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Department of Mechanical and Aerospace Engineering (DIMEAS)

Master Degree in Biomedical Engineering

Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) :

a non invasive technique to study cell-surface interactions

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Academic Year 2019/2020

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Abstract

The Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is an ultrasensitive mechanical sensing device capable of providing real-time, non-invasive measurements of changes in resonance frequency and energy dissipation responses due to cells immobilization onto the sensor surface. Most of its applications in cell research have been limited to the study of adhesive interaction between cells and the substrate surface and the evaluation of the effect of external stimuli on the adherent cells. The aim of this thesis work was to further exploit the capabilities of the QCM-D to study cell adhesion on different substrates. Firstly, it was introduced the mechanism of cell adhesion on the QCM-D sensor surface characterized by a three-phase response profile: cells adsorption, attachment and spreading, and remodelling. A state of art of QCM-D was also presented in order to show the biological applications of this technique, in particular the use of QCM-D to study cell adhesion on surfaces that are used in medical implants. The experimental section presented in the final chapter was used to evaluate the effectiveness of scaffold functionalization protocol ,adopted in a previous work, by reproducing each functionalization step on the gold sensor surface . The gold sensor surface was coated with Polycaprolactone (PCL) using the electrospinning technique to continue the previous work , in which *in vitro* models of human cardiac fibrotic tissue based on 2D and 3D bioartificial Polycaprolactone (PCL) scaffolds were produced.

Bidimensional scaffold were fabricated by electrospinning technique because of its ability in mimicking closely the hierarchical properties of the extracellular matrix (ECM) of the native tissue. Gelatin (G) was grafted on the scaffold surface by a two-step mussel-inspired strategy. At first 3,4-Dihydroxy-D,L-phenylalanine (DOPA) was polymerized on PCL scaffold (PCL-polyDOPA) and then G was grafted (PCL-polyDOPA/G). At last , a future experiment was set to monitor cell adhesion on the sensor surface coated with PCL and functionalized with poly-DOPA and Gelatine. This experiment should give informations about other aspects of cellular behaviour, such as cell morphology, cell signaling, all of which can potentially be applied to medical diagnosis and pharmaceutical development.

1.Introduction

Biosensors combine the selectivity of biology with the processing power of modern microelectronics and optoelectronics to offer powerful new analytical tools with major applications in medicine, environmental diagnostics, and foods and their processing industries. It is gaining importance and popularity over conventional analytical techniques because of specificity, low cost, fast response time, portability, ease of use, and continuous real-time signal. Biosensors offer rapid and accurate detection with minimal sample preparation. They are also amenable for online analysis.

1.1 The concept of biosensor

The concept of biosensor was pioneered by Clark and Lyons in 1962: they proposed the use of electrical detectors to immobilize enzymes in order to form enzyme electrodes. The first enzyme electrode was devised for monitoring glucose concentrations in blood using glucose oxidoreductase (GOD). GOD was held next a platinum electrode in a membrane sandwich (figure 1).

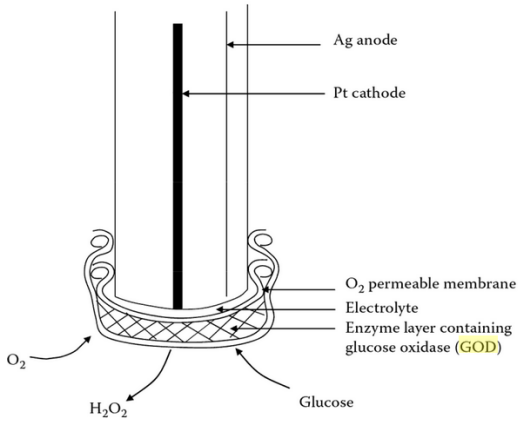

Figure 1: Structure of the platinum (Pt) electrode

The platinum electrode was polarized at +0.6 V and responded to the peroxide produced by the enzyme reaction with substrate [1].

$$Glucose + O_2 \rightarrow gluconic\ acid + H_2O_2$$

A biosensor is an analytical device that consists of a biological component coupled to a transducer that converts biochemical activity into a quantifiable electrical/optical signal. It consists of five parts (figure 2):

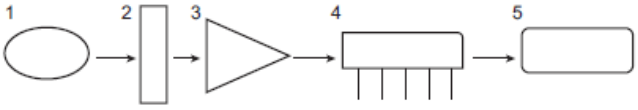

Figure 2: The schematic flow of a biosensor

1. *Biological recognition element* converts the analyte into product with or without reactant,

2. *Transducer* detects the occurrence of the reaction and converts it into an electrical, optical, mechanical, mass, acoustic, thermal, chemical, and magnetic properties,
3. *Signal conditioning circuit* is an associated electronics or a microprocessor that are primarily responsible for signal processing and amplification,
4. *In microprocessors* signal is digitalized and stored for further processing,
5. *Display* shows graphical/numeric real-time analyte concentration.

An ideal biosensor should have many of the following features:

- ✓ A biocomponent that is highly specific to analyte and enough stable to provide longer operational life,
- ✓ Minimal dependence of the reaction on parameters such as stirring, pH and temperature,
- ✓ A response signal that is accurate, precise, and reproducible with linearity over the useful analytical range,
- ✓ A high signal-to-noise ratio, with minimum noise,
- ✓ Cheap, small, portable and simple to use.

Among the various types of biosensors, acoustic biosensors offer the possibility to analyse cell attachment and spreading. This is due to their speed of detection, their real-time, non-invasive approach and their high sensitivity both to the mass coupling, and to viscoelastic changes occurring closer to the sensor surface . Quartz Crystal Microbalance (QCM) and acoustic wave (Love-wave) systems

have been used to monitor cell adhesion to different surfaces and record cells behaviour under various conditions. Particularly, the QCM sensor detects cytoskeletal rearrangements caused by specific drugs affecting either actin microfilaments or microtubules. [2]

1.1.1 Quartz Crystal Microbalance (QCM)

Quartz Crystal Microbalance (QCM) is a highly sensitive technique employed to study adsorption processes and mass changes at the interface between a gas or liquid environment and a solid material [3] . The signal transduction mechanism of the QCM technique relies on piezoelectric effect in quartz crystals, was first discovered in 1880 by the Curie brothers, via a pressure effect on quartz [4]. In 1959, QCM was firstly used in a sensing mode when Sauerbrey reported a linear relationship between the f decrease of an oscillating quartz crystal and the bound elastic mass of deposited material [5]. The main idea is to measure the frequency shift of quartz sensing electrodes in order to calculate the mass of the deposited material above the crystal [6] [7]. The QCM technique is based on the usage of a thin quartz crystal between two metal electrodes. The crystal is usually AT-cut, meaning that, if it lays on the XY plane, it makes an angle of about 35 º with the Z-axis (optic axis) (figure 3).

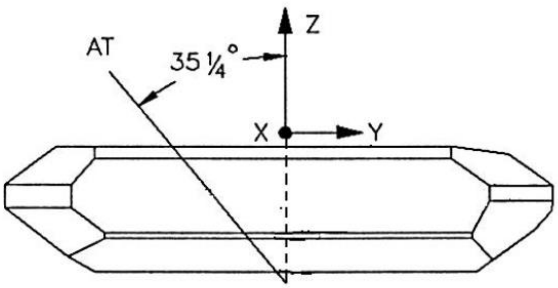

Figure 3 Scheme of an AT-cut quartz crystal

This type of crystal cut is employed, because it guarantees low temperature coefficient at room temperature (small changes in temperature only result in small changes in frequency). When an oscillating current is applied, the quartz crystal, which is a piezoelectric material, undergoes an in-plane shear mode oscillation (figure 4). Such oscillation is sensitive to the change in mass coupled to the sensor surface, which shift the resonance frequency of the oscillating crystal. This is due to the piezoelectric properties of the crystal and to its orientation. If a mass is attached to the crystal, the oscillation frequency f decreases (negative frequency shift). So, measuring the change in resonance frequency, it is possible to calculate the mass of a thin and rigid layer attached to the sensor surface, exploiting the Sauerbrey equation [8]:

Equation 1

$$\Delta f = -\frac{2f_0^2 \Delta m}{A\sqrt{\rho_q \mu_q}} = -C_f \Delta m$$

Where f_0 is the fundamental resonance frequency of the crystal (Hz), A is the area of the electrode *(c*m^2), ρ_q is the density of the quartz *(g/cm^3)* , $\mu_q$is the shear modulus of the mass*(g/(cm s^2))*, C_f integral mass sensitivity($m^2 Hz/g$), Δf is the shift in resonance frequency (*Hz*), i.e. change in oscillating frequency of the crystal, Δm is the change in oscillating mass per area(g/cm^2).

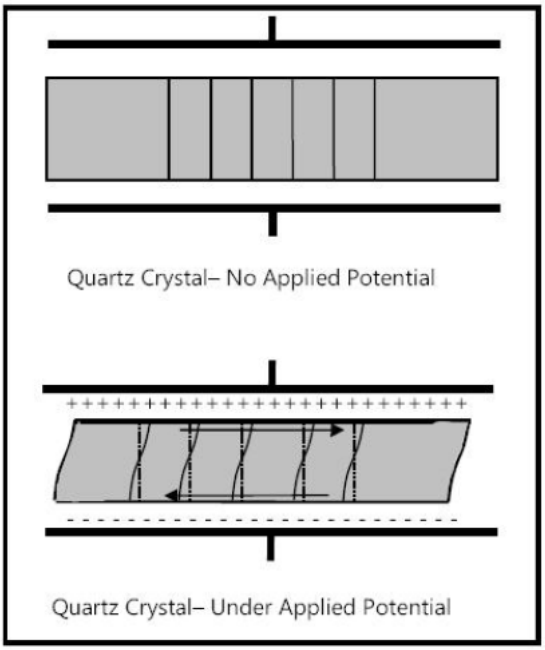

Figure 4 Scheme of thickness shear deformation of an AT-cut quartz crystal

This relationship points out that the frequency shift (Δf) is linearly dependent on the adsorbed mass. It is also possible to monitor the frequency shift for a harmonic overtone of the fundamental frequency and extract the mass change per area as [3]:

Equation 2

$$\Delta m = -\frac{C_{QCM}}{n}\Delta f$$

In which Δm is the amount of adsorbed mass, Δf is the shift in resonance frequency, C_{QCM} is a mass sensitivity constant (17.7 ng/cm^2 for a 5 Mhz crystal) and n is the resonance frequency overtone number. It is also possible to obtain the estimation of the thickness of the adsorbed layer of proteins [9]

Equation 3

$$h_{eff} = \frac{\Delta m}{\rho_{eff}}$$

Where $\rho_{eff}(g/cm^3)$ is effective density of adsorbed protein layer.

QCM method requires a short equilibration time (about 3-30 minutes) and assures hight accuracy. Oliveira et al. [10] demonstrate that QCM is an efficient and accurate method for studying the solubility of gases in polymers, in particular if compared to other methods like gravimetric or pressure decay.

However, the authors highlight the fact that it is necessary to assume that the adsorbed material spreads uniformly throught the crystal and vibrates syncronously with it. Another positive aspect of QCM technique is that it can be applied to several different liquids, being not necessary to isolate and purify the analyte. Beside, it allows to analyze conformational and functional changes of proteins in real time. [11] On the other hand, for a soft or thick layer of material bound to the crystal, the frequency shift due to the dissipation will be higher and the Sauerbrey equation cannot be applied. In these cases, the Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is a more suitable technique. [12]

1.1.2 Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

The Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is a lab-scale, fast analytical tool that can precisely monitor and quantify the amount cells adsorbed onto a polymer surface in real time. The QCM-D technique provides that, when the quartz crystal is immersed in a gas or aqueous solution, it is excited to oscillate at a fundamental resonant frequency, 5 Mhz. The Sauerbrey equation is valid if the adsorbed mass is evenly distributed, sufficiently rigid to have no energy dissipation, enough thin to have negligible internal friction, and has smaller mass compared to the mass of the crystal. However, if the adsorbed layer does not follow the oscillation rigidly, the energy

dissipation in the oscillation will be changed. In order to obtain mass change and structural properties of the adsorbed material layer, energy dissipation should be considered [9]. Dissipation factor can be defined as follows:

Equation 4

$$D = \frac{E_{lost}}{2\pi E_{stored}}$$

Where E_{lost} is the energy dissipated in one cycle of oscillation, E_{stored} is the total energy stored in the oscillator [13].

The most common viscoelastic model system used for estimating viscoelastic properties of adsorbed soft films in QCM-D measurements is the Kevin-Voigt model [9]. Spencer and Smith [14] found that the amplitude of a quartz crystal decays as an exponential sinusoidal, when the driving power to a piezoelectric crystal oscillator is switched off . The QCM-D system can determine the decay time (τ) and the resonance frequency (f) of the exponentially damped sinusoidal voltage signal over the crystal caused by switching of the voltage applied to the piezoelectric oscillator. The dissipation factor D, is inversely proportional to the decay time, (τ), as follows: [9]

Equation 5

$$D = \frac{1}{\pi f \tau} = \frac{2}{\omega \tau}$$

Where f (Hz) is the resonant frequency and τ (s) is the decay time. By recording the amplitude during the decay and numerically fitting the data to equation 5, both the resonance frequency and the dissipation factor of the crystal can be evaluated simultaneously. The high sensitivity of this approach, which is ng/cm^2 range in liquid, makes it possible to clarify material-cell interactions, including morphological and cytoskeletal changes of cell, during cell adhesion, spreading and growth processes [15].

1.1.3 Input parameters for QCM-D

In the Quartz Crystal Microbalance with Dissipation Monitoring apparatus, several parameters can be set and changed in order to optimize the detection of protein adsorption, cell adhesion or the properties of the adsorbed material under investigation.

Temperature In the Quartz Crystal Microbalance equipment, the temperature of the crystal chamber can be tuned, within certain limits. Some researchers chose a value of 37 ± 0.05 ºC to monitor cell adhesion on different materials [3] [8], others set a temperature of 22 ºC and 32 ºC, waiting 30 minutes after each temperature setting, for studying bovine serum albumin (BSA) adsorption and diffusion into hydrogel films [16].

Flow rate Analyte injection rate into the crystal chamber can also be tuned. Alf and co-workers [16] used a flow rate of 100 $\mu L/min$ for monitoring the

adsorption of several polymers onto the crystal. The same value was also employed to investigate thermo-responsive nanocomposites or polyelectrolytes [17]. Chung et al. [7] chose a value of 60 $\mu L/min$ to flow a solution with stem cells over a polymer or a crystal with adherent fibronectin. A flow rate of 30 $\mu L/min$ was used in other studies to investigate chitosan adsorption onto a QCM crystal and fibroblasts adhesion on the polymer layer [18]

Frequency range and number of overtone In Q-sense E_4 (Q-sense, Goteborg, Sweden) the resonance frequency of the sensor is 4.95 ± 0.05 MHz. Besides, it permits to measure changes in frequency in the range 1-70 MHz and up to the 13th overtone. For instance, some authors registered the 3rd overtone [3] [13], while others recorded four overtones at the same time [19]. As for the time required, Oliveira and co-workers [10] measured the values until the frequency was stabilized in a range of $\pm 2\ Hz$. Other authors [19] recorded the signal for 30-60 minutes. In some works, Δf and ΔD were measured every 15 seconds [8], while in others they were registered every minute. [20]

1.1.4 Applications of QCM-D

In general ,QCM-D can be employed to evaluate and monitor formation of polymer films, lipid bilayers or bacterial biofilms, protein and vesicle adsorption on a material, as well as cell adhesion and spreading on a surface [19].

Cell adhesion Understanding the mechanisms of cell-substrate interactions has provided the motivation for the development of biomaterials in medical implants and tissue engineering. The most important property of a biomaterial is its biocompatibility, which is typically evaluated based on how well cells adhere and grow on the material surface. QCM-D can be used to measure, not only the mass ,but also the viscoelastic properties of the adsorbed material and it can be applied to understand the mechanism of cellular behaviours [21]. Watarai et al. [12], evaluated the initial cell attachment behaviour, especially the strength of adhesion to the material surface by determining the slope of dissipation versus frequency (Df) plots using the QCM-D system. Chronaki et al [22], used QCM-D to distinguish the dynamic cell adhesion behaviour of human normal thyroid (Nthy) epithelial cells from poorly differentiated anaplastic carcinoma cells (ARO). Their results suggest that QCM-D is also a diagnostic tool.

Protein Adsorption When a biomaterial encounters blood it rapidly becomes covered with a layer of proteins followed by the initiation of blood coagulation, complement activation, inflammatory responses, and platelet activation. To prevent or reduce this effect, biomaterial surfaces may be chemically treated to minimize nonspecific protein adsorption [23]. Yafei Luan et al. [24],used three types of antifouling molecules to explore the applicability of QCM for the evaluation of the protein resistance of hydrophilic brush surfaces. It was found that the viscous nature of the hydrophilic polymer brushes may cause a hearing

loss in QCM measurement of protein adsorption. For a given polymer, there appears to be critical brush thickness beyond which the QCM chip cannot sense protein adsorbed on top of the polymer brush. Detection failure on longer polymer brushes may also due to water liberation upon protein adsorption. Qiang Chen et al. [25], developed an aptamer/protein/Apt-GNPs sandwich QCM-D biosensor for sensitive detection of thrombin. The amplification effect of Apt-GNPs on QCM-D, as both mass and viscoelasticity enhancers, was demonstrated in analytical method development for the first time. Their work extends the application of QCM-D biosensor, providing a promising approach for sensitive detection of proteins with two binding sites in biological simples.

Screening new antibacterial or drug candidates The uncontrolled use of antibiotics over almost a century has resulted in bacterial mutations and resistance towards existing treatment. As a consequence, many antibiotics have been rendered ineffective and there is now an urgent need to discover new classes of antibiotics to combat multi-drug resistant organisms [25]. Joshi et al. [28] reconsidered aminoglycosides as active antimicrobial agents/co-agents by providing detail of activity at the bacterial membrane. The two aminoglycosides, kanamycin A and neomycin B still offer potential as antimicrobial therapeutics for the future. In this context, QCM-D method illustrates great promise for screening new antibacterial or antiviral drug candidates.

Polymer Thrombogenicity The platelet adhesion and activation are frequently characterized to assess the thrombogenicity of biomaterials, which is a crucial step for the development of blood-contacting devices, such as endovascular implants [26]. Fatisson et al, used QCM-D chips coated with thrombogenic and non-thrombogenic model proteins to investigate polymer thrombogenicity. The responses of the sensor showed the predominant role of surface hydrophobicity and roughness towards platelet activation and thereby towards polymer thrombogenicity [27]. The QCM-D has interesting advantages compared to standards technique, such us scanning electron microscopy (SEM), which is commonly used for morphological studies. The QCM-D methodology does not require sample preparation and dehydration. Biological samples, such cells or proteins, can be studied in situ under wet condition. This technique also provides a real-time measurement of the morphological changes within a short time analysis. [28]

1.2 QCM-D as a tool to monitor cell adhesion

QCM-D sensors have been used for the study of eukaryotic cell adhesion. These studies took advantage of the sensor properties to detect mass adsorption in liquid and differences in viscoelasticity of the probed biological layer, and tried to provide new information on cellular behaviour in real-time. Particularly, the process of cell adhesion can be monitored, via the change in frequency, non-

invasively, and providing information about different molecular mechanisms responsible for cell attachment and spreading [2] .

1.2.1 Cell-extracellular matrix (ECM) interactions in vivo

For tissue engineering strategies, it is essential to know how cells can interact with ECM and transduce the information received by the extracellular molecules into an intracellular event. The identification of cell binding sites within extracellular molecules is a key step toward understanding the mechanism of cell-ECM interactions. Cells adhere to the extracellular matrix ECM (figure 5) via integrin-mediated adhesions that link the ECM to the actin cytoskeleton.

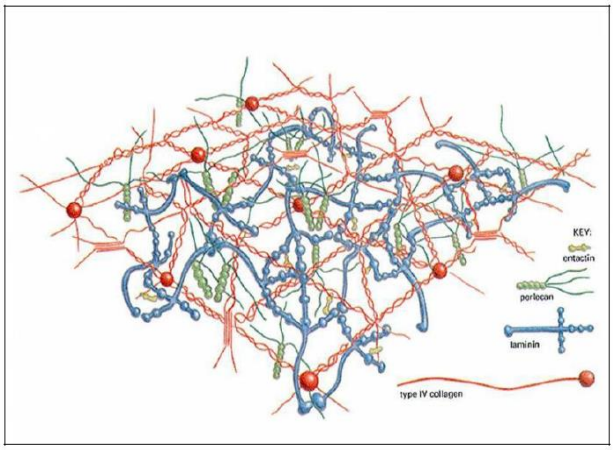

Figure 5 : The Extracellular Matrix (ECM)

Integrins are cell adhesion receptors that mediate the attachment of cells to the extracellular matrix but that also take part in specialized cell-cell interactions. Only a subset of integrins recognizes the sequence arginine-glycine-aspartic acid

(RGD) sequence in the native ligands. In some ECM molecules, such as collagen and certain laminin isoforms, the amino acid sequences RGDs are exposed upon denaturation or proteolytic cleavage, allowing cells to bind these ligands by using RGD-binding receptors [29]. Integrin heterodimers are composed of non-covalently associated α and β subunits. While β subunits seem to have a non-specific role in ligand binding activity, α subunits on the contrary confer high specificity on signal transduction.

As soon as ECM molecules bind to their specific integrin receptors, a change in cytoplasmatic domain of the receptors occurs, which associates the cytoskeleton at focal adhesions sites. There is a continuous crosstalk between cells and molecules of the extracellular matrix which leads to development of patterns, morphogenesis, differentiation and maintenance of the differentiated phenotype. There are at least two main ways by which the cell-ECM interactions can affect cell behaviour. One of these is the cell-ECM interaction which may directly regulate cell functions through receptors mediated by signals. The other is the ECM control on the mobilization of growth or differentiation factors, modulating cell-proliferation and controlling cell phenotype [30]. Martins-Green and Bissel [31] proposed that most processes, resulting from matrix-receptors interactions, fall into three categories which correspond to different developmental events: (I) cell adhesion/de-adhesion during migration mostly involving interactions via integrin and proteoglycan receptors and their

association with cytoskeletal elements, (II) expression of specific genes during differentiation involving interactions with basement membrane components via integrin receptors and with soluble factors via their receptors, (III) morphogenesis of epithelial organs involving cell death, and epithelial-to-mesenchymal transition that involve interactions with ECM. ECM undergoes remodelling via its receptors for enzymes, integrins, proteoglycans and soluble factors. Post-translational modifications also affect the ways in which ECM components interact each other and with cells. The degree of glycosylation of fibronectin and laminin and amount of calcium bound by thrombospondin have all been shown to modulate cell adhesion. [32]

1.2.2 The structure and function of the Extracellular Matrix (ECM)

The extracellular matrix (ECM) is composed of a great variety of molecules and includes collagen family, elastic fibres, glycosoaminoglycans (GAG) and proteoglycans, and adhesive glycoproteins. The different combination, immobilization, and spatial organization of these secreted substances give rise to different types of scaffold that characterize the different body tissues and organs [31]. Collagen (figure 6)is the most abundant protein in the vertebrate body which constitutes a heterogenous class of proteins. In addition to

mechanical and structural function, collagen plays an important role in determining cell attachment and spreading [33].

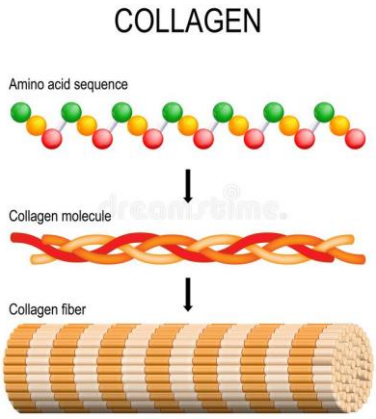

Figure 6: Structure of collagen

GAGs (figure 7) are linear polysaccharides formed by repeating disaccharide units that may contain sulphate groups. The most common GAGs associated with cells and basement membranes are heparans. Except for hyaluronan, all GAGs can be associated to a protein backbone and give rise to the so-called proteoglycans. Similarly, in other tissues such as skin, proteoglycans in association with other structural molecules form specific complexes responsible for hydration and spatial organization of ECM [31].

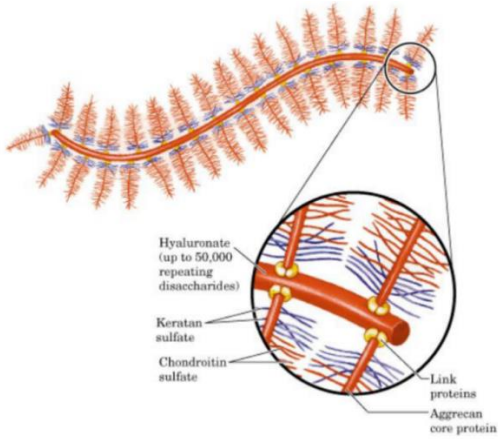

Figure 7 : Structure of a proteoglycan

Adhesive glycoproteins are a class of ECM molecules including interactive glycoproteins that exist in several variant forma and possess multiple binding domains capable of binding collagen and proteoglycans, as well as binding to the cell surface. Fibronectin and laminin belong to this class of ECM molecules. Fibronectin plays an important role in the cell attachment proliferation and differentiation [34]. Given its high cell binding affinity, laminin alone or in combination with other ECM molecules is widely used to coat cell culture dishes and plates to enhance cell attachment and spreading. [35] The elastic fibres are composed of an amorphous protein, elastin ,whose structural characteristic is elasticity. Cross-linking of these peptides by gamma irradiation gives rise to hydrogels which can be used as substrate for tissue reconstruction. [36]

1.2.3 Cell adhesion and spreading

Generally, cells interact with surfaces, either in vivo or in vitro, only via molecules on cell membrane. Under *in vivo* conditions, cells transmit extracellular or intracellular forces through localized sites at which they are adhered to other cells or an extracellular matrix. The adhesion sides are formed by the integrins to anchor the cell to a matrix or by adhesive molecules to bind other cells. Both the integrins and adhesive molecules are attached to the tensile member of the cytoskeleton, the actin filaments, through the focal adhesion (FA) complex, a highly organized cluster of molecules (figure 8). The cytoskeleton structure holds the nucleus and maintains the shape of the cell. As a pathway for transmission to the cytoskeleton, integrins play an important role in transduction through FA proteins connecting the integrin domains to the actin filaments to form the adhesion complex. Upon binding, integrins cluster into FA complexes that transmit adhesive and traction forces. The FA formation is important in cell signalling to direct cell migration, proliferation, and differentiation for tissue organization, maintenance, and repair. [37] There are also non-specific

interactions, based for example on electrostatic interactions between ionic or polar groups at the cell surface and the substrate [2]

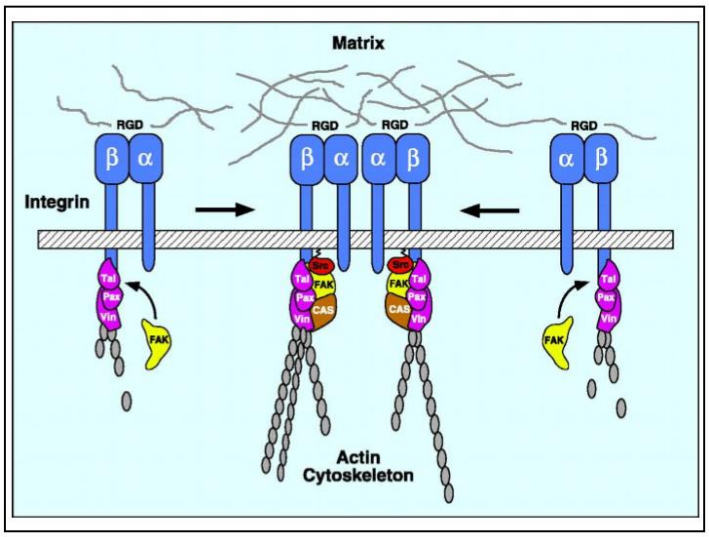

Figure 8 : Focal Adhesion

Under *in vitro conditions*, cells passively adhere and spread on the substrate, undergoing morphological alterations driven by passive deformation and active reorganization of the cytoskeleton [37]. The process of static *in vitro* adhesion is characterized by three stages: first a cell comes in contact with the substrate and loosely attaches onto the substrate surface. Second, the cell begins to flatten, spread its membrane and simultaneously form focal contact over the substrate surface. Lastly, the cell form focal adhesion complexes (FAs) that connect the extracellular matrix (ECM) with intracellular actin filaments, which securely

anchor the cell to the substrate surface. With exogenous stimulation and modulation the cell can de-adhere from the ECM (Figure 9) [38].

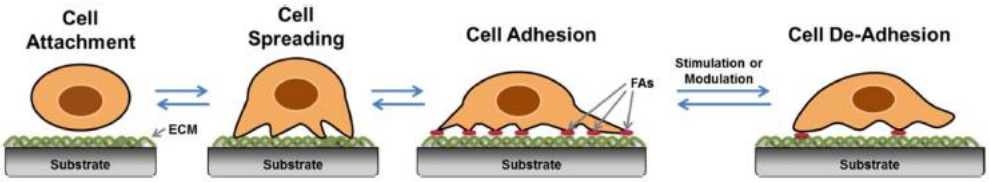

Figure 9: The process of cell-adhesion to a substrate

Cell spreading appears to be accompanied by the organization of actin into microfilaments bundles. The strength of adhesion becomes stronger with the length of time a cell is allowed to adhere to a substrate or another cell [39]. Cell interaction with artificial substrates is important for basic and applied biomedical research, e.g., in the development and modification of biomaterials for implants. Therefore, tools capable of monitoring the establishment and dynamics of cell-substrate contacts are extremely useful in many areas of interest. [2]

2. QCM-D: state of art

The purpose of this chapter is to explore the potential of using Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) based technique for the characterization of living cells behaviour during the process of adhesion to a surface. The evolution of the dissipation factor in combination with resonant frequency provides a fingerprint of the cell adhesion process, which is sensitive to properties of the surface as well as the type of cells.

2.1 Biological applications of QCM-D

The use of biological coating, such as enzymes and antibodies, was a natural progression from the initial piezoelectric sensor development. The first piezoelectric immunosensor for microbial pathogens was developed by Muramatsu et al. in 1986 [40]. Their piezoelectric crystals were coated with antibodies against Candida Albicans. The sensor proved to be specific with no detectable response observed with the other species tested (Saccharomyces cerevisiae). Conventional bacterial adhesion methods generally require laborious procedures and many hours or even days for complete analysis. The relatively large mass of bacterial cells, combined with the availability of antibodies to most species, means that piezoelectric immunosensor detection offers a very attractive alternative to microbiological methods, generally, giving results in minutes, with adequate sensitivity and selectivity. Su and Li [41]

developed a QCM immunosensor by immobilizing anti-Salmonella polyclonal antibodies on AT-quartz crystal with Protein A method and monitoring the oscillation frequency of the crystal in real time [41]. The direct detection of nucleic acid interactions based on the use of acoustic wave devices was first provided by Fawcett et al. [42] They described a quartz crystal microbalance (QCM)-based biosensor for DNA detection by immobilizing single-stranded DNA onto quartz crystals and detecting the mass changes after hybridization . Since this early work, several articles have appeared employing similar procedures, resulting in a microgravimetric measurements based on nucleic acid. In Hao et al. work's [43], a DNA probe functionalized quartz crystal microbalance biosensor was developed to detect B anthracis based on the recognition of its specific DNA sequences

2.2 The QCM-D in cell biology

In cell study area , QCM-D has mostly been used for the development of organic surface modifications, intended to functionalize substrates before cell seeding These studies have typically been focused on the adsorption of protein layers [23] [24], the formation of supported lipid bilayers ,or the properties of layer-by-layer (LbL) structure based on biopolymers [44]. However, QCM-D has also been applied as a tool for studying cell behaviour and their surface interactions [2],[12] , [22] and its development led to studies where Df plots (frequency plotted vs. dissipation) were introduced and used to derive information about

cell attachment to substrates[48]. These plots could provide unique signature or fingerprints, independent of the spatial distribution of cells over the sensor surface for different surfaces [45], and had distinct regimes, which were indicative of the properties of the surface. Thus, it was concluded that several different events could be detected in QCM-D, from initial cell binding through secretion of proteins, cell spreading, changes in adhesion, and changes in cytoskeleton [46] [9].

2.1.1 Cell adhesion on QCM-D sensor surface

It has been determined that stronger cell adhesion is associated with the clustering of focal adhesion complexes formed between cell surface integrin receptors and the ECM [37]. To probe the cell adhesion, the QCM-D relies on the penetration of the acoustic wave into the basal plane region of the cell. The depth of penetration δ can be determined from the following equation [47]

Equation 6

$$\delta = \sqrt{\frac{\mu}{\pi \rho f_n}}$$

Where μ ($g/(cm\ s^2)$)is the viscosity of the liquid, ρ (g/cm^3) is the density of the liquid, and f_n (Hz) is the frequency of n^{th} harmonic. The shear-mode oscillation of QCM is considered non-invasive for cell adhered to the sensor

surface because the lateral displacement of the sensor crystal rarely exceeds a nm during oscillation [47] . During cell adhesion , the resonance frequency of the piezoelectric crystal usually decreased as the result of accumulation of cell mass on the sensor surface [21] [19] [20]. Those studies showed that the relationship between the change of resonance frequency (Δf) and the change of cell mass (Δm) normally does not obey to the Sauerbrey equation. This can be explained by the fact that cells behave more like a soft material, than like a rigid mass on a QCM sensor surface [19] .For this reason, the Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is a more suitable technique for studying cell adhesion. In fact, this technique allows for simultaneously recording of (Δf) and (ΔD) for multiple harmonics ,which permit to better understand the viscoelastic properties of the adsorbed layer above the crystal [12].

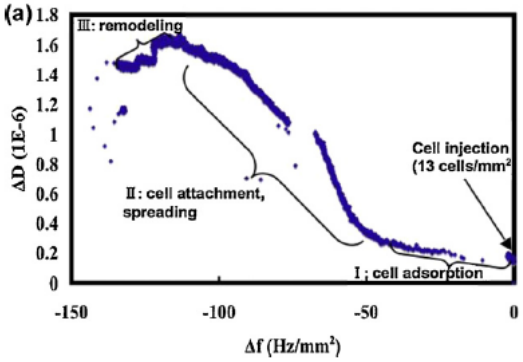

Figure 10 : The Δf and ΔD signals over time after the addition of fibroblasts onto nano-Ha coated Crystals

Dissipation versus frequency (Df) plot usually exhibits a three phases response profile: phase I, cells adsorption, phase II attachment and spreading of cells , phase III cells remodelling (figure 10). During the two initial stages, dissipation and frequency increase markely, which may be an early sign of cells approaching and attaching the surface (cells behave like a coupled oscillator [48]) . Then, Δf starts to decrease, and the cell spreading occurs, indicating that the cell come closer to the surface and create more attachment point. Then the cell, enter the third stage of cell spreading where the dissipation decreases rapidly together with an increase in frequency. In this phase cell undergo rearranging and lose a lot of adhesion points producing less but more mature focal adhesion. This phenomenon can reduce the mass coupled to the surface. In addiction the decrease in dissipation is a sign of the cells becoming more rigid as they organize their cytoskeleton. In the final fourth region of the Df-Plot both the frequency and dissipation decrease slowly, pointing toward the cells entering a form of steady state in spreading behaviour (*figure 11*). The continuous decrease in frequency might be a sign of the production of the extracellular matrix and/or serum protein adsorption [49].

2.1.2 Cell adhesion on different surfaces detected by QCM-D

The monitoring of cells adhesion mechanism on a surface plays an important role in the design of substrates for biotechnology and tissue-engineering

applications. Firstly, surface topography influences cells behaviour but also their organization at micro and nano-scale. Secondly, surface chemistry as well as surface energy influence the cell adhesion. Thirdly, cells never interact with a bare material but with materials on which proteins from biological fluids have been adsorbed [23]. The potential of biomaterials for supporting osteogenic differentiation and tissue-integration requires the attachment and spreading of bone-forming cells. The QCM-D sensor was used to compare the adhesion of pre-osteoblastic cells on *tantalum(Ta) and chromium (Cr) surfaces* [50]that are two medically relevant surfaces found in medical implants. The results showed larger frequency and energy dissipation shifts when cells adhered to tantalum-coated surface, suggesting that tantalum is a more biocompatible material than chromium .The signal change for frequency and dissipation was also used to indicate fibroblast attachment on tantalum and oxidised polystyrene surfaces coated by various proteins [51]. Osteo-pontin is a protein with great potential in inducing high osteo-conductivity and acts as a functional coating on orthopaedic implants. *Osteo-pontin adsorption on hydroxyl-apatite and gold* was investigated, as well as the attachment of human mesenchymal cells [52]. Cell attachment and spreading were found to be more efficient on the osteo-pontin /hydroxyapatite surface than on the osteo-pontin/gold surface and bare hydroxyapatite or gold surface. Self-assembled monolayers (SAMs) have been used largely in the recent year in order to elucidate the influence of surface chemistry on cell adhesion [53]. Kao et al. used QCM-D to study the NIH3T3

mouse embryonic fibroblasts adhesion on surface with *different ç-potentials.* Higher ç-potentials surfaces allowed the direct interaction between cells and surface resulting in a high adhesion and cell spreading while the electrostatic repulsions among surface and cells occurring in presence of lower ç-potentials required the formation of an ECM-like layer prior to cell adhesion and spreading [54] . In QCM observation, NIH3T3 cells adhered readily on NH2-rich surfaces, while the cell adhesion on COOH-rich surfaces was through the ECM coating. The surface potential affected the competition between the deposition of cells and ECM proteins through the difference between cell-surface and ECM-electrostatic interactions (figure 11)_ .

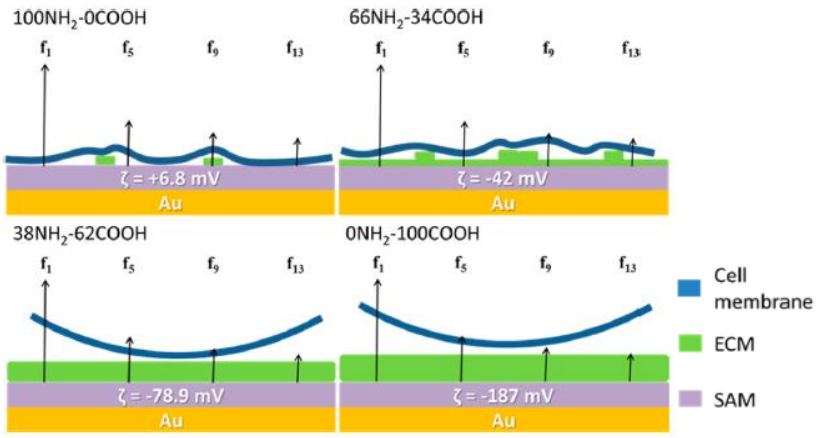

Figure 11: Illustration of the competition between cell and ECM protein deposition on serial surface potential.

With less electrostatic repulsion force between negatively charged cell membranes and NH2-rich surfaces, cells could readily adhere on surfaces with

little or no ECM coating, while the electrostatic repulsion preventing cells from adhering directly on negatively charged COOH-rich surfaces.

2.1.3 The strength of QCM-D to monitor cytoskeleton changes

The cells can be treated with a cytoskeleton perturbing agent to induce cytoskeleton changes. The induced cytoskeleton changes are expected to be sensed by QCM-D , as changes in viscoelastic properties of the layer of cells on the sensor surface [55]. Cytoskeleton is a dynamic network of interlinking protein filaments able to give cell its shape and to provide mechanical support, it is also involved in signal transduction. In particular, cytoskeleton of eukaryotic cells consist of three major classes of elements that differ in size and in protein composition. Microtubules are the largest type of filaments (diameter of about 25 nm) composed of a protein called tubulin and provide the basic organization of the cytoplasm. Actin filaments are the smallest type (diameter of about 6 nm) and allow cells to hold specialized shapes. They are also involved in cell movement. Intermediate filaments commonly work with microtubules, providing strength and support for the fragile tubulin structure. The shear elasticity of the cytoskeleton plays a key role in cell mechanical properties and thus the changes in cytoskeleton viscoelasticity can lead to changes in both ΔF and ΔD characteristics. In fact, the alteration in cytoskeleton stiffness or softness can be detected by QCM-D sensor even more than the changes in the surface mass (also dependent on cell density). When the cell are treated with a perturbing

agent (i.e. cytochalasin D), and the actin mesh is altered, the mechanical interaction between the cell and the protein layer changes , as well as the viscoelastic properties of the cell itself [21]. In fact, when cytochalasin D was added, there was a large dissipation increase upon cell adhesion and it was clear that cells interacted with the interfacial layer. Nowachy et al. [56] demonstrated that QCM-D is able to detect different cell behaviours in response to drug exposure. At low staurosporine (STS) concentration (figure 12), QCM-D is able to sense early cytoskeletal events associated with apoptosis, which are not sensed by the classical analytical techniques in cell biology within 4 h. So, they concluded that the energy dissipation shift could be used to track malignant (MCF-7s) mammary epithelial cells responses to STS.

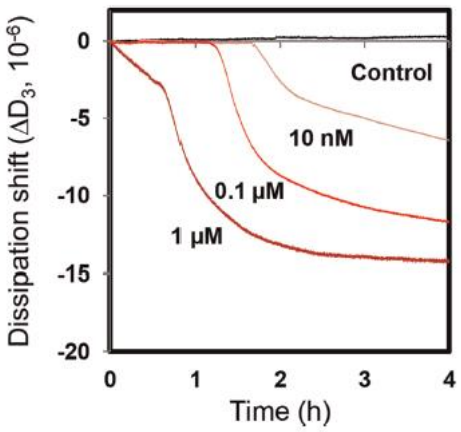

Figure 12: The QCM-D signatures obtained for low STS dose.

Fatisson et al. [57] used the layer by layer (LBL) assembly technique in conjunction with QCM-D to study the viscoelastic properties of the LBL films, which acted in synergy with blood platelets . The sequential deposition of biocompatible chitosan (CH) and hyaluronic acid (HA) polyelectrolyte on platelet with different morphogenesis was assessed, and the energy dissipation factors were measured for surface of various protein composition ratios HAS/HFN (figure 13).

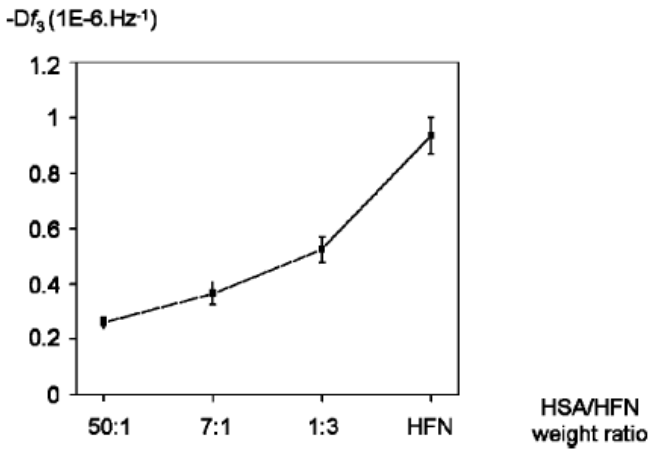

Figure 13: Plots of the Df3 of two CH/HA bilayer film deposited on the platelet sited on a QCM-D chip coated with various weight ratios of HAS/HFN

The change in energy dissipation can be directly associated with changes in the cell cytoskeleton because the cell population remains similar for all surface of various composition ratios.

2.2 Polymer deposition techniques on QCM-D

QCM sensors can be utilized to detect materials at shallow concentration levels by coating sensitive materials. The coating can adsorb analytes efficiently on QCM electrode. Generally, polymer deposition on QCM device occurs through drop casting, spin coating, or electrospinning [58]. Electrospinning technique has the advantage of allowing deposition of high surface area and high porosity polymeric material. It is a simple, reproducible, and continuous technique for the collection of nanofibers with controllable morphology starting from a solution of polymer.

2.2.1 Drop casting

For small substrates ($\sim 1 cm^2$), an easy deposition method is drop-casting-spreading a nanoparticles dispersion over a substrate and allowing it to dry under controlled conditions, i.e. pressure and temperature (figure 14). In principle, film thickness depends on the volume of dispersion and the particles concentration, both of which can be easily varied. There are also other variables that affect the film structure such as how well the solvent wets the substrate, the evaporation rate, the capillary forces associated with drying, etc. Generally, it is preferable to use volatile solvent, wet the substrate and are not susceptible to thin film instabilities. Organic solvent (hexane, toluene etc.) are often very good choices for nanoparticles with hydrophobic capping ligands. One drawback of drop-

casting is that under near ideal conditions, differences in evaporation rates across the substrate or concentration fluctuations can lead to variations in film thickness or internal structure [59].

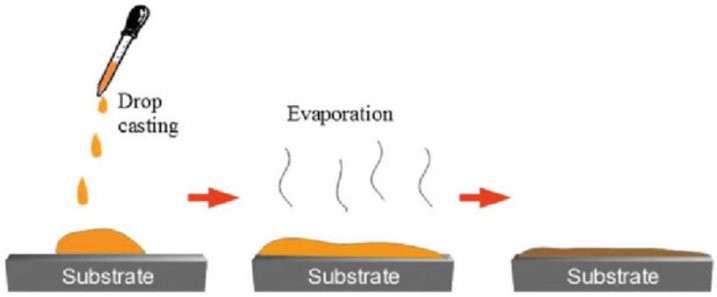

Figure 14 : illustration of the drop-casting process

Jin et al. [60] proposed a humidity sensor based on quartz crystal microbalance (QCM) using graphene oxide (GO) as a sensitive layer, and investigated the characteristics of sensor according to the shift of quality factor (Q factor) as well as the resonant frequency at different humidity (RH) , demonstrating a good prospect in humidity sensing application. The graphene oxide sensitive layer was deposited on the surface of Au electrode of the QCM with the thickness of 400 nm by dispersing GO solution drops on the surface and dried in air at room temperature.

2.2.2 Spin coating

Spin coating often provides more uniform thickness across the substrate compared with drop-casting and can accommodate much larger substrates. In this technique, a substrate is spun at high RPM and a volume of material with known particle concentration is dropped on the centre. Centrifugal forces lead to uniform spreading of the dispersion across the substrate, followed by evaporation of solvent to yield a thin particle film (figure 15). Film thickness depends on the dispersion concentration, volume, and the rotational velocity. Solvents other than water are favoured.

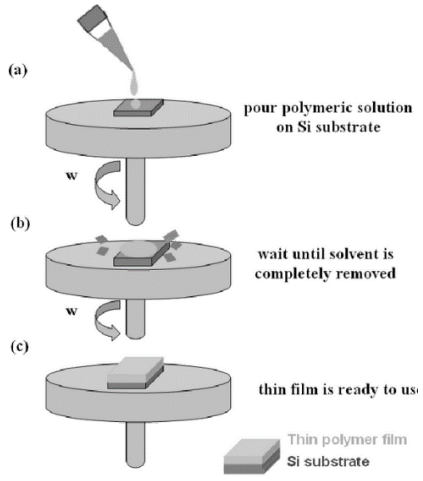

Figure 15: Spin-coating process

Spin coating is selected instead of inkjet printing for deposition of thin films to avoid the complexity caused by the additional ink components i.e. lubricants and

humectants such as ethylene glycol, propylene glycol and glycerol needed to make the PEDOT: PSS polymer and polyelectrolytes ink-jet printable [61] .

2.2.3 Electrospinning

Electrospinning is a voltage-driven process governed by the electrohydrodynamic phenomena where fibres and particles are made from a polymer solution. The most basic set up for this technique involves a solution contained in a reservoir (typically a syringe) and tipped with a blunt needle (for needle-based electro-spraying), a pump, a high voltage power source and a collector.

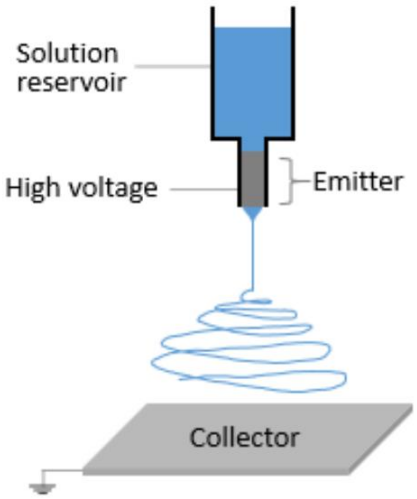

Figure 16: Typical Electrospinning set-up

The spinning process starts when the solution is pumped at a constant flow rate and a specific voltage is applied to create an electric field between the needle tip

and the collector. charges accumulate at the liquid surface. When the electrostatic repulsion is higher than surface tension liquid the liquid meniscus is deformed into a conically shaped structure known as the Taylor corner (figure 16). There are two ways nanofibers can be made through the electrospinning technique: needle-less and needle-based. In *needle-less electrospinning*, the starting polymer solution is transferred to an open vessel where the fibres are generated from a stationary or rotating platform. Mass production of material is one benefit in the needle-less electrospinning process but there are many disadvantages. Fibres morphology and quality are not precisely controlled, the raw materials that can be used are limited, which in turn limits versatile fibres production, and process parameters such as flow rate, cannot be controlled. In *needle-based electrospinning*, the starting polymer solution is typically contained in an air-tight closed reservoir, this minimizes and prevents solvent evaporation. This important difference allows for a wide variety of materials, including high volatile solvents, to be easily processed. There are many advantages of needle-based electrospinning, including flexibility inprocessing different structures like core-shell and multi-axial fibres. This distinction allows for active pharmaceutical ingredients (API) to be incorporated within a fibre. Additional advantages of needle-based electrospinning are tightly controlled flow rate, number of jets and minimizing solution waste [62]. Horzum et al. [63] reported a simple synthetic route to fabricate ZnO and CeO_2/ZnO nanofibrous mats and their sensing characteristics against volatile organic compounds such

as benzene, propanol, ethanol etc. The fibres were directly deposited on the crystal surface of a quartz crystal microbalance (QCM) using electrospinning technique.

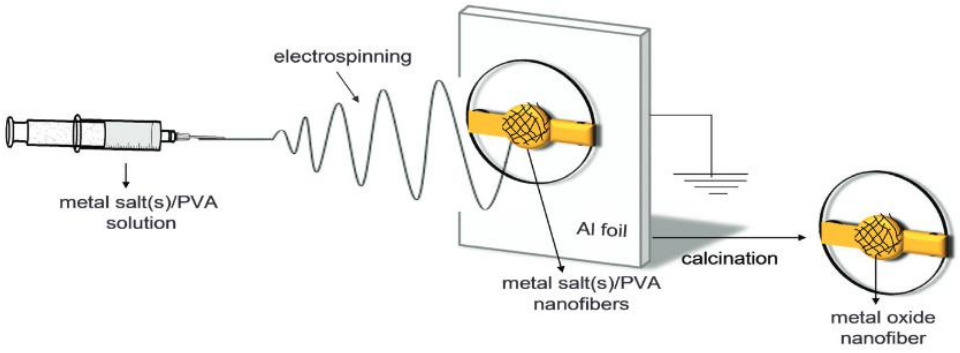

Figure 17: Scheme of the preparation of the electro-spun metal oxide fibre coated QCM

The viscous solution of PVA/metal salt precursors were loaded into a plastic syringe and connected to a high voltage generator (figure 17). As a counter electrode, a QCM crystal was removed from its electrodes and attached to the grounded aluminium foil. The electrospinning process was performed at 4 cm tip-to-collector distance for 15 min. A voltage of 10 kV was applied to the needler of the syringe.

2.3 Organic thin films

The modification of solid-liquid interfaces with polyelectrolyte multilayer films is a versatile tool to confer new functionalities to surfaces in environmentally friendly conditions. Indeed such films are deposited by alternate dipping of the

substrates in aqueous solutions containing the interacting species or spraying these solutions on the surface of the substrate [64]. Films produced from the alternated deposition of mutually interacting species can have similar properties to those of rigid and impermeable materials or to those of highly permeable gels [65].

2.3.1 Layer by layer (LbL) deposition

The LbL assembling approach is a simple and versatile deposition process with broad application in material science, for example in superhydrophobic surfaces, biosensors, implant coatings, semiconductors, fibre optics and drug-delivery devices. The whole process of multilayer structures build-up by LbL assembling, whole driving force, despite hydrogen bonding, covalent bonding, etc., is mainly electrostatic interaction between the oppositely charged species, usually consists of four steps (figure 18a) [64]. First (1) a cleaned, positively charged solid substrate is immersed into the solution of an anionic polyelectrolyte. Electrostatic force, collectively with adsorption, builds the first layer of multi-layer construction. After this, the removal of the excess and weak adsorbed polyelectrolyte from the surface is carried out by substrate rinsing with deionized water (2). A bilayer structure is achieved by immersing the substrate into the solution of the cationic polyelectrolyte (3). This step restores the original surface charge, and at the end (4) final rinsing removes polyelectrolyte exceeding.

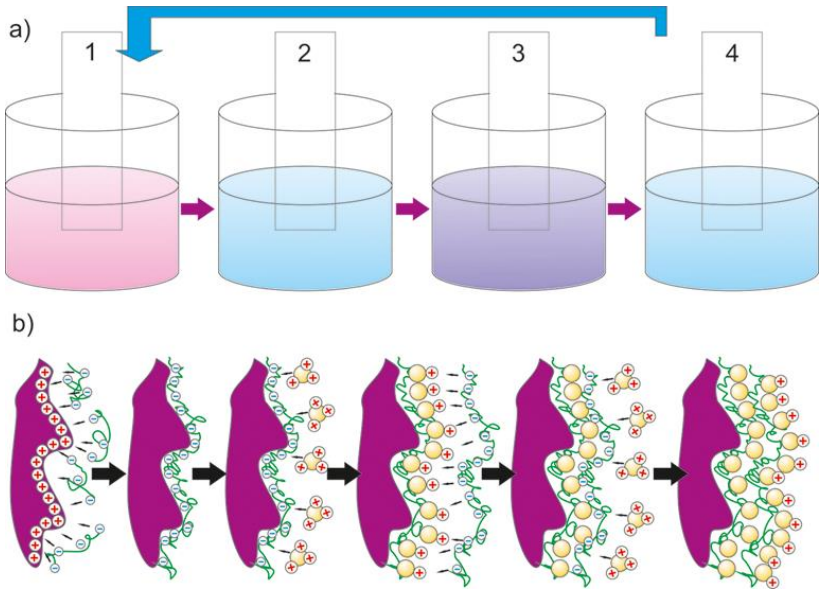

Figure 18: Schematic illustration of the LbL assembling process (a) and formation of shell structure on curved planes (b)

By repetition of these cycles, a desired number of layers can be achieved on curves planes, as shown in figure 18b, therefore the whole thickness of multilayer is easy controlled. The LbL method expands the possibilities of obtaining organic/inorganic films with high accuracy just by changing the number of multilayers, concentration and pH of the solution. QCM-D crystals have been applied to verify the biofunctionalization of nanoengineered surfaces through layer-by-layer technologies. Chen et al. [44], used an aptamer/protein/Apt-GNPs sandwich QCM-D biosensor for sensitive detection of thrombin. Firstly nucleic acid (TBA15) was immobilized on a crystal chip to form an aptamer monolayer for capturing thrombin. At last, TBA29-

functionalized gold nanoparticles (TBA29/GNPs) were introduced to form a TBA15/thrombin/TBA29-GNPs sandwich structure (figure 19).

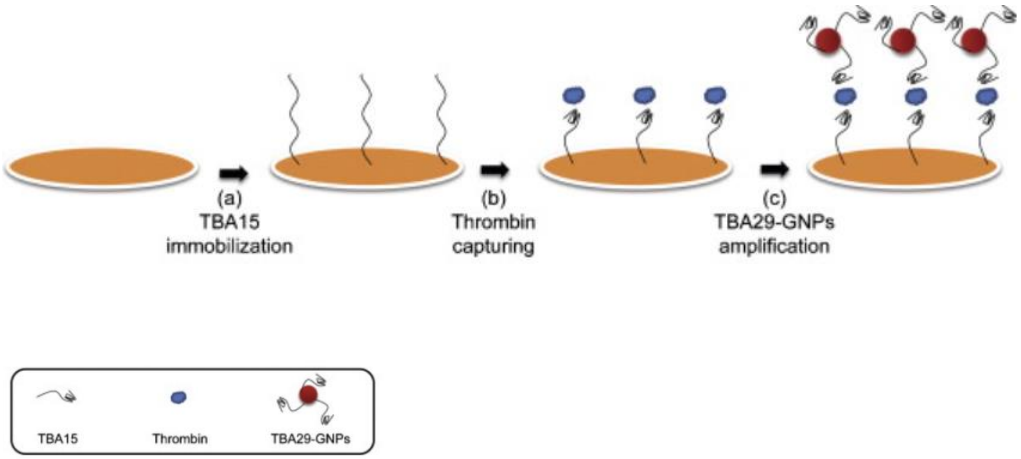

Figure 19: The schematic representation of the sandwich QCM-D biosensor for detection of thrombin

Wang et al. [66], prepared biomimetic multilayers based on layer-by-layer (LbL) assembly as functional films with compact structure. In situ layer-by-layer growth and cross-linking were monitored by the QCM-D to reveal the kinetics of the process and the influence of DOPA chemistry. The alternate polyethylenimine (PEI), Dopamine-modified poly(acrylic acid) (PAA/DOPA), Zinc (Zn)(or PEI, PAA, Zn as parallel sample) adsorption−rinse cycle was repeated to build multilayers on the crystal. In the experiments, for every layer construction PEI acted as player adsorbed onto the surface of gold crystal, providing a positive charged layer. Two anionic polymers PAA and PAA/DOPA were used, and different overtones were examined to understand the effect of DOPA during the deposition in the presence of cross-linker zinc. In the case of

the non-Dopa system (figure 20a), a typical deposition feature is notice, namely that mass uptake systematically increases after sequential flows of PEI, PAA and Zn^{2+}. After rinsing with the background buffer solution, the adsorbed mass of PEI, PAA, and Zn decreased, accompanied by the decreased dissipation with structure changes from soft, hydrated into a more condensed type (figure 20a').

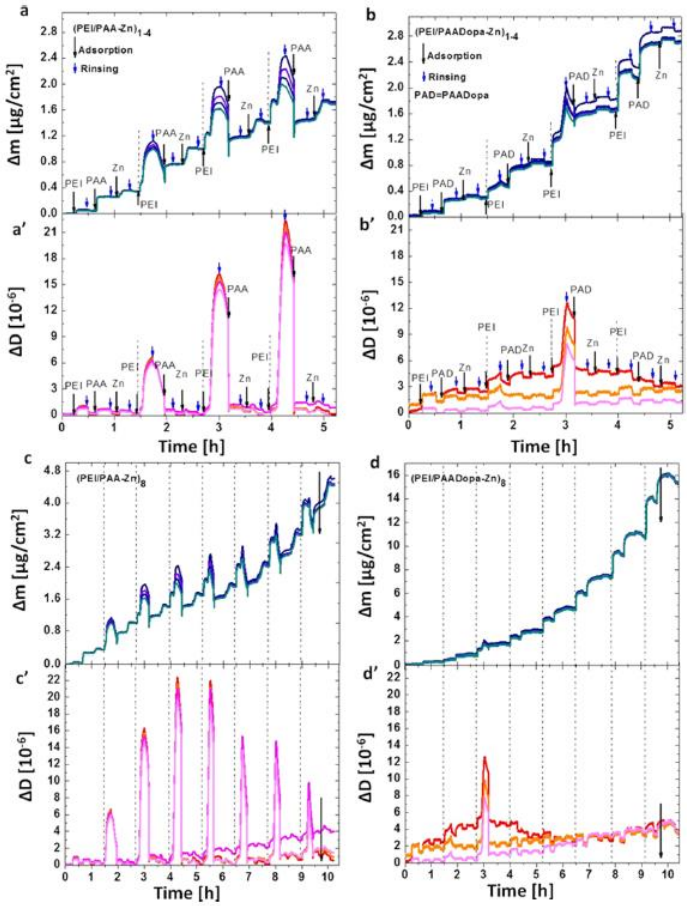

Figure 20: Shifts of mass and dissipation as a function of time for multilayers assembly of (a,a')(PEI/PAA-Zn), (b,b')(PEI/PAADopa-Zn), (c,c')$(PEI/PAA-Zn)_8$ and (d,d') $(PEI/PAADopa-Zn)_8$

The sudden increase of the mass is likely related to the layer swelling, which might occur during polyelectrolyte adsorption due to the formation of a soft and highly hydrated layer as shown from the spikes of dissipation (Figure 22a′) indicating a highly swollen structure. Upon rinsing, the weakly bound PEI can be washed off, and the following addition of PAA accelerated the dehydration process to form a relatively condensed structure, as reflected by the sudden drop of mass and dissipation. In contrast to the non-DOPA multilayers, modification of PAA backbone with DOPA groups generally eliminates the loosely adsorbed layers as most of the dissipation "spikes" disappeared during the adsorption (Figures 22b′ and 22d′). This is presumably because that higher adsorption of PAA/DOPA offers more hydroxyl groups as interactive sites to form coordination with zinc ions to form a more compact and stable network-like structure. This strong interaction between adjacent layers in addition to electrostatic complexation of polymers reduces the layer swelling.

2.3.2 Self-assembled-monolayers (SAMs)

Self-assembled monolayers (SAMs) are ordered molecular assemblies that are formed spontaneously by the adsorption of a surfactant with a specificity affinity of its headgroup to a substrate [67]. They are formed as a result of spontaneous self-organization of functionalized, long-chain organic molecules onto the surface of appropriate substrates into stable, well defined structure. As shown in

figure 21, the constituents of a SAM-molecule are : headgroup, chain or backbone, and end-group.

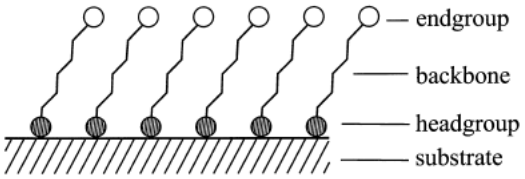

Figure 21: Schematic of SAM molecule

For a SAM to control friction, wear, and hydrophobicity, it should be strongly adherent to the substrate, and the terminal group (tail group or the group at the free end) of the organic molecular chain should be non polar. For strong attachment of the organic molecules to the substrate, the other end of the molecular chain (head group) should contain a polar end group. The substrate surface should have a high surface energy, so that there will be a strong tendency for molecules to adsorb to the surface. The surface should be highly functional, with polar groups and dangling bonds (generally unpaired electrons), so that they can react with organic molecules and provide a strong bond. Because of the exothermic head group-substrate interactions, molecules try to occupy every available binding site on the surface, and during this process, they generally push together molecules that have already adsorbed. The process results in the formation of crystalline molecular assemblies. The interactions between molecular chains have Van der Walls or electrostatic nature, with energies on the order of a few (< 10) kcal/mol, and exothermic [68]. SAMs are usually

produced by immersing a substrate in the solution containing precursor (ligand) that is reactive to the substrate surface, or by exposing the substrate to the vapor of the reactive chemical species. The backbone of a SAM is usually an alkyl chain ($C_nH_{2n\pm1}$) or a derivatized alkyl group. By attaching different terminal groups at the surface (or surface group), the film surface can be made to attract or repel water. The commonly used terminal group of a hydrophobic film with low energy surface, in the case of a single alkyl chain, is the non polar methyl group (CH_3). For hydrophilic film, the commonly used terminal group are alcohol (OH) or carboxyl acid groups (COOH). Surface active head groups most commonly used are thiol (SH), silane ($SiCl_3$), and carboxyl (COOH) groups. The substrates most commonly used are gold, silver, platinum, copper, hydrogen-terminated single crystal silicon (H-Si) and hydroxylate surfaces of SiO_2 on Si, Al_2O_3 on Al and glass [71].

Minsky et al [69], monitored the specific interactions of immobilized small hyaluronan oligosaccharides (sHA) by a quartz crystal microbalance with dissipation monitoring (QCM-D). The high-density presentation of sHA was achieved by modifying the reducing end of sHA with a short thiol linker (sHA-

eSH) and its subsequent immobilization on gold surfaces (figure22).

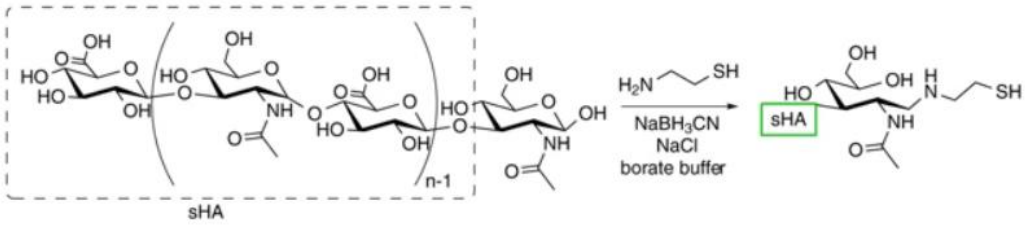

Figure 22: Thiol-end modification scheme for sHA (sHA-eSH)

Poly(l-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG), a widely used copolymer in biomolecular interface to create an inert background and control protein adsorption, was grafted on silica QCM-D sensor in flow mode.

2.4 Effects of surface coating on the cell adhesion process

Biomaterial surface properties firstly influence cell adhesion mechanism. The ability to closely mimic *in vivo* environment can potentially improve the biocompatibility of the biomaterial surface. In recent years, QCM has been used as a tool for the evaluation of real-time cell-surface interactions, because of its ability to monitor the cell adhesion process in a non- invasive manner. Tymchenko et al. [21] schematized the interaction between cells and QCM sensor surface through a *two-layer-model (*figure 23*)*.

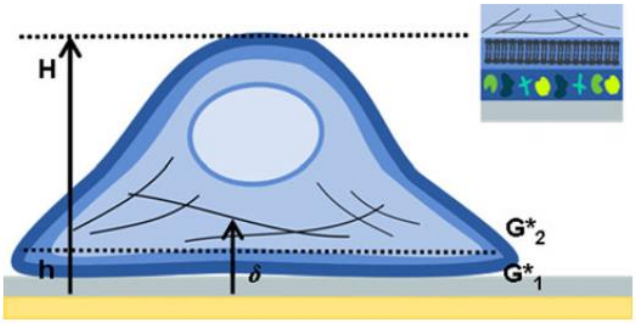

Figure 23:Schematic illustration of the two-layer-viscoelastic model suggested as a representation of QCM-responses

The interfacial layer between the cell and the sensor surface (G_1^*) represents the structure responsible for the cell adhesion to sensor surface, including the sensor coating, and material inside the cell associated with the cell membrane. The top layer (G_2^*) approximates the bulk properties of the cell. H identifies maximal cell thickness estimated at $\sim 10\ \mu m$,h the thickness of the interfacial layer and δ the penetration depth (sub-micro-meter)(equation 6).

2.4.1 Fibronectin adsorbed gold

Fibronectin is a very aboundant extracellular matrix (ECM) and plasma protein with a molecular weight of about 450 KDa. It has a dimer structure, being made up of two polypeptide chains. These chains are bonded by two di-sulphur bridges in the regions of C-terminals. Each of the chains is made up of about 2500 amino acids (figure 24)

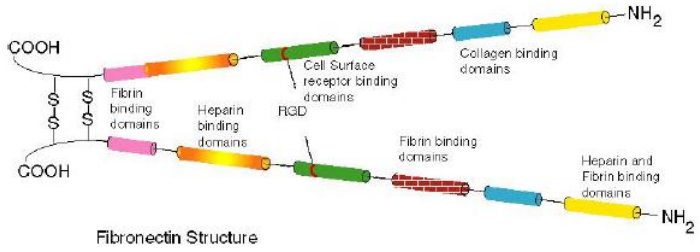

Figure 24 : Dimer structure of fibronectin

Fibronectin is an adhesion macromolecule that affects cell migration and organization. As a matter of fact, it contains the sequence arginine-glycine-aspartic acid (RGD), which is a receptor for the integrins. When the integrins bind to the RGD sequence on the fibronectin, they allowed cell-ECM adhesion. Kandel et al. [70], reported a new method of chemical immobilization to graft fibronectin onto various surfaces and demonstrate that QCM-D can detect viscoelastic changes in fibroblasts subjected to cytochalasin D, an actin depolymerizing agent, when plated on fibronectin coated crystal. A comparison of surfaces coated with various adhesive ECM proteins (e.g., fibronectin, vitronectin, laminin and gelatine) confirmed the role of these molecules in mediating cell-adhesion to the quartz surface via specific interactions with integrin molecules at the cell membrane [46] [9]

2.4.2 Hydrophobic and hydrophilic polymers coated gold

In general, a positive frequency shift was shown to occur for interactions involving hydrophobic surfaces, i.e., when either cells or sensor surface were

hydrophobic [45]. Under these conditions, a relatively firm elastic cell-surface connection is suggested to govern and, intuitively, it could be presumed that higher hydrophobic interactions may induced more water molecules to leave the surface, being replaced with the attached cell wall component. In the case when both surface and cells were hydrophilic it may be assumed that the connection was even weaker, i.e., negligibly elastic and was manly through viscous shear forces. Presumably, without an elastic bound to the sensor, the cells. Thus, compared with to other cases, the viscous shear and dissipation increased due to a thin liquid gap remaining between the cell and sensor surface. Lower and similar contribution to the increase in the sensor frequency is shown for the combinations of hydrophobic surface and hydrophilic cells as well as hydrophilic surface and hydrophobic cells. For the case of hydrophobic cells on hydrophilic surface elastic load dominates, as observed by the elevation of ratio $\Delta F/\Delta D$ as a function of n, the resonance frequency overtone number. In contrast, for the case of hydrophilic cells on hydrophobic surface, the connection between the sensor and the cells becomes more viscous as n increases. Finally, for the combination of hydrophilic cells and hydrophilic surface, $\Delta F /\Delta D$ is the lowest and the viscous connection dominates [45]

2.4.3 Influence of surface charge on cell adhesion

Poly-D-Lysine does not support integrin binding and focal adhesion formation, so the cells attached via non-specific electrostatic bonds between the positively

charged immobilized PDL and the negatively charged cell membrane heparan sulphate proteoglycans. Heparinase treated cells, which were less negatively charged, displayed lower adhesion to the PDL-coated surface than untreated cells as indicated by lower frequency and resistance shift [71]. Other studies confirmed that synthetically formed surfaces, such us poly-electrolyte films, where shown to promote the attachments and spreading of cells. The suitably of electropolymerized porphyrin and peptide modified poly-siloxane was assessed. Satriano et al [60], prepared artificial surfaces presenting a fibronectin-derived peptide adsorbed to poly-siloxane. The attachment and spreading of fibroblast cells were monitored, revealing different kinetics for the cell/substrate interaction. The QCM sensor was also used to monitor the formation of supported lipid bilayers on the sensor surface. This lipid layer was further tested towards its ability to promote or inhibit cell attachment, little or no attachment of cells on plain phosphatidyl-choline bilayers was observed, suggesting potential applications of promoting the biocompatibility of materials [72].

2.5 Cell culture on the sensor surface

Using QCM-D to design a cell detection platform is an important application of this device. It can be used to monitor the cell behaviour when cells are in contact with bioengineered surfaces and biomaterials, in fact it is able to identify time-dependent changes in cells attachment and morphological changes [18], to monitor the formation of mature focal adhesions (ΔD rapidly decrease

when cell spreading occurs) [49], and real-time detection of the earliest morphological changes that occur at the cell-substrate interface during programmed cell death [56].

Cells can be isolated from tissues for ex-vivo culture in several ways. Mononuclear cells can be released from soft tissues by enzymatic digestion with enzymes such us collagenase or trypsin, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that growth out are available for culture. For example, NIH3T3 fibroblasts (ECACC), were cultured in DMEM supplemented with 10 % calf serum (CS), 1 mM sodium pyruvate, 2 mM L-glutamine, and 1 % penicillin streptomycin in a humidified incubator (5 % CO_2 at 37° C), next the cells were removed by trypsinization and centrifugated for 3-4 minutes at 1000 rpm and resuspended in serum free DMEM to eliminate interactions caused by serum before deposition on the quartz crystal surface.

Next the cell were seeded in situ, and their attachment and spreading were followed in real-time by using a window-equipped QCM-D module mounted in a light microscope to study cell adhesion on a Collagen I functionalized sensor .

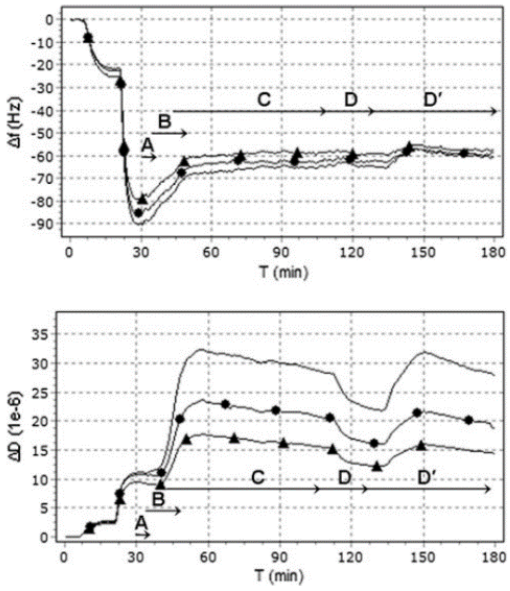

Figure 25 QCM-D frequency and dissipation versus time curves for NIH3T3 fibroblasts

The gold sensor surface coated with extracellular matrix protein (collagen I) was subjected to : cell seeding (at t= 30 min in figure 25), cell attachment and spreading (B, and C in figure 25). Reyes et al. [73], used bovine aortic epithelial cells (BAEC) to study cell adhesion on the gold sensor surface. The piezoelectric AT-cut quartz was covered with Zinc Oxide (Zn/O) nanostructure layer with a thickness of 500 nm(figure 26b) and exposed to UV light to make it super-hydrophilic.

The Zn/O nanostructure-QCM was then sterilized and deployed inside a Teflon cell-growth well to serve as the test device, while a sterilized standard QCM was

inserted in a similar Teflon cell-growth well to serve as the reference device. The two devices were filled with 50 µL of growth medium that was seeded with BAEC cells to have a $2.\ x\ 10^5$ cell/mL concentration, and then placed in a standard CO_2 incubator for an average of 50 h (figure 27 c).

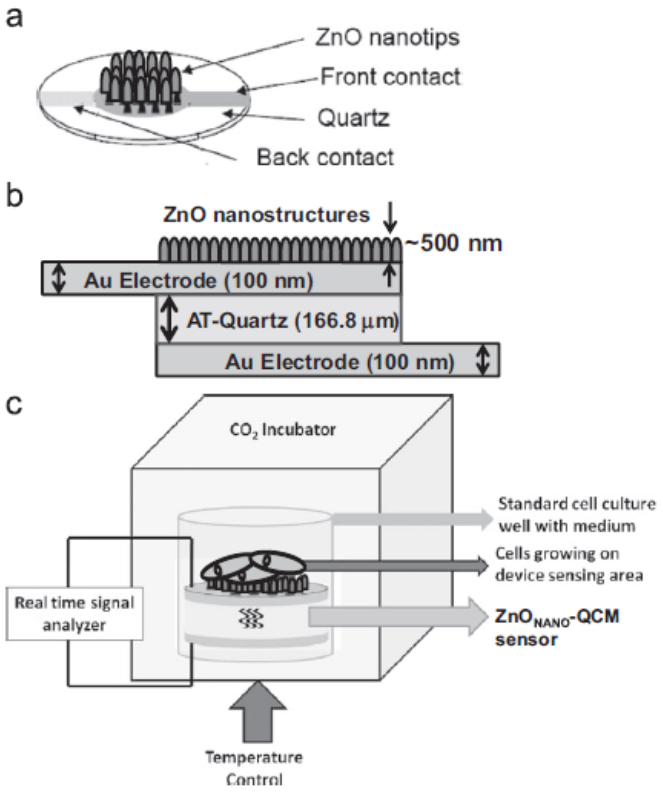

Figure 26 The Zn/O nanostructure-QCM

The Zn/O nanostructure-QCM was then sterilized and deployed inside a Teflon cell-growth well to serve as the test device, while a sterilized standard QCM was inserted in a similar Teflon cell-growth well to serve as the reference device. The two devices were filled with 50 μL of growth medium that was seeded with BAEC cells to have a $2.\ x\ 10^5$ cell/mL concentration, and then placed in a standard CO_2 incubator for an average of 50 h (figure 26 c).

The Zn/O nanostructure-QCM device was used to monitor adhesion and proliferation of the cells. The time-evolving resonance frequency shift δf(t)=f0−f(t) of both devices were monitored, where f0 is the resonant frequency of the device right after it has been injected with the cell-seeded medium and f(t) is the subsequent resonant frequency of the device after a time t (figure 28). The most evident feature of this plot was the enhanced sensitivity of Zn/O nanostructure-QCM over the standard QCM where the maximum frequency shift at confluence of Zn/O nanostructure-QCM was 10 times larger than the standard QCM. This can be attributed to the effective surface area made available for cell attachment to the ZnO nanostructures on the sensing area of the device.

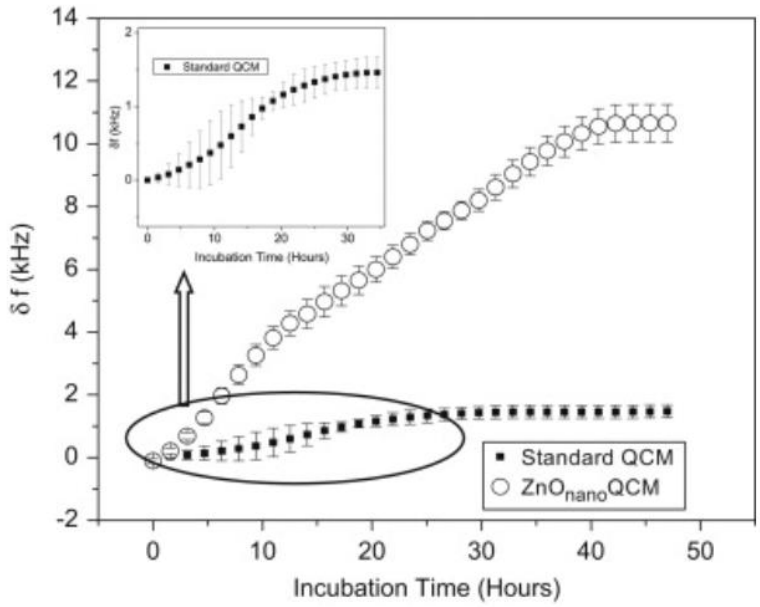

Figure 6 The time evolving frequency shift for the standard QCM (solid squares) and the ZnOnano-QCM (open cricles)

Nowacki et al. [56] seeded human breast adenocarcinoma cell-line MCF-7 on the QCM crystal surface as follow: the crystals were equilibrated in sterile Phosphate buffered saline (PBS)prior to deposition of 250 μL of complete medium on the gold surface. After 2 hours of incubation, the medium was removed and 30,000 cells in complete CO_2/independent medium (complete medium with 0.02 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)) were added on the gold surface of the sensor. Cells were allowed to attach for 24 h at 37ºC and under 5% CO_2 atmosphere. The MCF-7 cell layer was equilibrated 2 h in the QCM-D chamber under a flow rate of 12 μL/min of CO_2-independent medium at 37ºC .The equilibration medium was completed with Dimethyl sulfoxide (DMSO) at the same concentration and the silver

thiosulphate solution (STS) to avoid any artefact due to the DMSO. STS was injected at flow rate of 12 µL/min for 4 h . At the end of the experiment the sensor was removed and residual cells were trypsinized and counted, as well as the cell recovered in the collector to the chamber outlet. Yan et al. [74], designed a cell-based QCM as a drug screening tool. A Trypsin-EDTA solution was used widely for dissociation of tissues and cell monolayer. The silver QCM surface was first covered with a plasma polymerized film and photoinduced polymerization to render it resistant to solution and hydrophilic. The QCM surface was then cleaned by rinsing with distilled water and air drying. Next, the chip was sterilized by spraying it with ethanol and exposing it to UV light for 30 min.

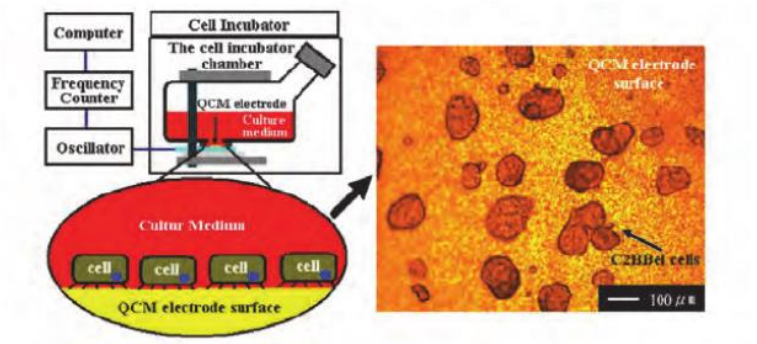

Figure 27 Schematic diagram of cell detection system hardware setup used for the QCM, and the condition of C2BBel cells growth on the QCM surface

After combining the cell incubator chamber and the QCM sensor (figure 27), 500 µl medium was added to the incubator chamber to complete the connection of chip circuit line and oscillator circuit. When turning on the system, testing

cells 1×10^5 were added until the system became stable. After achieving a stable signal, the cell incubator chamber was placed in a cell incubator to process the culture. Fig. 10 shows the results of applying a silver electrode QCM to cell detection. For untreated silver electrode QCMs, the frequency decreases quickly (Fig. 28 A), and becomes worse after seeding the cells (Fig. 28 B). The process of falling off decreases the chip's shaking ability, which causes the frequency to drop. Adding cells simply caused the cells to grow on the chip surface (Fig. 28 B). The frequency declines slowly at first, and then increases as the number of cells increases. After cell growth reaches a steady state, Δf also remains steady. As the medium nutrients run out, the quantity of dead cells increases, which cause the frequency to rise slowly.

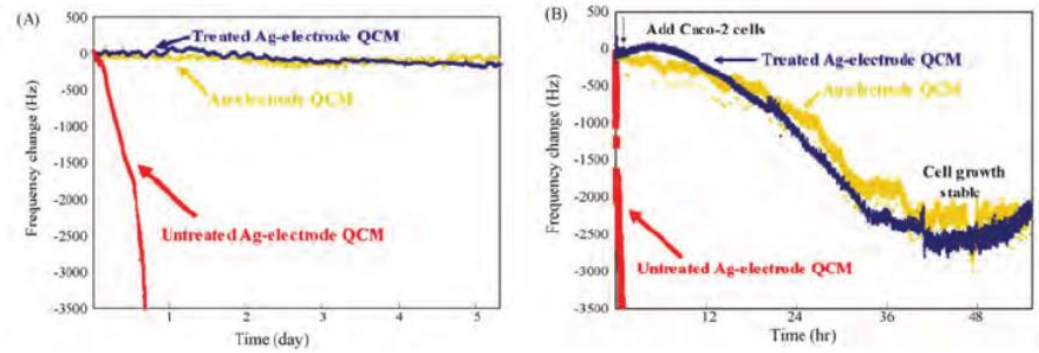

Figure 28 (A)Frequency change with time of QCM in response to the gold electrode, the silver electrode and the treated-silver electrode QCM. (B)Added cells on the gold electrode, silver electrode and the treated silver electrode QCM surface, respectively.

3. Experimental section

3.1 Experimental Background

In a previous work, *in vitro* models of human cardiac fibrotic tissue based on 2D and 3D bioartificial scaffolds were produced. Bidimensional scaffold were fabricated by electrospinning technique because of its ability in mimicking closely the hierarchical properties of the extracellular matrix (ECM) of the native tissue. G was grafted on the scaffold surface by a two-step mussel-inspired strategy based on : (i) 3,4-Dihydroxy-D,L-phenylalanine (DOPA) polymerisation (PCL-polyDOPA) and (ii) G graft (PCL-polyDOPA/Gel). In this experimental section, QCM-D was used to evaluate the effectiveness of scaffolds functionalization protocol (polyDOPA/Gelatin) by reproducing each functionalization step on the gold sensor surface.

3.2 Materials for QCM-D experiment

In general, degradable polymers are widely applied in biomedical engineering field especially for temporary device. The main advantage of synthetic degradable polymers is the opportunity to tune their mechanical properties and degradation rate. Besides, their chemical composition is known and well-defined. On the other hand, a drawback for the application of these polymers in tissue engineering is their low biomimecity for cell recognizing and adhesion [75]. In this work, a biodegradable polyester was considered: Poly-ε-

caprolactone (PCL). PCL thin nanofiber coating was collected on the QCM-D golden sensor surface by Electrospinning. Poly(3,4-Dihydroxy-DL-phenylanine) (polyDOPA) and type A Gelatin (G) depositions were monitored on the PCL coated gold sensor during the QCM-D experiment.

3.2.1 Poly-ε-caprolactone (PCL)

Poly-ε-caprolactone (PCL) is a hydrophobic semi-crystalline aliphatic polyester. It has a good biocompatibility, is resorbable and it has high thermal stability. So, it has been widely applied in the biomedical field. For instance, PCL is an important constituent of scaffolds for tissue engineering, it is used in drug release systems and to repair bone and cartilage. Because its degradation rate is slow, it is suitable to be used for long-term implants. However, copolymers of the monomer ε-caprolactone (figure 29) with other monomers are preferably employed when faster degradation rate is needed [79]. A summary of the main thermal and mechanical properties of PCL is presented in Table 1 [76] [77].

Property	Value
Melting temperature T_m	58-65 °C
Glass transition temperature T_g	-(65-60) °C
Degradation rate	about 24 months
Young's modulus	0.2-0.4 GPa
Tensile strength	20-42 MPa
Elongation at break	700-1000 %

Table 1: Summary of the main thermal and mechanical properties of PCL

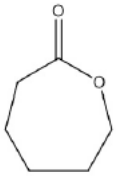

Figure 29 : Chemical structure of the monomer ε-caprolactone

3.2.2 3,4-Dihydroxy-L-phenylanine (DOPA)

3,4-Dihydroxy-L-phenylanine (DOPA) is a catechol amine secreted by mussels that can act as an adhesive under wet conditions. Catechol amines are responsible for mussel adhesion to inorganic surfaces, through the formation of hydrogen bonding or metal complexes and to organic surfaces, through covalent bond formation mediated by chinone groups. The chemical structure of DOPA is presented in figure 30 :

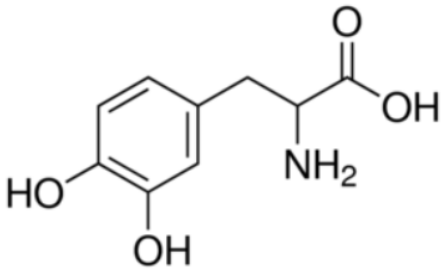

Figure 30 Chemical structure of 3,4-Dihydroxy-DL-phenylanine (DOPA)

Polydopamine (PDA), containing both catechol and amine groups, is a surface coating used to functionalize a wide array of material surface. The PDA is spontaneously formed by pH-induced, oxidative polymerization of dopamine-hydrochloride in alkaline solutions (pH>7.5) (figure 31) . To achieve PDA coatings, simple immersion of substrates in a dilute aqueous solution of dopamine (typically 2 mg/ml of dopamine in 10 mM TRIS buffer) results in spontaneous deposition of a thin PDA film [82].

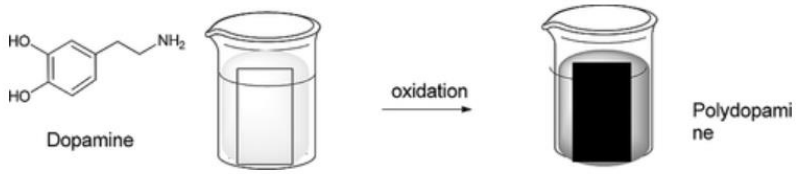

Figure 31 Oxidation of dopamine in polydopamine (PDA)

In solution, PDA build-up shares the first steps with melanin biosynthesis: the oxidation of dopamine to dopamine-quinone, its intramolecular cyclization,

oxidation to dopamine-chrome, formation of 5,6-dihidroxyindole(DHI), and further oxidation to 5,6 indolequinone (IDQ) (figure 32).

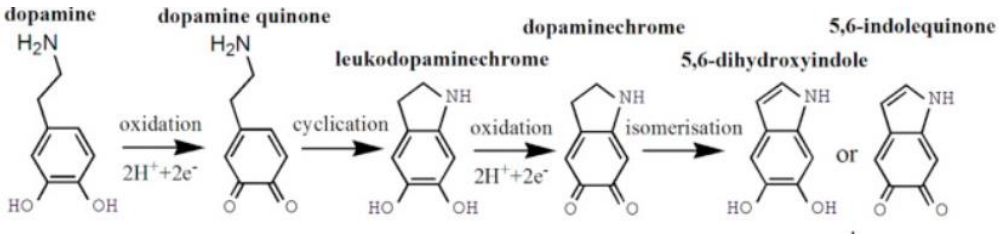

Figure 32 Scheme of DOPA reaction

PDA coating can be easily deposited on all types of inorganic and organic substrate and show controllable thickness and durable stability [79]. In addition, PDA possesses excellent biocompatibility and unique chemical structure containing many functional groups including catechol, amine, and imine, which can be used to covalently immobilize molecules and adsorb metal ions. Owing to these outstanding properties, PDA has been extensively used for modulating cellular and tissue responses to materials [80].

3.2.3 Type A Gelatin (G)

Gelatin is a biopolymer obtained by partial denaturation of the fibrous protein collagen at acid or alkaline pH. Gelatine derived from acid-treated processes are known as type A. The gelatin is normally extracted at pH above 4 as most of the proteins present in the raw material have isoelectric points in the range pH 4-5 and are thus least soluble during extraction. The final gelatin product is a mixture

of polypeptide chains with different molecular weights where the molecular weight distribution depends on the intra and inter-molecular covalent cross-linkages present in the starting raw materials, the proportion of unhydrolyzed cross-linkages left in the resulting gelatin as well as the resulting length of the polypeptide chains [81]. It is primarily used as a gelling agent in food, pharmaceutical, and cosmetic industries and it forms gel on cooling below 35 ºC. The gels formed by gelatin are transparent, elastic, and thermo-reversible in nature. Gelatin is highly hydrophilic and has good barrier properties (gas and liquid) in rubber nanocomposites. Its chemical structure is presented in figure 33:

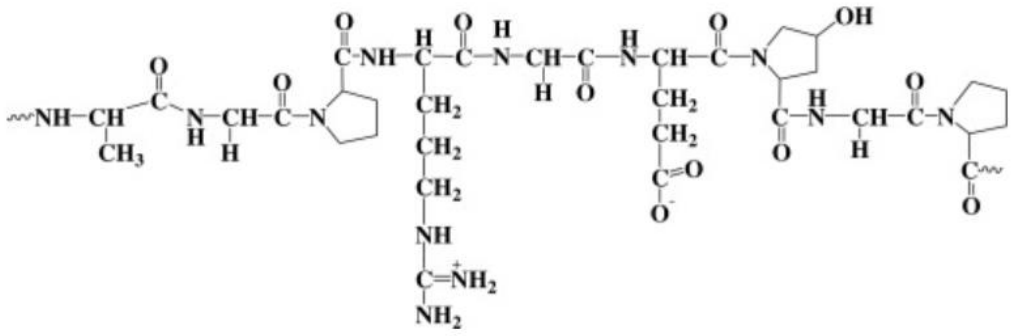

Figure 33 Chemical structure of Gelatin

A disadvantage for gelatin is that it degrades as a colloidal solution at or above 37°C, and gels at room temperature and lower. Nonetheless, these limitations can be overcome when used in conjunction with biodegradable synthetic polymers. In contrast to collagen, gelatin does not elicit any noticeable

antigenicity after implantation. For this reason, in addition to its similar physicochemical and biological properties to collagen, gelatin has been used as wound healing biomaterial in different forms, including electrospun nanofibers [82]. Electrospun gelatin/PCL and gelatin/PCL/collagen/elastin hybrid TEVGs were fabricated, and when compared to a non-gelatin TEVG in vitro, revealed better cellular attachment, migration, proliferation, and penetration processes, in addition to displaying excellent biocompatibility and mechanical properties [83].

3.3 Methods

3.3.1 Electrospinning of poly-ε-caprolactone (PCL) on the sensor surface

A 20% wt/v solution of (PCL) in a Chloroform/Formic Acid mixture (70/30 v/v) was prepared. Chloroform 99.8% and Formic Acid 98% were purchased by Sigma-Aldrich. Chloroform was chosen as primary solvent both because it is a good solvent for PCL and for its high volatility. Firstly, PCL pellets were dissolved in a proper volume of Chloroform. The prepared solution was stirred for about 3 hours, avoiding solvent evaporation. The addition of Formic Acid (reagent ACS > 96%) was required to enhance the polymeric solution conductivity. In fact, it increases the spinnability of the solution thanks to its polarity and high dielectric constant value. Consequently, this strong polar acid allows the deposition of fibres with a reduced diameter. After Formic Acid

addition, the 20% wt/v solution was stirred for about 40 minutes at 200 rpm to favour the complete Formic Acid dispersion.

The Electrospinning setup (figure 34)used for random fibers collection on QCM-D gold sensor includes:

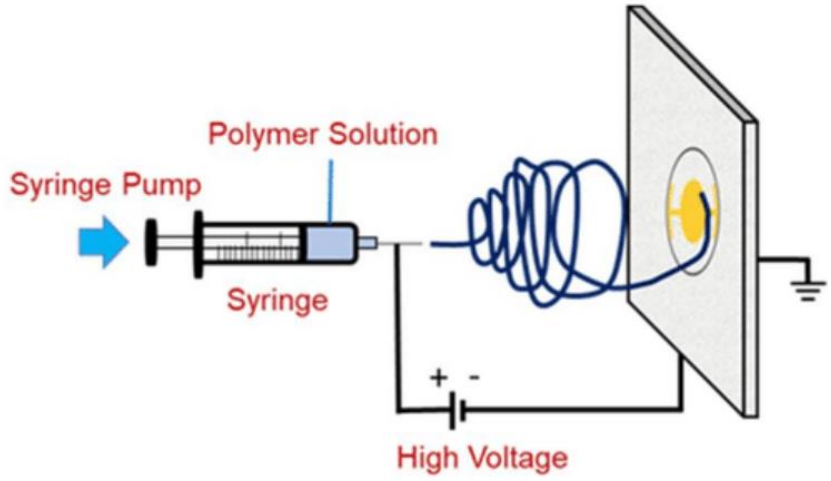

Figure 34 Electrospinning setup

1. A conductive flat collector covered by aluminium foil
2. A 5 mL glass syringe containing the polymer solution to be extruded through a 21 G needle;
3. A pump that applies a continues pressure to enhance solution extrusion;

4. A high voltage generator that contains the interface to set the process parameters for the voltage generation

QCM-D gold sensor was removed from its electrodes and was fixed to the grounded aluminium foil by scotch tape. The fibres were directly deposited on the crystal surface of using electrospinning technique. The polymeric solution of PCL was loaded into the glass syringe and connected to the high voltage generator. For the collection of random fibers on the sensor surface, the flat collector was grounded connected through a clamp, while the syringe needle was connected to the positive output of generator. The electrospinning process was performed at 12 cm tip-to-collector distance with a flow rate of 0.25 ml/h. A voltage of 15 kV was applied to the needler of the syringe.

3.3.2 Preparation of Gelatin and poly-DOPA solutions

Both Gelatin and poly-DOPA solution were obtained using Tris-HCl buffer solution as solvent. This alkalin buffer (pH=8.5) was mainly used to induce DOPA polymerization. To produce the alkaline buffer solution 10 mM, tris(hydroxymethyl)aminomethane (Tris) (ACS reagent, >=99.8%) was dissolved in double distilled water. Subsequently by the addition of HCl (1M), a pH=8.5 was reached (Tris/HCl solution). Gelatin (Type A bovin, Sigma-Aldrich, 2mg/mL) was dissolved in Tris/HCl solution at pH 8.5, at 40 ºC , under stirring for about 1 hour and half. For PDA coating, DOPA (Sigma-Aldrich, 2

mg/mL) was dissolved and polymerized in Tris/HCl solution at pH 8.5 with vigorous stirring (300 $r \cdot min^{-1}$) once it began to take on brownish colour.

3.3.3 (poly-DOPA/ Gelatin) deposition on the PCL-coated QCM sensor

QCM-D (QSense Biolin Scientific , Finland) was used to evaluate the effectiveness of gelatin grafting on polyDOPA precoating The compact chamber together with specialty modules enable (figure 35) simultaneous QCM measurements with microscopy, electrochemistry and ellipsometry .

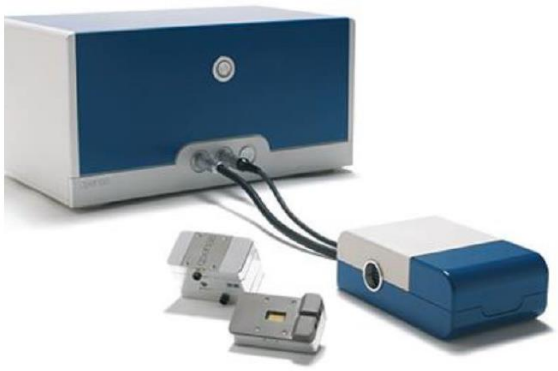

Figure 35 Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

QSense instrument track changes in frequency and dissipation when molecules bind and interact on the oscillating QCM-D sensor. Data collection at high sample rate from 7 harmonics of the fundamental frequency gives maximum input for data analysis.

Figure 36 Static module

The gold chip coated by PCL was placed onto the QCM sensor system , QCM-D sensor system was equipped with a static open module (figure 36) ,to pipette of solutions directly onto the chip surface, and temperature was set at 22 º C. After the baseline became stable, DOPA solution (300 μL) was placed on the sensor surface using a micropipette and left for 7 h, then DOPA solution was removed and tris/HCl solution was placed three times for 5 minutes. Finally, Gelatin solution (300 μL) was introduced and left for 16 h. Three tris/HCl washing steps were then performed, followed by a bi-distillated water final wash to remove the excess of salt. This experiment also demonstrated how washes can remove deposited material over the crystal.

3.4 QCM-D results and discussion

Weight deposition was recorded by a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). It was monitored real time two steps deposition of polyDOPA/Gelatin on the crystal coated by PCL. In this work, QCM-D measurements aims to evaluate qualitatively the efficiency of

polyDOPA coating in grafting Gelatin. In the previous work the same coating was deposited above a crystal without PCL electrospun nanofibers. This plot was obtained (figure 37):

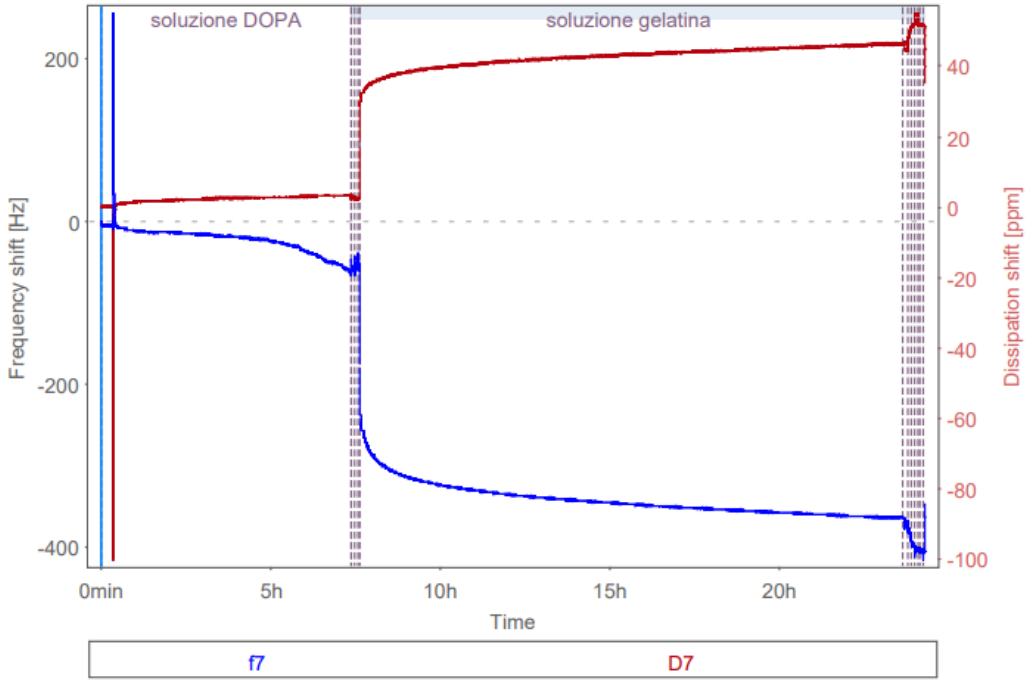

Figure 37 QCM-D deposition of PolyDOPA/Gelatin above the crystal without PCL

Time 0 corresponded to the introduction of DOPA solution. Time 7 corresponded to the removing of polyDOPA solution, subsequent 3 washes and introduction of Gelatin solution. Time 24 corresponded to the final washes. Frequency decreased during all the acquisition. These results demonstrated the effective mass deposition above the crystal. After the introduction of Gelatin

solution dissipation increased because Gelatin is a soft substrate with higther dissipation properties.

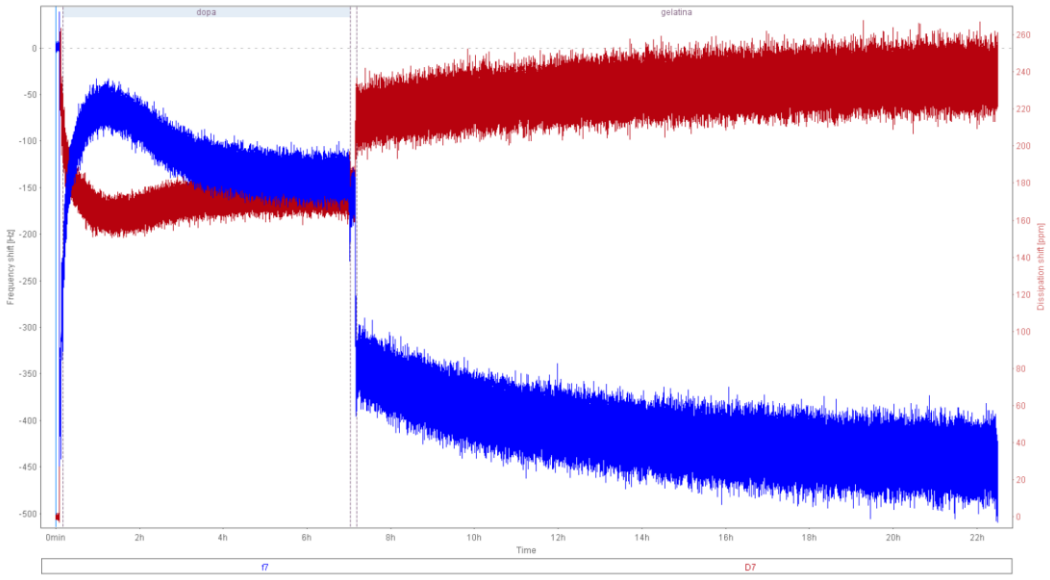

Figure 38 QCM-D of polyDOPA/Gel deposition above the crystal coated with PCL

Figure 38 shows frequency and dissipation curves for polyDOPA and Gelatin depositions on the sensor surface with PCL film ,obtained in this work. By comparison of this curves with the ones obtained from the polyDOPA/Gelatin depositions above the crystal without PCL, the two steps deposition of polyDOPA/Gelatin can be distinguished. In this case, both in frequency and dissipation curves a noisy signal was recording. For this reason a post-processing should be done to remove the noise from the signal. Alternatively a thinner layer of electrospun PCL nanofibers should be deposited on the sensor surface to obtain less noisy signals.. At time 0 DOPA solution was introduced and

frequency started to decrease during all the acquisition . This result demonstrated the effective mass deposition above the crystal . In particular, during the first 7 hours, the frequency decreasing demonstrates the polyDOPA deposition above the crystal. After 7-hours, polyDOPA solution was removed, sensor was washed, as reported in functionalization protocol adopted in the previous work , and Gelatin solution was added by a micropipette. Subsequently, frequency steeply decreases, and dissipation likewise increases in correspondence of Gelatin solution deposition. Dissipation change according to the substrate properties. Hence, during DOPA solution deposition, as polyDOPA is a rigid substrate, dissipation increase is neglible. On the other hand, during Gelatin grafting process, dissipation factor shifts is not negligible, because Gelatin is a soft material with viscoelastic properties. In both experiments looking at frequency variations, it was demonstrated how washes do not remove significantly deposited material. In particularly, in polyDOPA deposition, after washing there is a moderate frequency increase, while in Gelatin deposition there is a low increase of frequency due to the water swelling of Gelatin.

3.5 Conclusions and future developments

The work described in this dissertation demonstrated that the QCM-D is a highly sensitive, non-invasive, and label-free technique in studying cell adhesion. The ΔD-response of the QCM-D is an integrated functional output of the cell that is regulated by the network of cell signal and this makes it a novel approach for

dealing with the complexity of the cell. Another advantage of using this technique, as an approach to study cellular responses, is the QCM-D can offer lots of information with one experiment that is because it is a continuous monitor of cellular response in real-time. This capability can potentially save money and on reagents, such as ligands and modulators. In order to confirm the reliability of the QCM-D as a sensing platform for the adhesion of cells on implant materials, a future experiment should be done. This experiment should be a method to find ways to modulate the adhesiveness of cells to the substrate coated with PCL and functionalized with poly-DOPA and Gelatine. The QCM-D experiment should be conducted utilizing the open module setup instead of the typical flow module setup. The typical flow module setup procedure for cell adhesion experiments begins with rinsing of the sensor with fetal bovine serum (FBS)-containing cell medium to saturate the surface with FBS and other proteins. Then cells can be introduced slowly over the sensor for a period of time until they settle down onto the sensor surface. This can be followed by a constant flow of complete cell medium throughout the rest of the experiment. The open module setup procedure also begins incubating the sensor with complete cell medium. Next, the cell medium can be removed and cells can be seeded on the top surface of the sensor. The cells will then allowed to settle and attach for the duration of the experiment and the entire adhesion process will be monitored with the use of the QCM-D. The attachment and spreading of cells can be monitored for periods of 10-20 h to determine long term adhesion behaviour.

The ΔD response of the QCM-D should exhibit a three- phase response profile as presented in Chapter 1. To verify the three phases of the cell adhesion process, live cell images can be taken at various time point. A future study of other surface coating (metal, polymer and ceramic substrates) should be tested in order to verify the dependability of the QCM-D as a sensing platform for the adhesion of cells on implant materials.

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