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Effect of adsorption of charged molecules on the structure of Alginate gel

- A QCM-D study

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THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Effect of adsorption of charged molecules on the structure of Alginate gel

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Abstract

Natural origin, biocompatibility, low production cost and particularly ability of controlled release of the alginate gel are among the parameters which allow their utilization in pharmaceutical and food applications. For these purposes stability of gel, swelling and shrinkage due to the surrounding conditions like pH, temperature, ionic strength or interaction with molecules such as enzyme or substrate are essential in designing the material.

The aim of this work is to study the interactions of various molecules (organic cations) with gel structure by quartz crystal microbalance with dissipation monitoring (QCM-D). The surface of silica quartz crystals was first modified by an APTMS treatment and two main approaches were followed in this study; first covering the QCM-D crystal with a thin layer of alginate gel, drying and re-swelling of the gel during the experiments followed by the adsorption of the molecules of interest. Secondly, in situ formations of alginate gel on the surface of QCM crystal by a layer by layer built-up and studying the polymeric film when it is exposed to different changes. In both approaches the effect of surrounding conditions such as pH, temperature and ionic strength is presented. The effect of the adsorption of organic cations on the gel stability was also investigated and discussed.

Contents

Introduction

Alginate is a well known polysaccharide that can be mainly obtained from seaweeds [1,2]. In the presence of some cations such as calcium alginate can form a three dimensional network which contains high amount of water within its structure. The structure is then called hydrogel. One of the most important properties of alginate hydrogel is its ability of controlled uptake, release and retention of molecules due to the interactions of the macromolecular network with the diffusing or retained molecule.[3] This property and natural origin, biocompability and low production cost are among the main factors why alginate macromolecules are used in pharmaceutical and food applications as carrier for immobilizing cells, enzymes, proteins and for controlled release of drugs. [4] It can be also used as carriers for biocatalyst immobilization, or as absorbent particles in chromatographic processes.[5] Furthermore, degradation of polysaccharide due to microbial enzymes in the colon and large intestine allows drug release which is interesting for local therapies in colon cancer .[6]

Structural stability and mechanical properties of ionically crosslinked hydrogel is important for releasing drug and in tissue engineering. Swelling and deswelling of alginate hydrogel which affects this behavior has been studied before. Catherine K. Kuo and Peter X. Ma demonstrate that the swelling behavior is a function of initial crosslink density, alginate concentration and alginate chemical composition.[7] Furthermore, it has been shown that crosslinking ions being displaced by noncrosslinking ions, can potentially lead to the complete dissociation of the gel. In contrast, crosslinking creates elastic forces in the gel network that provide resistance to swelling tendencies.[7]

To be able to use a biocompatible material in applications the knowledge about their surface characteristics and gel structure when exposed to different molecules is needed. In order to have a deeper understanding about the gel behavior in liquid environment in different surrounding conditions, real time measurement in liquid environment is crucial. Moreover, the gel behavior through adsorption of molecules on the surface of gel is of interest. Real time study of such interactions can be performed by surface based biosensor techniques. Piezoelectric, optical, electrical, or electrochemical transducers convert surface interactions to readable output.[8] An example of such surface based techniques is a piezoelectric device called Quartz Crystal Microbalance with Dissipation monitoring.[9] It enables real time monitoring of resonance frequency change, Δf, of quartz crystal which corresponds to mass deposited on the surface of crystal. As a result, the technique can detect molecular adsorption/desorption events at the crystal surface. In addition to monitor change in resonance frequency, the energy dissipation, ΔD, induced by adsorption of molecule is monitored simultaneously through change in dissipation, ΔD. Change in dissipation is due to viscous losses in the layer deposited on the crystal surface and caused by the shear oscillation. [10] ΔD will provide information about viscoelastic property of the film formed on the surface of quartz crystal.

In order to study the behavior of the microgel structure, silica coated quartz crystals were used in QCM-D measurement. Due to existence of carboxyl groups in the structure of alginate, the polymer is negatively charged. On the other hand the silica coated surface of crystal is covered by hydroxyl groups which make the surface fully negatively charged. This cause electrostatic repulsion when alginate molecules come close to the surface of sensor. In order to overcome this obstacle a new protocol has been introduced in this work to modify the surface of crystal and make it positively charged. In the next step, two different approaches were followed to distinguish the difference between gel structure as a three dimensional structure and the surface of cross linked polymer strands. Surface chemistry of crystals was evaluated before and after any modification by QCM-D and AFM techniques. Swelling, shrinkage and change in gel structure were studied by QCM-D and compared with bulk experiments.

Aim of this work

The aim of this project is to explore the hydrogel structure in the nano and in the microscale to have deeper understanding of the microgel structure behavior when exposed to different conditions or different molecules adsorbed on the surface. The first part of this work is dedicated to study the gel stability and swelling/shrinkage behavior when different solutions are in contact with the gel. In the second part which mainly deals with QCM-D measurement microscopic behavior of gel in different ambient conditions such as pH, ionic strength, temperature is investigated. For this purpose two approaches were followed for studying the polymer film on quartz crystal: (i) Spin coating of gel on the surface of crystal (ii) Layer by layer adsorption of polymer on the surface of crystal with cross-linking stage with calcium ions. Finally, the gel stability through the adsorption of enzyme and several derivatives of an amino acid (arginine) is studied.

This thesis is divided into the following parts. The first chapter provides some background about alginate gel, ionotropic gelation, ion exchange in gel structure and techniques used for this study. In the second chapter, experimental set up and protocols are introduced. Chapter three mainly deals with the results obtained from the bulk experiments, the experimental route 1 and experimental route 2 in which the interaction of molecules and gel structure in micro and nano scale has been discussed. Finally, in chapter 4 the conclusion of this work is presented.

Chapter one

1.1 Background

Alginate is an anionic copolysaccharide consisting of (1→4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues (see fig 1).[11] The polymer is made of homopolymer sequence of mannuronic acid, M, homopolymer guluronic acid sequences, G, and mixed sequences of mannuronic acid and guluronic acid units, MG or alternating blocks. The distribution of blocks along the alginate molecules depends on the type of algae, age and part of algae from which alginate is extracted.[11] In general the alginate polymer is extracted from brown algae. Alginic acid present in seaweed is solubilized in dilute alkaline solution then treating with mineral acids and converted to a salt. Sodium alginate is the major form currently used. [12] Biocompatibility and non-toxicity of alginic acid and sodium and calcium salts are the main advantages that has made them being widely used in the medical, pharmaceutical, cosmetic, and food industry. In tissue engineering, alginate gels are used for cell encapsulation and drug delivery.[13,14] Alginate is an important material for wound healing, due to an ion exchange interaction between calcium in alginate and sodium in the blood and wound fluid.[15]

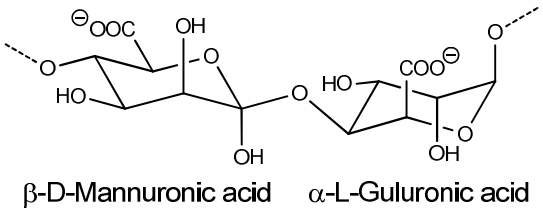


Figure 1. Structure of alginate , a copolymer containing M and G blocks

Depending on the concentration and molecular weight of the polymer, sodium alginate forms a viscous solution in water. Divalent ions (Ca^{2+}, Ba^{2+}, Fe^{2+}, Sr^{2+}, etc…) or trivalent ions (Al^{3+},etc…) act as cross-linkers and cause an ionic binding between G blocks in polymer chains and form three dimensional network. These cations form junction zones in which several polysaccharide chains are involved in. The "egg box" theory describes a model for binding each of divalent cations with two G blocks in two different chains. (See figure 2) [16] At moderate to high pH conditions negatively charged oxygen in polysaccharide molecule like hydroxyl oxygen, ether oxygens and carboxylate oxygens contribute in multiple bounding to aqueous cations.[11] G blocks in alginate strands have better affinity toward binding to calcium ions due to proper spacing and geometry.[17,18] The strength of gel is highly dependent on the G content of the alginate, the divalent cation, the weight and concentration of the polysaccharides.[11] Stabilized polymer chains entrap large quantities of

water and form three dimensional networks which is called hydrogel. The gelification process is highly depending on the diffusion of ions into the polymer network. Swelling and viscoelasticity of alginate structures are highly affected by the M/G ratio. M-rich alginate forms softer gel with lower porosity since the bound between the polymer chains are weaker and the flexibilities of molecules are higher.[12]

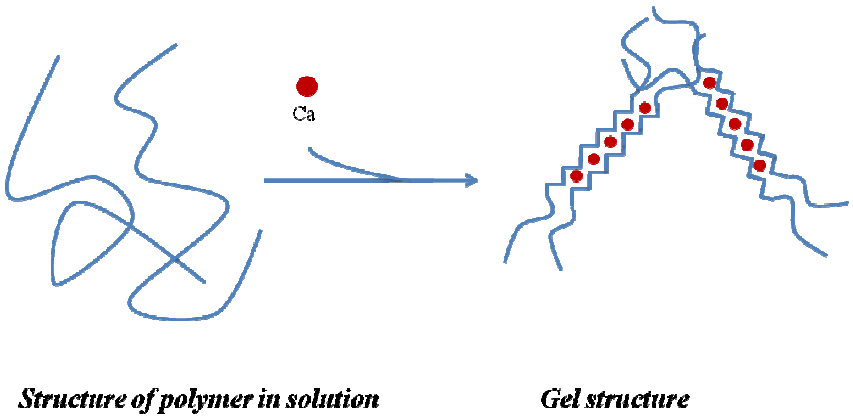


Figure 2. Gelification of alginate polymer in presence of calcium ions

As previously mentioned structural stability and mechanical property of calcium alginate gel are important in applications such as tissue engineering or controlled release of drugs. Consequently, knowing parameters which could affect swelling/shrinkage, degradability or any change in gel structure can be helpful in designing material for special purposes. One of the several reasons for the changes in gel stability could be the interaction of molecules or ions with polymer strands or gel structure. It has been observed that the rigidity of calcium alginate gel is decreased when it is exposed to sodium ions. In other word, exposure to sodium content solution leads to a softer gel.[19] Ion exchange between junctions in the so called "egg-box" and monovalent ions (Na^+) in solution is confirmed by RAMAN spectroscopy[3]. However, the interaction of cationic organics with gel structure and alginate polymer strands has not been investigated yet. In this work the effect of such interactions between simple cationic organic material like tetramethylammunium and some aminoacid derivatives such as Ac-Arg-OMe, L, D-BAPNA, H-Arg-βNa which are considered as substrates for trypsin on gel structure has been studied. Furthermore, the effect of enzyme (trypsin) on gel structure at room temperature and 37°C is also investigated.

1.2 AFM

Atomic force microscopy is one of the Scanning Probe Microscopy techniques (SPM) which is used for studying surface properties of material from the atomic to the micron level. The main components of this technique are a cantilever to which a sharp tip is attached, a piezoelectric scanner, a device for controlling the vertical position of probe which consists of a laser beam and photodiodes which is connected to the scanner and a positioning system

which brings the probe in the vicinity of the sample. Finally, a computing system converts the data into images. The principle is illustrated in figure 3.

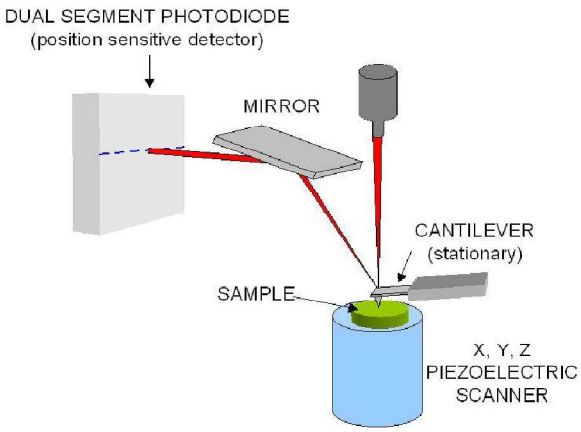


Figure 3. Scanning Probe Microscopy components

In this technique the surface of the sample is probed with a sharp tip which is attached at the end of a cantilever and is a couple of micron long and less than 100 Å in diameter. The force between the tip and surface cause deflection or bending of the cantilever. A detector which consists of a laser beam and a photodiode cells measures cantilever deflection and bending while the tip scans over the surface of sample. The detected laser beam on photodiode cells is converted to a topographical map of the surface by a computing system. Among different intermolecular forces, van der Waals forces between the atoms of the sample and the scanning tip are the main reason for bending or deflecting of cantilever.

Van der Waals forces depend on the distance between the tip and surface. According to figure 4 when the tip is very close to the surface most forces are sensed are repulsive and at a certain distance these forces are attractive. Depending on two different regimes of forces there are two modes of atomic force microscopy, contact mode and non-contact mode respectively.

In the contact mode the nature of the forces are repulsive as the distance between the tip and the surface is less than a few angstrom but in non-contact mode because of long- range van der Waals interactions, they are attractive.

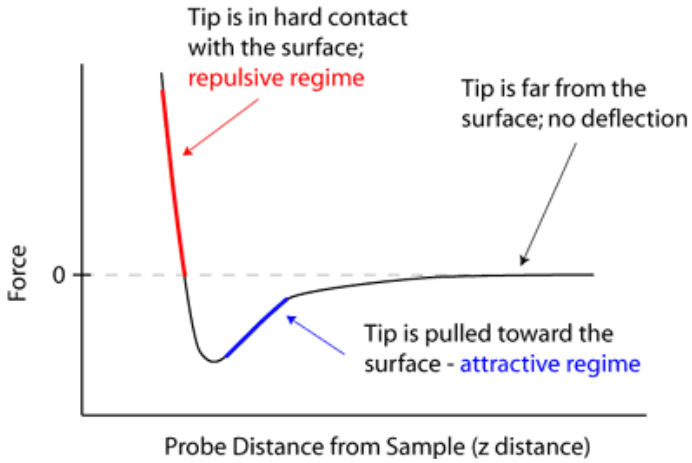


Figure 4. Interatomic force vs. distance curve[20]

In non-contact mode, usually a vibrating cantilever is used which oscillates at its resonance frequency, typically from 100 to 400 KHz. As the tip comes close to the surface the resonance frequency of cantilever changes. According to the spring constant of the cantilever and the gradient force applied to cantilever, the space between cantilever and surface is determined. By this mean, a topographical image of the surface is obtained. In non-contact mode, with the aid of a feedback loop which control the positioning of the scanner in the vertical direction, the resonance frequency and consequently the distance between tip and surface is kept constant. The amount of force between tip and sample in non-contact mode is about 10^{-12}N. This low value forces enables to use non-contact mode AFM to have topographical map of soft or elastic materials[20], as with present study.

1.3 QCM-D

Quartz crystal microbalance with dissipation is an ideal tool to study the adsorption of biomolecules and macromolecules like polysaccharides on the surface of a substrate in real time. Quartz crystal is a piezoelectric material which oscillates at its resonance frequency when an electric potential is applied over opposite sides of the crystal which is covered by gold electrodes. The form of oscillation depends on the direction of the crystal cut. To have thickness shear mode motion in quartz crystal, an AT-cut is used. The adsorbed mass and the changes in resonance frequency, Δf, have a linear relation, so-called the Sauerbrey equation.

$$\Delta m = (C_{QCM}/n)\,\Delta f \qquad \text{Eq. 1}$$

Where C_{QCM} (mass sensitivity) =17.7 ng.cm^{-2}.Hz^{-1} at f=5 MHz and n is the overtone number.

The Sauerbrey equation is valid for rigid, evenly distributed and thin adsorbed layers [21] However, it is not valid for non-rigid adsorbed mass, since soft film form a coupled oscillator which causes deviation from the linear relation between Δf and Δm. Furthermore, in QCM measurement, water or any solvent which couples to molecule due to hydration, viscous

drag or entrapped in the mass pores is sensed. In other words in QCM measurement, hydrated mass is measured.[21] When the QCM is complemented with dissipation measurement, Q-factor or D, information about viscoelastic properties of adsorbed mass will be provided. D describes the relation between dissipated energy and stored energy E_d and E_s according to [22]

$$D=E_d/2\pi E_s \qquad \text{Eq.2}$$

The energy dissipation factor increases during the adsorption of soft material on the surface which means that energy transfers from the oscillating crystal to the adsorbed film is partially stored due to viscoelastic property of the material. In a viscoelastic film energy dissipation and decay process is faster comparing to a rigid film. This results in an overestimation of the mass adsorbed on the sensor when Sauerbrey equation is used. In this case linear relation of shift in frequency and mass is not valid. A Voigt based viscoelastic film model, has been introduced by Voinova et al. to describe the propagation and damping of an acoustic wave in a viscoelastic adsorbed film in contact with Newtonian liquid under no-slip condition[23]. One of the benefits of QCM-D technique is the detection of mass deposition in liquid phase. For this purpose a physical model should be chosen to quantify the amount of mass adsorbed on the surface of oscillating crystal. Generally to main models describe the viscoelastic properties of the film which is used in modeling of viscoelastic material, Voight and Maxwell model. According to Voinova et al., the response of QCM can be compared to viscoelastic fluid and viscoelastic solid films in gas. For Maxwell fluid resonance frequency shift is the function of film shear elasticity G_M (Eq.3) while for Voigt solid it is the function of both viscosity and elasticity (Eq.4).[24]

$$\Delta f_M \approx -h\rho\omega/2\pi m_q\left\{1+h^2\rho\omega^2/3G_M\right\} \qquad \text{Eq.3}$$

$$\Delta f_V \approx -h\rho\omega/2\pi m_q\left\{1+h^2G\omega^2\rho/3(G^2+\eta^2\omega^2)\right\} \qquad \text{Eq.4}$$

If the mass adsorbed on the surface of crystal conserves its shape during adsorption and do not flow under shear deformation or if the mass is a polymer film which is far from the glass transition region then Voigt model can be applied to quantify the mass or thickness of the deposited film on the surface. Maxwell model is applied for polymer solution and polymers in the amorphous state and in near liquid-glass transition.[25] Since in this work the adsorption of macromolecules on the surface is studied, we have used Voigt model for calculation of thickness of adsorbed mass and interpreting viscoelastic property of the film in different ambient condition or in the case of adsorbing another molecules on the surface of film. The use of the Maxwell model was also evaluated.

Chapter two

2.1 Materials and methods

2.1.1 Gel components:

Alginate

Alginate sodium powder from brown algae was used in this work. It contains 30-40% mannuronic acid and 60-70% guluronic acid. The average molecular weight is 150000 Daltons. It was used directly in experiment without further purification.

GDL

D-(+)-Gluconic acid δ-lactone purchased from Fluka (sigma Aldrich) with purity of 99.0 %. GDL was used as an acidifier to dissolve calcium carbonate.

Calcium Carbonate

Carbonate calcium ‘’Mikhart 2’’ with purity of 99.1% was purchased from ‘’La Provencale’’. The range size is about 0-10 µm with a mean particle size of 3 µm.

Calcium chloride

96 % powder, anhydrous from sigma Aldrich.

2.1.2 Adsorbents:

Trypsin

Trypsin is a proteolytic enzyme that cleaves peptide bonds after lysine and arginine residues of proteins in order to give smaller polypeptide units. Chemical structure of trypsin is shown in figure 5. Trypsin (pI=10.9) is positively charge at pH < 10.9. Trypsin type 1 bovine pancreas (ref T8003) from sigma Aldrich was used in these experiments.

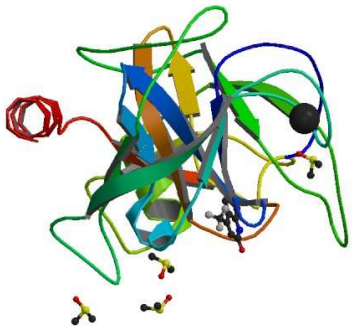


Figure 5. Trypsin complex with fluorine-containing fragment[26]

L-arginine

L-arginine is an aminoacid. The pK_a of aminoacid side chain of arginine 12.48. The chemical structure of L-arginine is shown in figure 6. D-arginine was purchased from sigma Aldrich (see fig. 6).

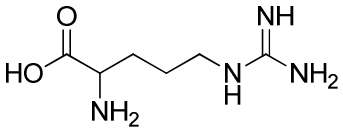


Figure 6. Chemical structure of arginine

L-BAPNA *(*N_α-Benzoyl-L-arginine 4-nitroanilide hydrochloride) and D-BAPNA (L-BAPNA enantiomer)**

L-BAPNA *(*N_α-Benzoyl-L-arginine 4-nitroanilide hydrochloride), a substrate of trypsin, and D-BAPNA (L-BAPNA enantiomer and a known inhibitor of trypsin) were purchased from Bachem (see fig. 7).

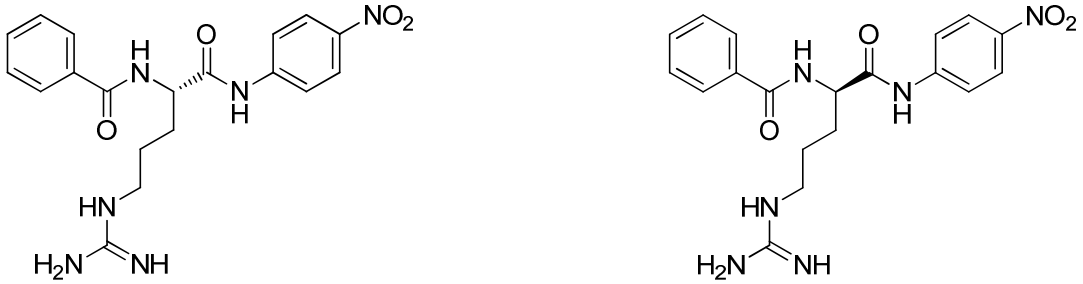


Figure 7. Chemical structure of L-BAPNA (left) and D-BAPNA (right)

H-Arg-βNa(L-arginine b-naphtalene)

H-Arg-βNa, a substrate for aminopeptidase B (arginine aminopeptidase) and cathepsin H, was purchased from BACHEM (see fig. 8).

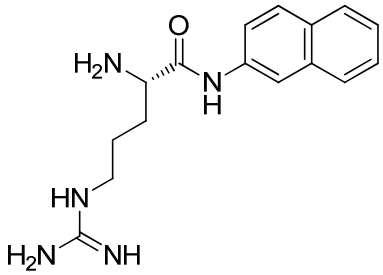


Figure 8. Chemical structure of H-Arg-βNa

Tetra methyl ammonium chloride (TMA)

Tetramethyl ammonium chloride (TMA), containing less than 2 % water from Fluka was used (see fig. 9).

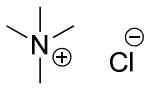


Figure 9. Chemical structure of tetra methyl ammonium chloride

Ac-Arg-OMe (Acetyl-L- Arginine Methyl Ester mono Chloride)

Ac-Arg-OMe, purchased from Bachem is a substrate of pancreatic and intestinal kallikreins from different species and it can also be hydrolyzed by trypsin. Chemical structure is shown in figure 10.

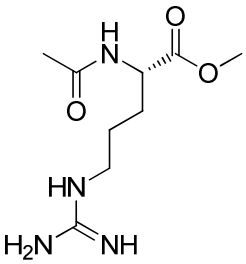


Figure 10. Chemical structure of Ac-Arg-OMe

2.1.3 Materials for crystal modification:

Toluene 99.9 % for HPLC and aminopropyltrimethoxysilane (APTMS) were purchased from sigma Aldrich. Ethanol 99.5 % analytical grade was used.

2.2 Surface treatment of the crystals

Silica dioxide (QSX 303, Q-Sense) coated crystals were first cleaned in UV/O chamber for 20 minutes. Then they were immersed in 2 % SDS solution for 20 minutes and then rinsed with MilliQ water and dried with nitrogen.

A 1 %(v/v) APTMS solution in toluene was prepared, and the crystals were immersed in this solution for 40 minutes then sonicated in toluene twice for 5 minutes then in pure ethanol twice for 5 minutes. After, they were put in an oven at 60˚C for 30 minutes. This procedure continued with sonication of crystals in pure ethanol for 5 minutes, and then after rinsing with milliQ water, they were cured in MilliQ water at 60°C for 30 minutes. Finally, the crystals were rinsed with MilliQ water and dried with nitrogen. After placing the crystals in QCM-D modules, all crystals were rinsed with HCl 1 mM to ensure that the surface was positively charged.

2.3 Gel preparation and bulk experiment

1.5 g of alginate solution 1 % (m/v) in MilliQ water was mixed with 550 µl GDL (D-(+)-Gluconic acid δ-lactone) solution (110 mM in water). 550 µl of a dispersion of calcium carbonate (50 mM in water) was injected into the mixture while the vial is vigorously shaken for couple of seconds. Immediately after the mixing, the gel structure is formed and after a couple of hours the whole content of calcium carbonate is dissolved and the blurry mixture turns clear and transparent.

2.4 Gel stability (bulk experiment)

For each set of experiment, four gel samples were prepared and were left at room temperature for one day to let the alginate gel form. Then different solutions including Tetramethylammoniumchloride (TMA), Ac-Arg-OMe, H-Arg-βNa, Trypsin and water were introduced over the gel surface. Sample set 1 contains 550 µl of water. Sample set 2 contains 550 µl of TMA (100 mM in water). Sample set 3 contains 550 µl Ac-Arg-OMe (100 mM in water). Sample set 4 contains 550 µl H-Arg-βNa (50 mM in water). Sample set 5 contains 550 µl L-Arginine (100 mM in water) and sample set 6 contains 550 µl Trypsin 10 mg/ml in water. The weight of the containers before and after introduction of solutions to gels was measured. Water leaked each day was removed by pipette to monitor the swelling /shrinkage behavior of gel. All samples were kept at room temperature.

2.5 Spin coating of the crystals by gel

1.5 gram of alginate solution 1 % (w/v) in MilliQ water were mixed with GDL (*D*-(+)-Gluconic acid δ-lactone) solution (110 mM in water) and then mixed with calcium carbonate dispersion (50 mM in water) before spreading over the surface of the spinning crystals. Spin coater device (POLOS MCD200 from SPS) was used for spin coating the APTMS treated crystals with gel. A two stage program was used for coating crystals. At stage 1, the rotating speed of the crystal is 1000 rpm for 1 minute and it is continued at higher speed (3000 rpm). Pre-gel mixture is injected over rotating crystal at speed 3000 rpm. The spin coating lasts for two minutes at 3000 rpm to allow water to evaporate from the surface and then crystals were dried at 37˚C for one day.

2.6 Characterization

AFM

Atomic force microscopy from NTEGRA probe Nanolaboratory with a 100µm scanner and silicon AFM probe with resonance frequency 300kHz (force constant 40N/m) and 150kHz (force constant 5N/m) suitable for tapping mode were used to obtain topographical image of a modified crystals after APTMS treatment and of the surface of wet and dry gel.

QCM-D

A Q-sense E4 instrument (Q-sense AB, västra Frölunda, Sweden) with four modules has been used. SiO_2 coated QCM crystals (QSX 303) from Q-sense were used in this study. In this work surface of crystals were modified with APTMS. A homemade auto sampler was used for injecting the different solutions including alginate, calcium chloride and Tris buffer in the measurement chamber.

Chapter three

Results

3.1 Bulk experiments – gel stability

To evaluate the gel behavior from a macroscopic point of view we performed an experiment regarding gel stability and swelling/shrinkage. The average amount of the gel in each set of samples was 2.57 gr. The two following graphs show the mass variation of the gel due to the introduction of different solutions including water, tetramethylammonium chloride, Arginine, Ac-Arg-OMe, H-Arg-βNa and trypsin solution over a gel during five days. Addition of water was done as reference. Figure 11 shows the gel shrinkage/swelling due to exposure to water, TMA, Arginine, Ac-Arg-OMe, H-Arg-βNa and enzyme. The first conclusion of this figure is the huge effect of H-Arg-βNa on the gel, leading to shrinkage of the gel up to 1.6 g. The other compounds have much less impact on the gel. TMA induces a loss of mass equal to 0.7 g while the other ones induce a loss of mass less than 0.3 g. It is remarkable that TMA and Ac-Arg-OMe induce the swelling of the gel on the first day and then shrinkage of it.

It was observed that the gel structure was disturbed when it was exposed to water for several days. Since all substances were dissolved in water, the graph related to water effect was subtracted from other graphs to have better understanding about gel stability when different compounds introduced over the surface of gel. Figure 12 shows relative swelling/shrinkage of gel in three following days to water due to exposure to mentioned substances. Almost the same conclusions can be made with figure 11, except that almost all compounds, with exception of H-Arg-βNa, induce swelling of the gel on the first day.

The specific effects will be discussed further and compared with the results obtained by QCM-D in chapter four.

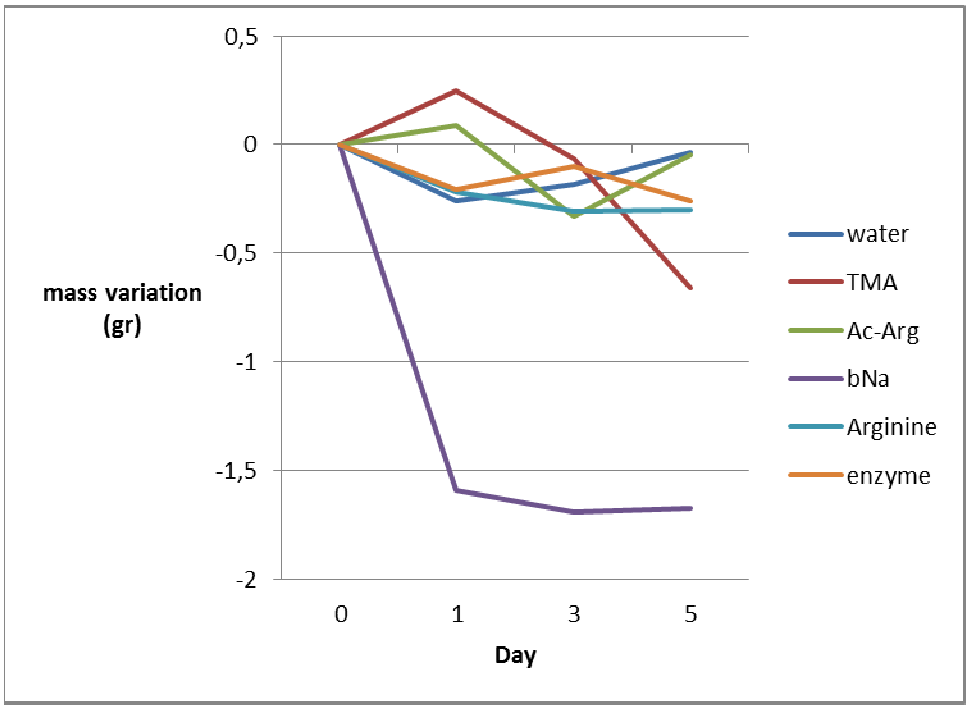


Figure 11. Gel stability due to exposure to water, TMA, Arginine, Ac-Arg-OMe, H-arg-βNa

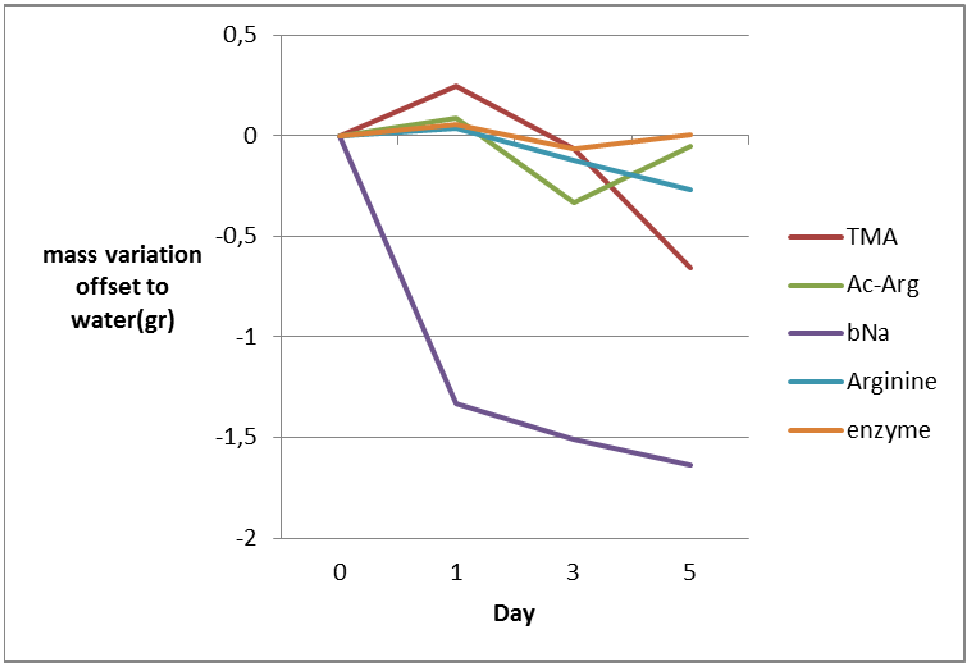


Figure 12. Gel stability relative to water for different solutions

3.2 Study of the gel structure on QCM-D surfaces

3.2.1 Surface chemistry of the crystals

Hydroxyl groups bound to the surface of SiO_2 coated crystals give the surface a neat negatively charge at relatively low pH. Alginate polymer chains are also negatively charged due to the existence of hydroxyl and carboxyl groups in the structure of alginate molecule (See fig.1). Obviously, alginate cannot bind to the SiO_2 surface and in fact the polymer is repelled from the surface due to electrostatic forces. As a consequence, the nature of the surface was changed from a negative charge to positive charge using aminopropyltrimethoxysilane (APTMS) (see fig. 13). A monolayer of APTMS covers the surface after this treatment.[27]

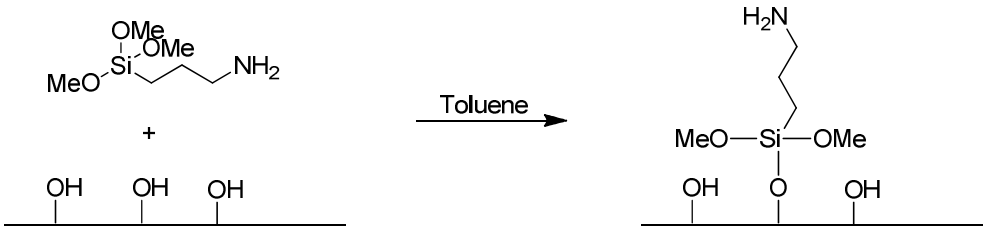
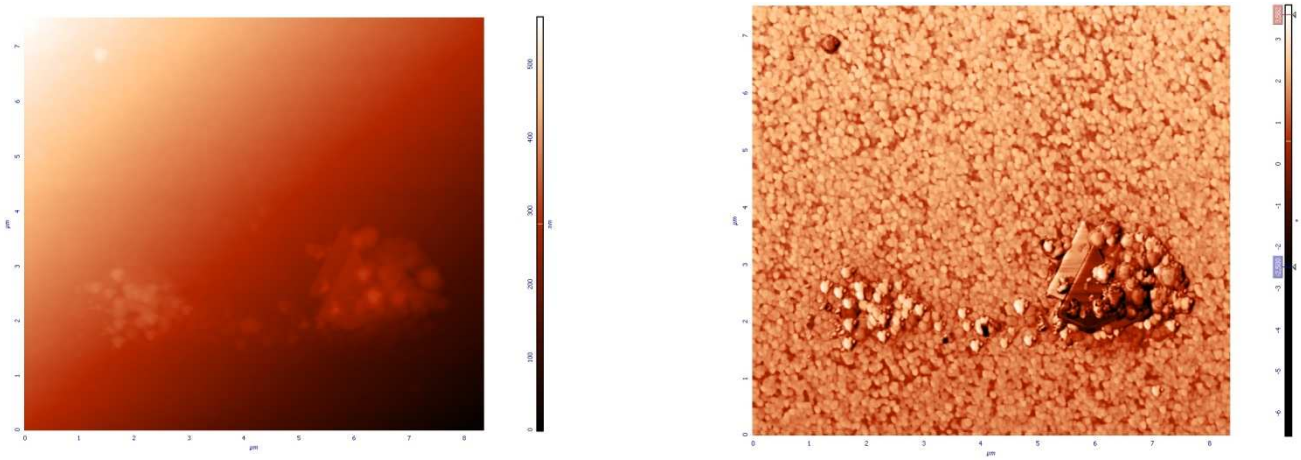


Figure 13. Surface treatment of SiO_2 coated crystal with APTMS

The surface modification was characterized by AFM. AFM images of both naked surface of silica and APTMS treated silica reveals that APTMS treated surface is rougher than bare silica. Average roughness of the bare silica surface is 90 nm versus 104 nm for APTMS



modified surfaces. (See fig. 14, 15)

Figure 14. AFM pictures of surface of bare glass(7×7µm). (left) height image, (right) phase image

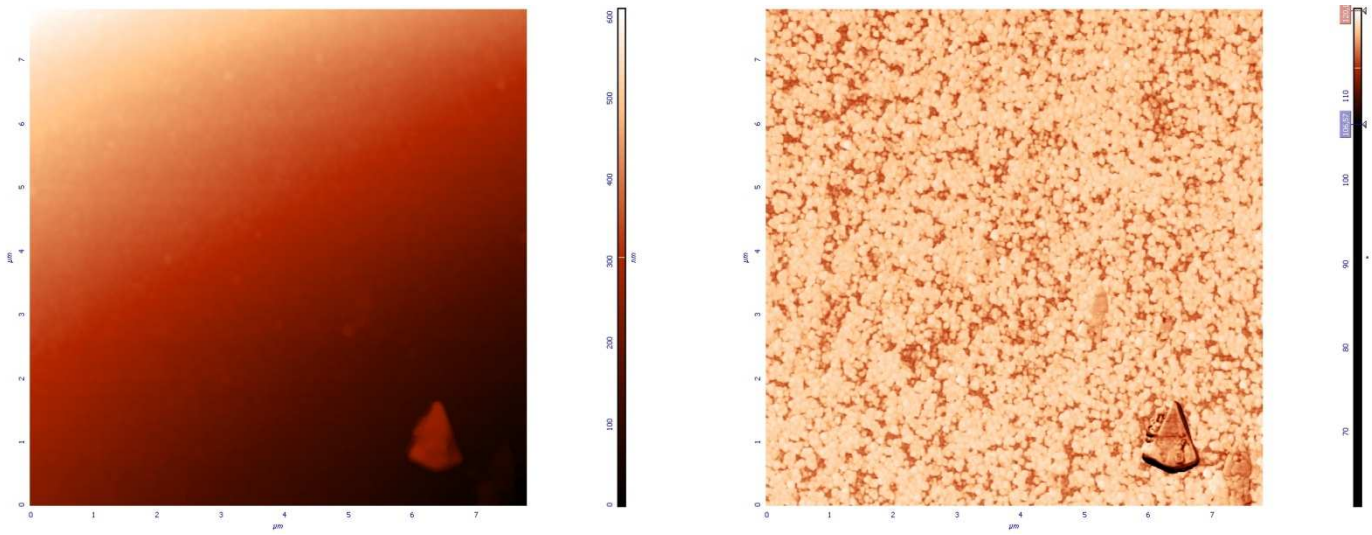


Figure 15. AFM pictures of surface of APTMS modified glass (7×7µm). (left) height image,(right) phase image

Average roughness of silica surface was increased after APTMS treatment which probably is due to the formation of aggregates of APTMS on the surface.

3.2.2 Surface chemistry evaluation

The main parameter in adsorbing a polymer film on a surface which should be considered is the competition among polymer chains, solvent and surface.[28] In order to have a strong adsorption of polymer chains on the surface, significant electrostatic forces between the polymer chain and the surface must occur. In the present case, curing APTMS modified crystal in water after all treatment steps, will cause further cross-linking APTMS molecules on the surface and the release of excess methanol molecules from un-reacted moieties which may interfere with the adsorption process in the experiments. As a result, a uniform positively charged surface is obtained (when pH is below 8-9). Moreover, it was proven that slightly acidic environment for APTMS treated surface is required to conserve the surface completely positively charged. The surface chemistry of the modified surface was evaluated by adsorption of positively charged enzyme on the surface in QCM-D. In order to examine this assumption, hydrochloric acid was used to turn the surface positively charged; thereafter, enzyme solution (1.1 mg in 25ml HCl 1 mM) was injected in the measurement chamber. As it is shown in figure 16 and 17, the positively charged enzyme is not adsorbed on APTMS modified surface which is protonated by hydrochloric acid while it is adsorbed on deprotonated APTMS modified surface.

Finally, it was observed that the solvent for alginate molecule can affect the adsorption remarkably. The alginate in Tris buffer 0.1M at pH 7.6 prefers to bind to the surface more than alginate in water. The reason is that at pH 7.6 the alginate chains have a neat negative charge. Hence, sufficient electrostatic force between the polymer chains and surface is provided in a way that polymer strands prefer to adsorb on the surface instead of remaining in the solution. The situation in water is reverse. The pH of MilliQ water is around 5, closer to the pK_a of carboxylate groups of the alginate. This reduces the electrostatic interaction and consequently reducing the driving force of adsorption. The polymer chains prefer to remain

in aqueous environment instead of adsorbing to the positively charged surface. Therefore, in all experiments in which adsorption from solution was needed Tris buffer 0.1 M pH 7.6 has been used.

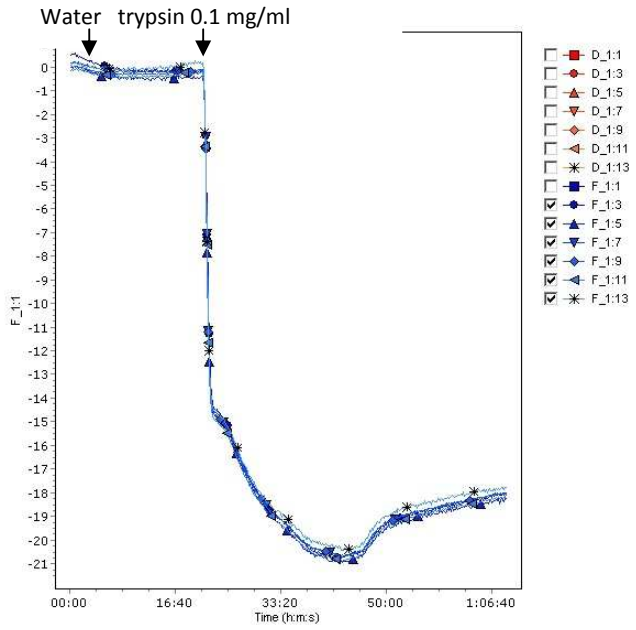


Figure 16. Adsorption of Trypsin on APTMS modified surface ***without using*** HCl

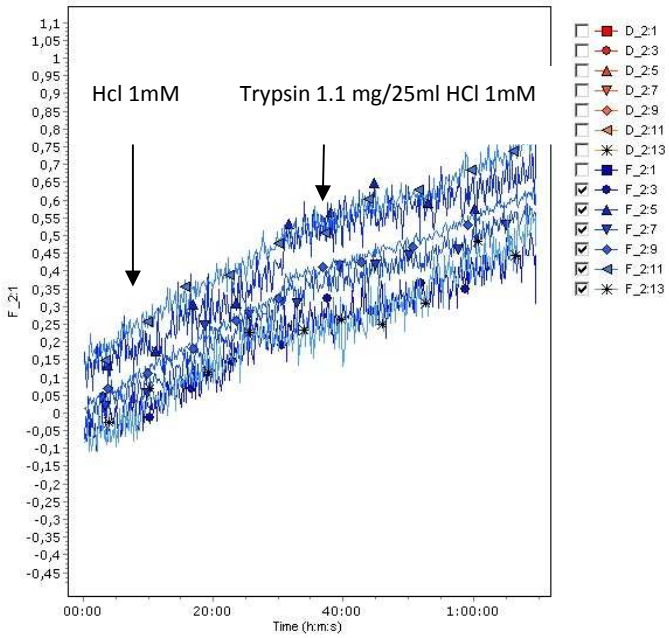


Figure 17. No adsorption of trysin on the APTMS modified surface ***using*** HCl

3.3 Route 1: Spin coating

Spin coated gel on surface

To understand the gel stability and swelling/shrinkage behavior on a microscopic scale, spin coated crystals with gel were prepared. Swelling of thin layer of gel on a QCM-D crystal when exposed to water is the first aspect of gel behavior in the QCM-D signal understanding. The variations in frequency and dissipation were monitored during swelling, as illustrated in fig. 18. The monitoring of Δf and ΔD at different overtones starts first in air, and then continued when water is introduced in the chamber. Large changes in Δf and ΔD are observed, resulting from two phenomena: the change of bulk from air to water and the swelling of the film.

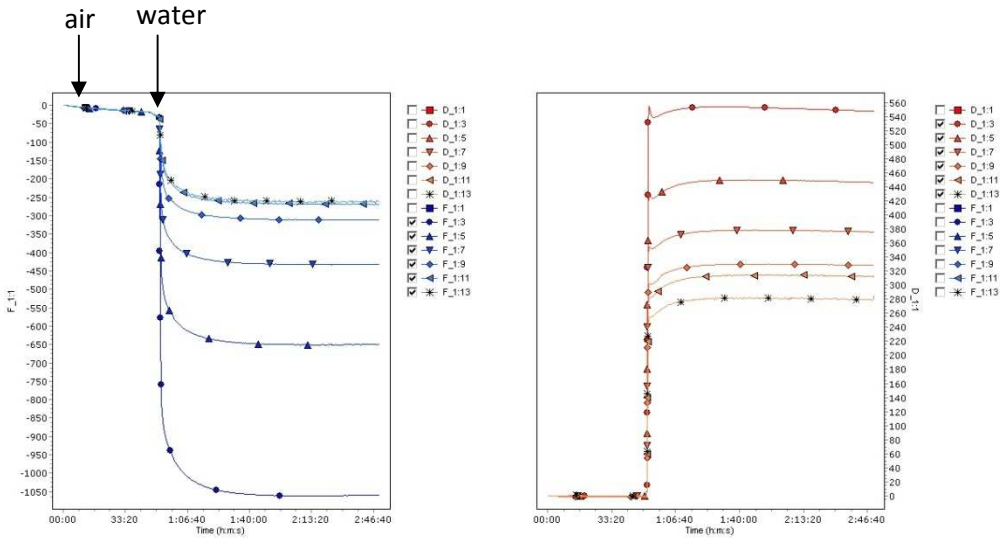


Figure 18. Variation in frequency (left) and dissipation (right) due to swelling

Considering that the film is rigid and evenly distributed over the crystal and using Sauerbrey equation the thickness of dried gel before swelling is determined to be about 9×10^{-2} µm. However, when it is exposed to water, Sauerbrey is not valid any longer, because water goes into the structure of the dried gel and form a viscoelastic film. This phenomenon can be interpreted by QCM-D measurement. A significant shift in frequency and a sharp increase in dissipation show that the crystal is highly damped (see figure 18). There are two reasons for damping crystal, first because of transfer from air to water and second due to the swelling of the film in water. To differentiate bulk effect from swelling behavior of gel we used a reference crystal to measure the overall shift in frequency and dissipation from air to water. Then the related amount was deducted from results of crystal covered by gel to have only the change in frequency and dissipation due to swelling. The results demonstrate that a

viscoelastic film is formed over the surface. Spreading signal with overtones (3, 5, and 7) showed that a soft film was formed. The thickness of film after eliminating bulk effect and using Maxwell model [24] is around 20 µm. The use of Maxwell model will be explained further in chapter four.

3.3.1 Ambient condition and gel behavior

pH effect

In order to study the effect of solutions at different pH on the gel, three crystals were spin coated with gel and one kept untreated and used as a reference. Initially the measurement started in air and then MilliQ water (pH around 6) was pumped through the chamber cell. After the swelling of the film, water at different pH values (5.17, 5.71, 7, 9.23 and 10) was introduced over the swollen gel. The pH of solution was adjusted with HCl 6N. Finally MilliQ water was used to rinse the surfaces. By increasing pH from 5.17 to 10, Δf increases which means gel shrinks. By changing the solution from 10 to 6.72 (pH of water) Δf is decreased back to initial value for swollen gel which means gel has reversible behavior. (See figure 19). Dissipation was few affected by pH changes. In that pH range, the structure of the film, in spite of its variation in thickness is not deeply modified.

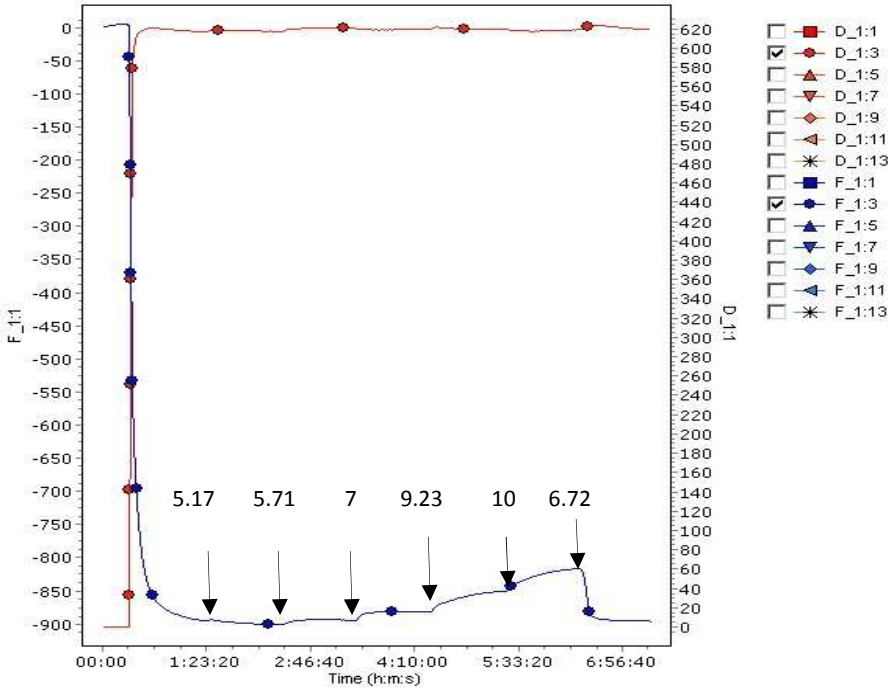


Figure 19. Variation of frequency (blue) and dissipation (red) for solutions at different pH

Ionic strength

The same approach as for pH effect was used to evaluate the effect of the ionic strength as shown in figure 20. The dried gel on the crystal swelled as it was exposed to water and the variation of frequency stabilizes after 40 minutes. Then solutions of sodium chloride at different concentrations (1, 5, 10, 15, 20, 25, 30, 35, 40 and 45 mM) were introduced into the system. Finally the surface was rinsed with MilliQ water. From fig.20, the effect of the ionic strength is obvious. The introduction of 1 mM of NaCl solution induces a significant change in

Δf and ΔD while the increase from 1 mM to 45 mM is of less amplitude. Finally after rinsing the film with water the frequency decreased which means that the film swells again but partly. The reduction of swelling can be described by Donnane equilibrium. Gel film acts as a semi-permeable membrane with immobile charge distributed inside its network. When a solution with large concentration of mobile ions is introduced over the gel, the concentration gradient between the core of the film and the solution acts as a driving force for entrapped water to displace from the network.[29] As illustrated in figure 20, gel has a partially reversible behavior when 45 mM solution is exchanged to water. Nevertheless, Δf and ΔD never returned to original values.

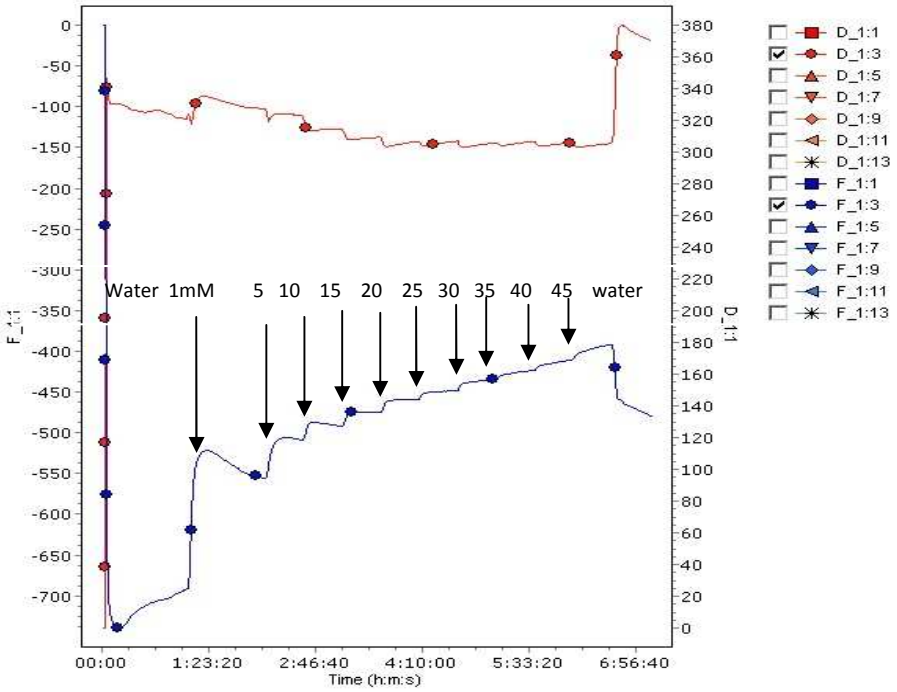


Figure 20. Variation of frequency (blue) and dissipation (red) for solutions at different ionic strength

In comparison with the effect of the pH, the changes induced by the introduction of a monovalent cation in the system must also be understood from a point of view where the dissociation of the gel can occur. This is fairly well supported by the variation of dissipation observed. During the course of the changes of ionic strength, larger effects were observed than for pH changes. After the final rinsing step, a large increase was observed, far from the original value. This is in agreement with a softer film, in other word, a film less cross-linked, i.e. dissociated.

3.3.2 Adsorption of organic cations on the surface of gel

In order to investigate the gel behavior when organic cations or positively charged molecules are introduced in the surface of the gel, we examined different solutions of organic cations: TMA, some arginine derivatives such as L-BAPNA, D-BAPNA, Ac-Arg-OMe, H-Arg-βNa. (See table 1). Finally, the effect of adsorption of enzyme as a large positively charged molecule on

the gel structure has been studied by QCM-D at different temperatures.

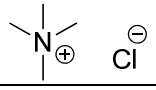
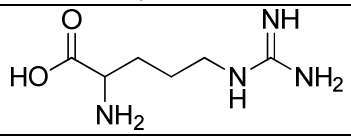
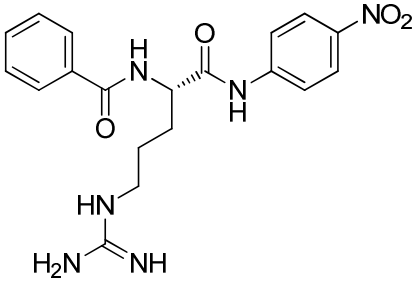
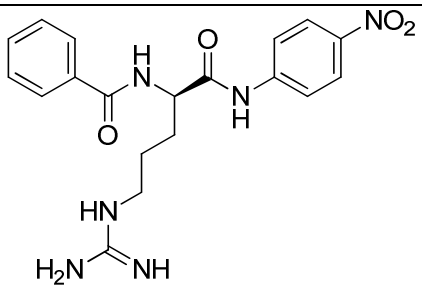
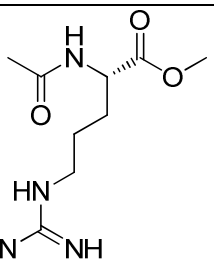
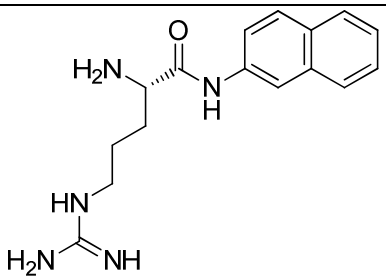
Name	Structure	Solubility/solvent
Tetramethylammonium		Soluble in water
L-Arginine		Soluble in water
L-BAPNA		Soluble in DMSO 5%
D-BAPNA		Soluble in DMSO 5%
Ac-Arg-OMe		Soluble in water
H-Arg-βNa		Hardly soluble in water

Table 1. Chemical structure of organic cations which were used in this study

3.3.2.1 Adsorption of substrate L-BAPNA and D-BAPNA - effect of the chirality

Spin coated crystals were used and the same approach as previously described was followed. Measurement started in air and then MilliQ water was pumped through the device resulting

in the swelling of the gel. Variation of frequency and dissipation due to swelling of film in water was recorded. The adsorption of L-BAPNA, one substrate of trypsin and D-BAPNA were monitored on the surface of the swollen gel. As L- and D-BAPNA are insoluble in water, an organic solvent dimethylsulfoxide (DMSO) was used to dissolve them. When stable baselines in frequency and dissipation for the swollen gel were obtained, the whole system was rinsed with a 5% (v/v) solution of DMSO to reach a new base line. Then, solutions of L-BAPNA or D-BAPNA in DMSO solution (5 % v/v) were pumped through the modules. Figure 21 suggests different mechanisms of adsorption for L- and D-BAPNA on the surface of gel. When BAPNA adsorbs on the gel, carboxylic groups in alginate molecule are neutralized by the positively charged part of BAPNA and hydrophobic part cause water repel from structure of swollen gel (see table 1). Consequently, the gel shrinks, resulting in a rapid increase of frequency shift and decrease in dissipation (see figure 21).

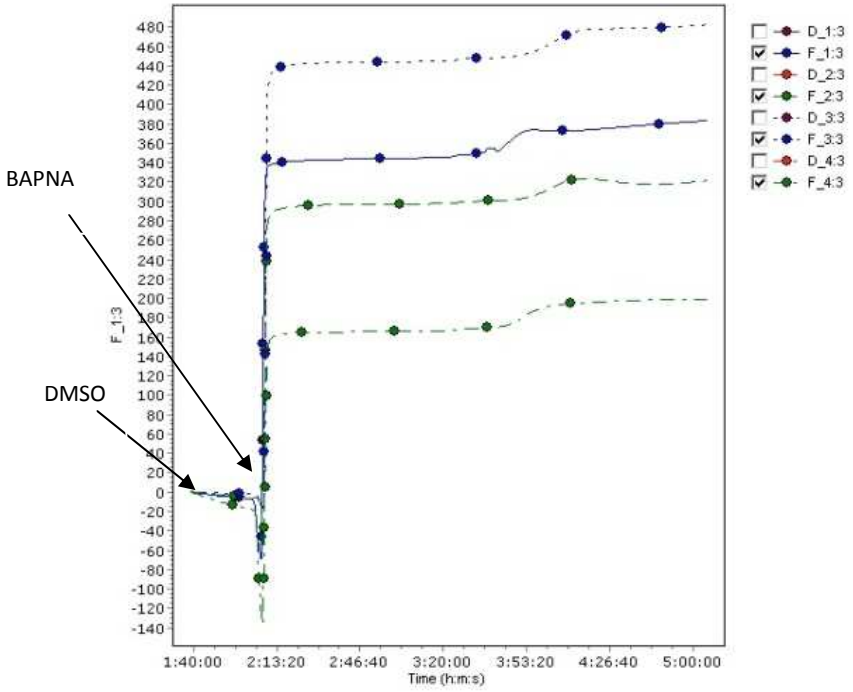
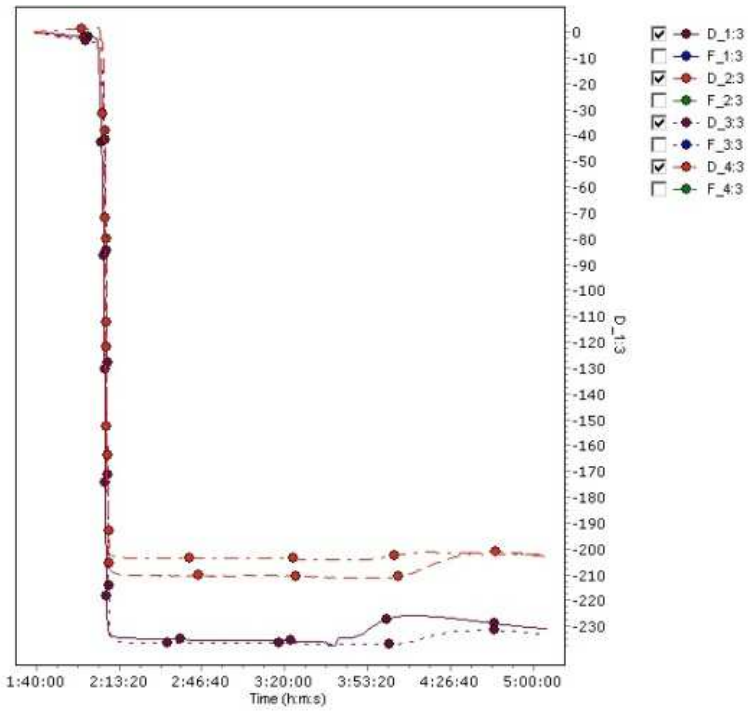


Figure 21. (a) Variation of frequency due to interaction of L-BAPNA (blue) and D-BAPNA(green) with the swollen gel

(a)



(b)

Figure 21. (b) Variation of dissipation changes due to interaction of L-BAPNA (dark red) and D-BAPNA (red) with the swollen gel.

This experiment aimed at investigating the effect of chirality on the adsorption profile on the gel. According to the observation presented above, there is a little difference between L-and D-BAPNA in adsorption pattern and the rigidity of the film but it is not remarkable enough to claim that chirality of molecules can affect the gel structure to large extent.

3.3.2.2 Adsorption of TMA (Tetra Methyl Ammonium)

The adsorption of TMA on the surface of gel was monitored. As stable baselines in frequency and dissipation for the swollen gel were obtained, a solution of TMA at concentration 4 mM was pumped through the modules. A sudden increase in frequency and a decrease in dissipation suggest (see figure 22) either gel shrinkage or a disturbance of the gel structure by exposure to the organic cation. As previously mentioned the alginate gel contains negatively charged moieties and when it is in presence of a solution containing cationic ions, electrostatic repulsion of the carboxylic groups in alginate structure are suppressed, thus reducing gel swelling and inducing the shrinkage of the gel. On the contrary of the effect of NaCl on the film, the rinsing step did not lead to any changes. This supports the hypothesis of a loss of a structure of the gel induced by counter ion of medium hydrophobicity, eventually because of an irreversible adsorption.

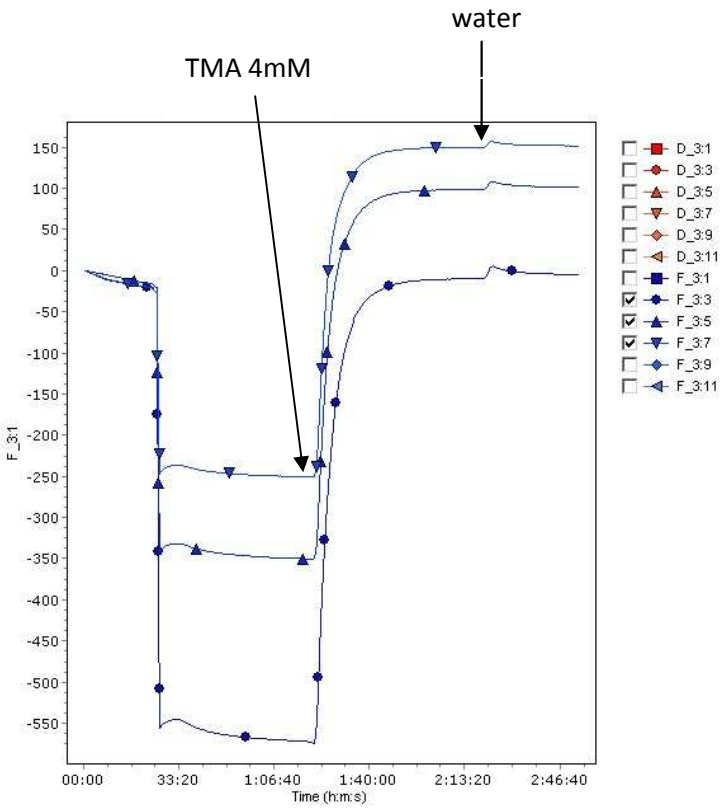


Figure 22.(a) Variation of frequency due to interaction of TMA with the swollen gel

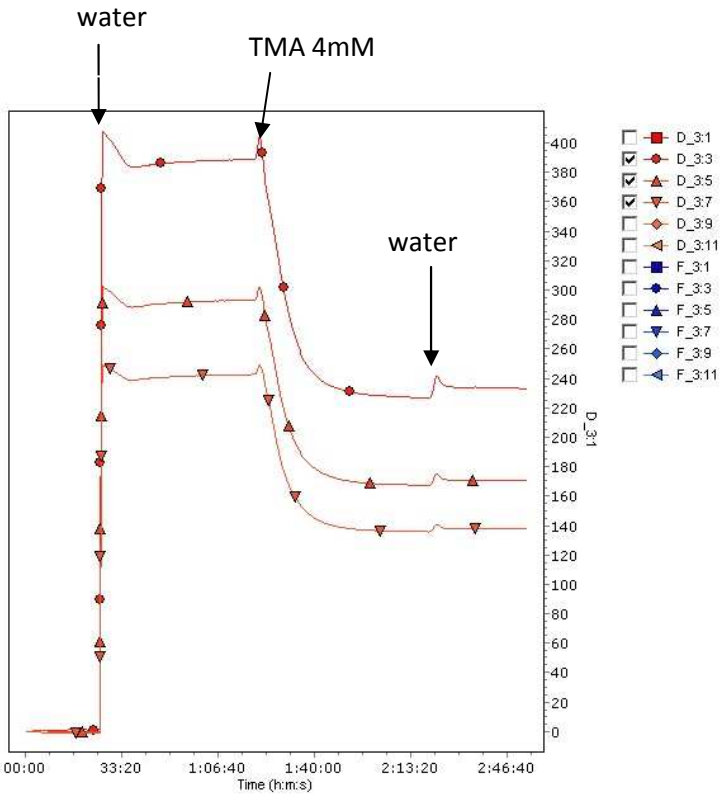


Figure 22.(b) Variation of dissipation changes due to interaction of TMA with the swollen gel.

3.3.2.3 Adsorption of L-Arginine

A solution of L-Arginine in water at 20 mM was pumped through the modules at a rate of 30µl/min when a stable baseline of the swollen gel was obtained. A sudden increase in frequency and a decrease in dissipation suggest (see figure 23b) either gel shrinkage or disturbance of gel structure by exposure to L-Arginine solution. Finally, the system was rinsed with water. The decrease in frequency and the increase in dissipation reveal that there are some residues on the surface which absorbs water when it is exposed to pure water. As previously mentioned alginate gel contains negatively charged moieties and when it is subjected to a solution with positively charged ions, electrostatic forces cause carboxylic groups in alginate structure to be covered by cationic ions resulting in a shielding of carboxylic groups and thus a reduction of repulsion of these groups which reduce gel swelling and stimulate gel shrinkage or deterioration of the gel network. (see figure 23) The adsorption is partially reversible.

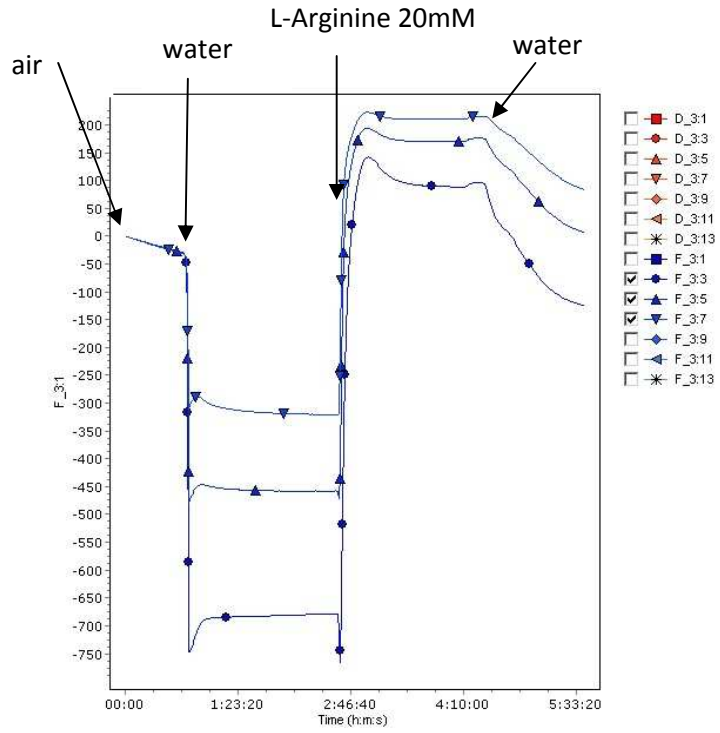


Figure23.(a) Variation of frequency due to interaction of L-Arginine with the swollen gel

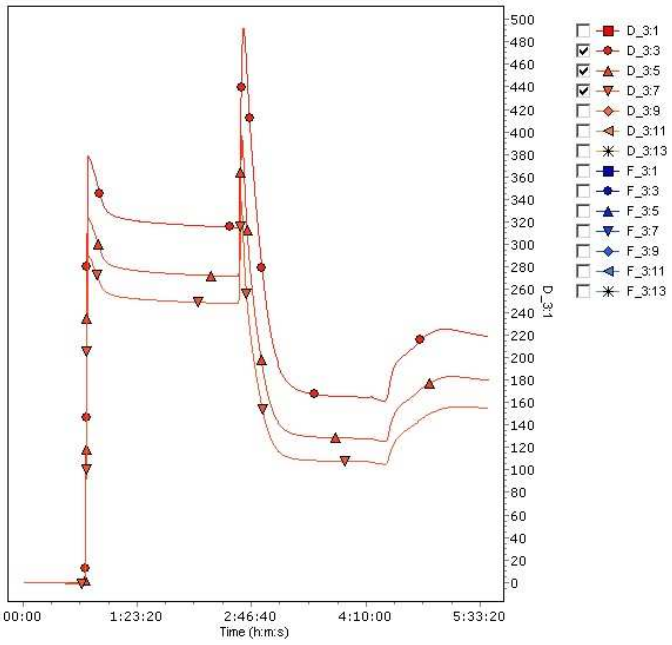


Figure 23.(b) Variation of dissipation due to interaction of L-Arginine with the swollen gel

3.3.2.4 Adsorption of Ac-Arg-OMe

Solution of Ac-Arg-OMe at a concentration of 4mM in water was pumped through the modules at a rate of 50µl/min when a stable base-line was obtained. As previously observed, the effects (see fig. 24) suggest gel shrinkage or modification of its structure when exposed to Ac-Arg-OMe. At last the system was rinsed by water. The increase in frequency can be described as water expulsion from the network due to adsorption of Ac-Arg-OMe molecules. However, dissipation remained nearly constant which means that the rigidity of the film has not been changed. Further information about viscoelastic property of the film is presented in section 4.2.1. (See figure 24b)

This behavior is very similar to one which observed before. (See previous section)

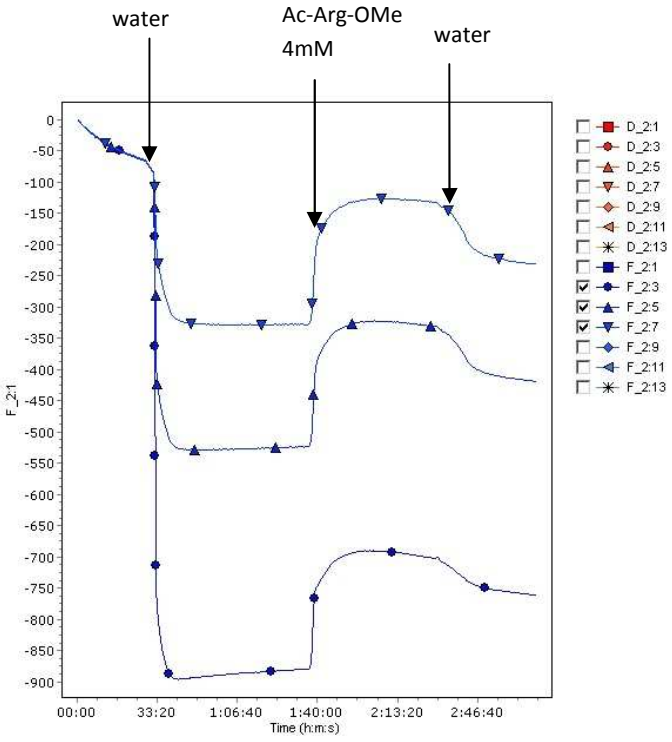


Figure 24(a). Variation of frequency due to adsorption of Ac-Arg-OMe on the surface of gel

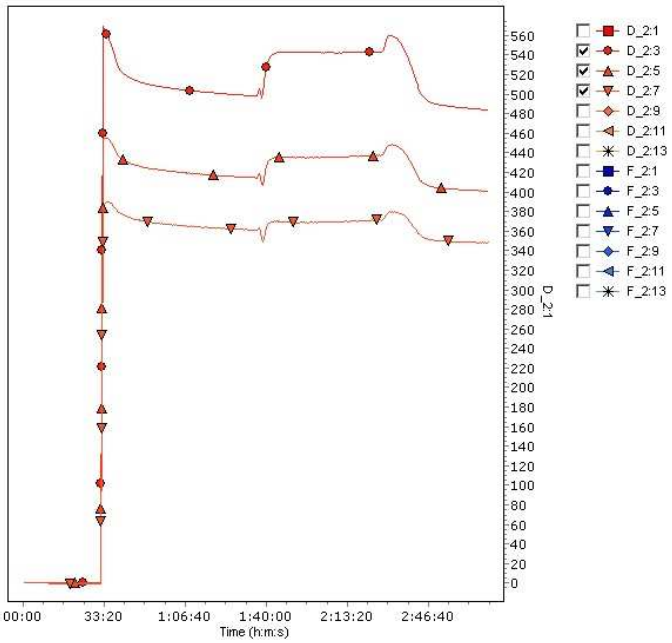


Figure 24(b). Variation of dissipation changes due to adsorption of Ac-Arg-OMe on the surface of gel

3.3.2.5 Adsorption of H-Arg-βNa

The same behavior was observed for H-Arg-βNa solution (see figure 25). Nevertheless, upon the rinsing the shrinkage was accentuated, as for a loss of material from the film.

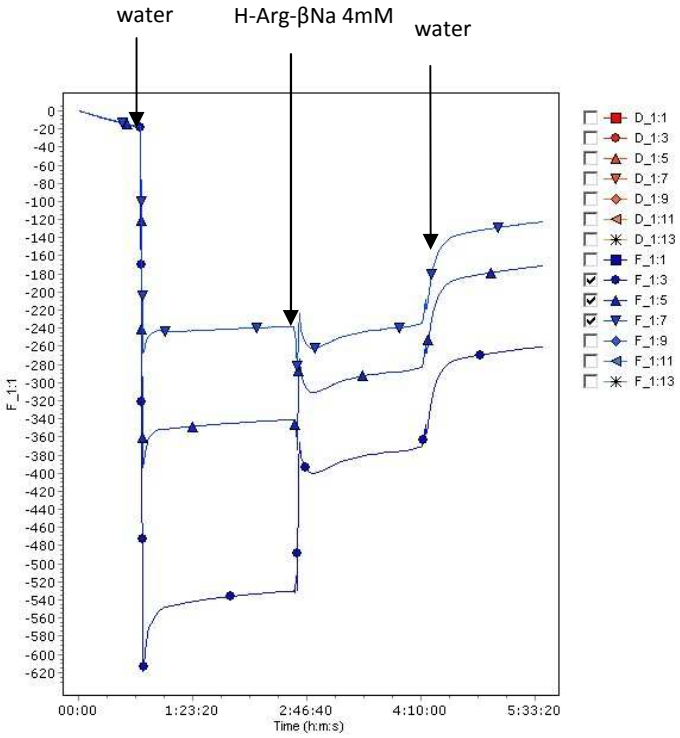


Figure25(a). variation of frequency due to adsorption of H-Arg-βNa

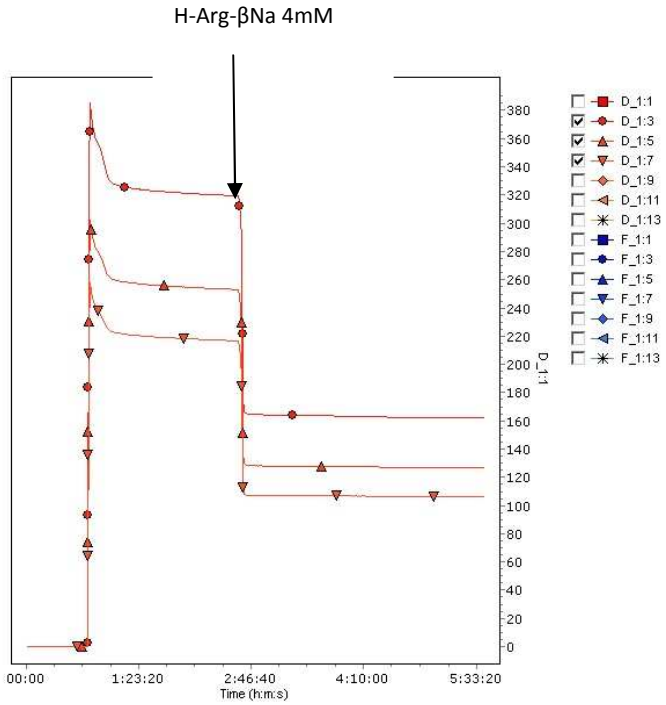
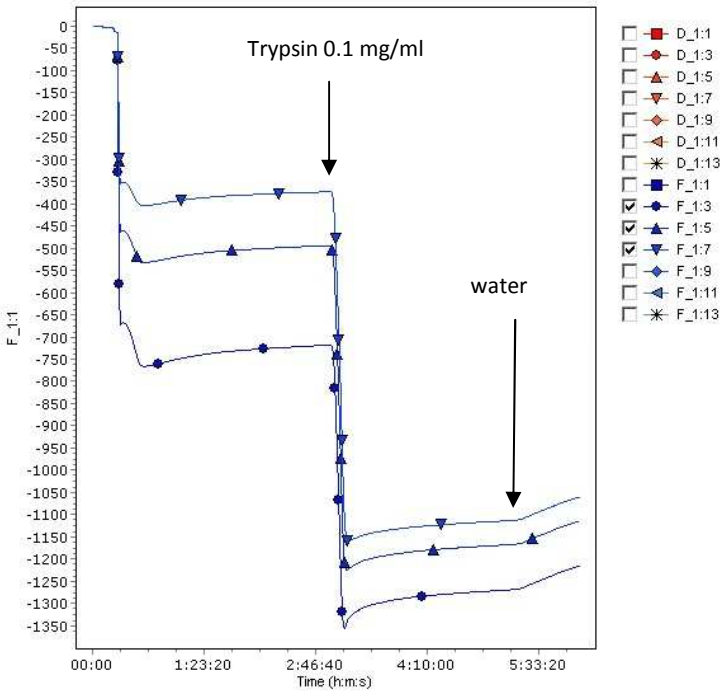


Figure 25(b). Dissipation changes due to adsorption of H-Arg-βNa on the surface of gel

3.3.2.6 Adsorption of trypsin at room temperature

As a complement of small organic cations, the interactions with large and positively charged molecules were investigated. Trypsin is a good example of such molecule. The experiment was carried out at room temperature.
Measurement was started in air and then MilliQ water was pumped through the cells resulting in the swelling of the gel. The variation of the frequency and the dissipation due to swelling of film in water was observed. After complete swelling, trypsin (0.1 mg/ml in water) was introduced in the system and followed by rinsing with water. A sharp decrease in the variation of frequency showed the strong adsorption of trypsin on the gel and desorption from the surface due to water rinsing was not remarkable (see figure 26a). The decrease of dissipation due to adsorption of enzyme suggested the formation of a more rigid film (see figure 26b).



Figureure 26(a). Variation of frequency due to adsorption of trypsin on the surface of the swollen gel at room temperature

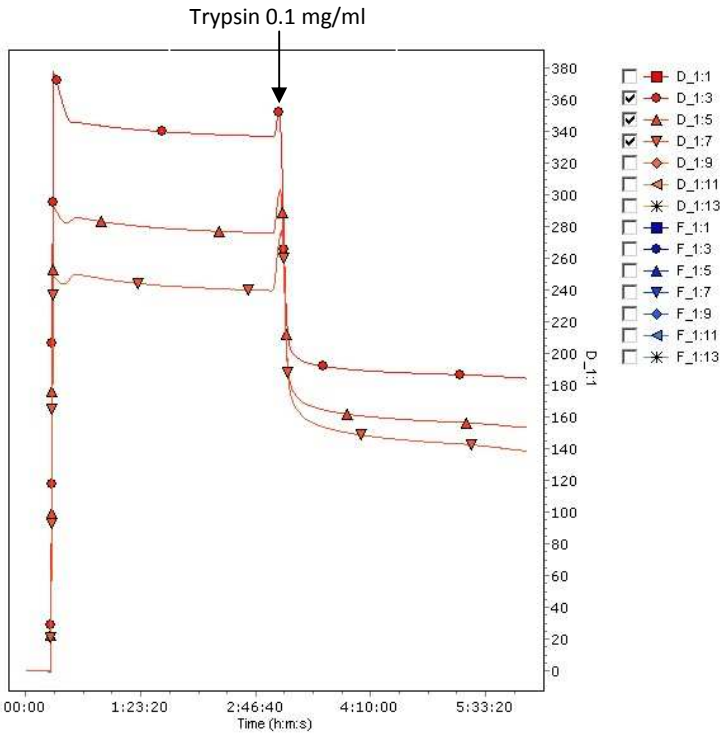


Figure 26 (b). Changes in dissipation due to adsorption of trypsin on the surface of the swollen gel at room temperature

3.3.2.7 Adsorption of enzyme at 37°C

Adsorption of the enzyme on the gel was monitored at 37°C on spin coated crystals. Measurement started in air and then MilliQ water at rate of 50µl/min was pumped through the device resulting in the swelling of the gel. The variations of frequency and dissipation due to the swelling of the film in water were observed. Trypsin (0.1 mg/ml in water) was introduced at rate of 30µl/min into the system, followed by a rinsing step. The sharp decrease in variation of frequency showed strong adsorption of trypsin on the surface of gel. Desorption from the surface due to rinsing with water was much more pronounced than at room temperature (see figure 27a). The decrease of dissipation due to the adsorption of enzyme on the surface of gel suggested the formation of a rigid film (see figure27b), as observed at room temperature.

Adsorption of enzyme on the gel is stronger at 37°C than at RT. Moreover, desorption followed by rinsing is more pronounced comparing to the room temperature. Further evaluation about rigidity of the film is presented in section 4.2.2.

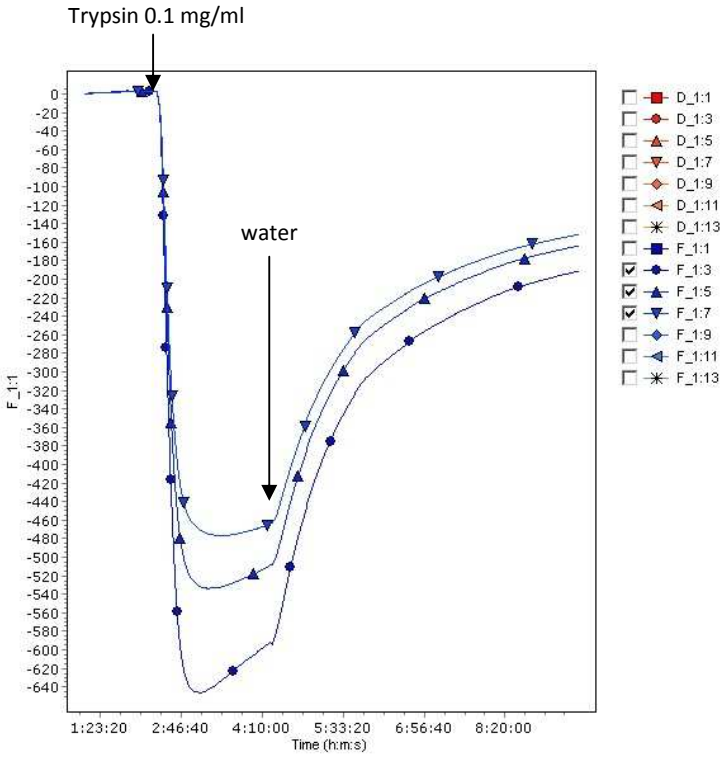


Figure 27 (a). Variation of frequency due to adsorption of enzyme on the surface of swollen gel at 37°C.(in this graph just adsorption of enzyme is shown. Data related to gel swelling has been removed)

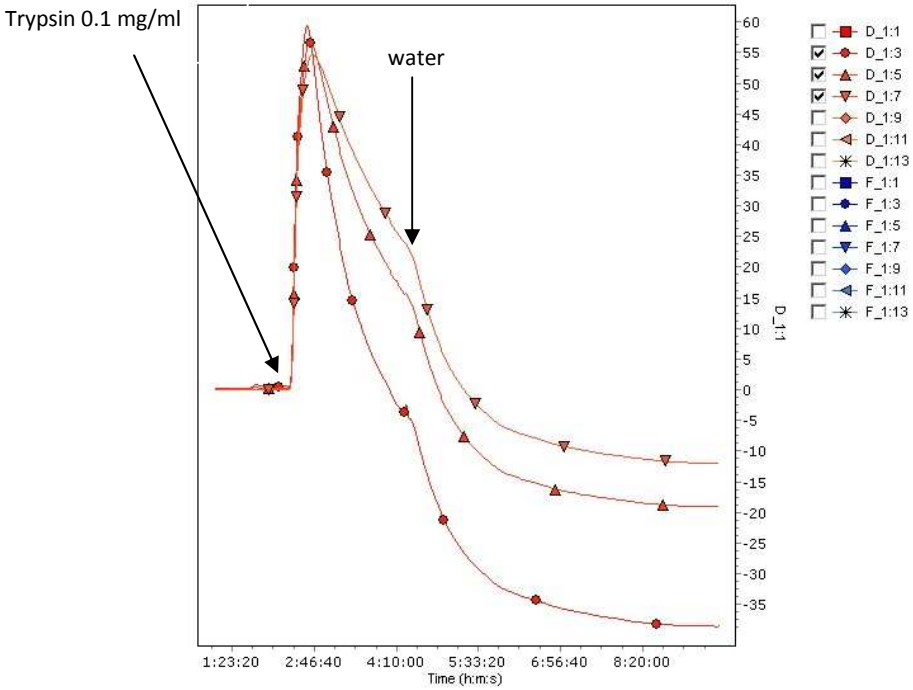


Figure 27(b). Changes in dissipation changes due to adsorption of enzyme on the surface of swollen gel at 37°C.(in this graph just adsorption of enzyme is shown. Data related to gel swelling has been removed)

3.4 Route2: Layer by layer formation of the film

In this approach layer by layer adsorption of polymer chain on the surface will result in a film of polymer chains on the surface of crystal. First, the polymer is adsorbed on the surface and then calcium chloride is injected to cross link the polymer chains. This procedure will result in a layer of cross-linked polymer film on the surface of crystal. The effect of the ambient conditions and adsorption of organic cations on the structure of polymer film was first studied.

3.4.1 LbL formation of the film

A film of alginate polymer was built on the surface of APTMS modified crystal by adsorption of alginate on the positively charged surface from 0.1 % (w/v) alginate solution in Tris buffer 0.1 M at pH 7.6. The procedure was followed by introduction of calcium chloride (50 mM in Tris buffer 0.1 M at pH 7.6) to the crystal that can cross-link alginate strands on the surface. This was continued by second flow of alginate solution and calcium chloride as a cross-linker. Each step was followed by rinsing with Tris for 3 minutes with Tris buffer 0.1 M pH 7.6 to avoid clogging in the tubing system. Finally, the system was rinsed with Tris buffer 0.1 M pH 7.6 (See figure 28). The overall shift in frequency was around 25 Hz which correlates to 44 nm, the thickness of polymer film when the data were fitted with the Voigt model.

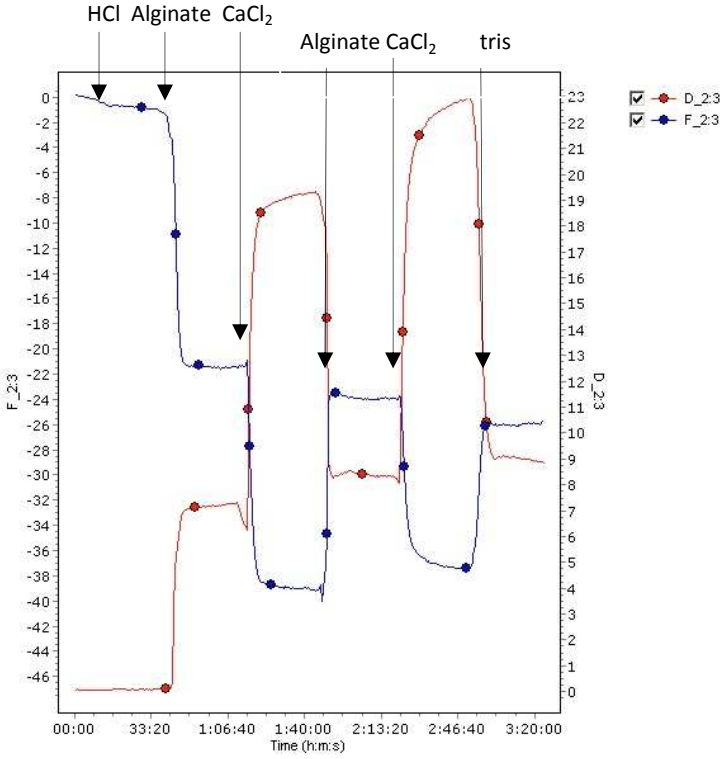


Figure 28. Built up of the polymer film. Blue curve: variation of frequency; red curve: variation of dissipation

3.4.1.1 Polymer film ambient condition

pH effect

A polymer film was built on the surface of the crystal as previously described. All solutions used during the film formation were in 0.1 M Tris buffer at pH 7.3. Then, 0.1 M buffer solutions at different pH (7.8, 8.25, 8.75 and 9.3) were pumped through the modules. The pH of solution was chosen in the range of ±1 of the pKa of Tris (8.06) ±1. By increasing the pH, both frequency and dissipation increases. It can be assumed that by increasing the pH, the polymer chains are less bounded to the surface so both frequency and dissipation increase. Conversely, at lower pH values polymer chains are tightly bounded to the surface. Reversible behavior of polymer film was observed in exchanging solution from pH 9.3 to 7.3, where frequency and dissipation shift decreased. See curves in figure 29.

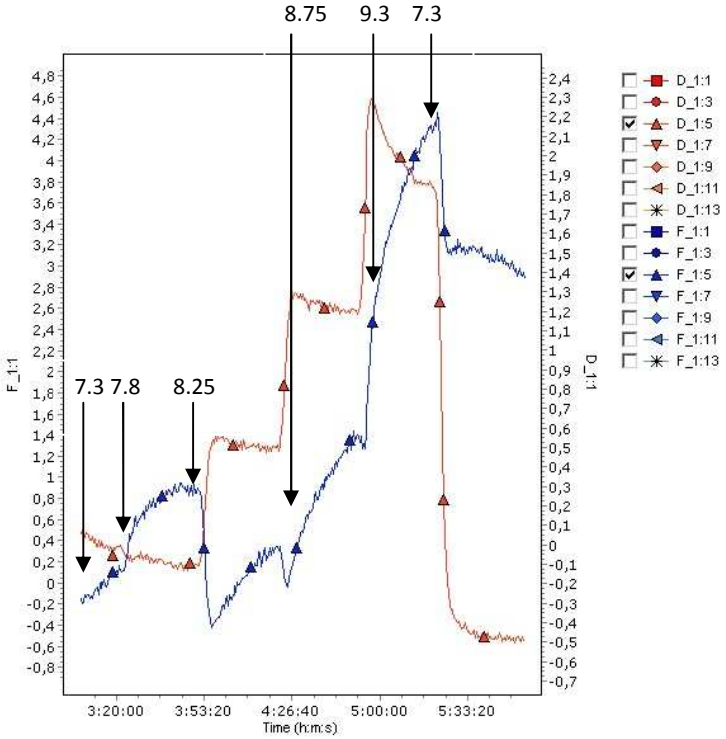


Figure 29. Variation of frequency (blue) and dissipation (red) due to interactions of polymer film with solutions at different pH. (For emphasis on pH effect, the rest of curve related to the building of the film has not been shown).

Ionic strength effect

A polymer film was built in Tris buffer 50mM at pH 7.6. Tris solutions at concentration 100 and 150 mM at pH 7.6 were introduced to the device, respectively. Finally the system was rinsed by 50 mM Tris solution at pH 7.6. Increasing the ionic strength of solution led to a decrease in frequency and an increase in dissipation which can be observed for a film swelling. It also shows a reversible behavior when solution was adjusted at 50 mM highly concentrated salt solution may have higher density and viscosity, which can induce artifacts known as bulk effect.[30]

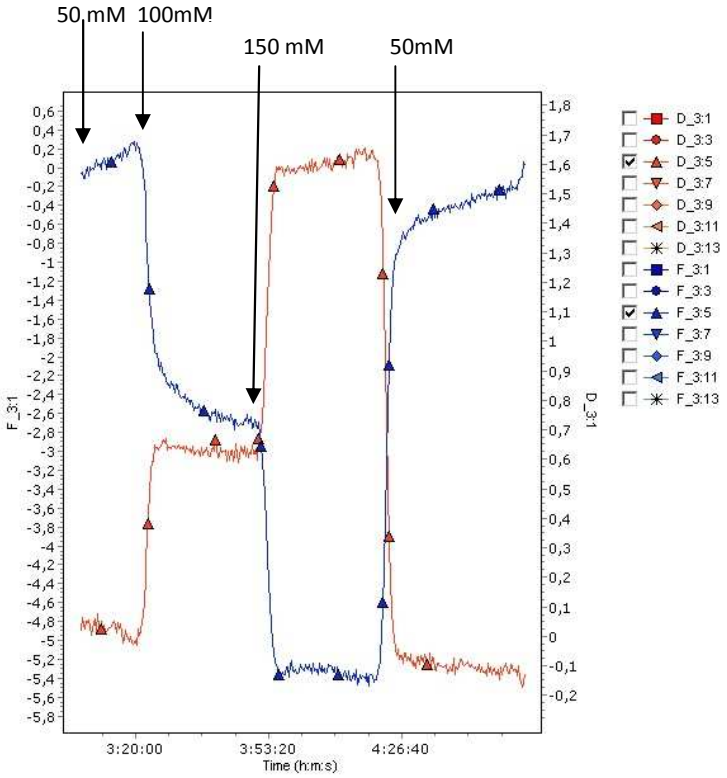


Figure 30. Variation of frequency (blue) and dissipation (red) due to ionic strength effect. (For emphasis on the effect, the rest of curve related to the building of the film has not been shown).

3.4.2 Adsorption on the surface of polymer film

3.4.2.1 Adsorption of L-BAPNA and D-BAPNA

The polymer film was built using 0.1 M Tris buffer at pH 7.6. DMSO (5% v/v) in water was pumped through the chambers. After reaching equilibrium L-BAPNA (4 mM in DMSO 5%) were pumped through two modules. Simultaneously, D-BAPNA (4 mM in DMSO 5 %) was pumped through the other two modules. Both L and D-BAPNA adsorb on the surface of polymer film. Variations of frequency and dissipation eventually did not stabilize. Finally, the four modules were rinsed with DMSO 5% v/v to monitor the desorption of L- and D-BAPNA from the polymer film. No significant difference was observed between adsorption of L-BAPNA and D-BAPNA in variation of frequency but dissipation changes was higher for L-BAPNA in one experiment (see figure 31).

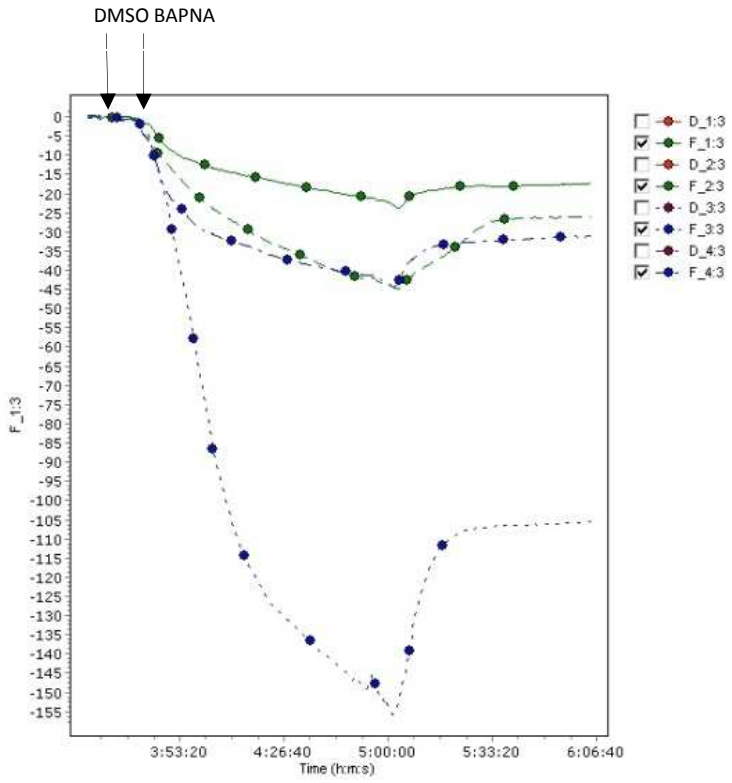
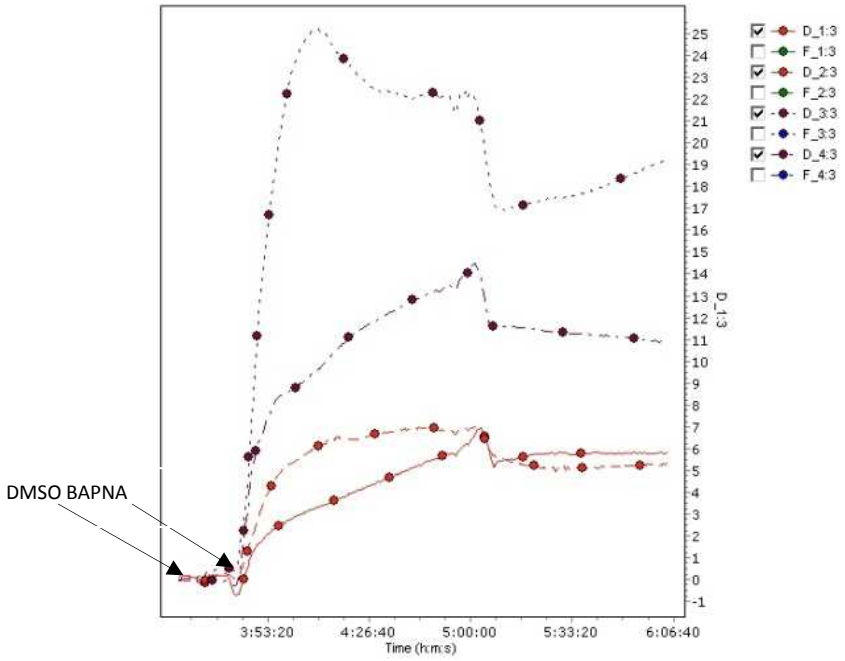


Figure 31. (a) Variation of frequency due to interaction of L-BAPNA (blue) and D-BAPNA (green) with polymer film on the surface of QCM crystal.



(b)

Figure 31. (b) Dissipation changes due to interaction of L-BAPNA (dark red) and D-BAPNA (red) with polymer film

3.4.2.2 Adsorption of TMA

The polymer film was built using 0.1 M Tris buffer at pH 7.6. TMA at concentration 4 mM in Tris was pumped through the modules. No adsorption was observed even at higher concentration of TMA (20 mM). (See figure 32)

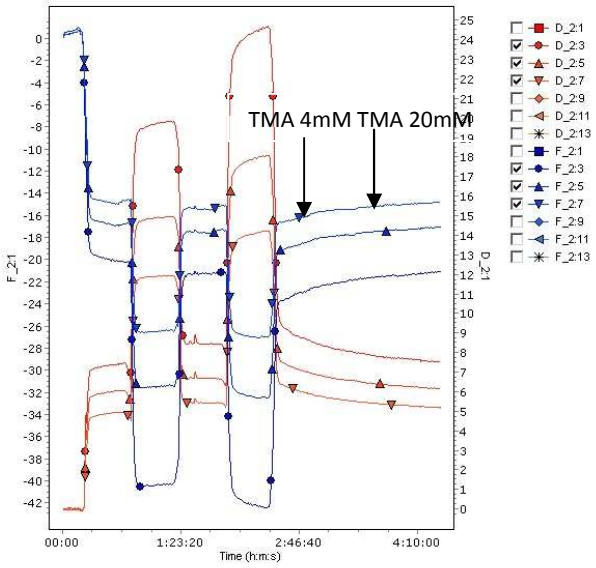


Figure 32. Adsorption of TMA (tetramethylammunium)

3.4.2.3 Adsorption of L-Arginine

The polymer film was built up using 0.1 M Tris buffer at pH 7.6. In this case the polymeric film consisted of two alginate layers. The upper layer is supposed to be non-crosslinked alginate layer. At the end the polymer film was rinsed with buffer. The solution of L-Arginine at 20mM in 0.1 M Tris pH 7.6 was pumped through the modules at rate of 50 µl/min. Small variation in frequency and dissipation can be considered as small adsorption on the surface of polymer strands. One can also consider that rinsing with buffer at the end can cause desorption from the polymer strands. (See figure 33).

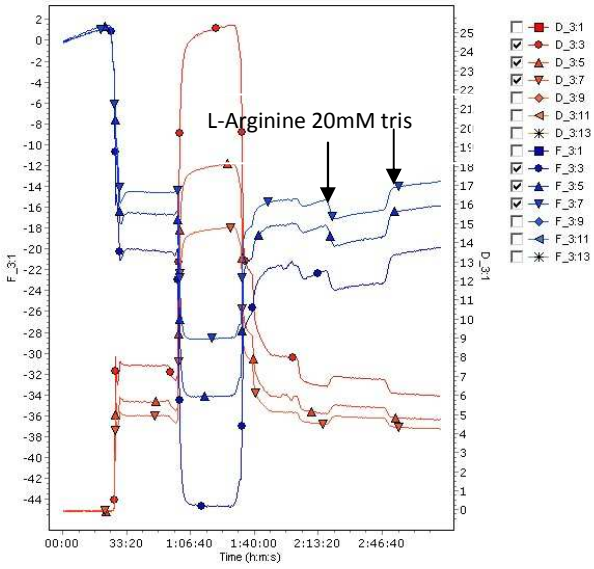


Figure 33. Adsorption of L-arginine on the surface of polymer film

3.4.2.4 Adsorption of Ac-Arg-OMe

The polymer film was built using 0.1 M Tris buffer at pH 7.6. The solution of Ac-Arg-OMe at concentration 4mM in Tris buffer 0.1 M pH7.6 was pumped through the instrument at a rate of 50 µl/min. No significant adsorption was observed. (See figure 34).

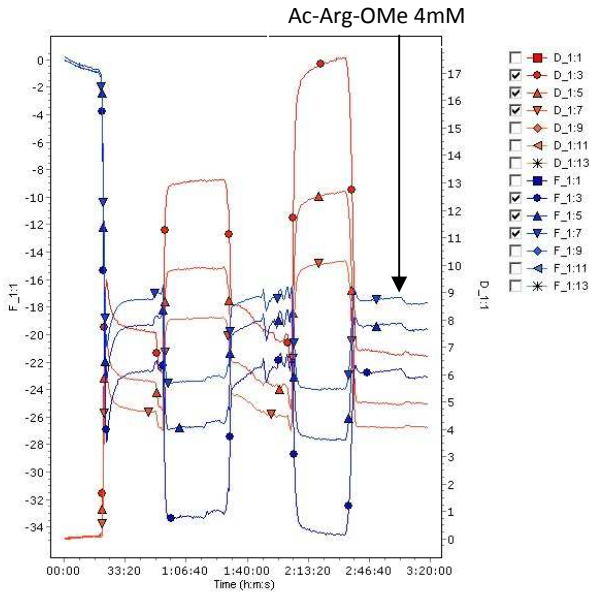


Figure 34. Adsorption of Ac-Arg-OMe on the surface of polymer film

3.4.2.5 Adsorption of H-Arg-βNa

The polymer film was built using 0.1 M Tris buffer at pH 7.6. The solution of H-Arg-βNa at 4mM in Tris 0.1M pH 7.6 was introduced on the crystals at a rate of 50 µl/min. At the end, the system was rinsed with buffer. The variation of frequency shows adsorption of the molecules on polymer strands. Dissipation changes are not remarkable. The adsorption was followed by rinsing the film with Tris buffer. A sharp peak in frequency and dissipation variation shows some film rearrangement and then is followed by desorption of molecules from the polymer chains as it is shown in figure 35, when the frequency shift increases and the dissipation decreases.

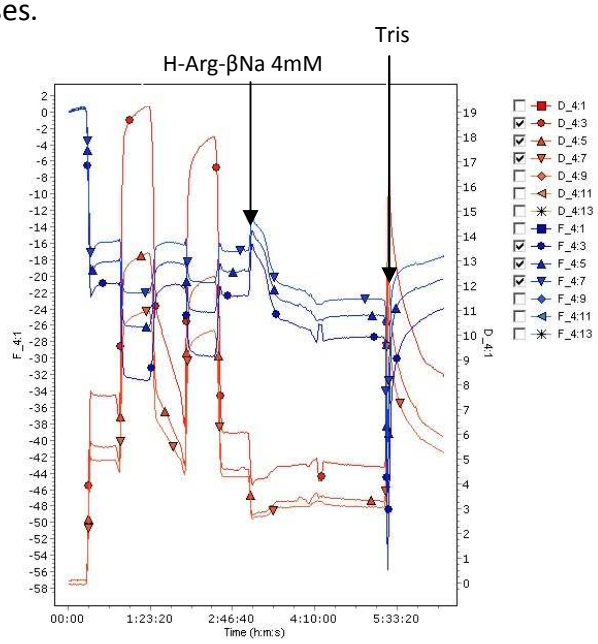


Figure 35. Adsorption of H-Arg-βNa on the surface of the polymer film

3.4.2.6 Adsorption of enzyme at room temperature

The polymer film was built using 0.1 M Tris buffer at pH 7.6. Trypsin at the concentration 0.1 mg/ml in Tris 0.1 M at pH 7.6 was pumped through the modules. A strong decrease in variation of frequency shows a remarkable adsorption of the trypsin to the film. However, rinsing the film with Tris causes the enzyme desorption from the polymer film and the frequency shift almost returned to the initial state, as shown in fig. 36. Dissipation did not return to the original value, although it was affected by the rinsing.

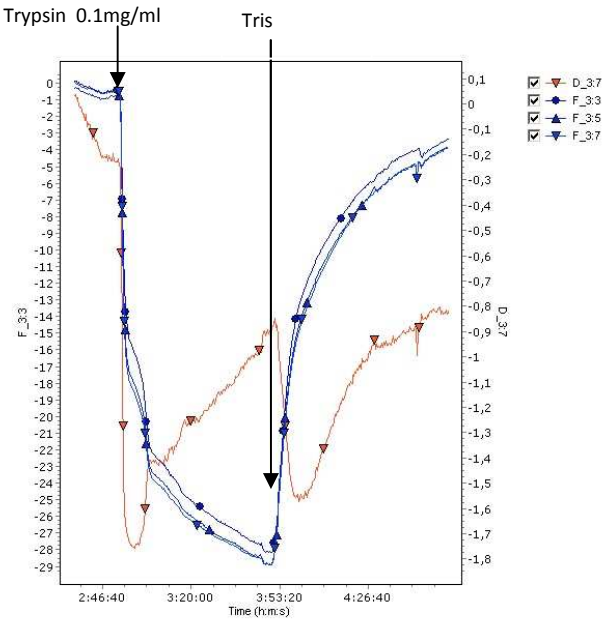


Figure 36. Variation of frequency (blue) and dissipation (red) due to adsorption and desorption of enzyme on the surface of polymer film (in this graph just adsorption of enzyme is shown. Data related to film formation has been eliminated)

3.4.2.7 Adsorption of enzyme at 37°C

The adsorption of enzyme on the surface of polymer strands was monitored at 37°C. The polymer film was built on the surface of modified silica crystals with APTMS at 37°C. The same procedure which has been described earlier was followed to form a film on the surface of the crystals at 37°C. Enzyme solution in Tris buffer (0.1 M pH7.6) at 0.1 mg/ml was introduced into the system at 37°C. A sharp decrease in frequency shows strong adsorption of enzyme. However, dissipation decreased as well, showing that the oscillating crystal is less damped due to enzyme adsorption. After about 45 minutes the system was rinsed with Tris buffer to monitor the desorption pattern. Figure 37 shows increasing frequency shift and dissipation when system is rinsed with buffer returning to the original values.

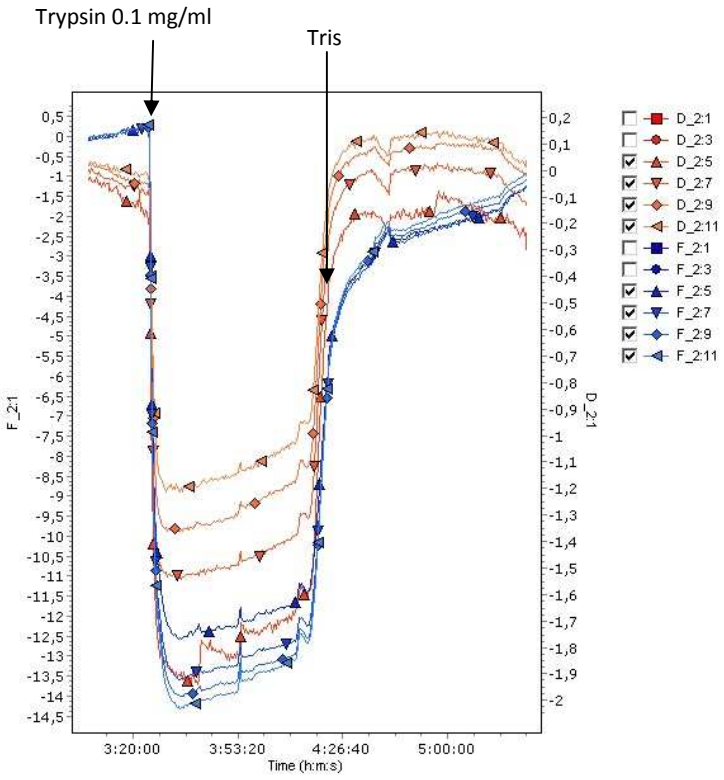


Figure 37. Adsorption of enzyme on the surface of polymer film at 37°C

Chapter four

Discussion

4.1 Thickness of the film

As previously mentioned in the introduction, there are two main models by which viscoelastic property of the material can be determined, Voigt and Maxwell model. Using these models the mass or thickness of the film on the surface of oscillating crystals can also be determined. The Voigt model is based on the description of the system by a dashpot and a spring mounted in parallel, as illustrated in figure 38a. In such case the paralleling of the viscosity (dashpot) and the modulus of elasticity (spring) allows a description of film with a solid-like character. The Maxwell model is fairly similar in the sense that it is composed of a dashpot and a spring, but mounted in series, see figure 38b. In rheology, it is usually used to describe fluid systems. In this work, two types of film were prepared, thick and thin films respectively. All data obtained by the two approaches were modeled with the two mentioned systems, which were embedded in the software of Q-sense. It was observed for spin coated system (thick film), the Maxwell model fits the data while for the second system, the polymer film (thin film), the Voigt model fits the data. In another meaning, swollen gel in spin-coated system is mostly viscoelastic fluid film while polymer film from layer by layer built up is a viscoelastic solid film. Calculated thickness in each system was highly dependent on quality of APTMS treatment and the surface of crystal. However, the average thickness of polymer film described by the Voigt equation is between 12 to 50 nm and for a swollen gel described by the Maxwell equation, the thickness is about 20µm.

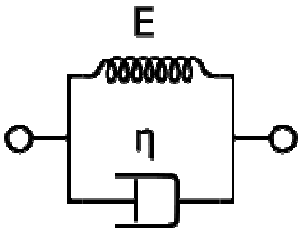


Figure 38 a

Figure 38 b

4.2 Comparison of the effect of adsorption of cations

QCM with dissipation monitoring provides information about viscoelastic properties of film on crystals. Due to viscous losses in the structure of a soft film, dissipated energy of the crystal is higher relating to the storage energy, which means that Q factor (damping) is higher for a soft film compared to a rigid one (Eq.2). Comparing $\Delta D/\Delta f$ for binding of different molecules on the surface of swollen gel or film of polymer enable us to have deeper understanding of the rigidity of the film over crystal in different surrounding stimuli or when it is exposed to different molecules.[31] QCM with dissipation enables us to interpret the change of viscoelastic property of formed film due to adsorption of different molecules in

two approaches which have been described earlier. Prior to this, the results from the QCM-D measurements were gathered in the following table (table 2). It aims at giving an overview of the results obtained.

	Gel				Polymeric film			
	During adsorption		After rinsing*		During adsorption		After rinsing*	
Molecule	Δf	ΔD	Δf	ΔD	Δf	ΔD	Δf	ΔD
TMA	increase	decrease	same	same	same	same	same	same
L-Arginine	increase	decrease	decrease	increase	decrease (slight)	increase (slight)	increase (slight)	decrease (slight)
Ac-Arg-OMe	increase	increase	decrease	decrease	same	same	same	same
H-Arg-βNa	increase	decrease	increase	same	decrease	decrease	increase	decrease
L-BAPNA	increase	decrease	increase	decrease	decrease	increase	increase slight	decrease slight
D-BAPNA	increase	decrease	increase (slight)	decrease (slight)	decrease	increase	increase (slight)	decrease (slight)
Enzyme at RT	decrease	decrease	increase (slight)	same	decrease	decrease	increase	same
Enzyme at 37° C	decrease	increase	increase	decrease	decrease	decrease	increase	increase

* relative to the adsorption step

Table 2. Variation of frequency and dissipation due to the adsorption of charged molecules on gel and polymeric film

In order to fully understand the pictures depicted by QCM-D measurements, it is necessary to keep in mind the fact that under the flow of positive organic cations the gel can undergo four different situations: nothing happens, the gel shrinks, the gel swells or there is dissociation. In the first case, there is neither interaction nor adsorption of the cation and the gel structure remains unchanged. This situation will of course induce no change in the frequency and dissipation response. This was never observed during this study.
In the case where the gel shrinks, the variation of the frequency of oscillation will be positive because of the loss of bounded mass (water released from the gel) while the dissipation will decrease, as a consequence of the film being more rigid. The reversibility of the phenomenon can be assessed upon rinsing. Furthermore, the monitoring of the adsorption on the polymeric film (i.e. the LbL film) gives details about the mechanism of the swelling.
Indeed, for the swelling of the gel, the variation of the frequency and dissipation observed would be the opposite, i.e., the shift of frequency will be higher and dissipation will increase.
The last case discussed, the dissociation of the gel, implies a partial or complete loss of the gel structure, leading in some case to the desorption of the gel from the surface. This effect results from a softening of the gel followed by desorption, that is observed as an increase in

frequency along with increase of the dissipation.
The case of the interaction with large macromolecules such as an enzyme has to be regarded as an exception. In this situation the adsorption, and then the resulting change of mass cannot be neglected and will induce significant change, as large as the one observed for the changes of the gel structure.

The changes observed in the QCM-D response for the gel for TMA, the smallest cation observed, seemed to indicate a strong change in the gel structure, irreversibly. The increase in Δf along with a decrease in ΔD can indicate a complete dissociation of the gel. The reason for this could be the strong osmotic changes in the solution because of TMA, since no adsorption could be seen in the polymeric film.

The situation in presence of arginine is fairly different, and it is possible to anticipate that the osmotic effect will be less. Furthermore, the study on the polymeric film seems to indicate that there is reversible adsorption. This adsorption changes the structure of the film which shrinks.

In comparison with arginine, Ac-Arg-OMe has amines and acid groups that cannot get charged, having only the group on the side chain available for electrostatic interactions. Despite a very weak adsorption of the molecule on the polymeric film, fairly important modifications were observed on the gel structure, which seems to undergo a reversible softening in presence of the cation. This softening could be explained by Donnane equilibrium, mentioned earlier.

H-Arg-βNa represents a more hydrophobic character because of the naphthalen ring, while it has a neat positive charge. In a sense, it has an amphiphilic character. On a polymeric film, the adsorption observed was reversible. As a consequence, this leads to the partial dissociation of the gel.

The last two molecules adsorbed, L-BAPNA and D-BAPNA, gave very similar results in both the gel and the polymeric film. Accordingly the gel seems to shrink as a result of the reversible adsorption of the cation (fairly hydrophobic) on the gel strands, without any calcium displacement.

The effect of the adsorption of enzyme on the gel at room temperature and 37 °C is the same. Decrease in frequency shift and dissipation shows that trypsin strongly adsorbed to the gel, leading to the formation of a more rigid film at room temperature. The adsorption of trypsin at 37 °C leads to a softer film. After rinsing the system at room temperature, less amount of material left the structure and the dissipation remained almost the same but at 37 °C, the rinsing triggered the release from the structure which is seen in the form of an increase in frequency shift and a decrease in dissipation. Therefore, temperature can affect desorption profile of trypsin drastically.
The decrease in the frequency shift and in the dissipation shows that positively charge trypsin adsorbed tightly on the polymeric film at both room temperature and 37 °C. Rinsing the system at both room temperature and 37 °C led to an increase in frequency shift and dissipation which means in both cases the adsorbed mass was removed from the surface.

We hypothesize that the increase in dissipation is due to the displacement of water into the structure, forming a softer film.
Comparing the adsorption and desorption profiles of trypsin on the gel and polymeric film shows that in both cases at two different temperatures, trypsin adsorbs but in the case of the gel we assume that the enzyme diffuse into the structure and make the gel structure more rigid. In this case diffused trypsin cannot readily be removed by rinsing but at higher temperature the process can be affected (see table 2). Assumingly, at higher temperature the affinity of trypsin to adsorb and remain within the structure is less compared to room temperature.

4.2.1 Variation of viscoelastic property of the gel

All data from QCM-D measurement related to adsorption on the surface of gel were offset at the point of stabilized signals related to the swollen gel. Only the adsorption process has been considered in this evaluation. To be able to compare the adsorption phenomena the fifth overtone of each experiment was used.

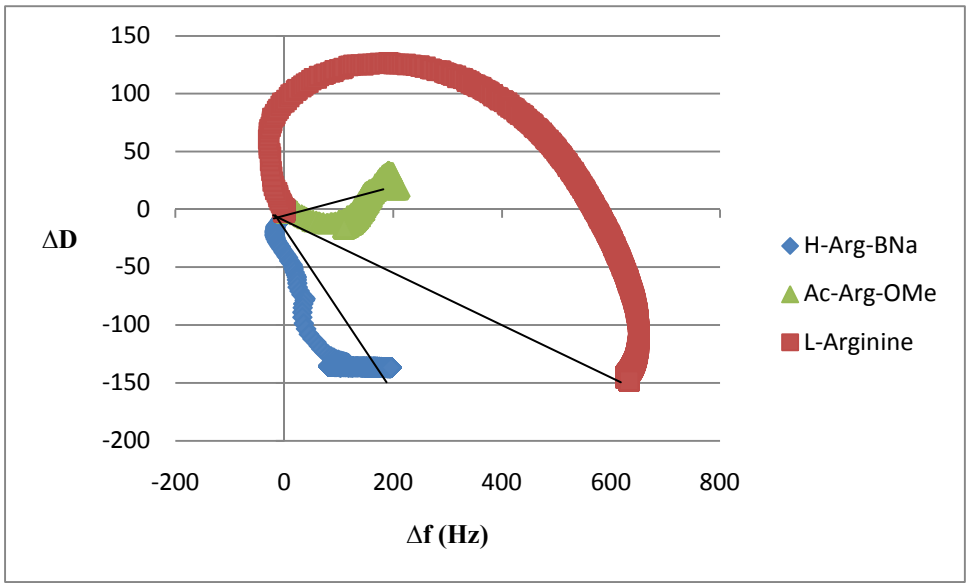


Figure 39(a). ΔD vs. Δf describing the effect of different molecules on gel stability

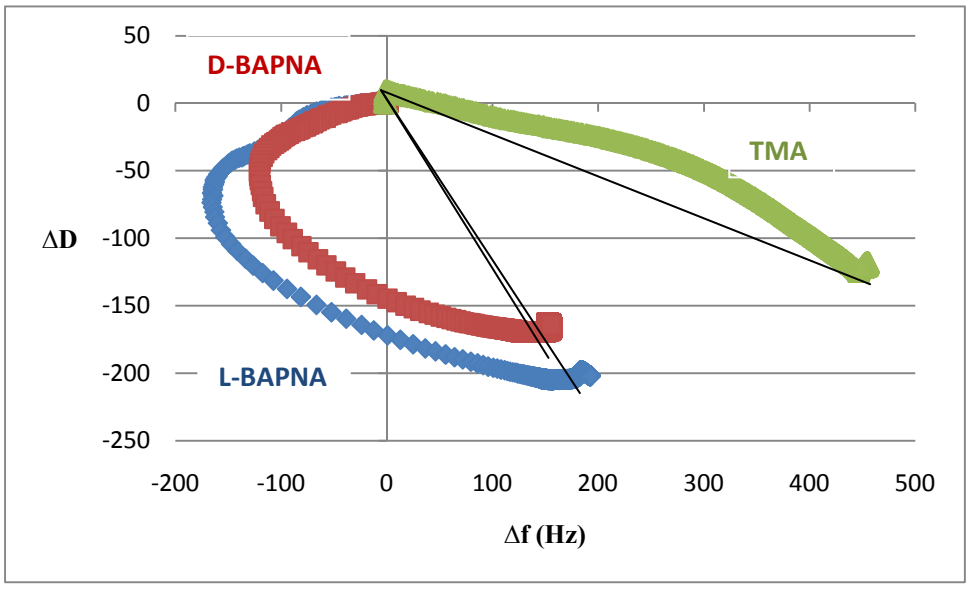


Figure 39 (b). ΔD vs. Δf describing the effect of different molecules on gel stability

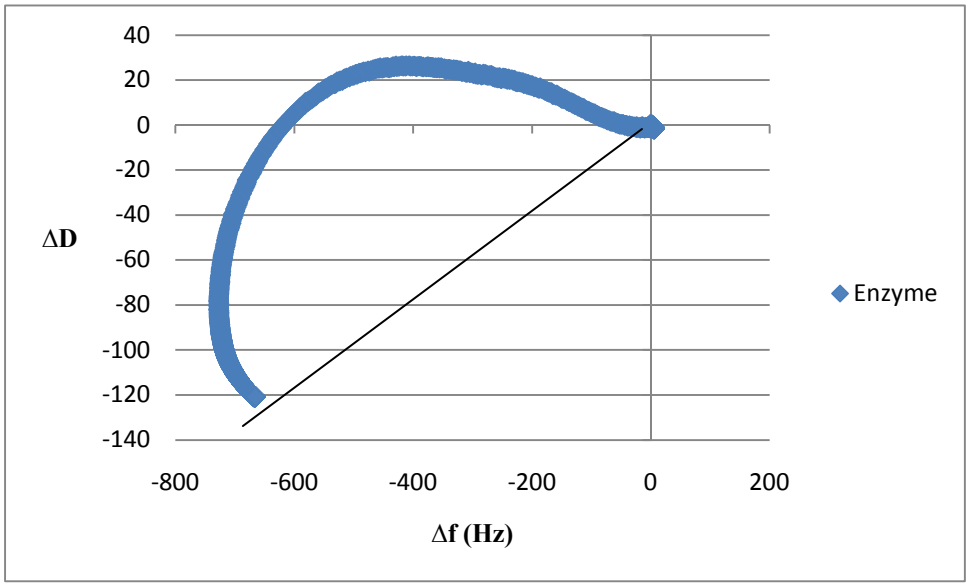


Figure 39 (c). ΔD vs. Δf for enzyme

Positive values for the frequency shift with decreasing ΔD for the adsorption of L,D-BAPNA, TMA, L-Arginine, H-Arg-βNa and Ac-Arg-OMe show a gel shrinkage or disturbance of the gel structure. Negative values of Δf on the initial stage of adsorption for L- and D-BAPNA shows adsorption of these molecules while decreasing dissipation can be considered as a release of water from the gel structure and film shrinkage. This observation is in agreement with bulk experiments.

Furthermore, graphs 39a, 39b and 39c clearly show changes in the film structure due to the interactions with the cations. However, if only the initial point and the last point of each adsorption considered, the slope for H-Arg-βNa is the highest among the others which means that remaining film on the surface is softer in comparison with others.

Negative value of the frequency shift for the enzyme with decreasing dissipation at last stage of adsorption shows that gel structure become more rigid due to the enzyme adsorption.

4.2.2 Adsorption of enzyme on surface of gel at different temperature

Negative values for shift in frequency with decreasing dissipation show that enzyme adsorb in the structure of the gel and that the structure becomes more rigid. Higher values in frequency in figure 40 suggest that at room temperature more enzymes absorbs in the film structure. Moreover, initial values of $\Delta D/\Delta f$ (slope of curve) at 37°C is higher compared to room temperature which means that gel structure is softer at 37°C. As it is clearly shown, rigidity of film in both cases increases by adsorption of enzyme. The gel becomes more rigid since the amount of adsorbed enzyme at room temperature is higher.

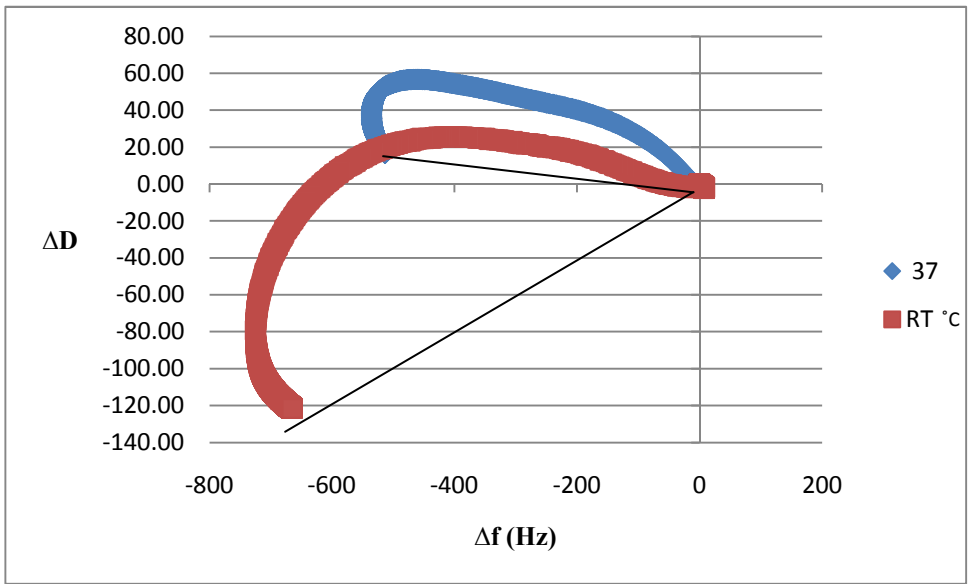


Figure 40. Comparison of adsorption of enzyme on the surface of gel at 37° C and room temperature

4.3 Variation of viscoelastic property of the polymer film

All data from QCM-D measurement related to the adsorption on the surface of the gel were offset at the point of stabilized signals related to the formed film. Only adsorption process has been considered in this evaluation. To be able to compare the adsorption phenomena the fifth overtone of each experiment was used.

According to obtained results which are shown in graphs 32, 33 and 34 TMA, L-Arginine and Ac-Arg-OMe do not adsorb on the surface of the polymer film while L, D-BAPNA and H-Arg-βNa and enzyme (trypsin) adsorb.

4.3.1 Adsorption of L,D-Bapna

Figure 41 suggests that D-BAPNA is less adsorbed in comparison with L-BAPNA. The thickness of the film on both crystals is estimated about 12nm according to the Voigt model. However, $\Delta D/\Delta f$ for D-BAPNA is higher, meaning that the rigidity of the film is lower.

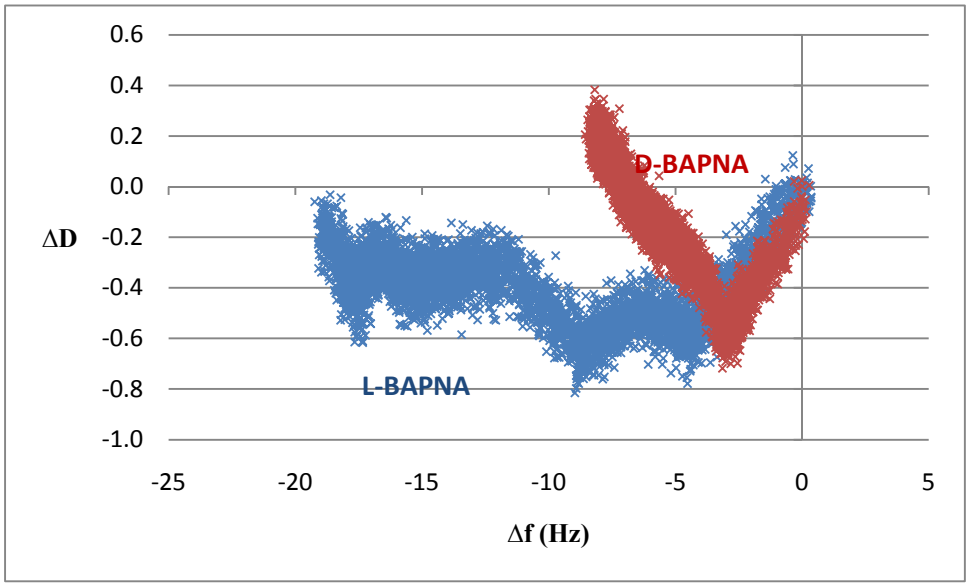


Figure 41. Adsorption of L- and D-BAPNA on the surface of polymer strands

4.3.2 Adsorption of H-Arg-βNa

First, an increase in frequency and a decrease in dissipation when the H-Arg-βNa solution introduced over surface of polymer strand show rearrangement of polymer strands in a way that polymer chains are about to leave the surface. After the rearrangement of film (point 1 in the figure 42), H-arg-βNa molecules adsorb on the surface of the polymer chains which can be seen as a decrease in frequency shift and an increase in dissipation changes, as shown in figure 41.

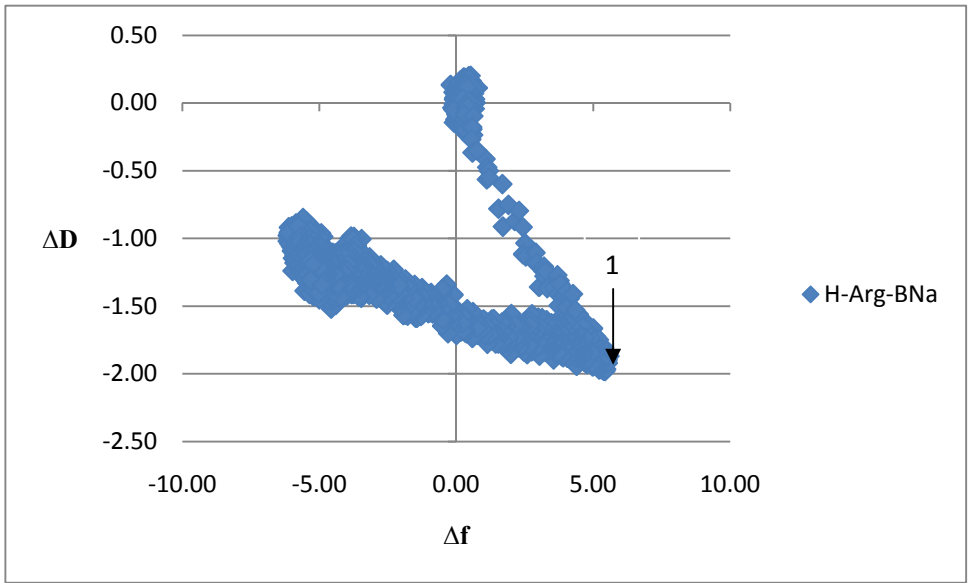


Figure 42. Adsorption of H-Arg-βNa on the surface of polymer strands

4.3.3 Adsorption of enzyme at different temperature

Comparing the mechanism of enzyme adsorption on the surface of polymer strands at two different temperatures shows that in both cases the adsorption of the enzyme causes a decrease of the dissipation and the formation of a rigid film on the surface of oscillating crystal. However, at 37 °C lower amount of enzyme is adsorbed. The slope of ΔD vs. Δf graph for adsorption at 37 °C is slightly higher compared with room temperature, which means that the binding of the enzyme to the polymer strands at room temperature forms slightly more rigid film. (See fig. 43)

According to the raw data for the formation of the film at different temperatures, the thickness of the film at 37 °C was less compared with the one at room temperature (results are not shown). Therefore, it can be assumed that the reason for less adsorption of enzyme at 37 °C would be due to less available surface of polymer strands.

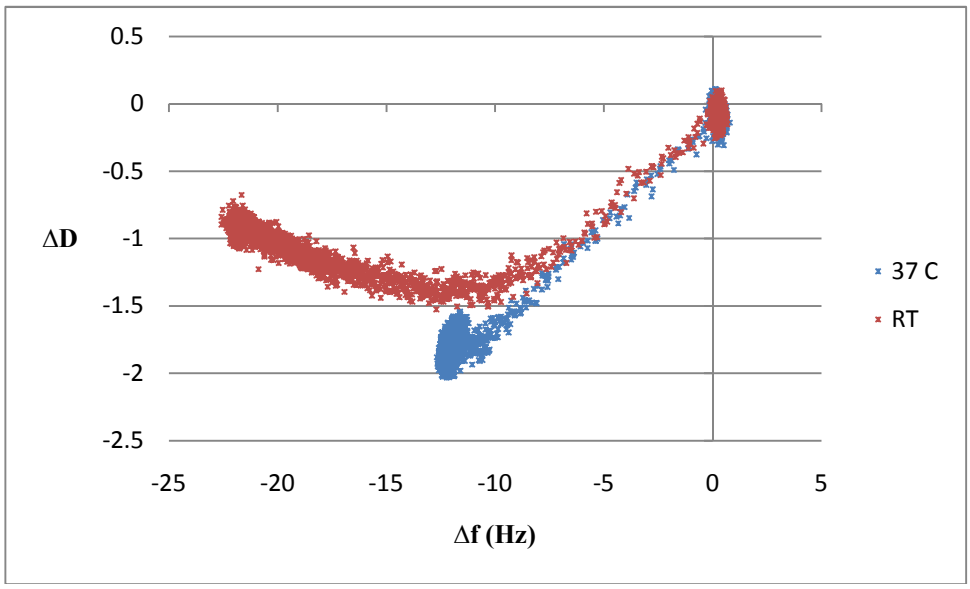


Figure 43. Comparison of adsorption of enzyme on surface of polymer strands at 37° C and room temperature

Conclusion

In order to study the effect of charged molecules on the gel structure with QCM-D two systems were developed: spin-coated gel and polymeric film. In the first one the effect of interaction of the charged molecules on the gel structure was studied and in the second, the interaction of polymer chains with charged molecules was of interest. Furthermore, the thickness of formed film in both cases was evaluated, using Voigt and Maxwell models. Moreover, viscoelastic properties of both gel and polymeric film while they were exposed to organic molecules were studied.

The results presented in this work show that, the swollen gel is a more fluid viscoelastic material while the polymeric film can be considered as a more solid viscoelastic material. Calculated thickness of the swollen gel in this study using Maxwell equation is about 20 µm and for polymeric film using Voigt model is about 44 nm. The results also show that the gel has a reversible behavior in different pH and ionic strength which can be described by Donnane effect.

It has been also investigated that organic cations which have no interaction with the polymeric film such as TMA and Ac-Arg-OMe and L-Arginine can disturb the gel structure reaching even dissociation. This effect can be explained by Donnane effect. On the other hand, cation such H-Arg-βNa which has amphiphilic property reversibly adsorbs on the polymeric film and cause the gel partially dissociates. The other two molecules L- and D-BAPNA which are hydrophobic adsorb on the polymeric film reversibly without calcium displacement and trigger the gel to shrinkage.

The effect of the adsorption of enzyme on the gel and polymeric at room temperature and 37°C is the same. The results show that at room temperature the enzyme adsorbs stronger on the gel rather than 37°C. Assumingly, affinity of trypsin to adsorb on the gel is less at 37˚C compared to room temperature.

Evaluation of ΔD/Δf of data reveals viscoelastic property of the film. Accordingly, adsorption of L- and D-BAPNA, TMA, L-Arginine, H-Arg-βNa, Ac-Arg-OMe soften the structure of the gel. The enzyme adsorption on the gel and polymeric film results in more rigid film due to diffusion of the enzyme into the gel structure at room temperature and 37°C. However, rigidity of the film is less at 37°C.

Based on these results, it can be assumed that organic cations and macromolecules interact with the gel structure which leads to variation of the gel stability. Furthermore, the hydrophobicity of organic cations plays a major role in the gel stability.

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