frac{\Delta F}{F_{\text{water}}} \quad \text{Equation 7.2.6}$$

where the shifts in inverse quality factor and frequency are defined as sample – water, and the shift in resonance frequency is considered negligible when calculating the ΔQ figure of merit.

Using a value of Q_{water} of 1600 based on experimental measurements, the shifts induced by the 1% change in permittivity give figure of merit values of 0.007 for ΔQ and 0.002 for ΔF . By this metric it can be seen that the shift in frequency is small compared to the resonant half-width than the quality factor shift, where a 0.7% shift in resonant half-width is predicted. The quality factor measurements also seem to result in lower standard deviations, possibly due to the high overall quality factor of the sensor, making the quality factor measurements potentially more sensitive and reliable than the resonant frequency measurements. Plotting the values of $\Delta F/F_{\text{water}}$ and $\Delta(1/Q)$ against each other as in Figure 7.2.8 shows more clearly the higher sensitivity in quality factor than resonant frequency in the high permittivity regime.

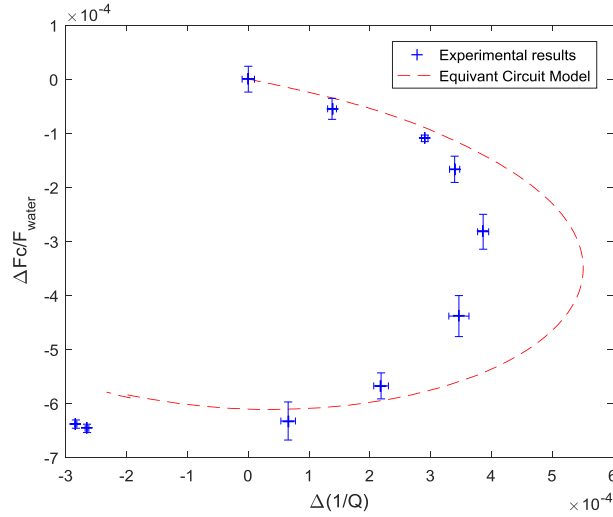


Figure 7.2.8 The normalised frequency and quality factor shifts from experiment (blue crosses) and the circuit model (red dotted line) plotted against each other, showing general agreement, especially at low concentrations (near to 0).

7.2.2.5. EXPECTED SENSITIVITY TO CHANGES IN PERMITTIVITY

The ECM can be used to evaluate the expected shift in quality factor for different values of real and imaginary permittivity, as shown in Figure 7.2.9. From this it can be seen that in the high permittivity regime, shifts in the quality factor are almost entirely due to changes in the real rather than the imaginary permittivity. This is to be expected if the coupled system is acting as a transducer, relaying information about the shift in frequency of the DSRR via shifts in quality factor of the DR. It is also especially apparent considering that the imaginary permittivity varies by only ~ 2.1 over a range of ethanol concentrations from 0 to 11.5% by volume, whereas the real part varies by ~ 14 . The shifts in frequency are much more affected by the imaginary permittivity, and have a complex dependence on both the real and imaginary.

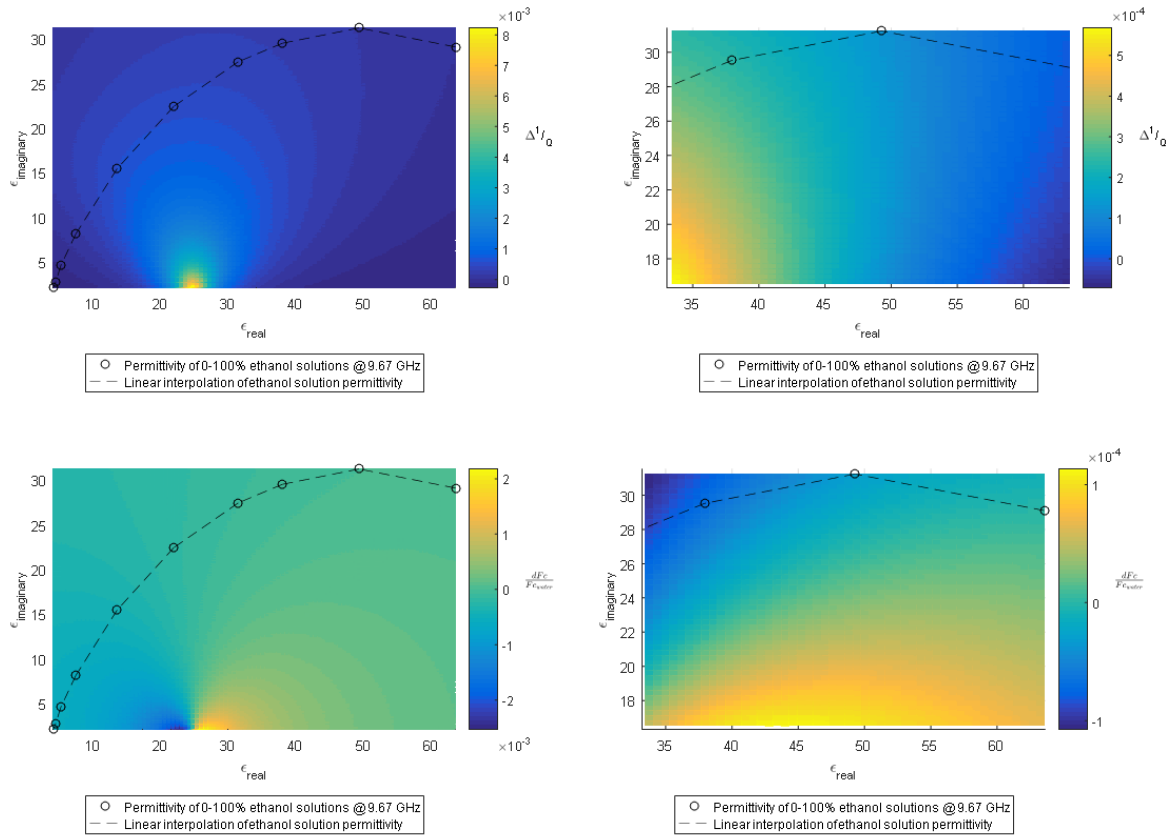


Figure 7.2.9: Colour map plots of the predicted quality factor and frequency shifts relative to water of a particular experimental configuration for a variety of real and imaginary permittivity values. The permittivity values of ethanol solutions, from literature,¹²⁶ used for experiments are marked in black circles with the dotted lines denoting a linear interpolation. Inverse quality factor shifts are plotted at the top, and normalised frequency shifts on the bottom. Plots on the right hand side focus on the high permittivity regime.

Looking at the dependence of the frequency shift on permittivity at this high permittivity regime as well as over a wider range of permittivity, it can be seen that unlike the shifts in quality factor, the shifts in frequency for this experimental configuration show both positive and negative shifts. For small changes around the permittivity of water, the direction of the shift depends on mainly the imaginary permittivity of the solution.

7.2.2.6. EXPECTED SENSITIVITY TO FILLING FACTOR

The ECM also showed that the system was sensitive to small changes in the geometry-only dependent “filling factor” of the DSRR, i.e. the volume of liquid interacting with the capacitive electric fields, and that varying this filling factor can shift the loaded resonance frequency of the DSRR dramatically. As can be seen in Figure 7.2.10, changes in the filling factor can move the loaded DSRR resonance frequency from one side of the DR resonance to the other, passing through a regime of maximum theoretical sensitivity where the two resonances are close together. This may

explain the difficulties experienced when trying to recreate experiments with different channels or in between re-positioning of the channels, which will be discussed in Section 7.4.1.5.

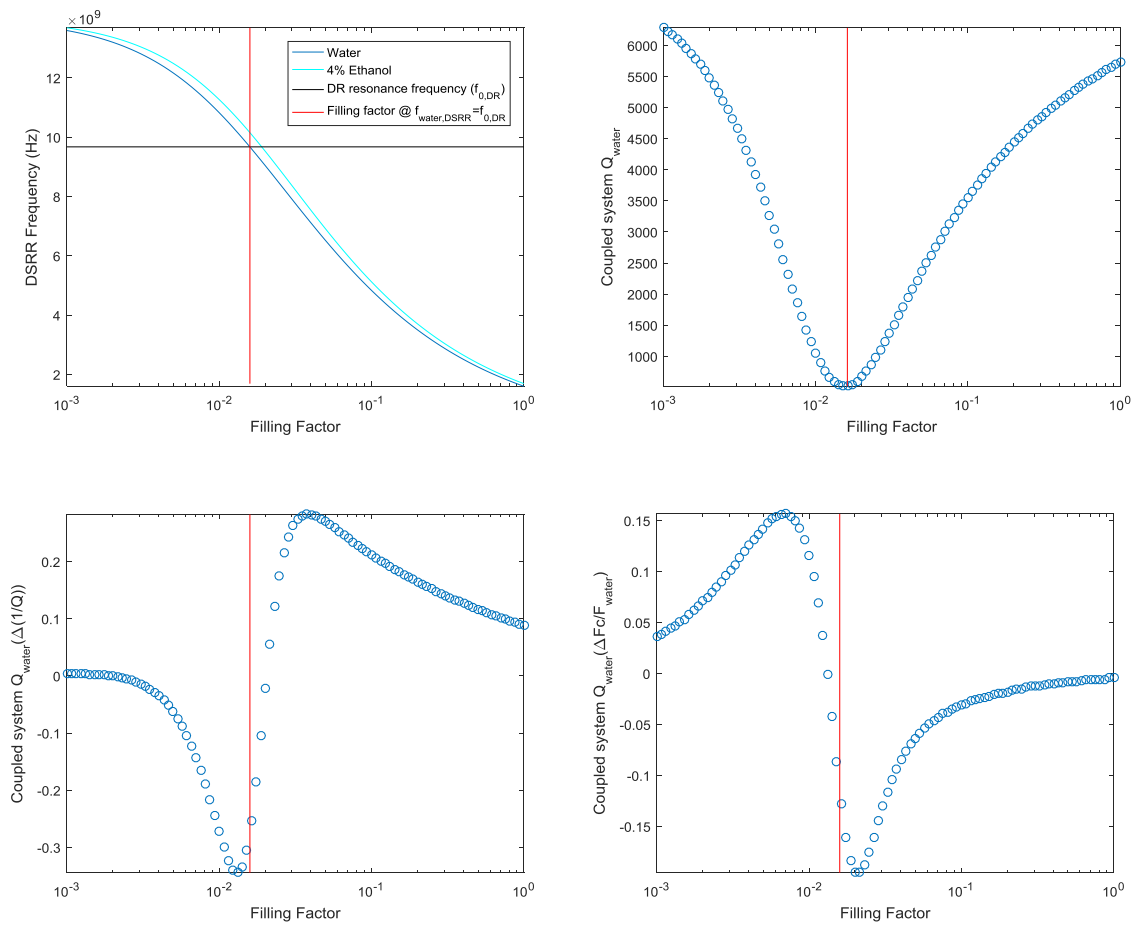


Figure 7.2.10: Top: Resonance frequency of the DSRR loaded with water ($f_{water,DSRR}$) and 4% ethanol in DI water from the ECM as a function of the geometry-dependent filling factor, showing the point at which $f_{water,DSRR}$ overlaps with the unloaded resonance frequency of the dielectric resonator ($f_{0,DR}$). The filling factor is varied from 0.001 to 1. The black horizontal line shows $f_{0,DR}$, while the red vertical line denotes the filling factor at which this crossover occurs (0.0159). On the right the quality factor of the coupled system is shown, with the filling factor resulting in $f_{water,DSRR} = f_{0,DR}$ corresponding to a minimum. Bottom: the predicted shifts in inverse quality factor and normalised frequency, quoted as the figures of merit as defined in Equation 7.2.5 and Equation 7.2.6, for the coupled system over the same range of filling factor, with the red vertical line again denoting the point at which $f_{water,DSRR} = f_{0,DR}$.

The ECM was developed after experimental results were obtained, and so has been used to retrospectively understand the operation of the system, but this understanding has revealed details of the current configuration and may be used to optimise future iterations of the sensor. For instance, Figure 7.2.10 shows a theoretical maximum sensitivity in $\Delta(1/Q)$ for a configuration where the loaded resonance frequency of the DSRR ($f_{water,DSRR}$) is slightly higher than the unperturbed resonance frequency of the DR ($f_{0,DR}$). This would result in a system in which increasingly concentrated ethanol solutions would move the loaded DSRR frequency increasingly further away from $f_{0,DR}$, resulting in very large, positive quality factor shifts of the coupled system. As has been

discussed, the current configuration is not operating in this optimum frequency window, instead showing negative quality factor shifts due to the loaded DSRR frequency lying below $f_{0,DR}$. The exact value of the filling factor cannot be measured, is permittivity-dependent, and so is determined via iterative least squares fitting of the model to experimental data. From experimental results of the current configuration, the value of the geometry-dependent filling factor was found to be 0.05, meaning that the system falls somewhere near the slightly smaller, positive peak in the plot of coupled system $Q_{\text{water}}(\Delta(1/Q))$ vs filling factor in Figure 7.2.10.

7.3. MEASUREMENTS USING COUPLED DR-DSRR SYSTEM

7.3.1. ETHANOL SOLUTIONS

The new system, with in-house-produced PDMS channels sealed onto DSRR substrates, was first tested using ethanol solutions of known concentration. An attempt at a systematic investigation of DSRR parameters was made, but problems with reproducibility of channel alignment, ethanol concentration uncertainty, and the design of DSRRs resulting in a lack of independent control of the different parameters made it difficult to compare different configurations and draw causal links between sensitivity and ring geometry.

In some cases, high sensitivity in frequency shift came at the expense of sensitivity in quality factor shift and vice versa. This is an effect predicted by the ECM, and as was discussed in Section 7.2.2, the configuration (filling factor) resulting in the maximum quality factor shift for small changes in permittivity away from that of water ($f_{0,DSRR,water} = f_{0,DR}$) results in the minimum frequency shift.

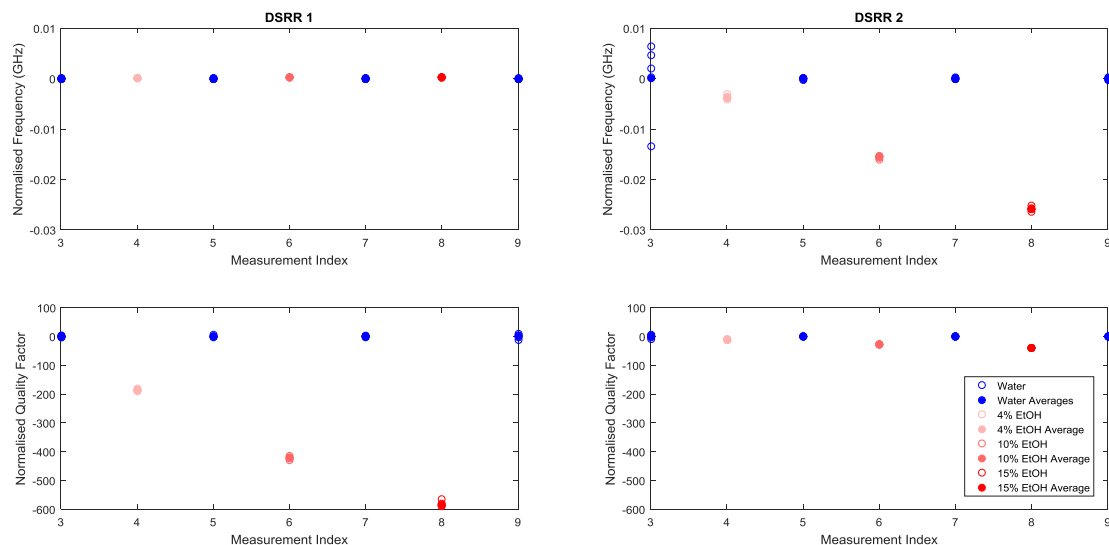


Figure 7.3.1: Example results of measurements of solutions of ethanol in water taken using two DSRRs of different geometries showing the relationship between high sensitivity in one measured parameter, and lack of sensitivity in the other. DSRR1 has an outer diameter of 6 mm, the largest ring studied, while DSRR2 has a smaller outer diameter but a wider gap.

The DSRR used in all further experiments was a compromise offering both high frequency and high quality factor sensitivity, but given recent insights from the ECM it may be preferable in future to focus on only one measured parameter, e.g. quality factor shift, and significantly increase the sensitivity by adopting an alternative ring geometry.

Although gold was initially intended to be used for the DSRR due to being chemically and biologically inert, copper was also trialled due to its high conductivity ($\sim 6 \times 10^7 \text{ Sm}^{-1}$ for copper and $\sim 4 \times 10^7 \text{ Sm}^{-1}$ for gold) and shown to be adequately stable and unreactive. In hopes of achieving the highest possible quality factor for measurements, a copper DSRR was used.

As will be discussed in Section 7.4.1.5, in order to maximise the electric field felt by the liquid sample the channel had to encompass the edge of the metal in the gaps. The first channels made were on the order of $\sim 150 \mu\text{m}$ wide (due to the size of objects to be used as negatives and the resolution of the available 3D printers), and therefore a DSRR with a gap separation of $\sim 100 \mu\text{m}$ was used.

In general, a thicker DSRR ($\sim 1 \mu\text{m}$ of deposited metal) was chosen due to the corresponding increase in interaction area between the electric field and the material in the channel that this would provide. Although rings of up to $\sim 7 \mu\text{m}$ of deposited metal were made, a balance had to be struck between using the thickest metal and sealing the PDMS channel around the gap, as well as maintaining a relatively high quality factor. Thicker rings also showed more dependence on changes in temperature, but given the nature of the experiments this effect can be mitigated against by taking

measurements relative to a background solution measured a few seconds before and after measurement of the target solution or cell. When dealing with flowing particles, these background measurements can be made constantly throughout an experiment, effectively removing the problem of temperature induced drift. Changes in the interaction volume will also affect the loaded resonance parameters of the DSRR.

The width of the rings would, again, affect the interaction volume and their loaded resonance, and a relatively large ring width of 500 μm was used so as to be significantly large compared to cells and allow multiple measurements of cells as they passed over the gap.

The effects of different ring diameters were harder to predict, though the development of the ECM may allow for elucidation of the effect ring diameter would have, since the diameter of the DSRR will determine its resonant frequency. In order to fully optimise the system, it may be worth a larger investigation into the relationship between a given DSRR's water-loaded resonant frequency (affected by the ring diameter, ring width, and metal thickness) and the resonant frequency of the DR. According to the ECM, there should be an optimal crossover between the two, which would result in the greatest degree of quality factor shift. In order to do this, however, a reliable method of determining the DSRR resonant frequency will need to be found, as simulation techniques have not reproduced experimental results, and direct measurements suffer from the aforementioned problems of inconsistency in experimental parameters such as channel alignment. In addition to this, the ECM may not tell the whole story of the sensor, being unable to reproduce results from a configuration which showed increasingly positive frequency shifts for ethanol solutions, rather than the expected negative shifts, alongside negative quality factor shifts. The complexity of the system as well as the difficulty in accurately defining its geometries makes analytical modelling non-trivial.

Many rings were experimentally investigated, and through a process of trial and error a configuration which showed sensitivity to small polystyrene particles was found and retained. Figure 7.3.2 shows the shifts in frequency and quality factor as well as phase and amplitude measured using the new coupled DR-DSRR system for solutions of ethanol in DI water over a range of concentrations. Phase and amplitude measurements were taken at a single frequency (the resonance frequency of the coupled system with water in the channel) to speed up measurements in preparation for real-time monitoring of flowing cells. These results show that, at low ethanol concentrations, the sensitivity shown in the resonant frequency and quality factor measurements taken over a frequency domain is also seen in the single frequency measurements, alongside a reduction in the variation of repeat measurements.

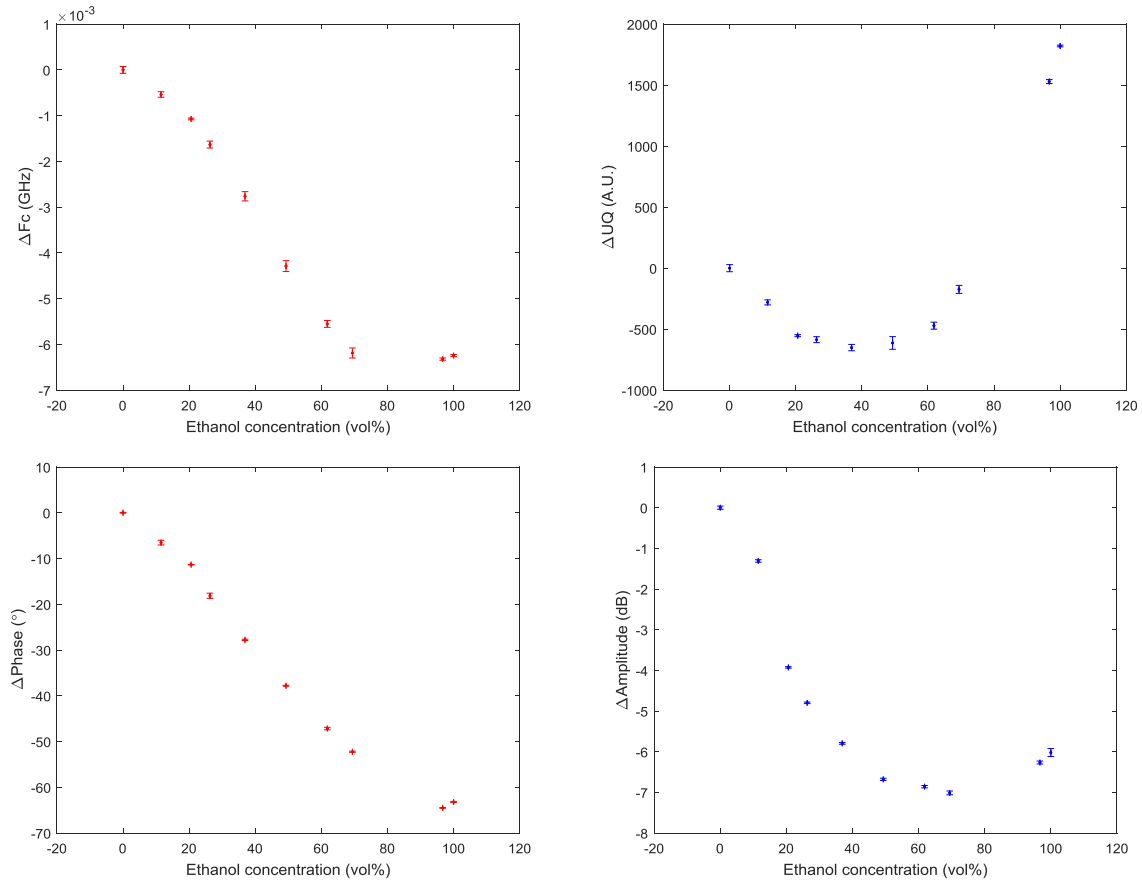


Figure 7.3.2: Plotted results of a range of ethanol in DI water solutions measured using the most promising set-up. Measurements are taken in both frequency sweep mode to give values of resonant frequency and quality factor, and continuous wave (CW) mode at the resonance frequency of water to give amplitude and phase data.

For future comparison of shifts induced, it is useful to convert phase and amplitude shifts into frequency and quality factor shifts. Assuming a Lorentzian response from the resonator, the shifts in frequency and quality factor can be linked to the shifts in phase and amplitude, respectively, via the following relations:

$$\Delta\varphi = -2Q_0 \frac{f_{\text{sample}} - f_0}{f_0} \quad \text{Equation 7.3.1}$$

Where phase shift ($\Delta\varphi$) is in radians, Q_0 and f_0 are the unperturbed quality factor and resonance frequency (resonance parameters with water in the channel) and f_{sample} is the resonance frequency of the perturbed state (ethanol solution in the channel). Assuming that any fractional change in Q-factor will be much larger than a fractional change in frequency the quality factor can be related to the amplitude shift via:

$$\Delta A_{dB} = 10 \log_{10} \left(\frac{Q_{sample}}{Q_0} \right)^2$$

Equation 7.3.2

where ΔA_{dB} is the measured amplitude shift in dB, Q_{sample} is the perturbed state quality factor and Q_0 is as before. These ethanol solution measurements can be used to test these relations between phase and frequency shift and amplitude and quality factor shift, using $Q_0 = 1500$:

Table 7.3.1: Measured amplitude and inverse quality factor shifts, as well as the inverse quality factor shift calculated according to Equation 7.3.2.

Shift relative to water	11.5% ethanol in water (vol %)	20.6% ethanol in water (vol %)
Measured ΔA	-1.31067	-3.928
Calculated $\Delta \frac{1}{Q}$ from ΔA	1.0825e-04	3.2147e-04
Measured $\Delta \frac{1}{Q}$	1.3774e-04	2.9018e-04

Table 7.3.2: Measured phase and normalised resonant frequency shift, as well as the normalised resonant frequency shift calculated according to Equation 7.3.1.

Shift relative to water	11.5% ethanol in water (vol %)	20.6% ethanol in water (vol %)
Measured $\Delta \varphi$ (°)	-6.5243	-11.3427
Calculated $\frac{\Delta f}{f_0}$ from $\Delta \varphi$	3.7957e-05	6.5989e-05
Measured $\frac{\Delta f}{f_0}$	-5.11e-5	-1.1248e-4

These equations reproduce the trends observed in the measured quality factor, but they are not an exact match. Due to the experiments measuring amplitude/phase and quality factor/frequency taking place on consecutive days and the lack of a calibration technique, it is possible that the phase stability of the instrument is not high enough to be able to accurately link one to the other. Further problems may be introduced due to the accuracy of the single frequency at which the CW measurements are taken. The accuracy of measured frequency and quality factor values may also be an issue, and depends on the IFBW, the number of points, and the method of quality factor determination.

For the phase measurements, although the absolute magnitude of the calculated values is similar, the equations are unable to reproduce the correct direction of the shifts. Due to the fact that there is no calibration technique done before measurements there is a significant effect on the phase from the long cables which is not corrected for. It is not reasonable, therefore, to assume that the frequency shift can be calculated from the phase shift, and in fact between experiments the phase shifts due to the coaxial cables may change, leading to unpredictable results. Calibration had not

been initially thought necessary due to the relative nature of measurements as well as the high quality factor of the resonator, but subsequent investigation of the phase measurements has shown that a calibration accounting for effects from the cables would be needed in future.

7.4. REAL-TIME MEASUREMENTS OF POLYSTYRENE SPHERES

Although ethanol experiments showed that the system was able to pick up small differences in the permittivity of the material in the sensing gaps, the capability of the system to pick up differences due to particles much smaller than the dimensions of the gaps needed to be assessed. Additionally, while the ethanol solutions would fill both sensing gaps, individual particles would interact with only one gap at a time, which could result in potentially different effects. For this reason, experiments were started using particles with a very different permittivity to that of water: polystyrene microspheres. At the operational frequency of the resonator system (~ 9.67 GHz), polystyrene has a relative permittivity on the order of 2.5,²⁰² while the DI water it is suspended in has a relative permittivity of 63.6 at room temperature.¹²⁶

While the system could be perturbed by a particle in either of the sensing gaps, the real-time measurement set-up was only able to record video of one gap. In order to correlate signals to passages of particles, the solutions had to be diluted enough to reasonably assume that particles were only in one gap at a time. This resulted in long periods of time looking for suitable particles to be measured, and (after considerable practise) later results of multiple measurements of each particle, rather than single measurements of multiple particles.

7.4.1. 25UM DIAMETER MICROSPHERES

7.4.1.1. FREQUENCY AND QUALITY FACTOR MEASUREMENTS

The first real-time monitoring script used to control the VNA and microscope camera took approximately 3 quality factor measurements per second. The quality factor was read from automatic marker data generated by the VNA at -3 dB from the peak frequency. The VNA carried out a sweep centred on the resonant frequency (~ 9.7 GHz), using 200 points, an IF bandwidth of 100 Hz and a power of 0 dBm. From the time-stamped video, the flow rate of the PS sphere can be calculated as approximately 500 $\mu\text{m/s}$.

The very first real-time measurements conducted on 25 μm polystyrene spheres showed a large peak and clear correlation between the passage of the spheres and the shifts in quality factor, an example of which is shown in Figure 7.4.1.

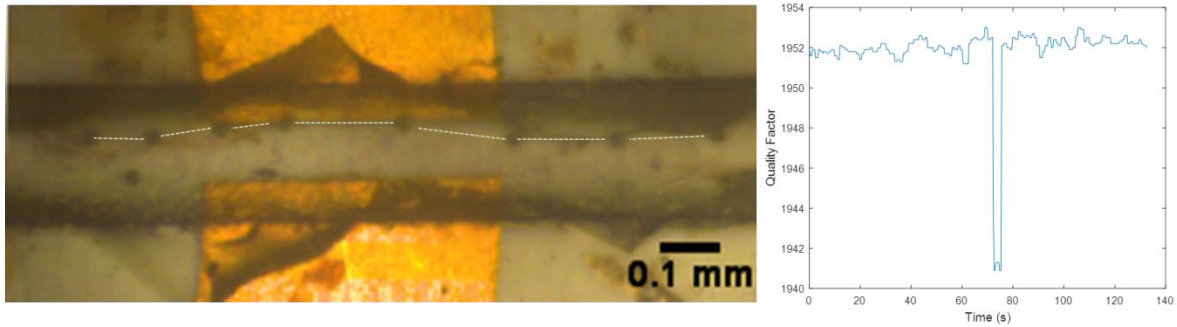


Figure 7.4.1: Left: composite image from microscope video feed showing the path of the bead through the capacitive gap. Right: plot of the quality factor against time, showing the decrease due to the passage of the sphere at just before 80 seconds. The composite image shows the position of the polystyrene microsphere at each measurement point (every ~ 0.3 s) during its passage through the capacitive gap, corresponding to the measurements of quality factor.

Despite the large shifts observed in Figure 7.4.1, measurements of the quality factor were slow and resulted in quite high levels of noise.

Following this promising result, the measurement set-up was changed to speed up measurements, increase precision and reduce noise. Measurements of amplitude and phase at a single frequency were made, rather than reading peak frequency and quality factor from the -3 dB markers from the VNA.

7.4.1.2. AMPLITUDE AND PHASE MEASUREMENTS

The new measurement set-up increased both the speed of measurements and precision of the data. With only one frequency point being measured rather than a sweep of frequencies, measurements can be carried out much quicker, and since the measurements do not rely on the two -3 dB markers from the VNA to ascertain the quality factor, instead recording the absolute values of amplitude and phase directly, the error in measurements is theoretically reduced. In order to reduce noise, holders for the large, heavy coaxial cables were made to minimise mechanical drift of the system. Single frequency measurements also allow for a much lower IFBW to be used without significantly lengthening measurement times, which should reduce instrument noise inherent to the measurements.

In addition, the channel was replaced with a newly cast channel in hopes of achieving a better seal around the metal, after problems with leakage. Unfortunately this resulted in a reduction of sensitivity. The effect of different channels and alignments on the level of sensitivity of the system is discussed further in Section 7.4.1.5.

The VNA was set up in continuous wave mode at the resonance frequency of the water-loaded coupled system, with the IFBW set to 10 Hz and the power at 0 dBm. A sample period of 0.2 seconds was used for both the VNA and the high speed camera. As shown in Figure 7.4.2, measurements carried out using single frequency measurements show the same correlation between sphere passages and signals recorded in both amplitude and phase, as well as a clear correlation between the amplitude and phase measurements.

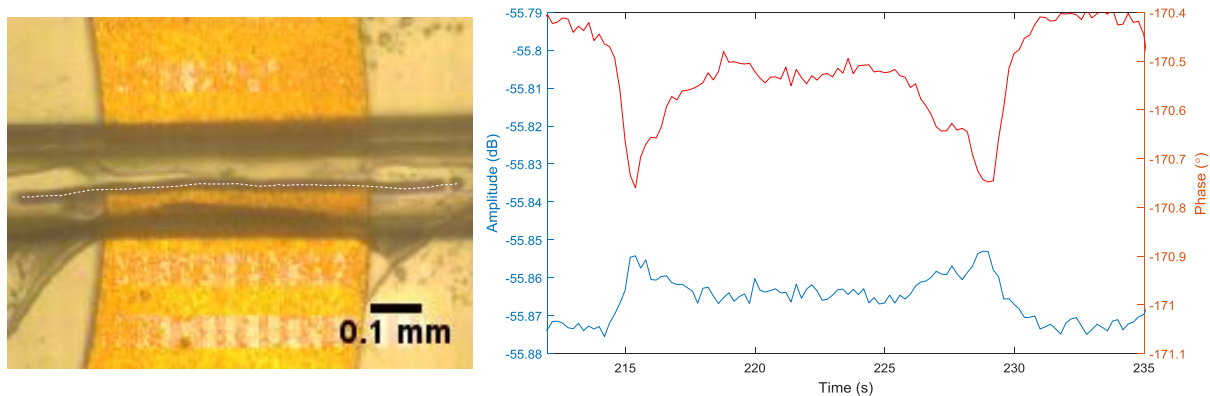


Figure 7.4.2: Example of CW results showing on the left the composite micrograph of a particularly slow moving 25 μm sphere passing close over the edge of the metal (path highlighted in white dashed line), with the corresponding phase (°) and amplitude signals (dB) shown on the right.

As can be seen from Figure 7.4.2, the re-aligned system showed positive amplitude shifts for the passage of polystyrene spheres. Given the previous negative quality factor shifts for ethanol solutions, negative amplitude shifts were expected. This discrepancy may be due to the new channel or its realignment significantly altering the filling factor of the DSRR, as discussed in Section 7.2.2.6, or could be due to effects from the other capacitive gap.

7.4.1.3. SENSITIVITY TO SPHERE PATH

As can be seen from Figure 7.4.3, the observed shift in both amplitude and phase increases the closer the path of the particle is to the edge of the metal in the capacitive gap. This is to be expected, since the strength of the electric field decreases with increasing distance from the metal surface, as can be seen in Figure 6.2.9.

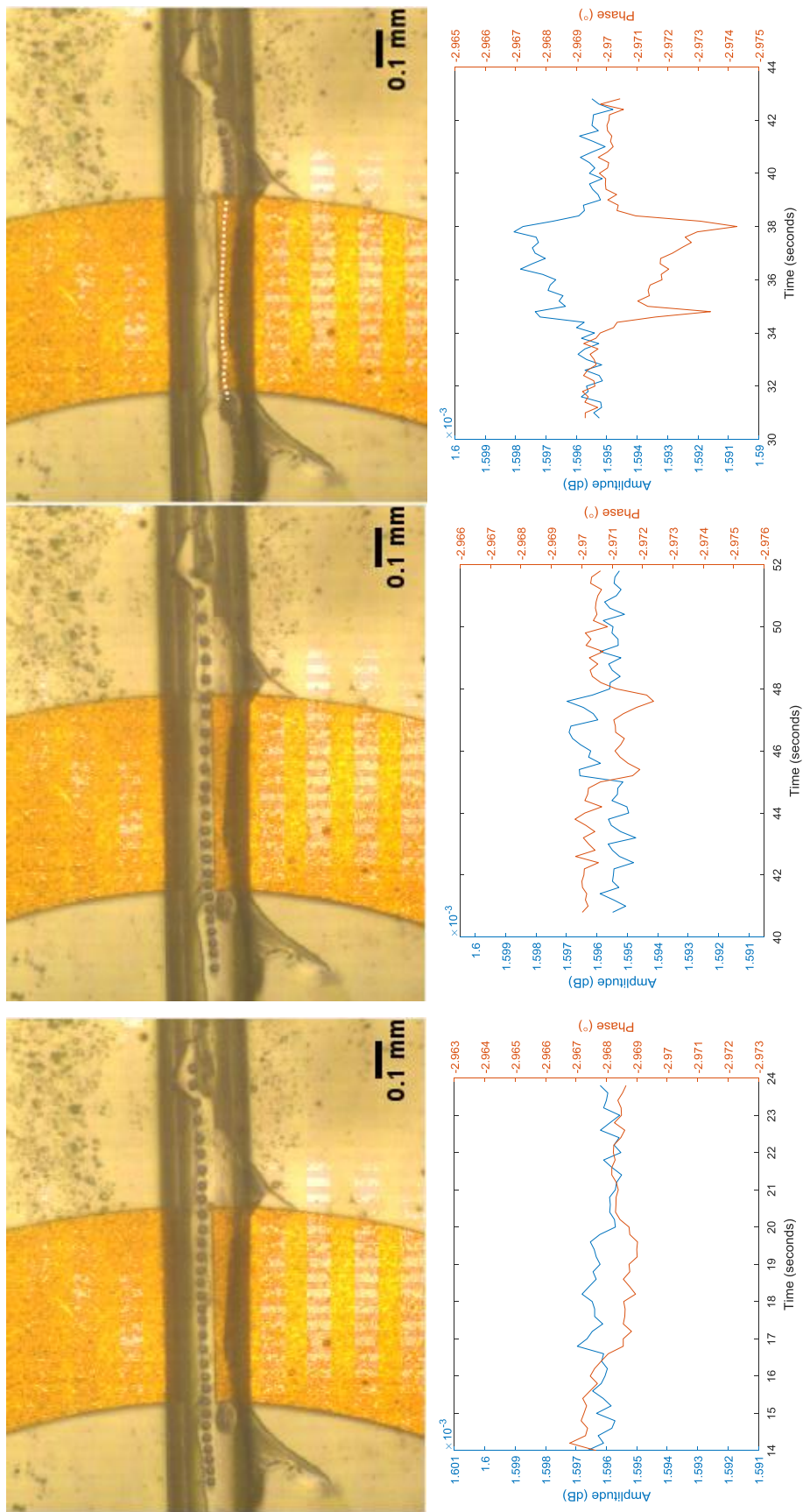


Figure 7.4.3: Composite images of microscope video feed showing the passage of $25\text{ }\mu\text{m}$ PMs through a capacitive gap and the resultant signal in both amplitude and phase. From left to right the sphere passes closer to the exposed metal surface, with the right hand example showing the passage directly over the metal, making the sphere difficult to see, so that the path has been highlighted with a white dotted line. All plots are shown on the same scale, with an amplitude range of $0.01\text{e}^{-3}\text{ dB}$ and a phase range of 0.01° .

7.4.1.4. SENSITIVITY TO SPHERE HEIGHT

Due to the microscope arrangement, it is not currently possible to directly measure or visualise the height of the particle in the channel. However, the vertical distance of the particle from the metal will have an effect on the sensitivity of the measurements in the same way as its lateral position. Figure 7.4.4 shows two measurements of the same polystyrene sphere, with similar lateral paths across the sensing region. It can be seen that the two beads reflect the light of the microscope camera differently, suggesting they lie at different heights in the channel. From the observed signal it can be assumed that the lighter one lies lower in the channel, closer to the metal surface, due to the larger signal observed as it passes.

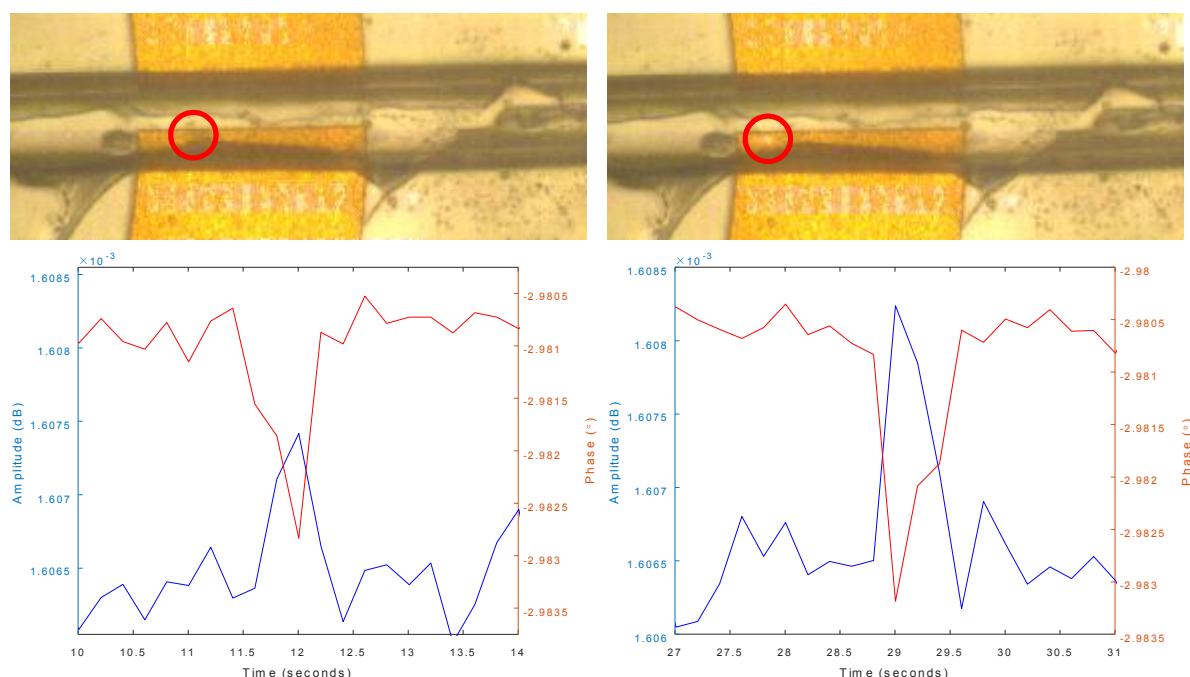


Figure 7.4.4: Two passes of the same sphere with similar lateral paths with potentially different heights within the channel. Top: Microscope images of the sphere positioned over the metal showing different optical properties. Bottom: Real-time amplitude and phase measurements for the two passes. Both plots have the same amplitude and phase intervals on their axes (Amplitude: 0.0025×10^{-3} dB, Phase: 0.0035°).

7.4.1.5. SENSITIVITY TO CHANNEL ALIGNMENT

After these measurements, attempts were made to improve the system by adding an outlet tube. This led to flow problems, as the high pressures required to push the liquid vertically upwards out of the outlet tube meant that cells could not be controlled or moved adequately slowly. In order to remove the outlet tube and return to a working system, the PDMS channel had to be removed and soaked in acetone. In an attempt to regain the sensitivity of the very first measurements carried out on PMs, the original channel was re-aligned and re-sealed over the same DSRR. Despite keeping all

other parameters the same, the change in alignment between the gaps and channel resulted in a complete disappearance of the sensitivity of the system, even to ethanol solutions. This could have been due to the apparent sensitivity of the system to small changes in the interaction volume, or “filling factor”, as identified by the ECM (see Section 7.2.2). From examining the alignment, shown in Figure 7.4.6, it seems that there may also be another important difference between the two set-ups arising from the channel re-alignment. The channel used for these experiments was cast from a $\sim 200\mu\text{m}$ outer diameter glass capillary, which gives the channel a cylindrical shape with ledges of PDMS encroaching from the sides into the bottom of the channel, as shown in Figure 7.4.5.

As explored through CST MWS simulations (Section 7.2.1), if this PDMS covers the surface of the edges of the metal in the capacitive gaps, it partially screens the liquid from the electric field and particles are not able to pass directly above the metal, which could explain the sudden and dramatic loss of sensitivity. CST MWS simulations show a decrease in maximum electric field strength in the channel by almost a half when the channel is sealed with the edges of the gap covered in PDMS, compared to when the liquid in the channel passes directly over the metal.

As can be seen in Figure 7.4.6, the set-up with no sensitivity has more of the metal covered by the bottom of the PDMS channel. It was thought that the covering of PDMS would reduce the electric field interacting with the liquid, reducing the sensitivity of the sensor, as the most successful set-up shows a lot more uncovered metal. For this reason, attempts were made to align one gap in the DSRR with a section of the original channel which had straight, clear sides, and after multiple manual re-alignments of the channel, sensitivity was eventually regained and further measurements were carried out on PMs.

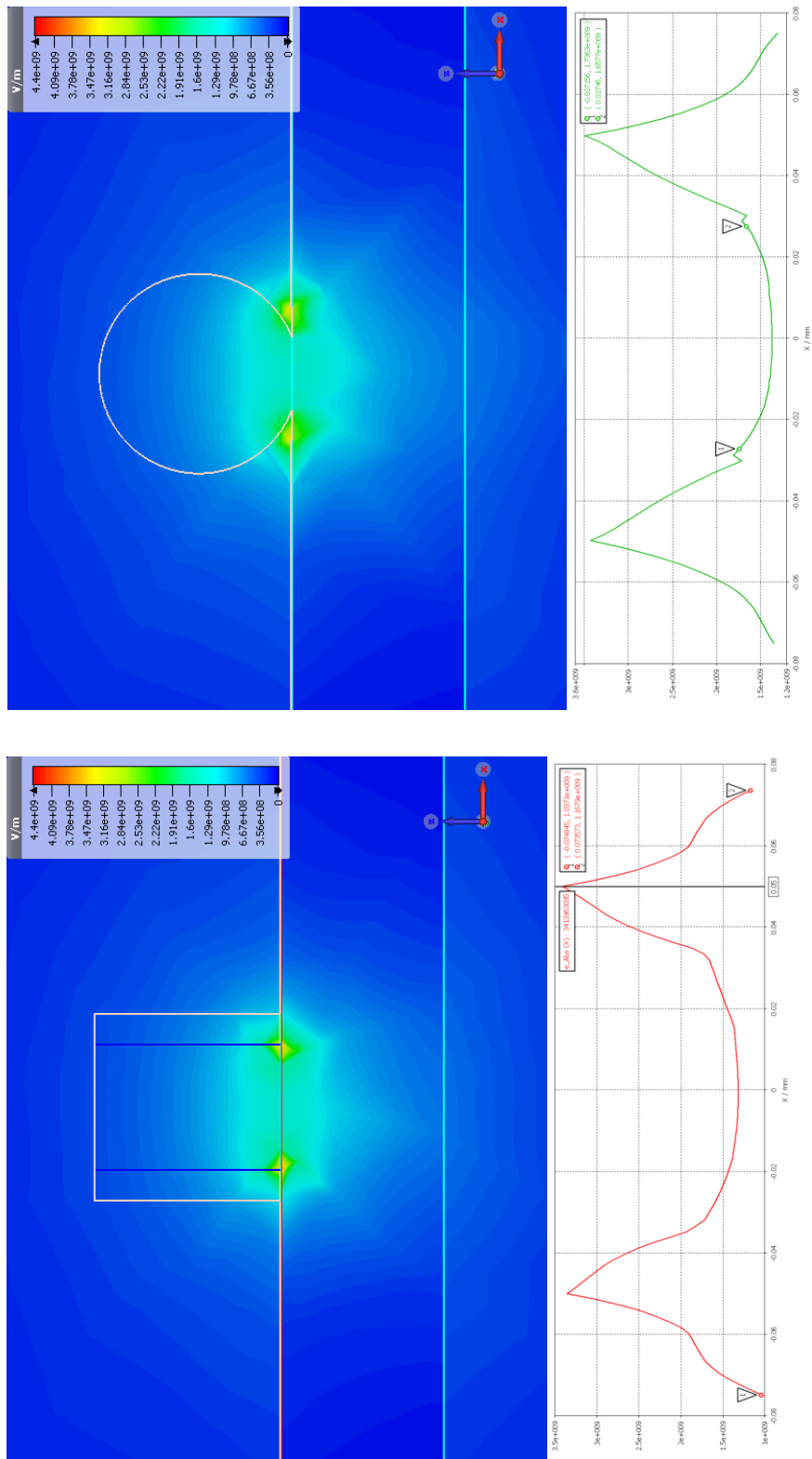


Figure 7.4.5: Top: cross sections of CST MWS simulations of two different channel geometries showing a cut through the channel at the midpoint of a sensing gap, with the electric field strength in Vm^{-1} plotted as a colour map. Bottom: evaluation of the field strength at a line across the bottom of each channel, with markers one and two denoting the channel walls. On the left a rectangular channel which passes over the metal is simulated, while on the right a cylindrical channel of similar dimensions is simulated, with the edges of the channel wall curving inwards, sealing over the metal edges of the gap.

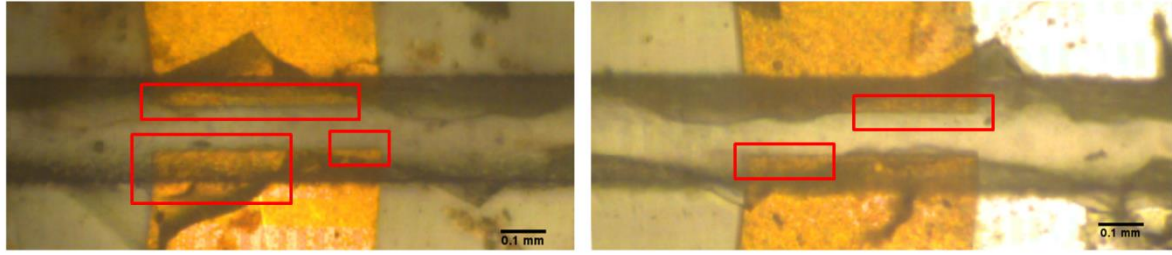


Figure 7.4.6 Stills from videos of two different experimental set-ups to compare the channel alignment and the effect this has on sensitivity. On the left is shown the alignment that gave good sensitivity, the results shown in Figure 7.4.1. On the right, the same channel has been removed and re-sealed over the same DSRR, resulting in a different alignment and loss of sensitivity. Red boxes highlight uncovered surfaces of the metal DSRR, with the rest of the metal covered in PDMS.

7.4.1.6. OPTIMISED SET-UP

Once the system had been stabilised and some sensitivity restored, as many measurements as possible were made without any further changes, so that they could be directly compared. After considerable practise with the pressure pumps, multiple measurements of individual particles could be made. All data were processed the same way to remove background drift and calculate the average shifts in amplitude and phase normalised so that measurements of the background deviated around zero. A full explanation of the processing methods trialled and chosen for use can be found in Appendix 2. Figure 7.4.7 shows an example of these first measurements made with the sensitivity re-gained and the process of carrying out multiple passes of a single sphere as well as assigning signal and background data points, and processing the resulting trace to remove the background drift. From the video stills on the left hand side of the figure it can be seen that the bottom of the PDMS channel was no longer sealing on top of the metal, and that some of the edge of the metal was protruding into the channel and therefore in contact with the sample.

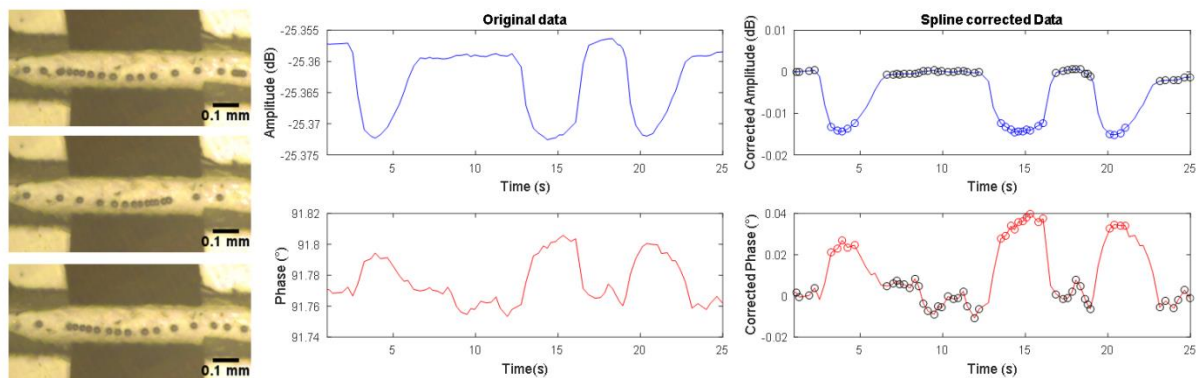


Figure 7.4.7: Example results from the second iteration of the set-up, showing the sensitivity re-gained. On the left are composite images from the microscope video showing recorded passages of an individual 25 μm polystyrene sphere. In the middle are the raw data traces of amplitude and phase, as measured and collected from the VNA. On the right is shown the same data after background removal, with black and coloured circles signifying background and signal data points, respectively.

Once the data have been processed in this way, the individual normalised signal and background data points for amplitude and phase can be plotted against each other and the average shifts ascertained. Figure 7.4.8 shows the results of three different experiments taken over a period of four months with the same set-up using the same commercially available 25 μ m polystyrene spheres plotted this way. From repeat measurements, the system gives average shifts in amplitude and phase of 0.013 dB and 0.03°, respectively, for 25 μ m polystyrene spheres.

It seems that multiple re-alignments of the channel in order to try to restore sensitivity resulted in a change in sign of the shifts observed in both amplitude and phase. This is understandable given the sensitivity of the system to the alignment of the channel and the effect this has on the overlap of the two resonances.

Although the average values for amplitude and phase are similar for all three experiments there does seem to be a difference in how spread out the data are. It generally seems that the more individual spheres are measured, the greater the spread of the data. Breaking down these results into individual spheres shows that some spheres result in higher shifts in both amplitude and phase, which may be due to some spheres being closer to the bottom of the channel. Spheres giving larger shifts would often be slower and more difficult to move when being manipulated, which could suggest they were closer to the bottom of the channel. Some spheres also result in a wider spread of shifts, which may be due to spheres settling out while being manipulated, which was also noticed during measurements. There is also an increase in the standard deviation of background (water) measurements when more measurements have been taken, which suggests that a large sample of spheres needs to be measured in order to get results representative of all paths taken through the sensing gap.

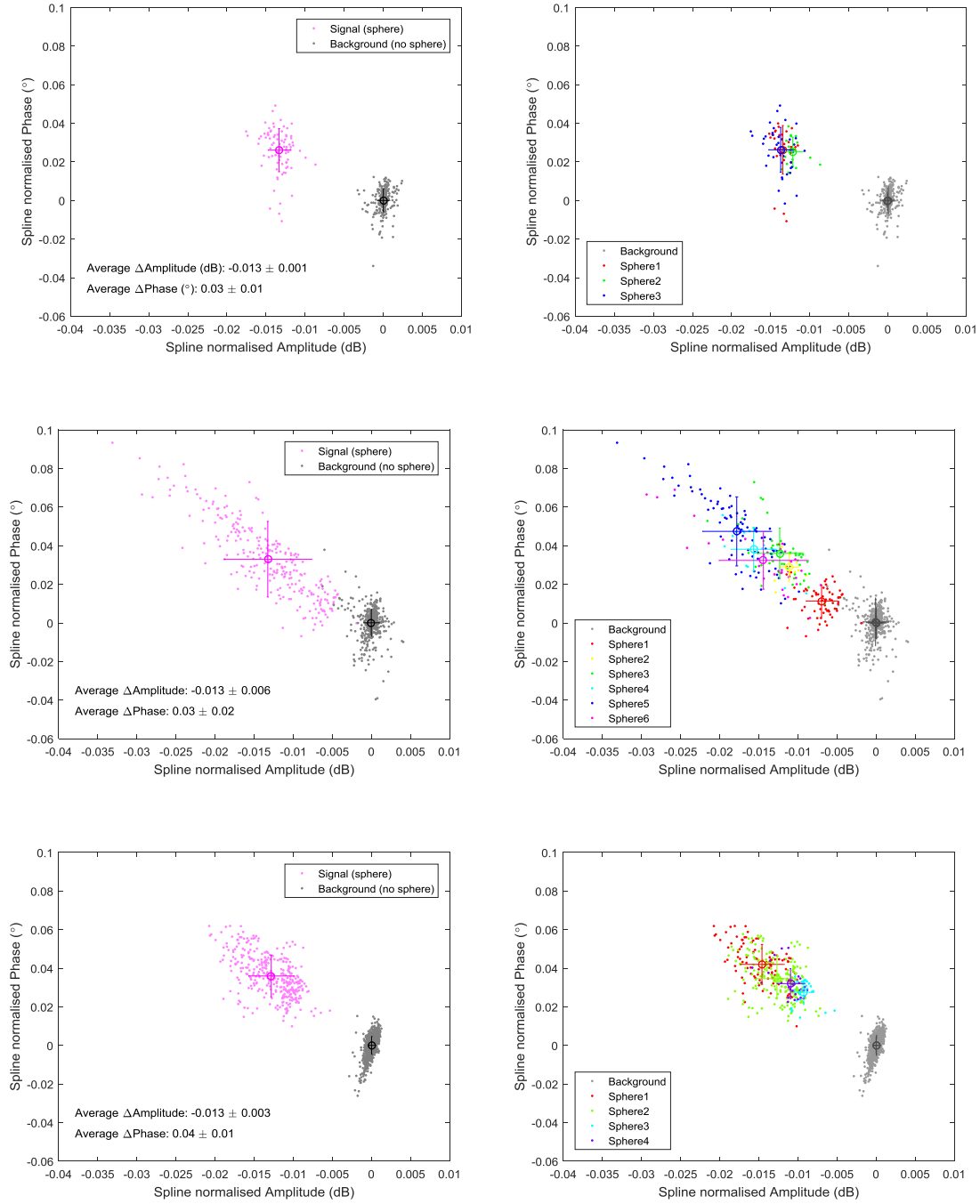


Figure 7.4.8: Progression, from top to bottom, of 25μm sphere measurements over time plotted as normalised phase against normalised amplitude (as processed according to Appendix 2), showing similar average shifts in both. On the left side, pink dots represent measurements made as the sphere passes through the sensing gap, while grey dots represent measurements made of the background water with no sphere in the gap. The average values for amplitude and phase for both sphere and background measurements are marked using pink and black circles, respectively. The right side shows the data split into individual spheres measured, with coloured dots representing measurements made as the sphere passes through the sensing gap, while grey dots represent measurements made of the background water with no sphere. Total lengths of all error bars denotes two standard deviations of the data.

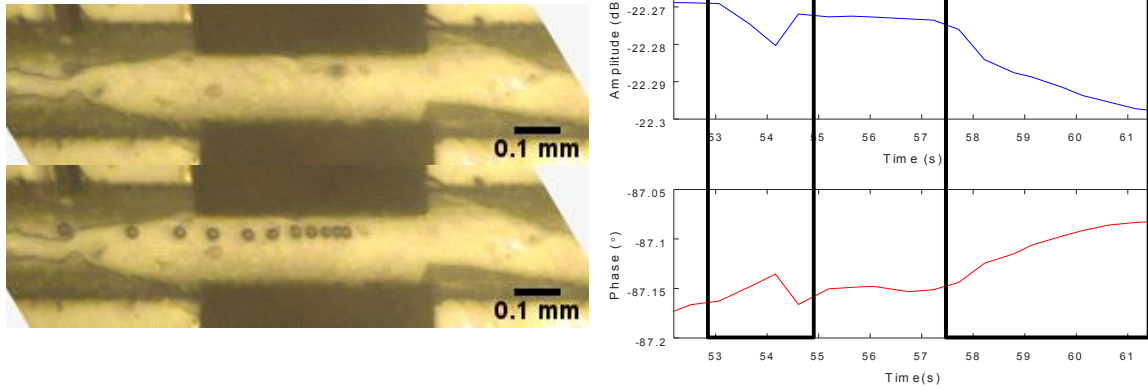


Figure 7.4.9: Measurement of a PS sphere settling out. On the left are composite microscope images showing a cell passing (top) then passing and settling out on the base of the channel (bottom). On the right the real-time amplitude and phase measurements, with the two sphere passages outlined in black squares.

Given the sensitivity to bead path and height in the channel, re-design of the channel dimensions and alignment may improve the repeatability of measurements. This is discussed in Section 8.2.1.

One experiment carried out between the first and second results shown in Figure 7.4.10 demonstrates the erratic nature of the phase measurements, which for one measurement show shifts in the opposite direction. It is not clear under what conditions the phase shift is negative or positive.

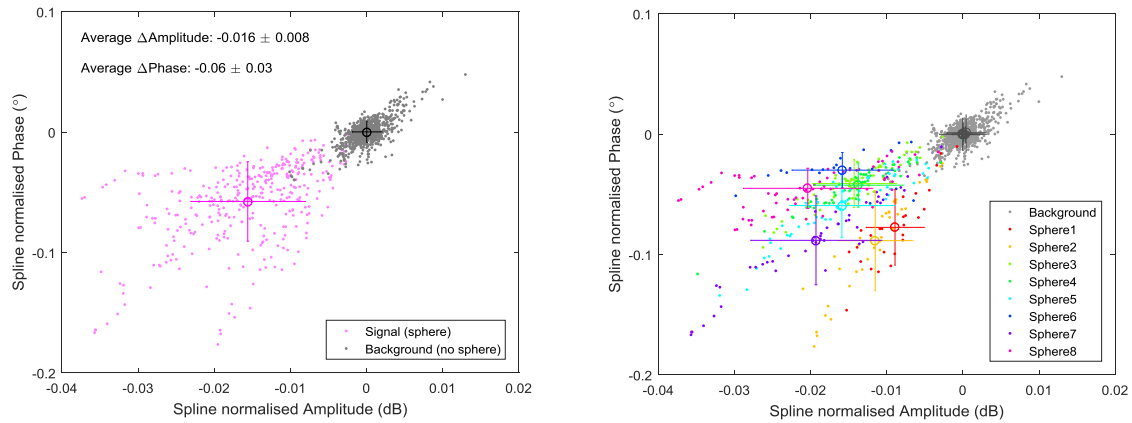


Figure 7.4.10: Left: All corrected amplitude and phase data points plotted against each other for 8 PMs measured showed as pink dots. Background measurements are shown in grey, while the mean of both the signal and background measurements are denoted by pink and black circles, respectively. Right: Data are split into individual spheres measured, with coloured data points denoting signal measurements and grey or black denoting background. Circles represent the means of the data. The total length of all error bars denotes two standard deviations of the data.

Although the amplitude shifts seem repeatable within experimental configurations, and according to the ECM depend quite trivially on the real permittivity of the material in the capacitive gap, the

situation for the phase shifts is not so clear. Added to this is the complication of the phase shift due to the coaxial cables, which makes the measured phase shift difficult to understand.

The absolute values of the amplitude and phase shift are slightly higher in this experiment than in the previous three, which may be due to the presence of air bubbles in the sensing region during measurements. While trying to keep the system and the alignment of the channel as unchanged as possible, the PDMS was not removed from the ring for cleaning. This experiment suffered from a build-up of debris and the formation of bubbles in the channel which were difficult to remove. As shown in Figure 7.4.11 these bubbles stuck to the channel walls and grew slowly. The effect was seen over the length of the channel, including the capacitive sensing region, where their effect is not understood. It is not clear from the images what height in the channel the air bubbles formed and it is possible that, rather than occurring at the bottom of the channel and blocking the electric field from the metal, they are higher up and cause a change in both the path of the spheres as well as the filling factor of the channel. This could result in spheres passing closer to the bottom of the channel as well as creating a smaller sensing volume, both of which would causing an increase in the shifts observed, possibly increasing the sensitivity of the system. The changing size of the bubble (growing as well as washing away) may also explain the large spread of data observed for sphere measurements during this experiment.

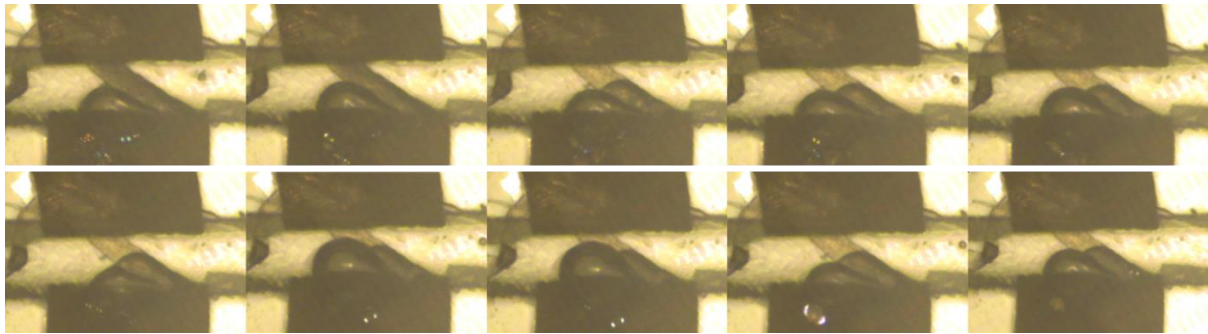


Figure 7.4.11: Stills taken from each measurement video showing the presence and growth of an air bubble in the sensing region for every sphere measured. Progression in time from left to right and top to bottom.

Even though the formation of stable air bubbles in the sensing region may result in increased shifts this effect is not controllable or repeatable, thus resulting in data that cannot be directly compared from one experiment to the next. For this reason, data from this experiment will not be used for comparison of results. However, for future measurements it seems as though reducing the interaction area by using a channel with a smaller height may have a similar positive effect on sensitivity. This is discussed in Section 8.2.1.

Efforts were made to clean the channel and soak the PDMS without altering the alignment of the channel over the DSRR whenever bubbles were observed. For later measurements, background liquids were left to flow for some time before measurements were taken, in efforts to clear bubbles that would form.

7.4.2. 11 μ M DIAMETER MICROSPHERES

In order to gauge the dependence of the signal on the size of the particle, 11 μ m PMs (the smallest available) were tested under the same conditions as the 25 μ m PMs. Example data are shown in Figure 7.4.12, while average results are shown in Figure 7.4.13.

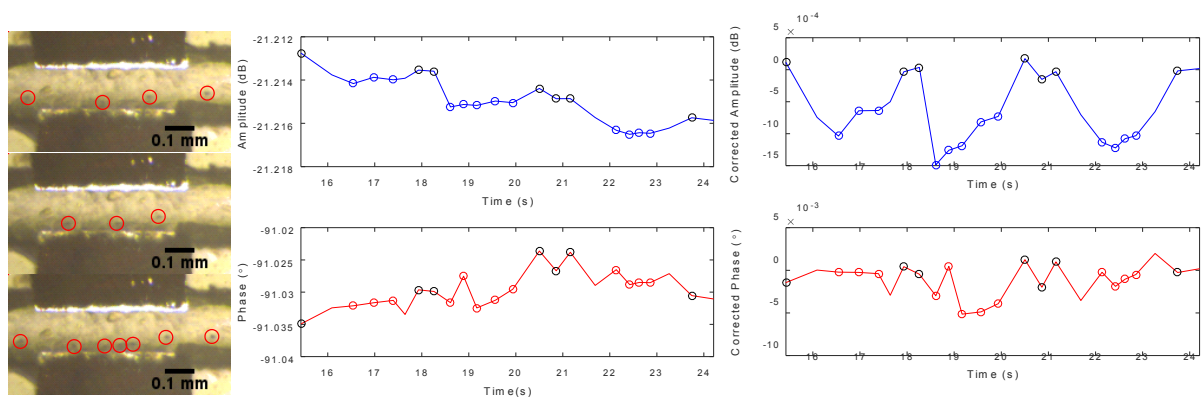


Figure 7.4.12: Example traces obtained from 11 μ m PS sphere measurements. On the left are composite images from the microscope video showing recorded passages of an individual 11 μ m polystyrene sphere. The positions of the sphere are highlighted in red circles for clarity. In the middle are the raw data traces of amplitude and phase, as measured and collected from the VNA. On the right is shown the same data after background removal, with black and coloured circles signifying background and signal data points, respectively.

The 11 μ m spheres were more difficult to control than the larger spheres, moving much quicker for the same pressure changes, and showing more signs of settling out. This may be due to the experimental set-up; since these spheres are smaller and more difficult to see either with the camera or by the shift they induce, those which are flowing closer to the bottom and therefore are slower and induce larger shifts may be the ones that are spotted. Spheres closer to the bottom are of course more likely to settle out sooner than those further up in the channel. The process of controlling the spheres to pass multiple times backwards and forwards over the gap may also slow them down further and result in them settling out.

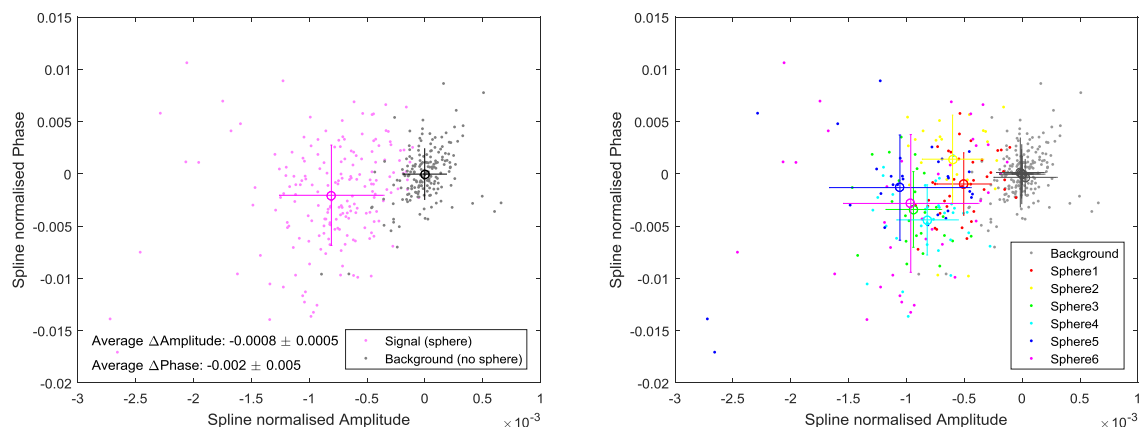


Figure 7.4.13: Graphs of normalised phase and amplitude measurements for 11 μm PS sphere experiments. On the left all data points are plotted for all spheres measured as dots, while the mean is denoted by a circle, with the average shifts and errors quoted as ± 1 standard deviation of the data. On the right each individual sphere measured is plotted separately as dots, with the means denoted by circles. Total length of all error bars denotes two standard deviations of the data.

As could be expected, the smaller spheres result in smaller shifts than the larger ones (see Table 7.4.1), with the phase measurements exhibiting both positive and negative shifts, suggesting that these data points may not be significantly out of the noise floor of the measurement system. Shifts in phase were small compared to shifts in amplitude, showing both positive and negative individual phase measurements. This may be symptomatic of the lower sensitivity of the phase measurements compared to the amplitude measurements, with the phase signals perhaps not strong enough compared to the background variation to be reliable. Across 6 spheres, the system gives average shifts in amplitude and phase of -0.0008 dB and -0.0020° , respectively, for 11 μm polystyrene spheres.

7.4.3. PS SPHERE SUMMARY

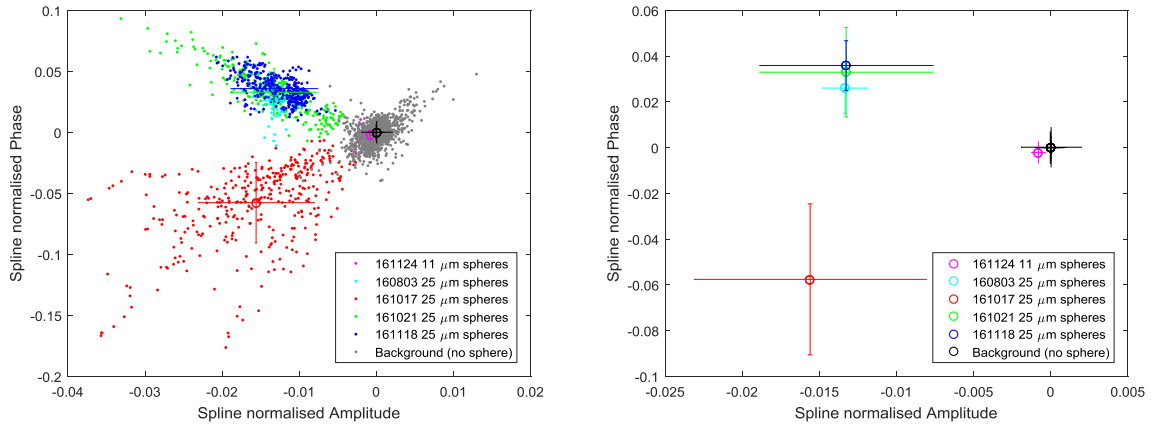


Figure 7.4.14: Plots of all PS sphere measurements, showing 25 μm experiments alongside 11 μm experiments. On the left all data points are plotted as coloured dots, with background measurements in grey, while the means are denoted by circles. On the right only the means and standard deviations are shown, for clarity. Total length of all error bars is equal to two standard deviations of the data.

The phase and amplitude shifts induced by the different polystyrene spheres measured are shown in Figure 7.4.14. As would be expected, and as can be seen from Table 7.4.1, larger spheres result in larger shifts in both amplitude and phase.

Table 7.4.1: Summary of the average shifts in both amplitude and phase of polystyrene spheres measured using the same set-up. Errors are quoted as $\pm$ one standard deviation of the data, and values of the means are quoted to a significance level according to their error.

Experiment date	160803	161017	161021	161118	161124
Diameter (μm)	25	25	25	25	11
Average phase shift $\pm \sigma$ ($^\circ$)	0.03 ± 0.01	-0.06 ± 0.03	0.03 ± 0.02	0.04 ± 0.01	-0.002 ± 0.005
Average amplitude shift $\pm \sigma$ (dB)	-0.013 ± 0.001	-0.016 ± 0.008	-0.013 ± 0.006	-0.013 ± 0.003	-0.0008 ± 0.0005

Considering the instability in the phase in this experimental configuration relative to the amplitude, and the lack of understanding of its origin, amplitude shift data are focussed on when comparing results. According to perturbation theory, the change in loss induced by each passing PS sphere should be proportional to the volume integral of E^2 (electric field) over the sphere volume, assuming that the electric field is homogeneous over the volume of the sphere. If E is considered to be constant then the change in loss should be proportional to the volume of the spheres. This relationship is based on cavity perturbation theory, so the coupled sensor may deviate from this relation, but from the ECM sensitivity analysis discussed in Section 7.2.2.4 it can be seen that at low ethanol concentrations the correlation between inverse quality factor shift and volume of ethanol does remain linear, as shown in Figure 7.4.15.

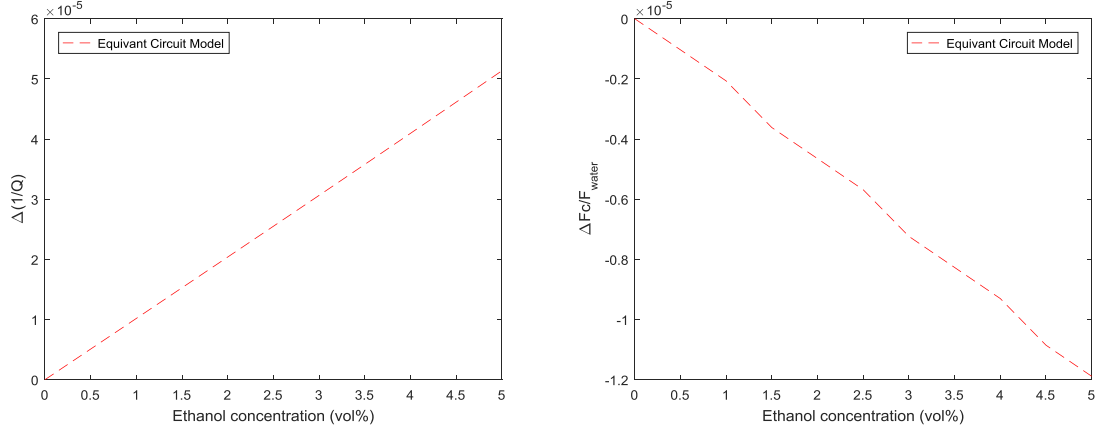


Figure 7.4.15: Plot of the ECM interpolated values of inverse quality factor and normalised frequency shift for very low concentrations of ethanol in deionised water, showing a linear relation.

Looking at the average ratio of the losses (or reciprocal quality factor shifts) calculated from all amplitude signal shifts of the two sphere sizes according to Equation 7.4.1, it can be seen to correlate roughly with the ratio of their volumes, within the 1σ error margins (where $\sigma = 1$ standard deviation, calculated according to the standard non-linear combination of errors, assuming a covariance of 0).

$$\frac{loss_{25\mu m}}{loss_{11\mu m}} = \frac{\frac{1}{Q_{25\mu m}} - \frac{1}{Q_0}}{\frac{1}{Q_{11\mu m}} - \frac{1}{Q_0}} = \frac{\frac{Q_0}{Q_{25\mu m}} - 1}{\frac{Q_0}{Q_{11\mu m}} - 1} = \frac{\sqrt{\frac{A_0}{A_{25\mu m}} - 1}}{\sqrt{\frac{A_0}{A_{11\mu m}} - 1}} = \frac{10^{\frac{-\Delta A_{25\mu m}}{20}} - 1}{10^{\frac{-\Delta A_{11\mu m}}{20}} - 1} = 17 \pm 12 \quad \text{Equation 7.4.1}$$

$$\frac{Volume_{25\mu m}}{Volume_{11\mu m}} = 12 \pm 1.21 \quad \text{Equation 7.4.2}$$

This is to be expected, since the low permittivity polystyrene spheres should, like ethanol, contribute to the displacement of water, thus decreasing the losses of the measured volume, with smaller spheres inducing a smaller change in the losses than larger spheres.

7.5. REAL-TIME MEASUREMENTS OF CULTURED CELL SUSPENSIONS

Once the system had proven successful with the large polystyrene spheres it was tested with cells. Cultured nucleated cells, available from collaborators were used: mouse myoblast cell line (C2C12), human mesenchymal stem cells (HMSCs) and human monocytes. Measurements of different cell types were carried out over a period of almost three months.

7.5.1. MOUSE MYOBLAST CELL LINE (C2C12)

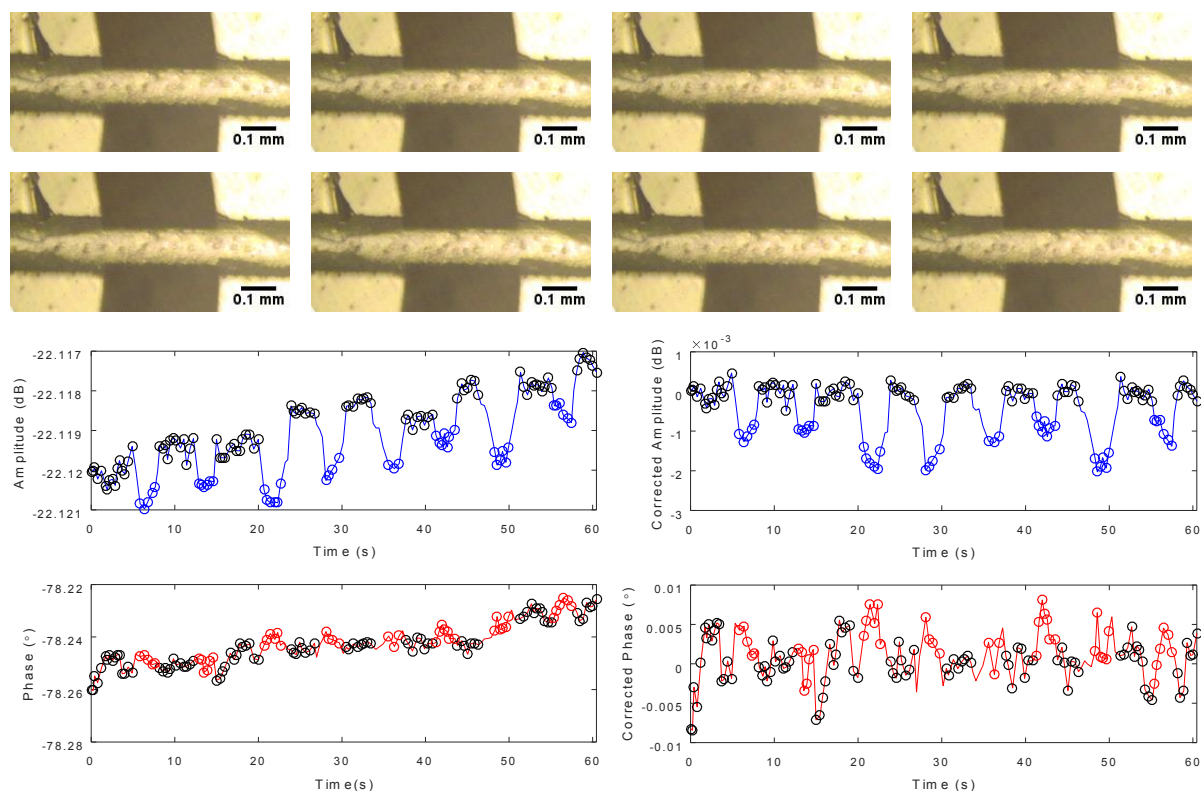


Figure 7.5.1: Results showing the correlation between C2C12 cell passages and signals observed. Top: Composite microscope images of each individual passage measured, in the order left to right and top to bottom. Bottom: Both the raw and processed amplitude and phase data, with data traces shown in blue and red for amplitude and phase, respectively. Black circles denote measurements of the background solution, while coloured circles represent measurements taken with the cell in the capacitive gaps. The graphs on the left show the raw data, while those on the right show the shifts after removal of background drift according to Appendix 2.

The first biological cells measured were mouse myoblast cells from the C2C12 cell line. Myoblast cells are the precursor to muscle cells (myocytes). The C2C12 cell line is a subclone of the mouse myoblast cell line established by Yaffe and Saxel obtained from injured thigh muscle of a mouse population genetically modified to develop a muscular dystrophy disorder.²⁰³ These cells, although immortal, are from normal origin and should not show any abnormal physiology associated with cancer. The C2C12 cells measured had an average diameter of $\sim 32 \mu\text{m}$ according to analysis of footage taken with the high speed camera. The same correlation between signal and cell passage that was observed with polystyrene microspheres can be seen with the C2C12 cells, as shown in Figure 7.5.1.

Repeat measurements of 5 different cells gave average shifts in amplitude and phase of -0.0015 dB and 0.006° , respectively, as shown in Figure 7.5.2.

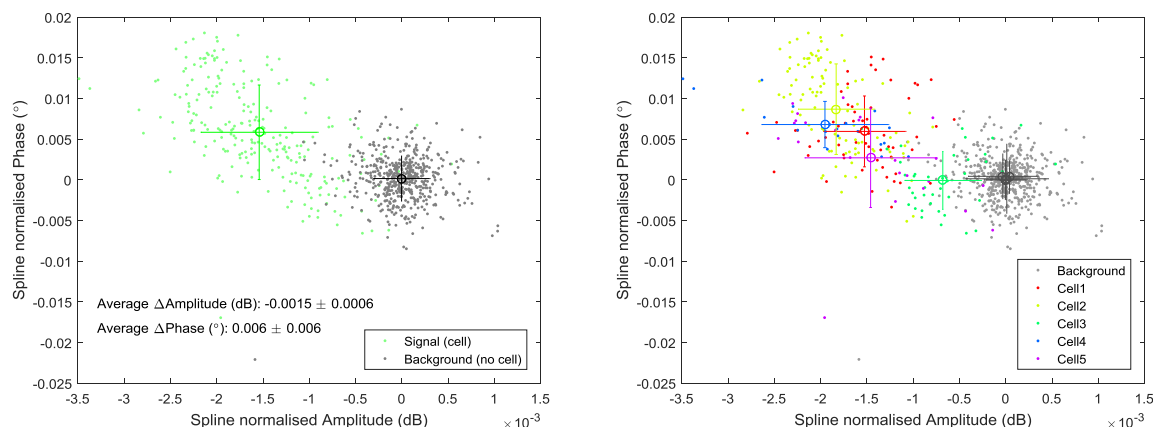


Figure 7.5.2: Processed and plotted amplitude and phase results from C2C12 cells measured. On the left all the results are plotted together with the average shifts displayed, while on the right results from each individual cell measured are plotted in separate colours with the total length of error bars denoting two standard deviations.

Although a lot of variation in signals can be ascribed to differences in cell height and path during passages, it can also be expected that biological cells will show significantly more heterogeneity in both size and composition compared to the polystyrene spheres. However, the standard deviations of both the amplitude and phase data for the cell passages are 6 times lower than that of the PMs. This is probably due to a decrease in standard deviation overall, with the standard deviation of the background measurements also reduced by a factor of 4 for this experiment compared to the average for PMs. This may be due to the smaller difference in permittivity between the cells and the background PBS solution, with variations in composition and cell path therefore not producing as large an effect, or due to the fact that the higher permittivity cells do not induce as significant a deformation of the electric field.

7.5.2. HUMAN MESENCHYMAL STEM CELLS (HMSCs)

Following the first successful biological cell measurements, the same system was tested with a different type of cell, cultured human mesenchymal stem cells, to see if the cell types could be differentiated based on their physiological differences. Commercially available primary human mesenchymal stem cells, taken from the bone marrow of normal volunteers, were used for these experiments. In contrast to the C2C12 cells, these cells are both of human origin, and of mesenchymal phenotype. According to our collaborators, HMSCs are the largest cell type to study, and therefore were a promising target for the system. However, according to camera footage taken during measurements, these cells had an average diameter of $\sim 30 \mu\text{m}$, similar to that of the C2C12 cells. Figure 7.5.3 shows the cells in the sensor and an example signal trace.

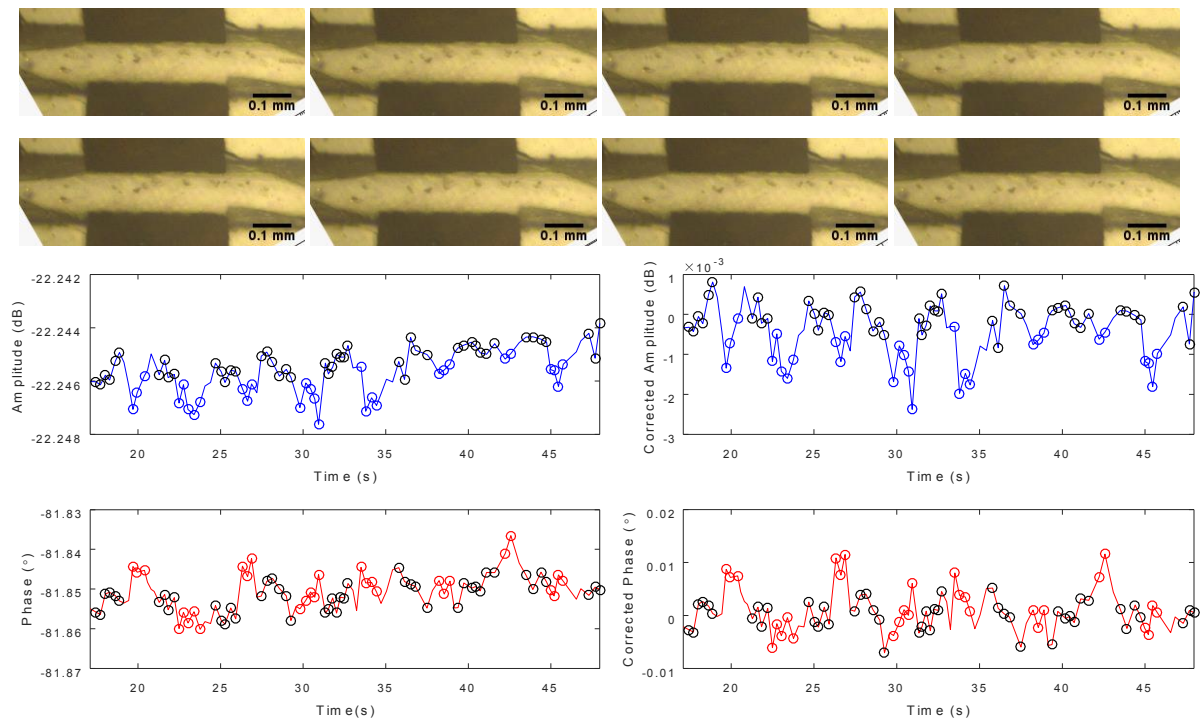


Figure 7.5.3: Results showing the correlation between human mesenchymal stem cell passages and signals observed. Top: Composite microscope images of each individual passage measured, in the order left to right and top to bottom. Bottom: Both the raw and processed amplitude and phase data, with data traces shown in blue and red for amplitude and phase, respectively. Black circles denote measurements of the background solution, while coloured circles represent measurements taken with the cell in the capacitive gaps. The graphs on the left show the raw data, while those on the right show the shifts after removal of background drift according to Appendix 2.

Repeat measurements of 7 different cells gave average shifts in amplitude and phase of -0.0011 dB and 0.0042° , respectively, as shown in Figure 7.5.4.

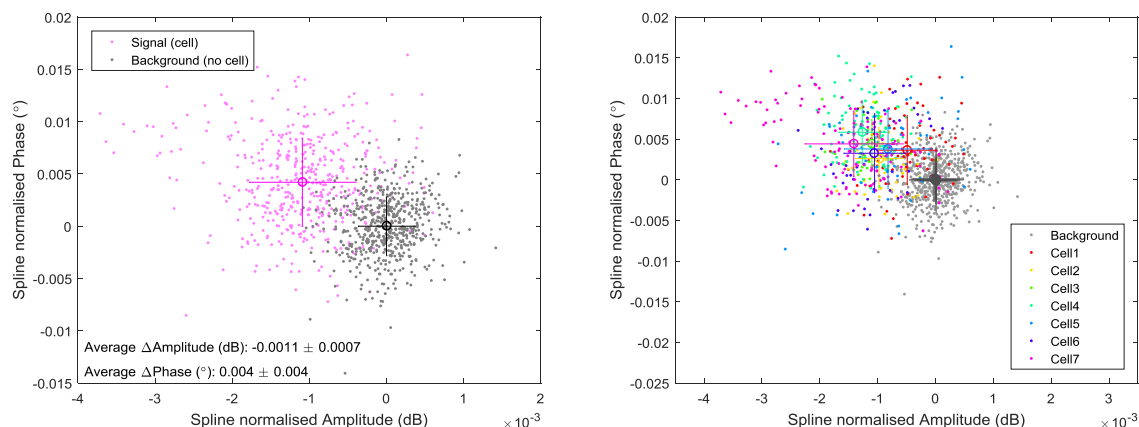


Figure 7.5.4: Processed and plotted amplitude and phase results from human mesenchymal stem cells measured. On the left all the results are plotted together with the average shifts displayed, while on the right results from each individual cell measured are plotted in separate colours with the total length of error bars denoting two standard deviations.

7.5.3. HUMAN MONOCYTE LINE

Human monocytes were the last cell type to be investigated. Monocytes are a type of white blood cell, and so are a relevant cell type for the proposed application of the sensor. Monocytes are the largest of the white blood cells and have large, lobed nuclei. These cultured monocytes were, on average, $\sim 27 \mu\text{m}$ in diameter, making them the smallest cells measured.

The monocyte cell line differs from the other two measured in that the source of the cells is a patient with acute monocytic leukaemia, which explains the cells lack of senescence. Although the primary cells will have been cancerous in nature, the cell line derived from these cells is widely used as a model for normal monocytes. It is not easy, therefore, to speculate whether these cells could have some physiological difference to “normal” cells that could be detected by our system, without testing the primary cells, which is impossible.

As the last cell type to be measured, the monocyte experiment suffered from air bubbles seen in previous measurements. In order to be able to collect results, air bubbles that were noticed were given time to clear and any data collected which showed air bubbles in the gap were discounted.

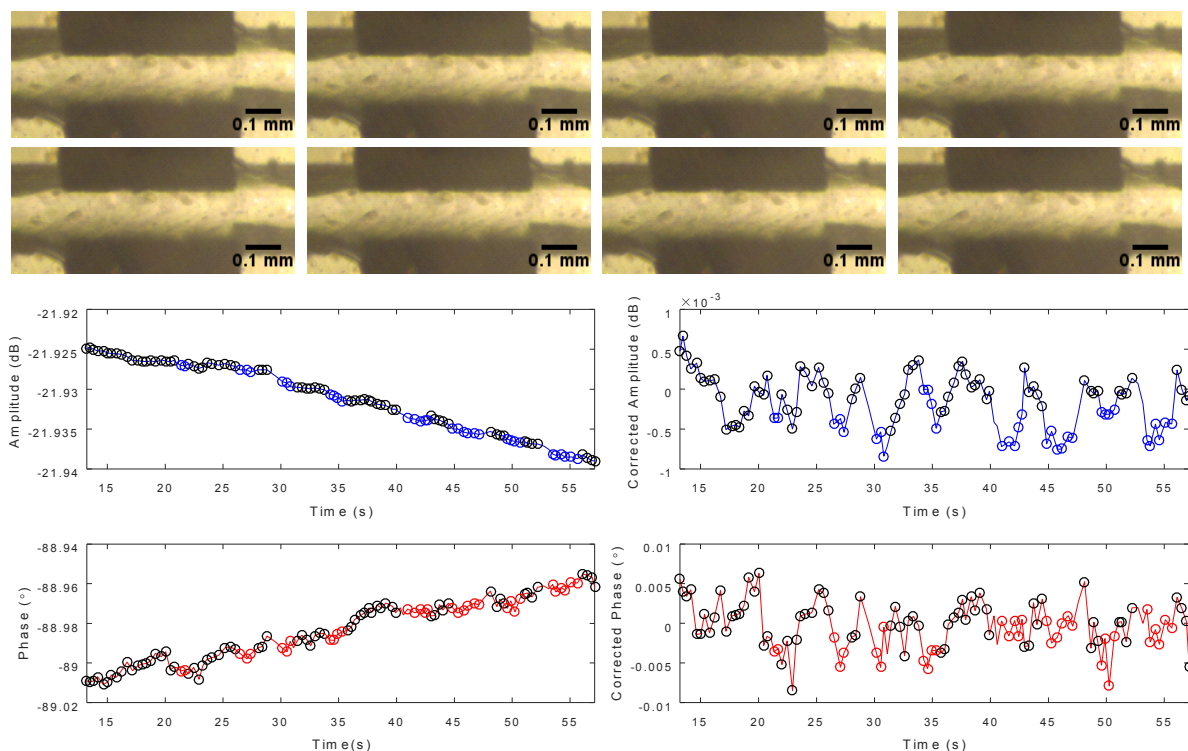


Figure 7.5.5: Results showing the correlation between human monocyte passages and signals observed. Top: Composite microscope images of each individual passage measured, in the order left to right and top to bottom. Bottom: Both the raw and processed amplitude and phase data, with data traces shown in blue and red for amplitude and phase, respectively. Black circles denote measurements of the background solution, while coloured circles represent measurements taken with the cell in the capacitive gaps. The graphs on the left show the raw data, while those on the right show the shifts after removal of background drift according to Appendix 2.

On average, repeat measurements of 7 different cells gave shifts in amplitude and phase of -0.00067 dB and -0.0054° , respectively, as shown in Figure 7.5.6.

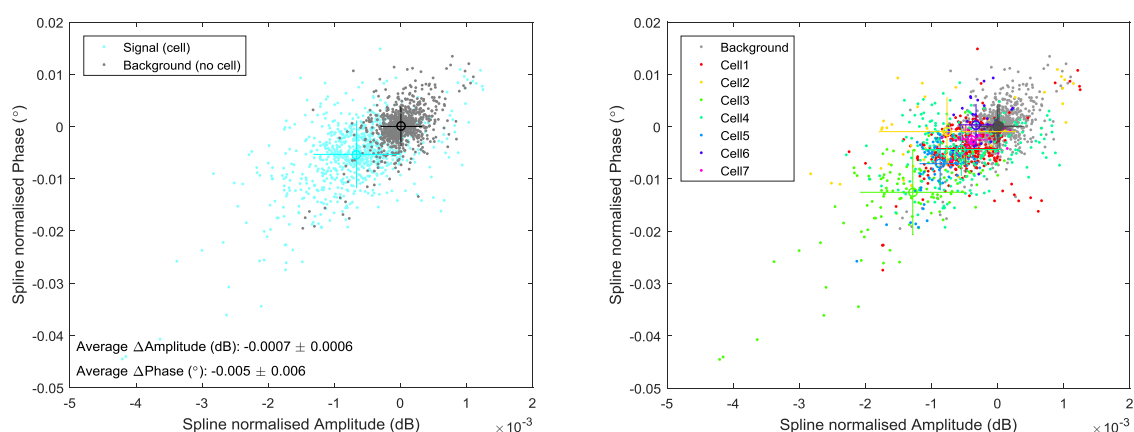


Figure 7.5.6: Processed and plotted amplitude and phase results from human monocytes measured. On the left all the results are plotted together with the average shifts displayed, while on the right results from each individual cell measured are plotted in separate colours with the total length of error bars denoting two standard deviations

7.5.4. BIOLOGICAL CELLS SUMMARY AND DISCUSSION

Given the seemingly erratic nature of the phase shifts and the lack of understanding of its physical cause, only results of the amplitude shifts of the different cell types are compared. Histograms of the amplitude shifts of the different cells have been drawn up in Figure 7.5.7 showing the shifts of the different cell populations.

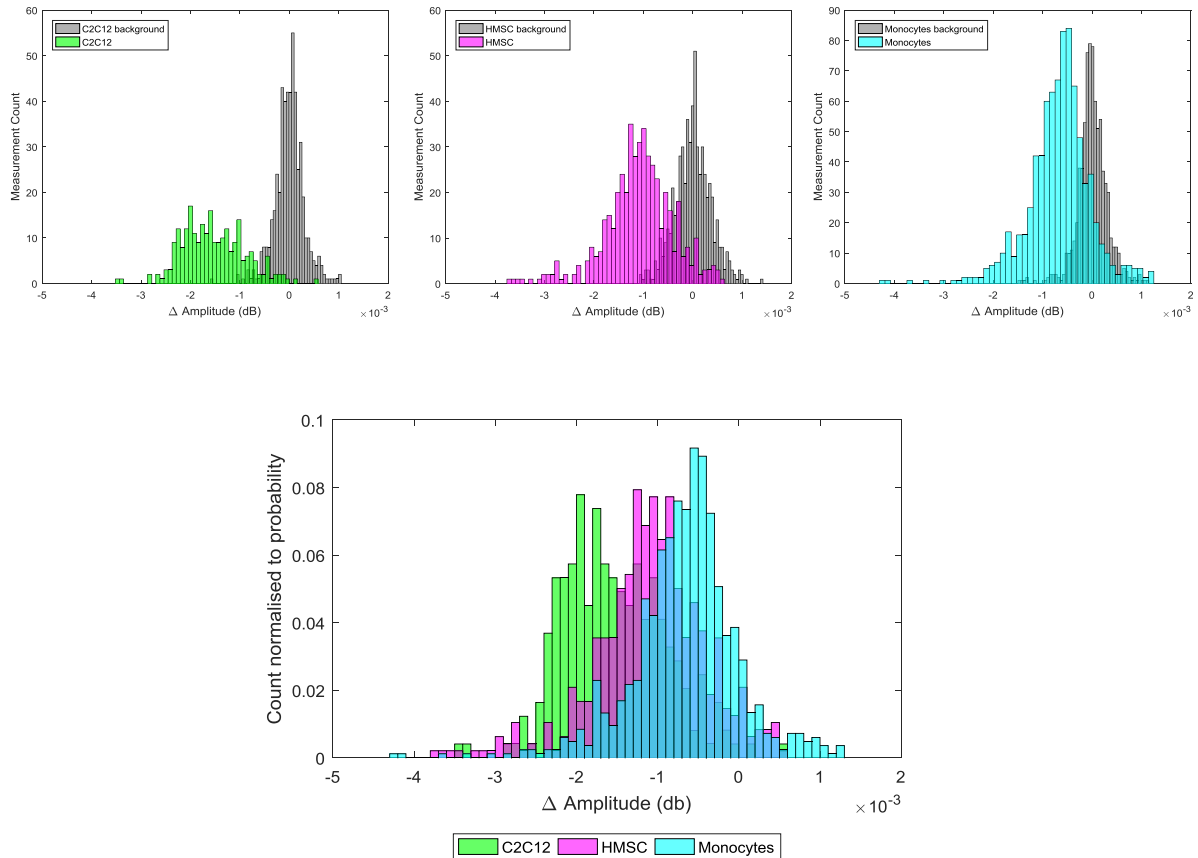


Figure 7.5.7: Histograms of observed amplitude shifts of the different cell types measured, relative to the background PBS (top row) as well as relative to each other (bottom row).

In order to ascertain whether differences in the signals observed could be correlated to physiological differences between the cells, the size of each individual cell measured was determined from the microscope video stills of each cell passage. The method is fully explained in Appendix 3, and although it is not perfect, it does allow cell size analysis to be done for the individual cells measured. The sizes of the different cell types estimated by analysis of the video footage of cell passages can be compared to the shifts to ascertain if there is any correlation.

Table 7.5.1: Comparison of average cell diameter as ascertained by video footage analysis and average induced amplitude shift for each of the three cell types measured. Sizes are quoted to 0 decimal places as the size of each pixel is $\sim 3 \mu\text{m}^2$

Cell type	C2C12	HMSC	Monocyte
Average diameter from video $\pm \sigma$ (μm)	33 ± 3.0	30 ± 3	26 ± 4
Average amplitude shift $\pm \sigma$ (dB)	-0.0015 ± 0.0006	-0.0011 ± 0.0007	-0.0007 ± 0.0006

The data can also be broken down into individual cells measured, and the mean size of each individual cell can be compared to the average amplitude signal generated by its passage, as shown in Figure 7.5.8. If cell size is a factor in the observed shifts, results can be normalised for size differences, and remaining trends in the results could be attributed to other differences between the cells.

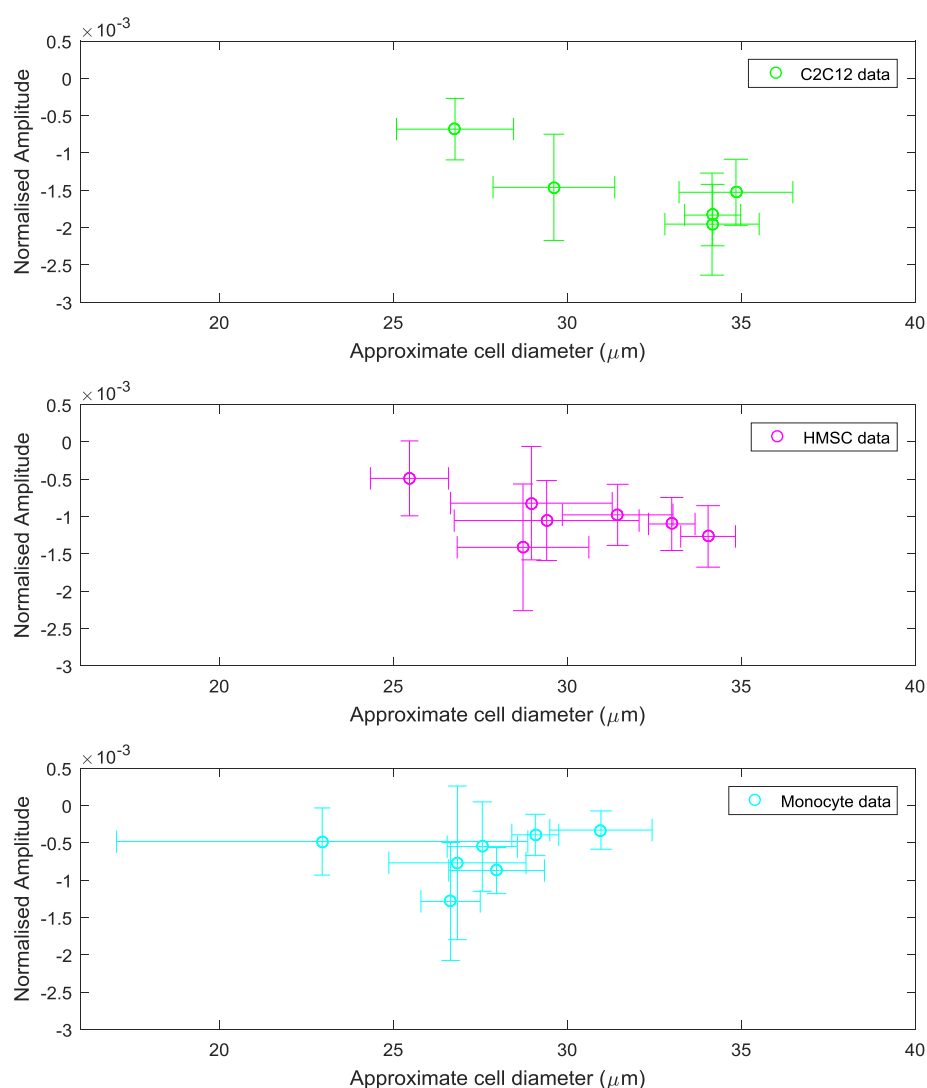


Figure 7.5.8: All biological cell data plotted as a function of approximate cell size, as determined from video analysis. The total lengths of the error bars represent 2 standard deviations of the size and amplitude data.

Although this method of cell size determination accounts for the cells actually measured, it is not a precise approach, resulting in some values with large standard deviations due to varying cell height and position in the channel as well as the limited resolution of the camera. The monocyte data show the largest variation in estimated cell size, which may be due to their small size overall combined with the resolution of the camera (3 μm pixels).

From Figure 7.5.8 it seems that for the C2C12 and HMSC data there may be some slight dependence of the strength of the signal on size of the individual cells, but this does not hold true for the monocyte data. For the C2C12 and HMSC measurements, in general it seems that a larger cell results in a larger shift in amplitude. This is to be expected, with larger cells displacing a proportionally larger amount of water from the sensing volume. The high frequency sensor can penetrate inside the cell and interact with the cell contents, meaning that larger cells should be analogous to a more concentrated solution of lipids or proteins in water.

As discussed in Section 7.4.3, according to perturbation theory, the average shift in reciprocal quality factor (the ratio of the change in loss induced by the cell, as calculated using Equation 7.5.1 which is a rearrangement of Equation 7.3.2, assuming $Q_0=1500$) should scale with volume of the cell. Therefore, as shown in Figure 7.5.9, a simple plot can be drawn up to see if there is a correlation between the average size of the cells and the average quality factor shift induced in the sensor.

$$\Delta\left(\frac{1}{Q}\right) = \frac{1}{Q_{\text{water}} 10^{\frac{\Delta A_{dB}}{20}}} - \frac{1}{Q_{\text{water}}} \quad \text{Equation 7.5.1}$$

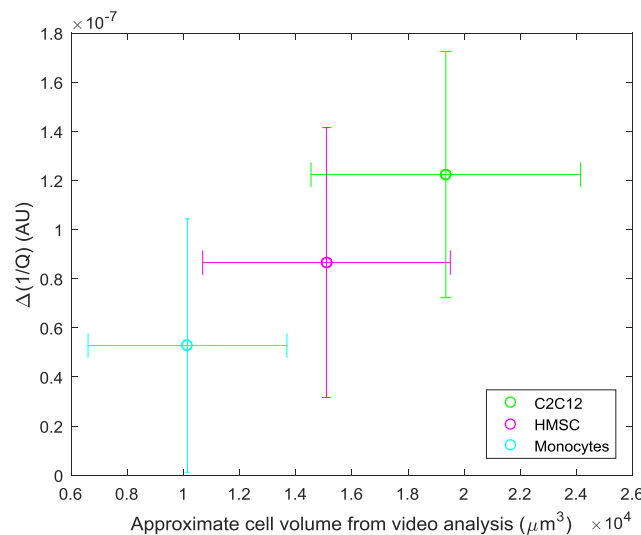


Figure 7.5.9: Reciprocal quality factor shifts calculated using average amplitude shifts plotted against average cell volume calculated from the diameters determined by video analysis. Total length of error bars denotes two standard deviations, with standard deviations directly determined from an array of values of cell volume and $\Delta(1/Q)$ derived from piece-wise calculations of all cell diameter and all amplitude shift data, respectively.

From this plot it seems like the calculated average reciprocal Q shifts are well correlated with the estimated size of the cells, with the larger cells inducing a larger shift in amplitude, as expected. This suggests that, rather than differences in cell content, size variation between cell types dominates the differences in magnitudes of the induced shifts. In order to assess whether the sensor can differentiate between the cell types due to any differences in their content, these results must be corrected for size. Since the amplitude shift is related to the inverse quality factor shift according to Equation 7.3.2, and the relation between the losses and the volume of the non-water present in a sample was shown to be linear in Section 7.4.3, results can be corrected for size using Equation 7.5.2, which relies on this proportional scaling of the change in losses induced by the cells and their volume:

$$\Delta A_{corrected} = 20 \log_{10} \left[1 - \left(1 - 10^{\frac{\Delta A_{dB}}{20}} \right) \left(\frac{diameter_{ref}}{diameter_{cell}} \right)^3 \right] \quad \text{Equation 7.5.2}$$

The averages of both the amplitude shifts, ΔA , and estimated cell diameters, $diameter_{cell}$, for each cell type must be used, rather than individual measurements, since these data are not paired. An arbitrary reference cell diameter, $diameter_{ref}$, of 30 μm , which falls within the range of cell diameters measured for all cell types, is used to normalise all measurements. Table 7.5.2 shows the size-corrected amplitude shift values for three cell types.

Table 7.5.2: Values of corrected amplitude shift for three cell types measured to 4 decimal places shown next to the uncorrected amplitude shift values. Due to the unpaired nature of the data, standard deviation of the corrected amplitude shift data is calculated according to standard non-linear combination of errors.

Cell type	C2C12	HMSC	Monocytes
Average amplitude shift $\pm \sigma$ (dB)	-0.0015 $\pm$ 0.0006	-0.0011 $\pm$ 0.0007	-0.0007 $\pm$ 0.0007
Corrected amplitude shift $\pm 1\sigma$ (dB)	-0.0012 $\pm$ 0.0006	-0.0011 $\pm$ 0.0007	-0.0010 $\pm$ 0.0011

When arithmetically corrected for size, the differences in shifts of amplitude for the three different cell types are smaller, while standard deviations remain relatively large. This is especially apparent for the monocyte data, due to a large spread in cell size combined with a small amplitude shift. Although the shifts are much smaller, the same trend is seen, with C2C12 cells inducing the largest corrected amplitude shift, and monocytes the smallest. This may suggest some small dependence of

the amplitude measurements on the contents of the cells. In order to investigate this further, Raman spectroscopy was carried out on the three cell types measured.

7.5.5. WATER CONTENT ANALYSIS BY RAMAN SPECTROSCOPY

Raman spectroscopy was subsequently carried out to more accurately determine differences in water content of the three cell types investigated. Although it is not trivial to give absolute values of water content for each cell type, relative water content can be estimated by the ratio of the broad –OH stretching vibration peak at $\sim 2700\text{--}3900\text{ cm}^{-1}$ and the --CH_3 symmetric stretching peak at $2800\text{--}3020\text{ cm}^{-1}$.^{204,205} The technique for measuring of cell hydration employed has been previously published looking at skin tissue hydration and offers a robust approximation of relative water content as well as quantitative analysis as confirmed by comparison to X-ray microanalysis,²⁰⁶ but has also been demonstrated on water concentration studies in eye lenses and corneas.^{206,207} Although the Raman spectra of cells are complicated due to the varied mix of organic constituents and different molecular environments of bonds, a good estimate can be obtained by discounting the fingerprint region and focussing on the –OH stretching peak and the –CH₃ symmetric stretching vibration peak, since these two peaks are well defined with little crossover, unlike the peaks in the fingerprint region. By integrating the Raman signal over the region from $3350\text{--}3550\text{ cm}^{-1}$ and $2910\text{--}2968\text{ cm}^{-1}$, effects from fluctuations of the peaks and the contribution from the 3329 cm^{-1} N-H vibration of proteins are minimised.

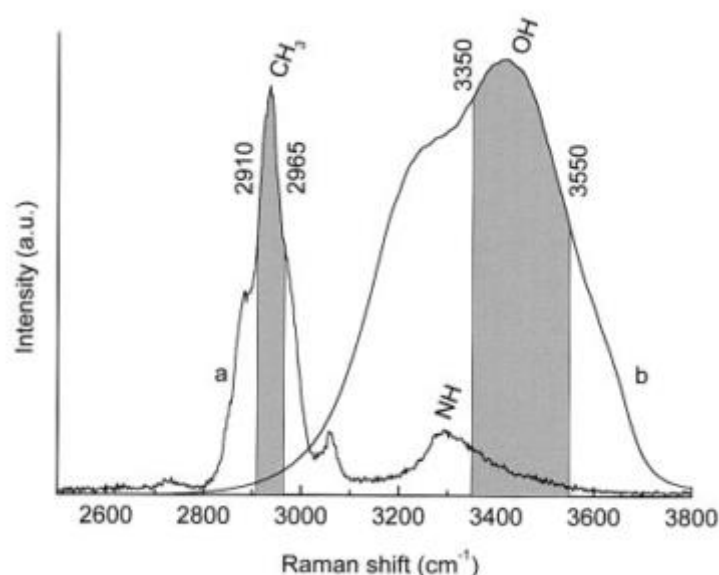


Figure 7.5.10: Raman spectra of pure water (a) and freeze-dried stratum corneum (b) reproduced from Caspers et al²⁰⁶ demonstrating the regions of interest for relative water:lipid ratio analysis.

Around 4-8 individual cells of each cell type suspended in medium (high glucose serum-free, phenol-free DMEM with 10% foetal bovine serum, 20mM HEPES buffer and 1% penicillin streptomycin) to ensure cell viability for the duration of measurements were seeded onto MgF_2 substrates. A confocal Raman microscope (alpha300R+, WITec, Ulm, Germany) equipped with a water immersion objective lens was used to optically identify the cells and the cell area was scanned in a raster sweep pattern, with spectra from -130 to 3673 cm^{-1} taken every $10\mu\text{m}$ in the x and y direction using a 532 nm YAG laser at 35 mW power. Larger cells, like mesenchymal stem cells, therefore have larger sets of spectra. A mask, defined by the $-\text{CH}_3$ symmetric stretching peak, is used to ensure that only Raman information from the cell, rather than the surrounding medium, is considered. Thus only spectra with an identifiable lipid/hydrocarbon peak are taken, and those without are ignored. Before this analysis is done, spectra are subject to background removal and area under the curve normalisation.

All spectra are similar, but differences in the relative intensity of the $-\text{OH}$ and the $-\text{CH}_3$ peaks between cell types can be seen. Figure 7.5.11 shows the mean and standard deviation of the spectra of different cell types acquired.

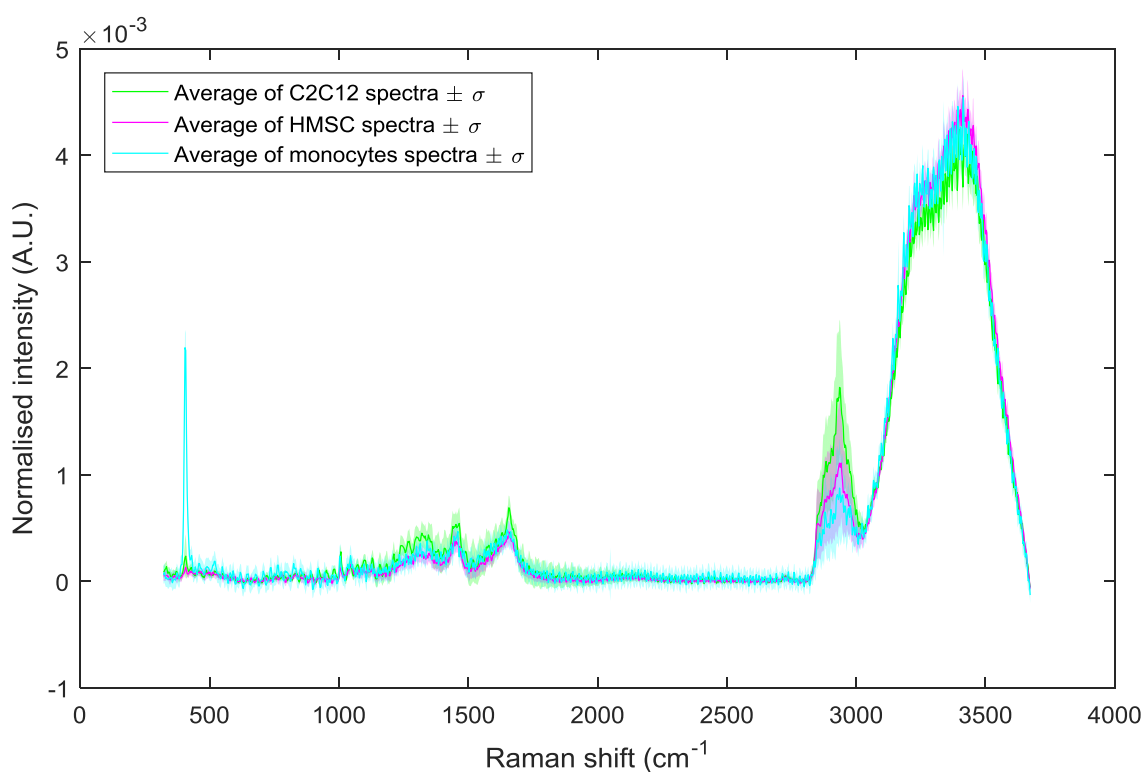


Figure 7.5.11: Mean and standard deviation of Raman spectra for all three cell types analysed, plotted as normalised intensity against Raman shift in wavenumber. The mean values are plotted in opaque colours while one standard deviation is shaded in transparent colours on either side of the mean.

At first glance it seems that the monocytes have the highest intensity of the -OH band, while the C2C12 cells have the lowest. Analysing the relative integrated intensity of the two regions of interest shows variation between individual cells measured, as well as between cell types, as shown in Figure 7.5.12.

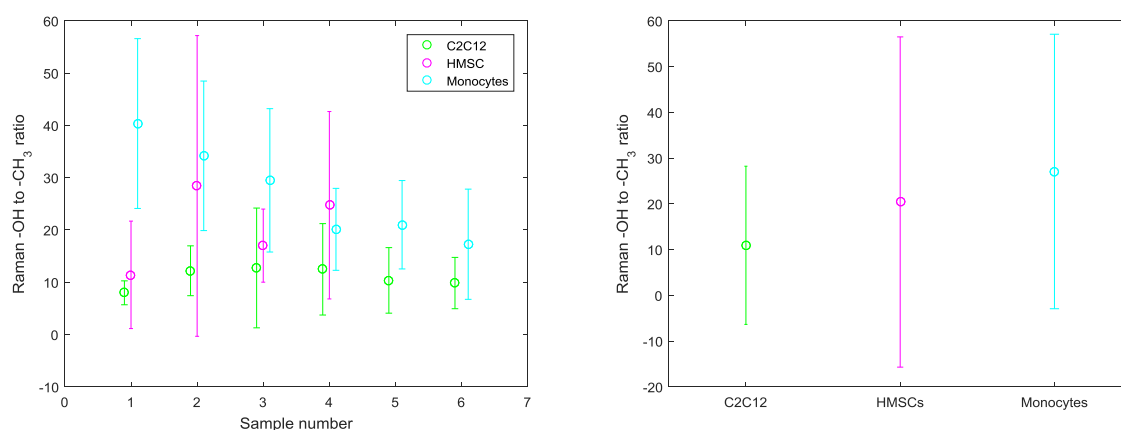


Figure 7.5.12: Left: Average -OH to -CH_3 ratio values for the individual cells of each cell type measured, with circles denoting the mean and the total length of all error bars denoting two standard deviations of the data. Right: OH to -CH_3 ratio values averaged across all cells measured, with circles denoting the mean and the total length of all error bars denoting two standard deviations of the data.

It can be seen that the variation in the HMSC data is large, and that the mean values also vary widely. This may be due to the large size of these cells as well as their tendency to spread out on the MgF_2 substrates used for analysis. Thinner areas of the cell may result in higher contributions from surrounding aqueous medium, while thicker areas may result in the opposite. The other two cell types remain balled-up during analysis and their ratio data show less variation. On the whole it appears that the C2C12 cells have the lowest -OH to -CH_3 intensity ratio, indicating low relative water content, while the monocytes show the highest ratio, indicating the highest relative water content. The average values of this ratio for each cell type can be plotted against the average amplitude shifts obtained by the microwave sensor, as shown in the left of Figure 7.5.13. It can be seen that the amplitude shift observed agrees well with the Raman ratio, with the cells with the highest ratio (most water) inducing the smallest amplitude shifts, and vice versa, as could be expected. In order to directly compare results between cells, the size corrected average amplitude shifts, as calculated in Section 7.5.4, can be plotted against the ratios, as shown in the right of Figure 7.5.13. This plot shows a similar distribution of points, with a general trend of higher Raman ratios correlating to smaller amplitude shifts, within the error margins. The Raman ratios are not an absolute measure of water content, so a mathematical analysis of the sensitivity of the system to changes in permittivity or water content, or a comparison to polystyrene sphere data, cannot be made.

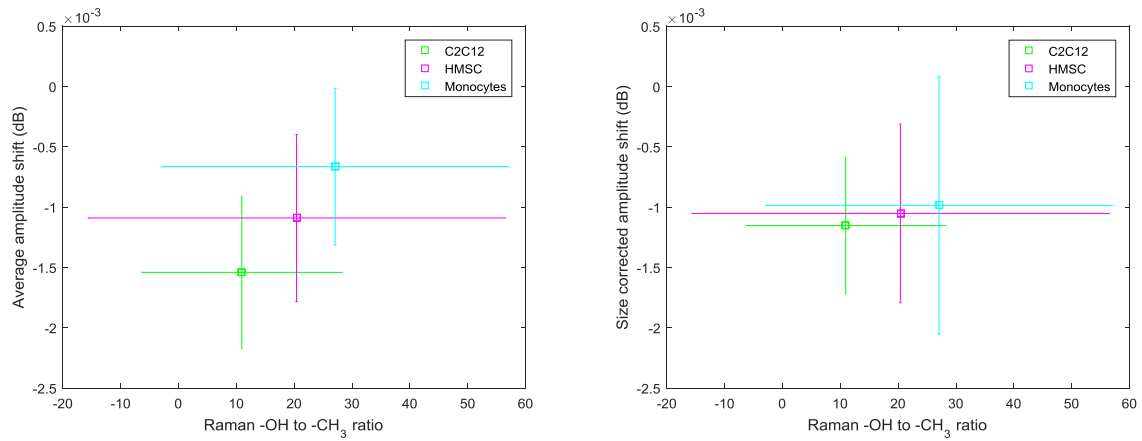


Figure 7.5.13: On the left, the average measured amplitude shift of all cells measured is plotted against the ratio from Raman analysis of the same three cell types. On the right the size corrected amplitude shift calculated in 7.5.4 for all cells measured is plotted against the ratio from Raman analysis of the same three cell types. . The mean of measurements is marked with squares, while the total length of error bars denotes two standard deviations.

Overall, it can be said that both techniques suggest that the C2C12 cells have lower water content than the other two cell types, while monocytes have the highest. It is possible that the microwave sensor is, therefore, capable of differentiating cell types based on overall water content, however experiments will need to be repeated for this to be considered conclusive.

7.6. SUMMARY

7.6.1. POSSIBLE SENSITIVITY ACHIEVABLE

If results from the very first successful real-time measurement of 25 μm polystyrene spheres (Section 7.4.1.1) are converted from quality factor shift to normalised amplitude shift according to Equation 7.3.2 (using a value of Q_{water} of 1951.9, which is the first of the background measurements of water taken during this sphere pass), this can be compared to the later results to ascertain whether significant increases in sensitivity are possible by altering the set-up. This calculation, presented in Figure 7.6.1, shows that the initial experiments recorded the equivalent of a shift in amplitude of -0.05 dB, more than three times larger than the largest shift recorded in equivalent subsequent measurements.

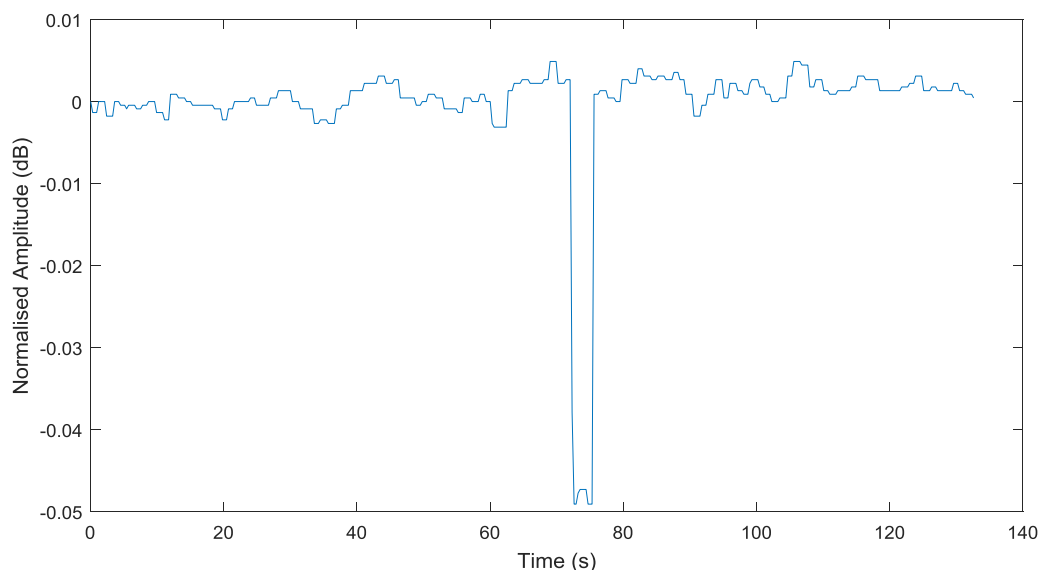


Figure 7.6.1: Quality factor data from the first real-time measurements of 25 μm polystyrene spheres converted into amplitude, showing the large shift achieved.

7.6.2. EFFECT OF PARTICLE SIZE AND COMPOSITION

As could be expected, results from the 25 μm and 11 μm polystyrene sphere experiments show that the larger particles result in larger shifts, with the difference in magnitude of the shifts correlating, roughly, to the difference in volume of the two sphere sizes measured. When comparing the biological cell measurements to similarly-sized polystyrene sphere measurements, it can be seen that the lower permittivity material particle results in far larger shifts than the higher permittivity biological cells. It is to be expected that larger permittivity deviations away from the background liquid (water/PBS) would lead to larger shifts in the observed signal.

Examination of both the sphere and cell results together, as shown in Figure 7.6.2, shows that the biological cells, despite being more similar in size to the 25 μm PMs, result in shifts more similar to the shifts induced by the 11 μm PMs. This proves that the response of the system is not simply dependent on the size of the particle, as a lower frequency sensor would be. The higher frequency of the microwave sensor allows the electric field to short the capacitive membrane, penetrate into the cell and interact with the contents, resulting in lower shifts than the equivalently sized polystyrene particles.

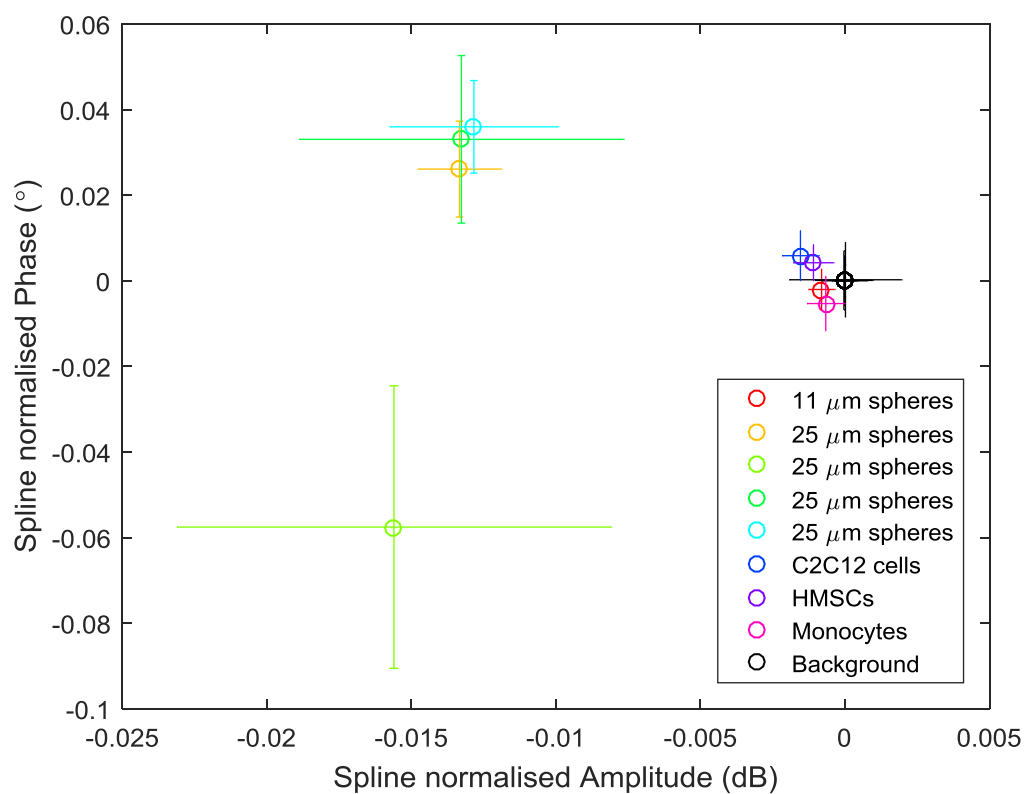


Figure 7.6.2: Average normalised phase and amplitude measurements of all polystyrene sphere data and biological cell data, with error bars denoting standard deviations of measurements. Total length of error bars denotes two standard deviations of the data.

8. CONCLUSIONS

8.1. MAIN FINDINGS

A new microwave sensor, based on coupled resonators (DR-DSRR) which offered higher sensitivity and greater field confinement than a dielectric resonator perturbation approach was constructed. Following initial measurements of well-characterised solutions of ethanol in water, the dependence of the response of the resonator system could be linked to the permittivity of the solution in the capacitive gaps of the DSRR, using an analytical model derived from LCR resonant circuit theory and perturbation theory. This equivalent circuit model showed that the new system relies on the coupling between the DR and the DSRR, with its sensitivity arising from the overlap between the two resonances. This sensitivity surpasses that of an equivalent dielectric resonator alone, and the system maintains a high quality factor even when the sensing region is filled with water, thus avoiding the problems faced by split ring sensors, allowing sensitive and precise measurements to be made on flowing, microscopic objects within a liquid medium.

The new DR-DSRR system showed significant and measureable changes in its resonance properties upon the passage of flowing polystyrene microspheres of both 25 and 11 μm diameters through the capacitive sensing element. Single frequency measurements at the peak of the resonance were taken in order to speed up data acquisition, with values of amplitude and phase of the transmitted signal recorded. It was shown that the amplitude measurements could be used to calculate an approximate equivalent inverse quality factor shift, so that changes in the system could be related to changes in loss. Inconsistency in the phase measurements between seemingly equivalent experimental set-ups was seen, which was later thought to be due to a lack of calibration to account for the significant effects of the cables. The relationship between the loss of the system and the volume of the spheres analysed was shown to be roughly linear, as predicted by the circuit model.

Measurements of three different types of cultured cells were carried out, which showed the same dependence of amplitude on cell passages through the capacitive sensing region. Although the system showed a signal dependence on volume of polystyrene sphere particles, dissimilar results for particles of different composition but equivalent size (cells vs polystyrene spheres) suggest that the strength of the signal is determined by the material of the target particle as well as its size, in contrast to well-established lower frequency techniques.

Analysis of this data showed some differences in the amplitude signals obtained between the three cell types. To ascertain whether this could be due to differences in composition, the data were normalised for the size of the cells, as determined by analysis of video footage of the cells in the microfluidic channel. After size normalisation, small differences in amplitude shifts between the three cell types were still observed, indicating potential differences in composition.

Due to the frequency used for measurements, the response system was thought to be correlated to the amount of water in samples, with large changes in water content resulting in large changes in resonance properties of the sensor. To test this theory, the three cell types measured were also analysed with Raman spectroscopy in order to ascertain an approximation of their average relative water content. Comparison of this water content value with the size-normalised amplitude shift values showed similar trends, indicating that the cells which induced the largest shifts had the lowest relative water content, while the cells that produced the smallest shifts had the highest relative water content. This preliminary evidence suggests that the sensor system is capable of detecting small differences in water content between different cell types. Given the link between cell hydration and carcinogenesis, this is a diagnostically valuable metric to measure, and this microwave technique offers a label free, fast and non-destructive method to determine cell water content.

8.2. FUTURE WORK AND OUTLOOK

While repeats of measurements will be required to validate the results presented in this thesis, there are also many things that could be done to improve the current system.

8.2.1. OPTIMISATION OF THE SENSOR

Given the problems with the phase measurements, it has become clear that it would be necessary to implement a calibration of the system before measurements if the phase information measured is to be used. It is possible that there is clinically useful information contained within phase measurements which, if calibrated, could help to separate measurement points of different cell types. A calibration would be straightforward to implement, but would require significant movement of cables after calibration, which could result in a small amount of phase noise from the cables, as well as additional contributions from the semi-rigid cables used to make the coupling loops. It would have to be seen whether these remaining effects would be small or large in comparison to the effects of the resonator and the shifts in frequency induced by cells.

An initial parameter that could be changed in order to increase sensitivity is the gap width of the DSRR. A smaller gap would result in a higher capacitance, and a stronger field in a smaller area. If it could be ensured that cells could pass through this high field region, and that the inter-resonator coupling was not adversely affected by the change, this would result in larger shifts for the same changes in permittivity.

There are quite large spreads in the data for cell measurements, so increasing the precision as well as the sensitivity of the system should make any differences in signals between different cell types more obvious. Optimisation of the sensor is key if the sensor is to be used for the proposed application of CTC enumeration and monitoring. Stabilising the sensor by reducing the contribution to the signal variation from things like the path taken by the cell or events in the other sensing gap and pressure changes in the channel should allow for variations within cell populations measured to be due to mostly innate cell heterogeneity, and variations between cell populations to be due to their physical differences.

Any changes made to the system will have to be investigated experimentally, using ethanol solutions and polystyrene microspheres or biological cells, since the ECM alone cannot predict sensitivity to cell-sized objects.

It is also possible that measurement of the quality factor using a VNA is not the best approach. Ongoing work in collaboration with NPL is investigating the use of an interferometer-like system for highly accurate determination of small changes in the resonance parameters of the coupled system.

8.2.1.1. TUNING THE SENSOR (ECM)

Earlier measurements taken with a different experimental set-up suggest that the current configuration is not the most sensitive achievable. As discussed in Section 7.2.2.6, according to the ECM, the sensitivity of the system could be theoretically significantly increased by designing a configuration in which the water-loaded DSRR resonance frequency matched almost exactly the unloaded DR resonance frequency. Although this would decrease the operating quality factor, leading to less precise measurements of resonance frequency, for quality factor or single-frequency amplitude measurements this may not be such a problem if quality factor shifts are at a maximum. This improvement may also come at the expense of frequency shift sensitivity, as seen in Section 7.3.1, but as discussed in Section 7.4.1.6, since the frequency shifts are significantly smaller than quality factor shifts in the high permittivity regime, and frequency-related phase measurements showed inconsistency in direction, which is so far not well understood, it is reasonable to focus

optimisation efforts on the amplitude (or quality factor) shifts, so any loss in sensitivity or stability of frequency measurements may be ignored.

This improvement could be achieved by alteration of the geometry of the DSRR, or by varying the filling factor of the DSRR by the use of different channels of different shapes and sizes. It is not straightforward to predictably and controllably alter the filling factor, however, and a combination of insights from the ECM to ascertain whether an increase or decrease in filling factor or DSRR is necessary, and experimental trial and error to determine which channels result in this change would be needed. Attempting this improvement requires a method of production of channels with well-defined geometries, which is discussed below.

8.2.1.2. MODELLING

To account for significant field deformations and the effect of asymmetric loading of the DSRR, it would be useful to further explore modelling of the coupled system. It is possible that numerical field analysis of a single cell inside a capacitor could be carried out with CST MWS to determine the changes in electric field distribution, which could then be implemented into the circuit model. Similarly, the ECM could be used in conjunction with finite element modelling (FEM) analysis to build a realistic model of the coupled system, in order to account for the unknown geometric quantities of filling factor and inter-resonator coupling.

The ECM could be tested by carrying out measurements of solutions in which the real or imaginary permittivity only is changed, e.g. solutions of salt and sugar, to ascertain whether the predicted relation between resonance properties and permittivity of the sample holds true. This work is currently ongoing.

8.2.1.3. FABRICATION OF WELL-DEFINED CHANNELS

As discussed in Sections 7.4.1.3 and 7.4.1.4, the sensitivity and selectivity of the system is limited by the effects on the signal strength of differing lateral and vertical positions of the particle in the channel, which is often influenced by the presence of pockets of air in the sensing regions. The production of carefully designed channels could lead to greater control over the path particles take over the sensing region, as well as a reduction in air bubble formation.

The first and most obvious change that could be made in efforts to combat these issues would be to use well defined and easily controlled 3D-printed moulds for PDMS channel fabrication. With the use of such moulds, it would be theoretically straightforward to decrease the height of the channel,

therefore bringing cells closer to the metal and increasing sensitivity and repeatability of cell height. However, use of PDMS channels made from 3D printed moulds such as the one illustrated in Figure 8.2.1 has resulted in a dramatic loss of sensitivity compared to the original capillary-moulded channel used. This is thought to be due to the effect the different channel shape and size has on the filling factor of the DSRR, shifting its loaded resonance out of the frequency window that produces high sensitivity. Given the difficulty in modelling this property, experimental evidence will have to be collected to ascertain whether this is a viable option. Decreasing the channel height in order to control the cell path may also result in an increase in channel blockages, which could be a considerable problem for a system aiming for high throughput measurements.

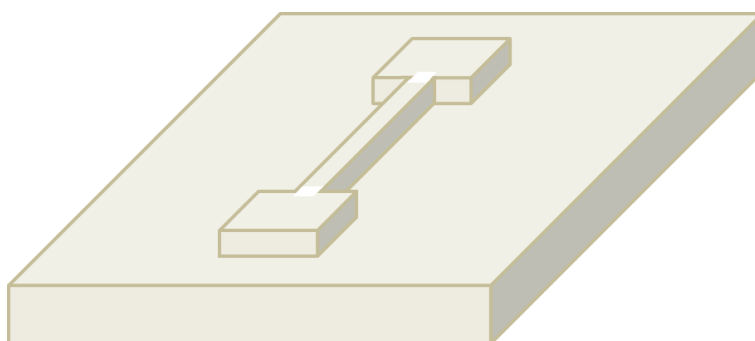


Figure 8.2.1: Schematic of 3D printed mould for PDMS channel casting.

The sizes of channels made using 3D printed moulds are limited by the resolution of the printer. Although sub-micrometer resolution is achievable with some 3D printing techniques (e.g. multiphoton photopolymerisation), the combination of small and larger feature sizes makes this an impractical fabrication method due to the cost and time. For higher throughput 3D printers (e.g. PolyJet) the achievable size resolution is around 100 μm , making this the smallest channel reasonably achievable. There are some ways to get smaller channels, such as printing at a 45° angle, but channel sizes of 50 μm or below may not be possible using this technique. Other methods, such as deep reactive ion etching (DRIE) of silicon offer higher resolution for making moulds but are considerably more expensive and may therefore not be suitable for the fast prototyping necessary for the trial and error style experimental approach needed to find the optimum channel geometry.

PDMS is gas permeable, and as such allows both air and water vapour to pass through it. After the sealing process water evaporates through the PDMS, bringing the surface of the PDMS channel mould in good contact with the glass below, allowing them to adhere. Over time air permeates through the PDMS and air bubbles become more of a problem during measurements. The movement of liquid through the channel pulls small pockets of air further into the channel, resulting in growing air bubbles at points along the channel near areas where air has accumulated underneath

the PDMS. This was particularly a problem in the capacitive sensing gaps, where even small volumes of air could have a huge effect on the capacitance of the gaps and therefore the measured phase and amplitude of the whole system. The exact effect on the phase and amplitude of the sensing system is not trivial to understand, but empirically it seems that, as well as changing the resonant frequency and quality factor of the coupled system, the presence of air in the sensing region can affect the magnitude of the shifts induced by the passage of a cell, and therefore may well contribute to imprecision in repeat measurements.

The channel used in most of the results was cast from a cylindrical capillary. As well as the problems of the PDMS curing under the capillary and then sealing over the metal, as described in Section 7.4.1.5, removal of the PDMS from the capillary results in a very rough edge to the channel, with a lot of small tears along the length of it. Looking at the measurements which suffered from the formation of bubbles in the gaps it seems that they often occur at the same places, on tears in the side of the channel. It may be that these tears act as nucleation points or points of entry for air bubbles into the channel. Using rectangular moulds results in channels with much smoother sides and may help to mitigate the problem of air bubble formation.

8.2.1.4. DIELECTROPHORETIC FOCUSING

Aside from altering the dimensions of the channel, a more complicated method to control the height of cells in the channel that would not have the undesired effects on the filling factor would be to use dielectrophoresis (DEP) to force flowing cells into the most sensitive region of the gap. In theory, this could provide better height control of the cells and therefore more reliable and consistent measurements. However, the dielectrophoretic force felt by each cell depends strongly on membrane effects and its permittivity, which may vary significantly both within and between cell populations. The frequency used would have to be tuned in advance to exert a particular force on a cell in order to move it closer to the metal, as it is possible that this frequency could produce the opposite force, or no DEP force on a different cell type of a similar size, resulting in an additional mechanism of height uncertainty. This may not be a problem as long as the result is repeatable, but could result in a loss of sensitivity for a critical cell population, and would make modelling of the system for optimisation even more complex. In theory the two gaps in the DSRR would allow for sensing to take place as the cell flowed through the first gap, followed by a dielectrophoretic force to be applied to the cell for separation purposes.

8.2.1.5. THE SECOND CAPACITIVE GAP

A radical redesign of the alignment of the microfluidic channel over the sensing gap could achieve a much higher sensitivity and repeatability of measurements. As shown in Figure 8.2.2, aligning the channel(s) perpendicular to the capacitive gaps rather than parallel ensures that cells always pass over the most sensitive part of the gap, the edges of the metal, and removes the error in measurements due to differences in the lateral position of the cell during passages.

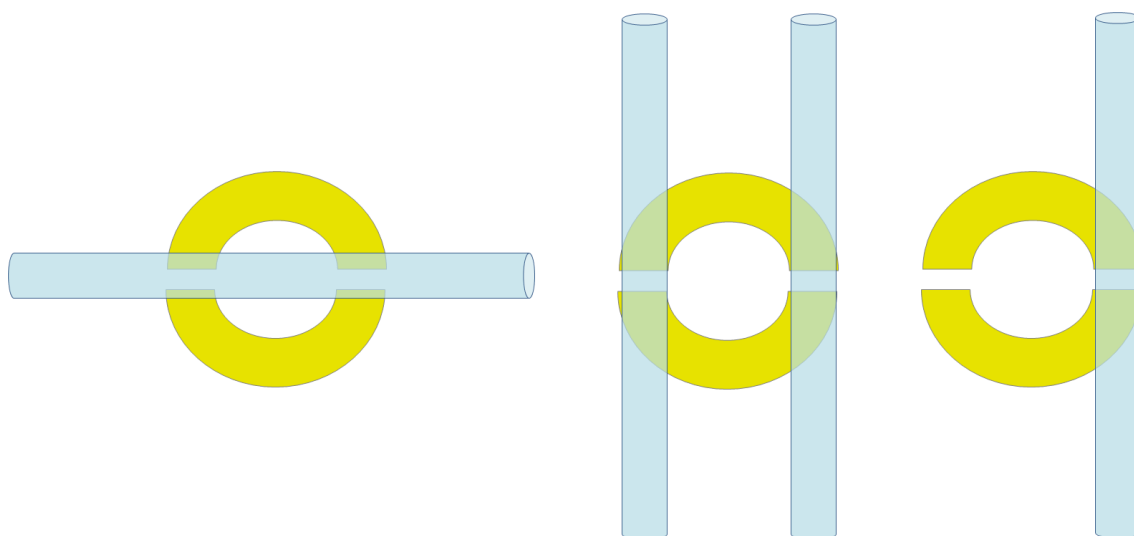


Figure 8.2.2: Schematic showing current channel and DSRR alignment (left), and two possible alternatives: either two rotated channels, one aligned over each gap (middle), or one rotated channel over one gap with the other sealed over with PDMS.

This would also eliminate the uncertainty associated with having two sensing regions. With one sensing region either blocked with PDMS or constantly filled with only the background solution, any deviations in signal can be more reliably assumed to be due to cell passage events in the other.

Clearly, simulation and experiments would need to be carried out in order to ascertain whether covering the second sensing region with PDMS or filling it with background liquid would result in higher sensitivity. This will depend on the geometry of the ring, with the currently studied rings falling into a useful resonant frequency window, with respect to the DR, only when both gaps are filled with high permittivity liquid. It is possible that simulations could be carried out to find the window of DSRR dimensions that would allow similar interaction between the two resonances with one of its gaps covered in PDMS. This may prove to be a preferred set-up, since the use of PDMS in the sensing region should result in lower losses in the DSRR, and therefore in the system as a whole. The ECM would need refining in order to account for the asymmetry that this possibility would result in, as in its current form it could not be used for predicting whether this configuration would be better, and some combination of simulations and the ECM would be required to provide a starting point for experimental testing.

8.2.1.6. CONTINUOUS PUMPING

In order to collect sufficient data to draw conclusions, multiple cell passages were measured for each cell. The changing of direction and speed will have some effect on the amplitude and phase measurements, with changes in speed potentially altering the height of the cell in the channel, and the changes in pressure used to control the cells potentially resulting in bulging of the flexible PDMS channel. Ideally cells would be measured as many times as possible during only one cell passage. In order to achieve this, a steady speed of cells of sufficient separation will be required. The speed will have to be high enough to ensure no settling out, and measurements may have to be sped up in order to achieve this. A reliable way of ensuring that cells are roughly evenly dispersed through the channel will have to be implemented, which may result in very low concentrations, again necessitating high flow rates and therefore fast measurements. Perhaps something similar to the sheath flow focussing system used in flow cytometry could be used to ensure a uniform height for cells in the channel and a high speed.

8.2.2. DATA PROCESSING

Ideally the processing of sensor output would be automatic, without the need for human identification of “background” and “signal” data points. With increased sensitivity and better control over the other gap, and perhaps with continuous flow measurements, the background drifts could be significantly reduced. Raw amplitude and phase data could then be fed into a clustering algorithm to determine the average shifts. This would remove any kind of error or bias inherent in manual data processing, hopefully making the system more robust.

This technique has been tried on 25 μm polystyrene sphere results with consistent background drift, and has shown to give similar results to the manual processing, as shown in Figure 8.2.3. However, this technique cannot be used to process data where the two populations are very close and less well defined, or when there is a large degree of sudden background drift.

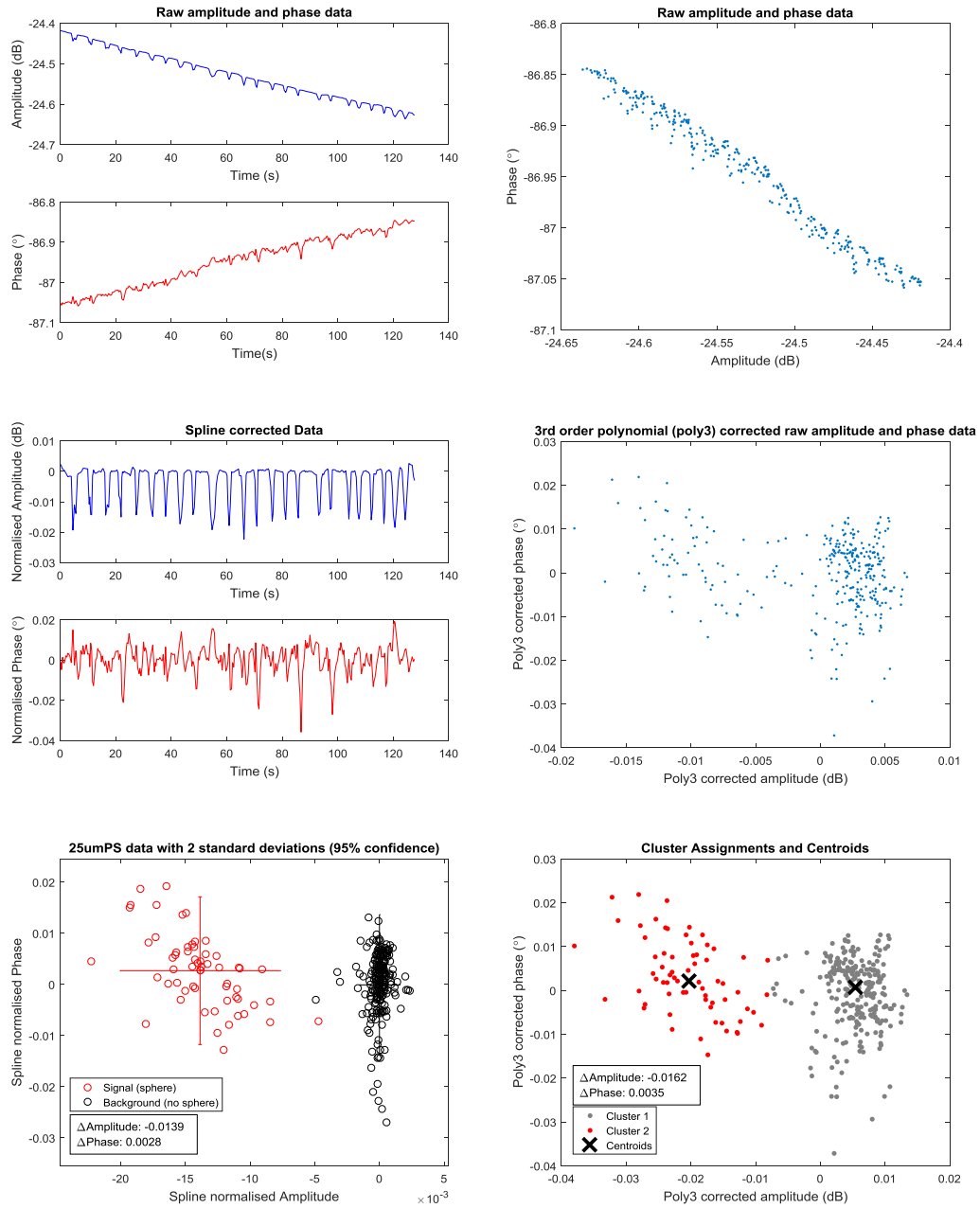


Figure 8.2.3: Comparison of “manual” data processing (left hand side), where reference and signal data points are assigned manually, before background correction, and use of a *k*-means clustering algorithm to define reference and signal data points without any manual assignment, in which background removal is done via a polynomial fit to all data before the algorithm is applied. The top two graphs show the same raw data for the two methods, below them the data after background removal is shown for the two methods, while at the bottom the results of the two different methods are shown, with the results of average amplitude and phase shifts.

From this comparison it can be seen that for this case the two methods give similar results, though the position and spread of the points is quite different, and inclusion of all data points for the clustering method results in greater overlap.

8.2.3. TESTING

Since the aim of the sensor is to detect circulating tumour cells, the first tests would be carried out on cultured cancer cell lines, e.g. MDA-MB-231 (breast cancer), which are available in the department from a collaborator. Comparing results from cancerous cells to those from non-cancerous, nucleated circulating cells should give an indication as to whether the sensor can be used to identify and separate malignant cells. Characteristic regions of a phase vs amplitude shift scatter plot could be defined for malignant and benign cells (c.f. cytometer system). Using well defined cell populations should also allow the rate of false negatives and positives to be ascertained, and this can be compared to other techniques.

If the sensor is able to differentiate between normal and malignant cells with significant accuracy it will be tested using whole blood samples spiked with varying amounts of cultured cancerous cells. This work will require the development of a pre-separation technique to remove red blood cells as well as possibly the plasma and smaller leukocytes. Dilution factors will be explored. A thorough investigation of white blood cells will also have to take place. This work should enable the quantification of the limit of detection (LOD) of the system and the cell recovery level, which can be compared to existing gold standard techniques such as flow cytometry and CellSearch, to ascertain whether CTCs are viable targets for the system. The final stage of testing would involve metastatic patient samples. Validation of CTC enumeration would be achieved by comparing our results to flow cytometry.

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APPENDIX 1: DERIVATION OF THE EQUIVALENT CIRCUIT MODEL (ECM)

DSRR CIRCUIT MODEL

As part 1 of the ECM, an LCR resonant circuit according to Fig. 1 is used to describe the resonance frequency, f_{DSRR} , and quality factor, Q_{DSRR} , of the DSRR.

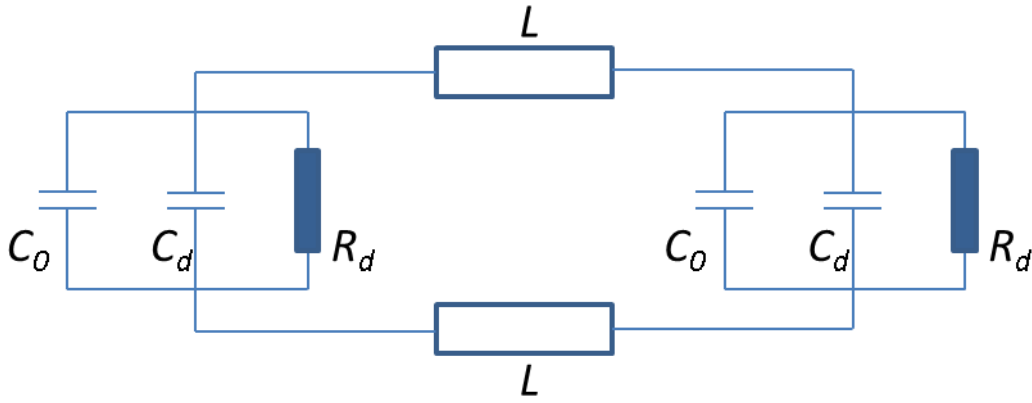


Fig. 1: Equivalent circuit for modelling the resonance parameters of the DSRR with capacitors partially filled with a sample

where L denotes the induction of each half ring, C_0 the capacitance of each gap due to the portion of the electric field outside the microfluidic channel, and C_d the capacitance due to the electric field within the liquid-filled microfluidic channel, while R_d is a parallel resistor accounting for the dielectric losses of the liquid.

A permittivity-dependant filling factor, κ , is introduced to describe what portion of the capacitive field is stored in the liquid in the microfluidic channel:

$$\kappa(\epsilon') = \frac{C_d}{C_d + C_0} = \frac{1}{1 + \frac{1}{\epsilon'} \left[\frac{1}{\kappa_0} - 1 \right]} \quad \text{Equation 0.1}$$

where κ_0 is a “geometric filling factor” accounting for the case of an empty microfluidic channel ($\epsilon' = 1$ and $\epsilon'' = 0$):

$$\kappa_0 = \frac{\frac{C_d}{\epsilon'}}{\frac{C_d}{\epsilon'} + C_0} \quad \text{Equation 0.2}$$

assuming that capacitance due to the field outside of the microfluidic channel is unchanged, and that the presence of the liquid in the channel does not alter the field distribution in the gap. This is a perturbative approach and is therefore valid for small changes in permittivity of the liquid in the gap. The value of κ_0 is dependent only on the geometry of a given channel-capacitor assembly, and is therefore varied as a fitting parameter.

The evaluation of the resonance parameters of the DSRR equivalent circuit according to Fig. 1 therefore results in equations for the resonance frequency:

$$\frac{\omega_{DSRR}^2}{\omega_{DSRR0}^2} = \frac{\varepsilon' - \kappa(\varepsilon')[\varepsilon' - 1]}{\varepsilon' [1 + \kappa(\varepsilon')^2 (\tan \delta)]} \quad \text{Equation 0.3}$$

and quality factor of the DSRR:

$$\frac{1}{Q_{DSRR}} = \kappa(\varepsilon') \tan \delta + \frac{1}{Q_m} \quad \text{Equation 0.4}$$

where Q_m is the quality factor of the DSRR accounting for ohmic losses due to the resistivity of the metal of the ring. This value is obtained from simulations of the DSRR alone, and is large compared to the Q due to dielectric losses of the liquid. These equations obey the trivial limit, $\kappa = 0$, $\omega_{DSRR} = \omega_{DSRR0}$ and $Q_{DSRR} = Q_m$.

COUPLED RESONATOR CIRCUIT MODEL

Part 2 of the ECM the equivalent circuit of the coupled resonator system is considered according to Fig. 2.

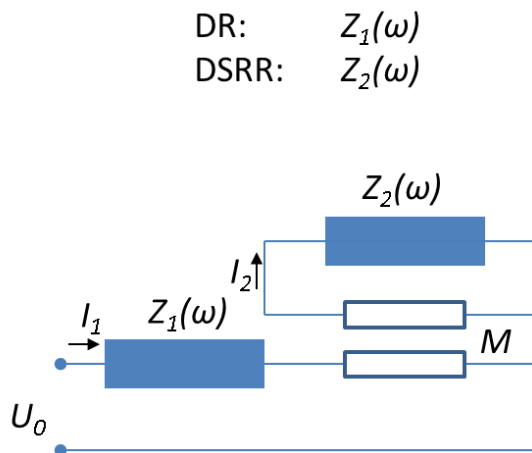


Fig. 2: Equivalent circuit model for the coupled resonator system with $Z_1(\omega)$ and $Z_2(\omega)$ representing the complex impedance of the DR and, DSSR respectively.

Both resonators are described by their normalised complex impedances, $z_i(\omega)$, where $i = 1$ for the DR and 2 for the DSRR, of a serial resonant circuit.

$$z_i(\omega) = \frac{Z_i(\omega)}{Z_{Li}} = \frac{1}{Q_i} + i \left(\frac{\omega}{\omega_{ri}} - \frac{\omega_{ri}}{\omega} \right) \quad \text{Equation 0.1}$$

where Z_i is the impedance of the resonator, Z_{Li} is the line impedance of a transmission line formed by the inductor and capacitor of each resonant circuit, Q_i and ω_{ri} denote the resonant frequency and quality factor of the DR ($i = 1$) and DSRR ($i = 2$).

The two resonators are inductively coupled together, so a dimensionless mutual coupling coefficient can be defined as:

$$k^2 = \frac{M^2}{L_1 L_2} = \frac{\omega_{r1}}{Z_{L1}} \frac{\omega_{r1}}{Z_{L2}} M^2$$

where M is the mutual inductance. The value of k is a fitted parameter.

In order to work out the resonance properties of the coupled system according to the circuit model in Fig. 2, the frequency-dependent effective normalised admittance $\left(\frac{1}{z_{eff}(\omega)} \right)$ of the circuit is calculated:

$$\frac{1}{z_{eff}(\omega)} = \frac{Z_{L1}}{Z_{eff}(\omega)} = \frac{1}{z_1(\omega) \left(1 + \frac{\omega^2 k^2}{\omega_{r1} \omega_{r2} z_1(\omega) z_2(\omega)} \right)}$$

The resonance parameters of the coupled system can then be found from the frequency and absolute magnitude of the maxima of $|z_{eff}(\omega)^{-1}|$.

APPENDIX 2: REAL-TIME DATA PROCESSING

As the DR-DSRR microwave dielectric sensor is a new technology, there is no predetermined method for analysing the results. The processing approaches tried and the reasoning for the final approach used are discussed in this appendix. In order to aid comparisons of methods, unless stated otherwise the example data for each method are the same and correspond to measurement of a C2C12 cell.

For all possible processing methods of real-time results the first step is to cross-reference the video with data from the VNA to determine which amplitude and phase data points correspond to spheres or cells interacting with the capacitive gaps (“signal” points), and which correspond to measurements of only the background solution (“reference” points). This is done by walking through the stills captured by the camera and the corresponding amplitude and phase measurements frame by frame, making note of the measurement indices denoting the beginning or end of a period of signal or reference. This indices data is then stored and various MATLAB scripts are then written which use the indices and output data files to process the data to get average values, cell by cell trends and visualisations of the shifts and the spread of the data etc.

ORIGINAL PROCESSING METHOD

All data points were split into either reference or signal. The averages of all individual reference and signal measurements were calculated and the differences between each signal and the reference measurements either side of it evaluated, as shown in Fig. 3. The overall average of these individual averages was then used as the amplitude or phase shift for that particular target.

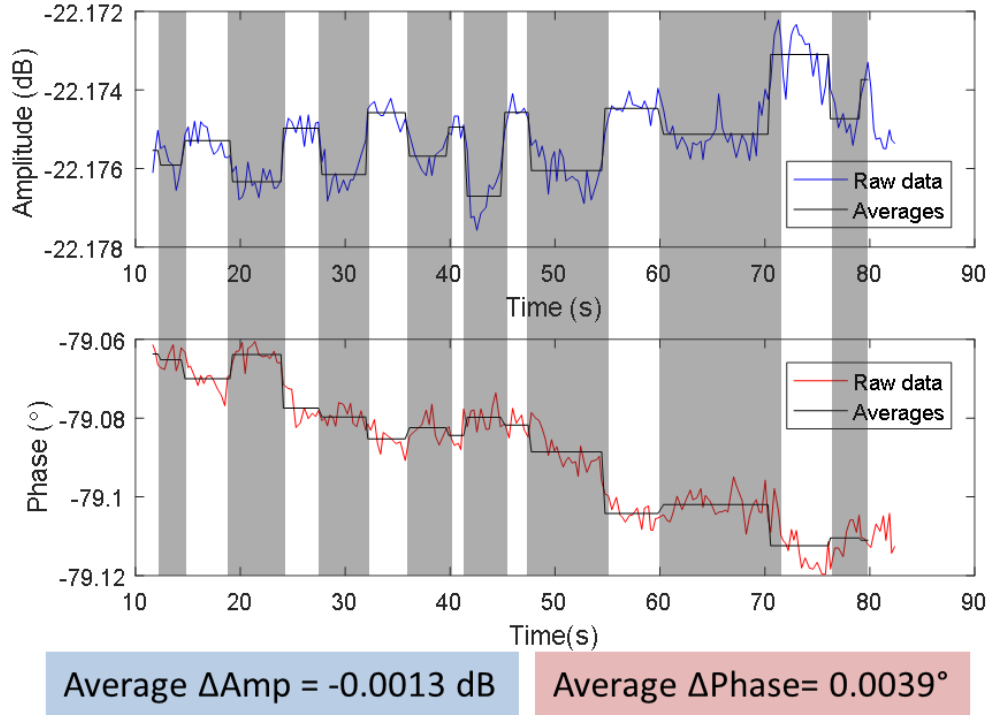


Fig. 3 Example results from multiple measurements and passages of a single C2C12 cell. The amplitude measurements are plotted above in blue, while the phase measurements are below in red. The black lines on both plots represent the averages taken of both signal and amplitude data points, while the grey areas denote cell passages as determined by cross-referencing with the video footage. The average amplitude and phase shifts are displayed on the graphs in units of dB and $^\circ$, respectively.

Although this approach is intuitive, it nullifies the effects of long term system drift and results in a defensible average value for each measurement, it does not lend itself to an obvious way to present or visualise the data and its spread, making comparisons between different targets or experimental set-ups difficult.

It is always difficult to determine at what point the data is to be deemed signal rather than background, and vice versa. Including all points in this approach makes this even harder. Transition points, where the particle or cell is approaching the gap and the amplitude or phase starts to change were omitted in later processing methods in order to make the distinction between background and signal clearer.

SECOND METHOD

The first data visualisation technique tried was a histogram approach, plotting the amplitude and phase measurements separately, alongside measurements of the background. In this way the spread and average of the data could easily be seen. In order to be able to plot the data as histograms, it had to be normalised to get rid of background drift. This histogram presentation was based on an

approach employed in a previous work looking at small shifts away from a reference measurement.²⁰⁸

POLYNOMIAL BACKGROUND SUBTRACTION

Initially a polynomial fit to the reference data was subtracted from all measurements to result in values of amplitude and phase shifts normalised to 0, as shown in Fig. 4. The degree of the polynomial was determined by the goodness of the fit to the background data, and depended on the shape of the drift.

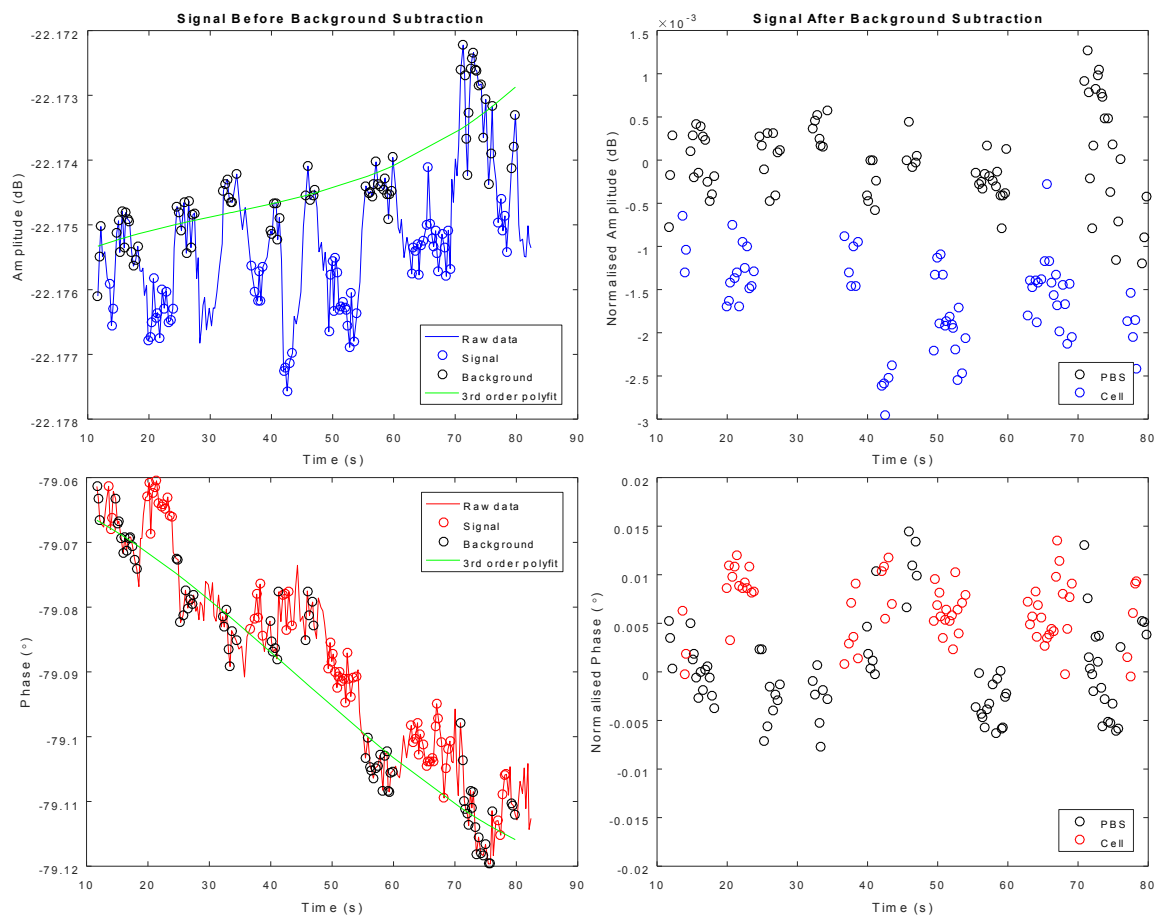


Fig. 4: The same example results as Fig 3 showing the polynomial fit method of background subtraction. On the left are the raw amplitude (top) and phase (bottom) measurements with signal data points marked with coloured circles and background measurements marked with black circles. The polynomial fit to the background data is shown as a green line on both amplitude and phase plots. On the right the data points after background subtraction are shown, again with coloured and black circles denoting signal and background data points, respectively.

HISTOGRAM VISUALISATION

In this way, all individual signal and reference measurements could be plotted as a distribution of phase or amplitude measurements, allowing clear visualisation of the separation between signal and

reference, as shown in Fig. 5. The average shifts were then calculated by applying a “normfit” function to the data to obtain the mean and standard deviation of the signal and reference data, and the difference found. This was then used to define a Gaussian curve to be overlaid on the histogram as a representation of the data.

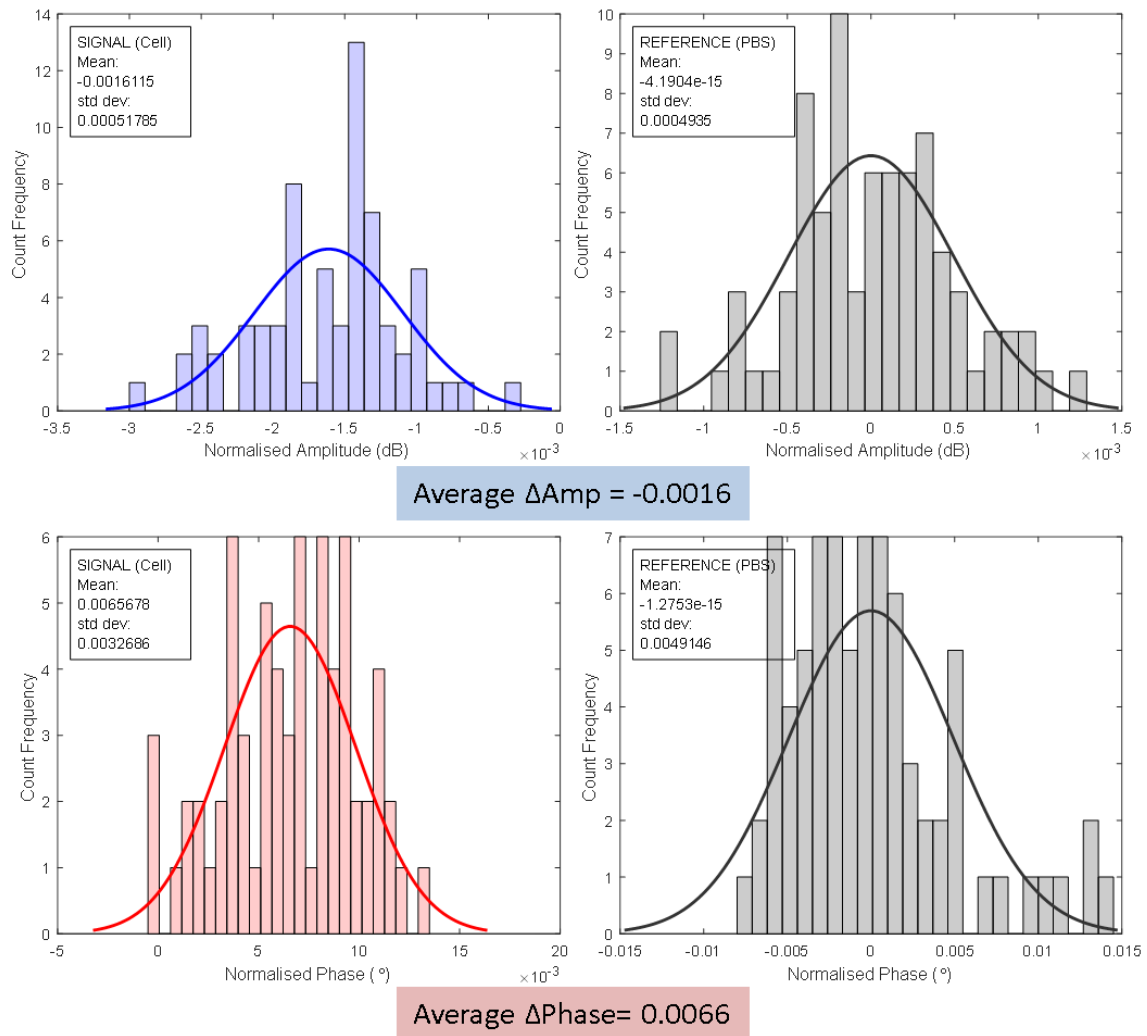


Fig. 5: Data points from Fig 3 plotted as histograms. On the left the signal data points are plotted for the amplitude (top, in blue) and the phase (bottom, in red). On the right the background data points for amplitude (top) and phase (bottom) are plotted in grey. Overlaid are the Gaussian distributions from the normfit to each data set. The average shift values calculated from the mean of the normfits are displayed underneath the relevant graphs.

Although this method gave a good idea of the spread of all the data, by including all measurements rather than averages, it relied on a Gaussian-fit of all data, which may not be valid for these data sets, especially for the small number of values collected during individual measurements. Mean and standard deviations could be calculated without assuming a normal distribution of data, but without assuming a distribution representative curve for easy comparison cannot be plotted.

This approach also deals with the amplitude and phase shifts completely independently, despite their obvious relation. An approach more similar to that seen in flow cytometry, for example, would enable simple visualisation of whether the combination of amplitude and phase shifts may differ between cell types, and possibly allow regions of an amplitude vs phase shift plot to be identified for specific cell characteristics.

The use of a polynomial fit for background subtraction, although intuitive, failed for the many datasets which suffered from sudden changes in the background. These changes may be due to mechanical perturbations (a cable being accidentally knocked, the table moving during control of the pumps using a keyboard), bubbles in the channel moving, bubbles at the output bursting or the pooled liquid moving, events in the other capacitive gap, or the constant and sudden changes in pressure used to control the particles resulting in deformation of the channel. This meant that on top of long term constant drift from, for example, temperature change or mechanical settling, there were smaller, shorter term and sudden steps in the background measurements, as demonstrated in Fig. 6.

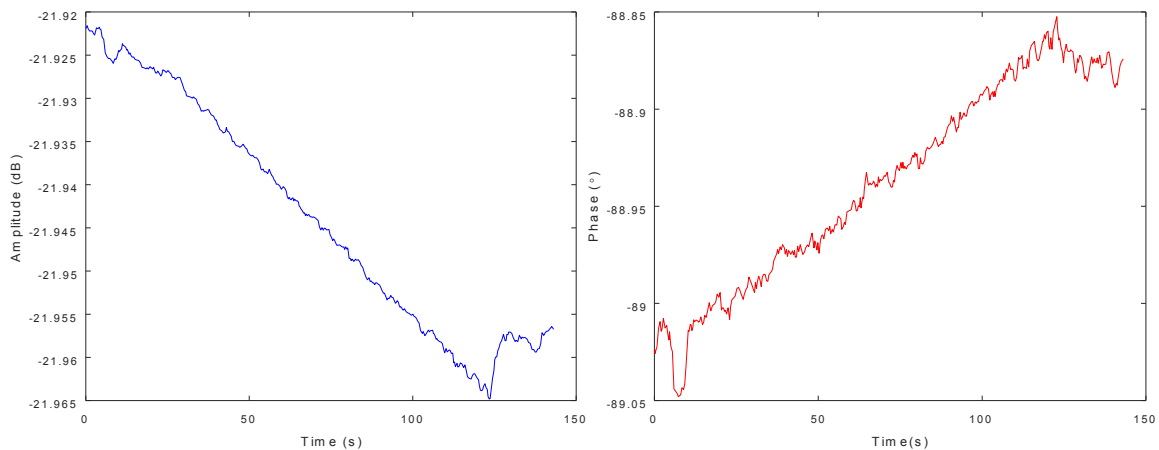


Fig. 6: Alternative example amplitude (left) and phase (right) results from monocyte measurements, which show a sudden step in both amplitude and phase towards the end of the measurements.

Fitting a polynomial to the background data and subtracting it resulted in erroneously large shifts in some places combined with a large standard deviation of both the signal and reference measurements, as shown in Fig. 7, due to its inability to follow these shorter term drifts.

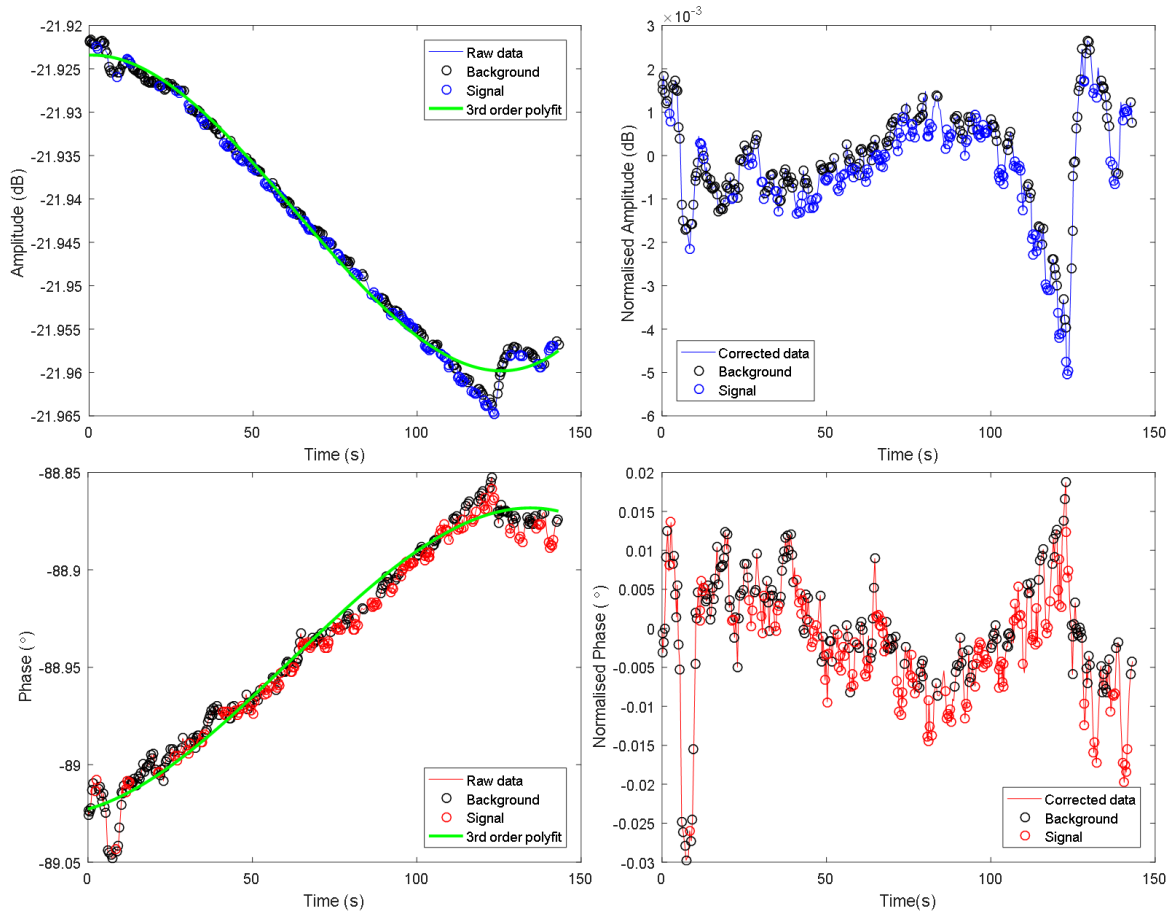


Fig. 7: Example results from Fig 6 showing the limitations of the polynomial fitting approach to background subtraction. On the left are amplitude (top, in blue) and phase (bottom, in red) measurements, with the raw data plotted as coloured lines. Background and signal data points are shown as black and coloured circles, respectively, while the polynomial curve fitted to background measurements is shown in green. On the right the data after background subtraction are shown, with the background and signal data points again shown in black and coloured circles, respectively. A large drift can be seen at the end of both plots.

Theoretically, the background measurements should always be approximately the same. While systematic variation of the background is expected due to instrumental imprecision and from other sources, large and sudden sloping changes are not.

THIRD AND FINAL PROCESSING METHOD

Although it would be possible to process sections of data sets separately, each with their own polynomial background subtraction, due to the manual nature of the data separation this would be overly time consuming, and subject to human bias. This would also result in multiple very small sets of data that might vary between them due to the goodness of the polynomial fit, and the inherent variation of their background. Ideally, all data would be processed identically so that they could all be directly compared. An automated method was preferred, and so background data were fitted

with a piece-wise cubic spline interpolation rather than a polynomial. A linear interpolation method was also considered, but the smoothing properties of a spline seemed, theoretically, more likely to reflect the pattern of drift of the system.

SPLINE FIT FOR BACKGROUND REMOVAL

In order to fit a spline to the background data without artificially reducing its variation to 0, the average of each set of background measurements was found, and a spline function fitted to those, as shown in Fig. 8. In this way, the random variation within background measurements was retained, while both long and shorter term drift was corrected for. In order to avoid large oscillations at the end of the data sets, the first and last background averages were duplicated and appended either side of the data in place of enforcing particular end point slopes.

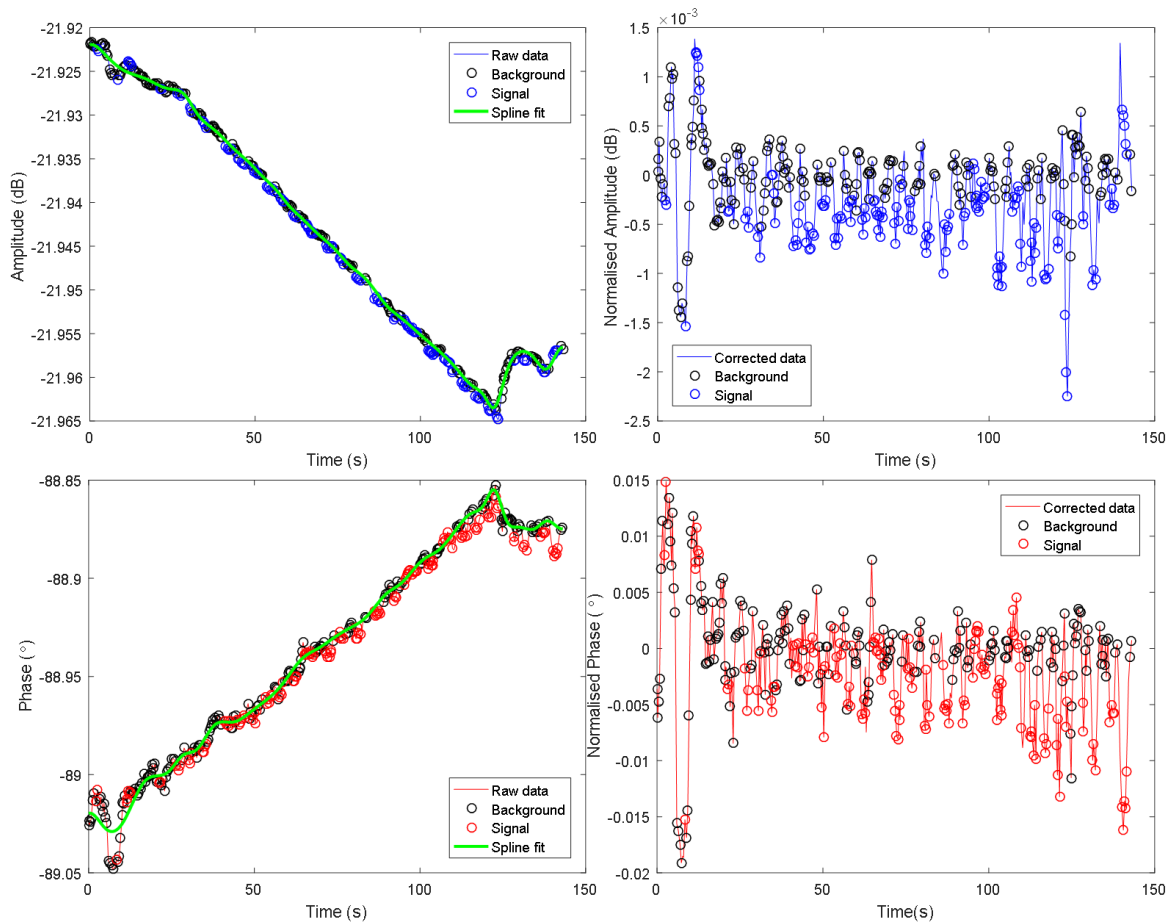


Fig. 8: Example results from Fig 6 showing the improvement offered by the spline interpolation approach to background subtraction. On the left are amplitude (top, in blue) and phase (bottom, in red) measurements, with the raw data plotted as coloured lines. Background and signal data points are shown as black and coloured circles, respectively, while the spline interpolation of background measurements is shown in green. On the right the data after background subtraction is shown, with the background and signal data points again shown in black and coloured circles, respectively. The large drift at the end of both plots has been removed.

Initially, there was concern that this approach would still artificially reduce the variation in the background measurements, or even perhaps increase it due to oscillations between interpolation points. In order to assess the validity of this method, several experiments were done to determine the variation of blank measurements without the influence of changing pressure, events at the other capacitive gap, human-caused mechanical wobbles or events at the outlet. This was done by leaving the system to continuously measure the amplitude and phase over more than two hours whilst pumping the background solution through at a constant low pressure (5 mbar), and collecting the output with a stationary absorbent tissue. This background data was then treated with a polynomial background subtraction to remove the obvious slow moving drift effects, as shown in Fig. 9.

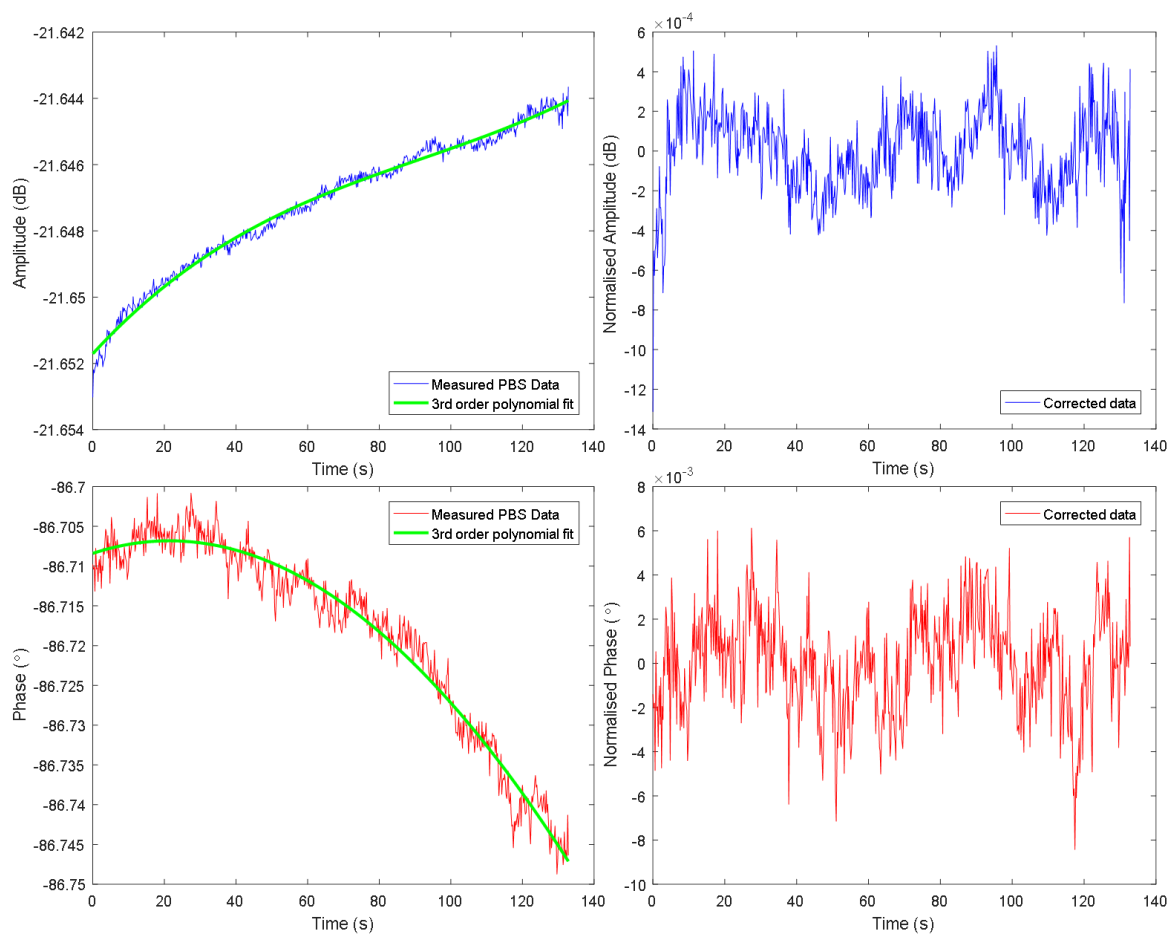


Fig. 9: Measurement of the background drift intrinsic to the system. On the left the raw amplitude (top, in blue) and phase (bottom, in red) values are plotted against time, with the polynomial used to fit the drift shown as a green line in both. On the right, the normalised amplitude (top, in blue) and phase (bottom, in red) after the drift has been subtracted.

The small variation in the blank measurements (the variation that could be expected without the variables inherent in measurement) was resembled more closely by the spread of the background data after spline background removal than the slightly wider spread obtained if a polynomial fit was used, as shown in Fig. 10, even when comparing measurements that did not have such obvious leaps

as those seen in Fig. 7 and Fig. 8. This suggests that there may be a lot of less noticeable, smaller, experimentally caused variations occurring between background measurements that are not addressed by polynomial background subtraction, and that the spline-based approach is better for accounting for this as well as for temperature drift.

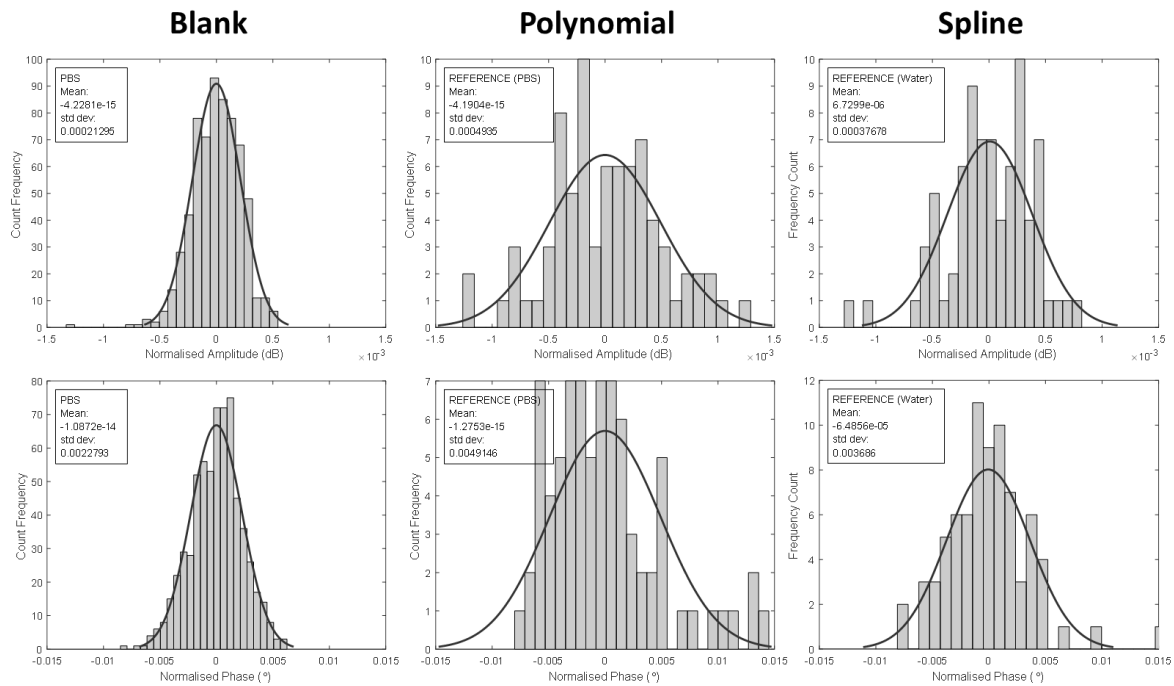


Fig. 10: Histograms showing the comparison between the variation in blank measurements expected, and the variation obtained by the two different background removal approaches discussed. On the left are histograms of the normalised amplitude (top) and phase (bottom) measurements from Figure 7, showing the expected variation of the background. In the middle are histograms of the normalised amplitude (top) and phase (bottom) measurements from Figure 2, a 3rd order polynomial fitting to the background measurements of the C2C12 cell experiment. On the right are histograms of the normalised amplitude (top) and phase (bottom) measurements from the same data as Figure 2, but with background removal achieved via a spline interpolation rather than a polynomial fitting. It can be seen that the standard deviations of the spline processing are most similar to that of the blank measurements, and especially in the phase show a more normal distribution shape.

Indeed, when applied to data sets with very little background variation aside from long term drift, the spline interpolation approaches a linear or polynomial fit, as shown in Fig. 11.

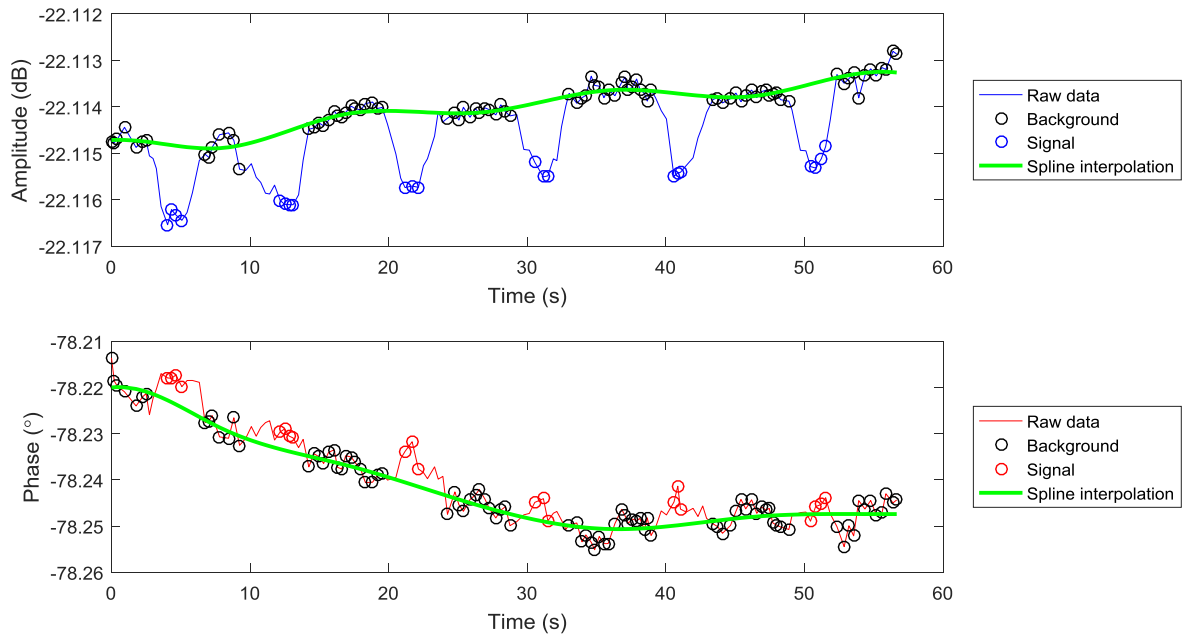


Fig. 11: Example output of a different C2C12 cell to that from Fig 3, showing very little sudden variation in the background measurements. On the top the amplitude measurements are shown, with the blue line denoting the raw data, while on the bottom the phase measurements are shown in red. Black and coloured circles denote background and signal data points, respectively, while the green lines denote the spline interpolation used for background removal. It can be seen in the phase plot that the spline fit is very smooth for data with little variation.

Once a spline processing method had been agreed as reasonable, a global Matlab script was created which could be used on any set of data, so that all data were processed in exactly the same way with minimal human interference, allowing for direct comparison of processed data.

SCATTER PLOTS

In order to link the measurements of amplitude and phase together, instead of plotting results as histograms the data could be plotted against each other as a scatter plot, with markers for the mean and error bars denoting the standard deviation of the data, as shown in Fig. 12. This enabled visualisation of the overlap between the background and signal measurements, as well as giving a 2-dimensional format, like flow cytometry, to identify areas of correlated amplitude and phase shifts that might correspond to characteristic physical properties of different cells.

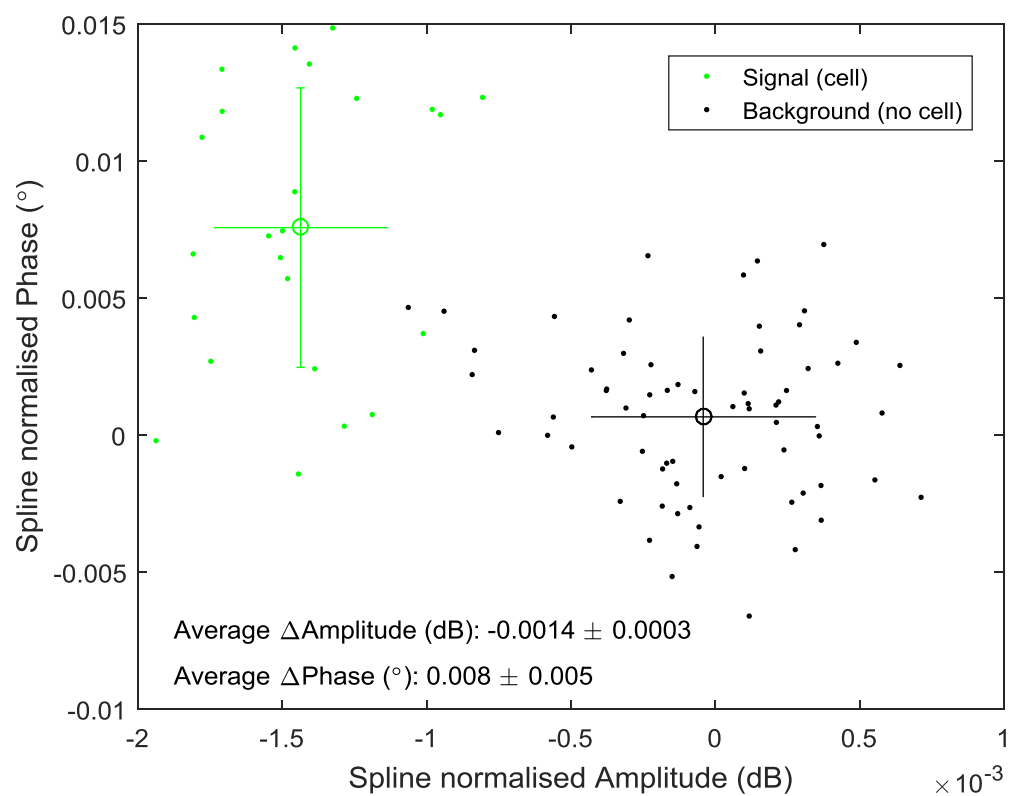


Fig. 12: Results from the C2C12 cell looked at in Fig 3, showing the scatter plot approach to data visualisation. The spline corrected amplitude and phase shifts are shown plotted against each other for every data point as green dots, with the background measurements shown in black dots. The mean values for both the background and signal measurements are shown as black and green circles, respectively, while the total length of the error bars denotes two standard deviations of the data.

APPENDIX 3: CELL SIZE DETERMINATION BY ANALYSIS OF REAL-TIME VIDEO FOOTAGE

In order to approximate the size of cells measured the real-time video footage from the measurements was analysed using ImageJ software. This appendix describes how this was done.

PREPARATION OF FOOTAGE

The MATLAB monitoring script embedded captured stills from the camera in a figure. Output files therefore had to be cropped and rotated. The scale was set by measuring the length of a known feature size (the width of the ring at the gap, 500 μm) in pixels and using this to define the pixel size.

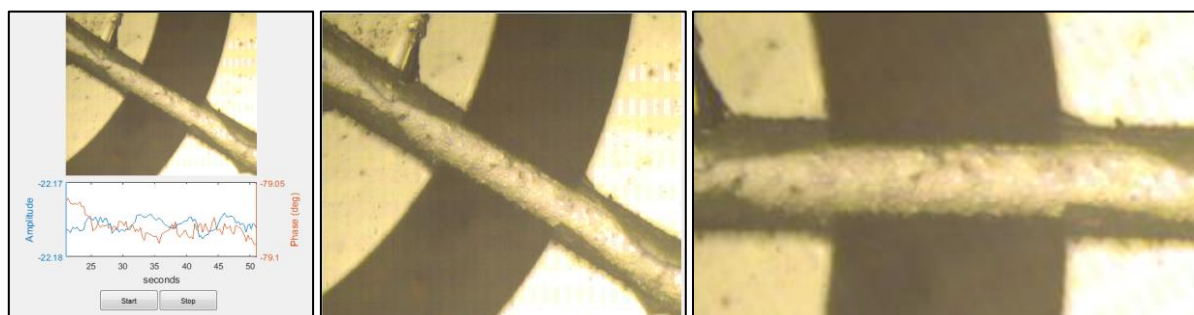


Fig. 13: The process of preparing the images, showing the output of the real-time monitoring script (left) and the cropped (middle) and rotated (right) microscope images.

BACKGROUND REMOVAL BY COMPOSITE IMAGE CREATION

The video footage of each cell passage during a measurement was looked at frame by frame, and notes taken of the frame numbers that correspond to a full passage of the cell from one side of the field of view to the other. Substacks (a series of stills) of each passage were then made from the prepared video and two composite images of each were made. The “background” composite image was made by using the median Z-projection function, which takes the median of the intensities of all pixels throughout the stack to create an average image, essentially an image of the pixels that do not change through the stack. Another composite image which highlights the changing pixels in the substack only was made using the minimum Z-projection function, resulting in a composite image of the cell as it passes through the gap, showing its position at every frame. The background image was inverted and added to the image with the cell passage to create an image of the cells only, as shown in Fig. 14.



Fig. 14: The background composite image of a cell passage (left), the median composite image of the same cell passage (middle), and the resulting image of the cell only (right).

THRESHOLD ADJUSTMENT

The image of the cell passage was then converted into an 8-bit format, reducing the image to greyscale. A thresholding function was then used to simplify the image further into black and white, with images of cells highlighted. The threshold could be varied incrementally to try to achieve the most reasonable looking cells. Too high a threshold resulted in significant interference from pixels unrelated to the cells, while too low a threshold resulted in the disappearance of pixels corresponding to the cells, as shown in Fig. 15.

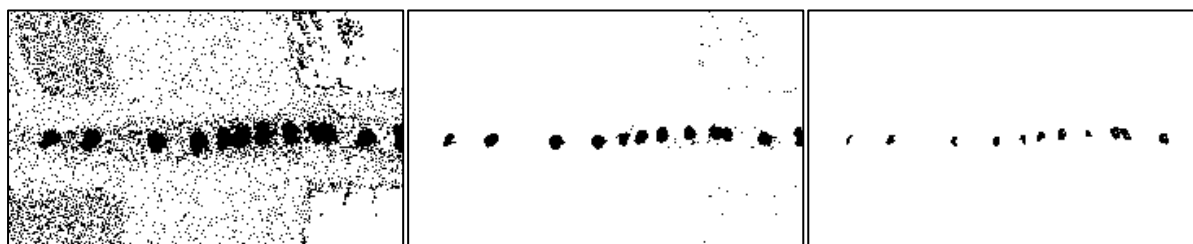


Fig. 15: Examples of threshold adjustments. A threshold setting which is too high (left), too low (right) and about right (middle).

Due to the interference from the top of the channel and the limited contrast between the cells and the surrounding medium, the edges of cells were poorly defined. The different paths taken during different passages and by different cells, as well as changes in lighting from experiment to experiment resulted in small variations of the threshold value needed to produce similar results, and so thresholding was done manually for every cell passage, with manual cleaning up of the background followed by a watershed function used to separate cell images that were too close, or a close function to fill in partial outlines of cells.

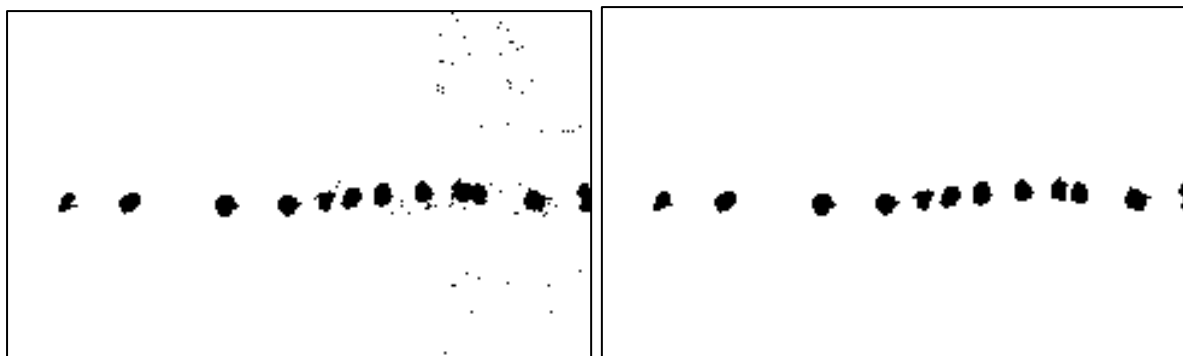


Fig. 16: The threshold adjusted 8-bit image (left) and the image after use of the watershed function and cleaning up of the background noise (right).

DETERMINATION OF AREA

The resulting cleaned-up image was then analysed using a particle analysis function that recognises circular objects and calculates their area in pixels, as shown in Fig. 17. The degree of circularity required to be measured could be varied and a range of 0.5-1 was used. Objects identified were analysed in mm^2 thanks to the calibration done earlier. Measurements corresponding to parts of cells rather than whole cells were discarded, while all others were exported and averages found for each cell pass. The diameter of the cells was calculated directly from the area.

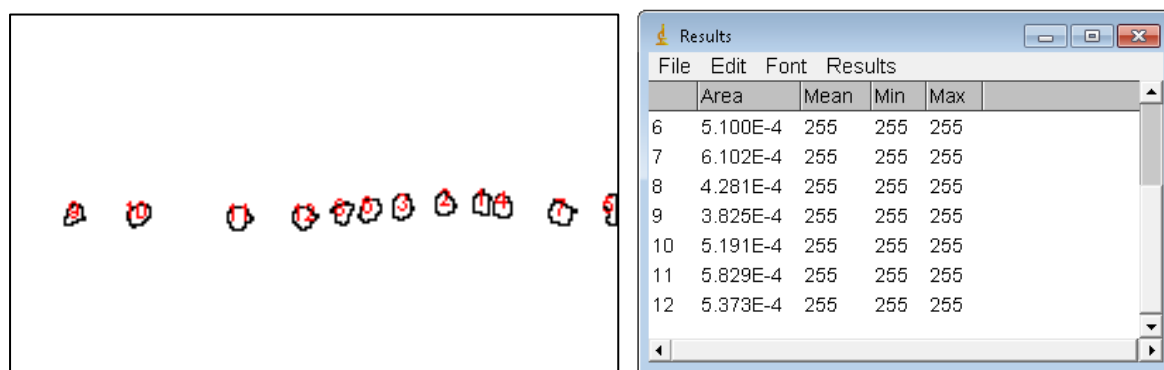


Fig. 17: Numbered objects identified for analysis by the particle analysis function, with the outlines shown in black (left) and the areas of each object exported as a table (right).