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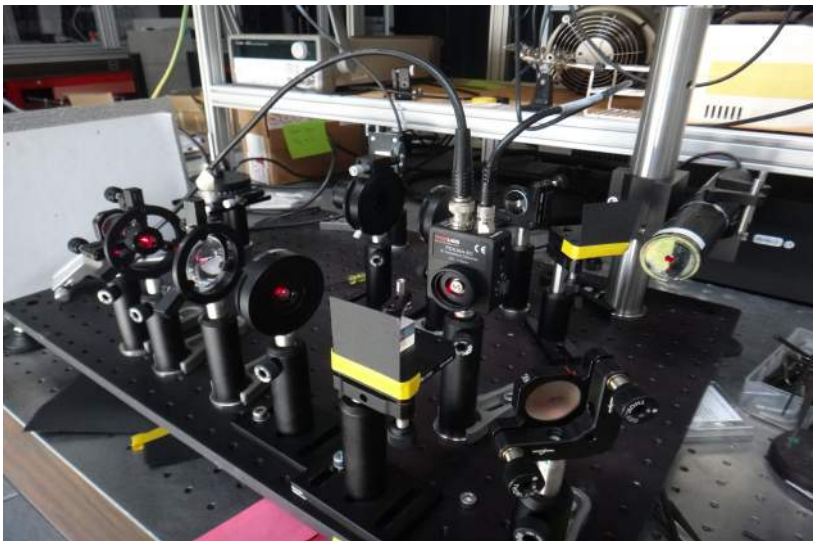
Functionalizing Nano Imprinted Cantilever for Meat Degradation Measurements



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Ying WANG

Abstract

Today, more than 30% food is wasted all over the world and cause a huge energy loss and serious environmental problem like carbon dioxide emission. Compare to crop, meat products need much more money and energy in producing, storage and transport, and wasted meat products impose heavier burden to environment. If the freshness of meat can be tested, it will be much more economic and time efficient for both meat supply industry and customer.

Micro and nano cantilevers have been well studied in recent years, many researches about biosensors based on those cantilevers were developed in the past decade. Micro and Nano cantilevers provide a promising way in sensing a very small amount of chemical or biological molecules. The subsequent bending or shift of resonance frequency of cantilevers will give a relative accurate results.

In this master project, we try to verify an idea that using such a micro cantilever to test the amount/concentration of cadaverine - one of the most common biogenic amines during meat spoilage and increase its amount over time. Functionalizing a micro cantilever is aiming to make a selective surface for cadaverine. Once cadaverine molecules bond to the cantilever, its resonance frequency which is proportional to effective mass will change. The resonance frequency is easily detected by AFM or possibly a simplified and cheap optical setup. In this way the cantilever is able to detect very low concentration of cadaverine and therefore gives a result of meat freshness.

This master thesis consists of 5 chapters. The first chapter will give a literature review of this project which contain food spoilage process and the related principle of cantilevers. The following chapter is the main part which focus on some details about functionalization, including chemical selecting, test method and result comparison. Next chapter will give several possible ways of building a setup to measure the resonance frequency of cantilever, in this part we will also show a possible optical setup. Chapter 4 is the part of measurements and results, where will also discuss the possibility of future application of the idea. The last chapter will discuss and conclude the result and give some reasonable future schedule.

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Chapter 1 Introduction

1.1. Background of Food Waste

Recent research indicates that 30%-50% of all produced food never have a chance to be hold in a plate on table[1]. Compare to products like fruits, vegetables, etc. in the food chain, the meat and dairy products from primary, secondary or even tertiary consumers as mammals, poultry and fishes consume mush more energy and resource. That also explained why with the same amount of food, meat is much more expensive than vegetables and fruits if we ignore the geographical restriction and other economic issue.

Apart from economic expense on meat industry, meat products need a much more complicated and strict conditions during storage and shipping. The bacterial and virus contained in meat is a bigger and more complex threaten to human beings compare to crop. According to some rough estimation from World Health Organization, nearly 75% new diseases are from animals to human and most of them were from table in the recent 10 years[2].

In addition, the decomposition of meat is a long process creates products like carbon dioxide which contribute a lot to Greenhouse effect and therefore add burden to our fragile environment. More seriously, the rotten meat contain some unknown virus without proper dealing will cause a widely spreading disease easily.

1.1.1. Meat Degradation and Toxic Products

Due to the complexity in meat industry supply chain, in this section we ignore animal disease and their health condition effected by environment pollution, only consider the protein decomposition during meat degradation to be the measurement of meat freshness testing.

The nutrients in fresh meat is mainly protein (usually 15%-30%, depend on different meat) and some fat. Certainly it also contain rich minerals, carbohydrate and a lot of water[3]. Protein are large biological molecules which is formed by a group of amino-acid with very similar structures[4]. The spoilage of meat during slaughtering, shipping, processing and storage can be basically divided into three mechanisms: microbial decomposition, lipid oxidation and autolytic enzymatic[5]. The three phases are illustrated in figure 1-1. The microbial or bacterial in meat or from environment will metabolize and produce different proteinases to decompose protein into different phases and thus to get energy for their proliferation. The group of proteinases endopeptidases will firstly decompose protein to peptides and soon they will be further broken down to amino acid by exopeptidases or endopeptidases. Those free amino acids will get through decarboxylation by amino acid decarboxylases and results in producing some toxic amines.

Early research indicates that the concentration of some amines such as cadaverine will increase during storage while the others like spermidine and spermine keep constant or decrease the amount[7][8][9] over time. The toxic effect of amines does not only varies by their own, but also is influenced by other

amines and other produced compounds. Certainly the storage or process condition is very essential to the results. For example, by interfering with the amino oxidases, the putrescine and cadaverien can enhance the toxic effect of tyrmine and histamine[10][11][12].

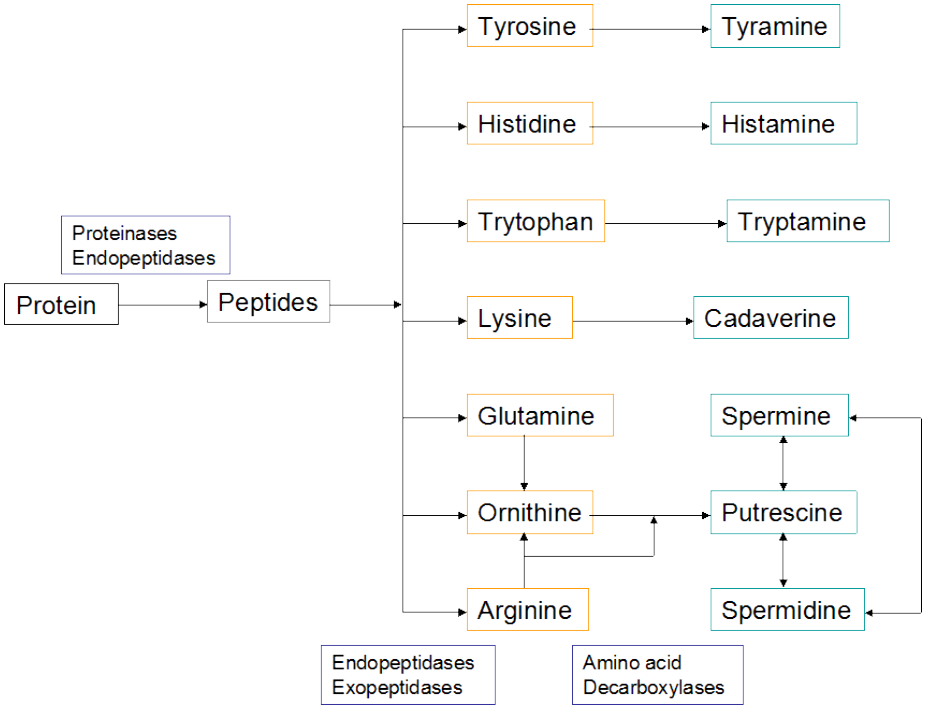


Figure 1-1. Formation of biogenic amines[6]

1.1.2. Selected Candidate - Cadaverine

Since the meat waste is a big problem all over the world, a fast and easy way of sensing the quality of meat during process or storage becomes more and more necessary. If the testing result of the meat is not very fresh, then the meat should be sold nearby instead of long-time transport or storage.

Apparently the toxic amines are the target for such a testing. Since after the decomposition of proteins, a lot of amines will exist in the old meat as shown in figure 1-1. The most common biogenic amines we could find in meat products are tyramine, cadaverine, putrescine and histamine[5]. However there are several requirements to choose the specific amines to be detected.

Firstly, the amount of such an amines should increase over time. The amines which decrease or remain constant over time will increase complexity of measurement calculation and comparison, or even cause a failure. Secondly, the toxicity of the amines should not be very strong during the experimental period for some safety reasons. And also, the amines should be now easy to fabricate and purchase in the market, certainly it should not be very expensive for low-cost consideration. In addition, it is better that the amines to have some odor to ensure the sensitive of such an sensor - if people can smell the meat is bad, it is already too late, therefore such an sensor should be able to detect a much lower concentration of the amines. Consider all the requirements, finally cadaverine was chosen as the amines to be detected in this project.

Cadaverine is the product from a specific amino acid called lysine after decarboxylation[13]. It always

comes with putrescine during the meat spoilage process. The formula of cadaverine is $NH_2(CH_2)_5NH_2$ and it has strong unpleasant odor[14]. Cadaverine can be absorbed through skin, eyes and mouth, the symptom of poisoning to human is coughing, shortness of breath, headache, nausea and etc. Tests from Wistar rats indicates that the no-observed-adverse level was 180mg/kg body weight per day for cadaverine[15] .

1.2. Solution for Meat Spoilage Detection

In order to make an efficient meat supply chain from industry to customer, a lot of methods have been applied to build a system for food freshness testing. Meat storage and meat products process is a traditional industry, a lot of findings from our ancestors have already prolong the fresh time of meat, like keeping them in a cold and dry environment. With the technology development of freezing and vacuum packaging, the meat can be stored for a much longer time and therefore people can eat meat from different place. But still, meat production is always in a long process and supply chain, there are so many chances and possibilities for fresh meat to get contaminated before they finally reach to customers' stomach.

1.2.1. Existing Ways for Meat Spoilage Detection

The most easy and common way to detect freshness of meat is just use senses of human beings like to see color by eyes, smell by nose or taste by tongue, it is a quite traditional way but the disadvantage is obviously lacking of the accuracy[16].

Temperature and PH value can be also used for meat detection, as we all known that the meat without any processing will degradate and give a change of temperature and PH value inside. However due to meat storage technology development this method is more commonly used to detect the storage standard condition instead of the quality of meat itself[16]. That is why recently more and more experts focus on amines detection for meat degradation.

1.2.2. Existing Methods for Cadaverine Detection

As the detection candidates for meat spoilage, cadaverine and other common meat biogenic amines have been studied for several decades. High concentration of cadaverine does not need any sensor, just a breath can solve the problem (due to the strong odor). However for relative low concentration which is undetectable for human nose, a sensor is needed. Several existing equipments and solutions are developed during recent decades.

Capillary electrophoresis(CE) is a relative cheap and simple technology which let electrophoresis performed in a capillary tube[17][18][19], as shown in figure 1-2. Electrolyte is filling in the capillary tube and the ends of tube is absorbed in two buffer reservoir. When a high voltage is applied to the ends of capillary tube, different molecules with their own velocity (depends on molecular weight) will be separated by the height of tube[21]. The sample injection can be done by replacing one of the buffer reservoirs[20].

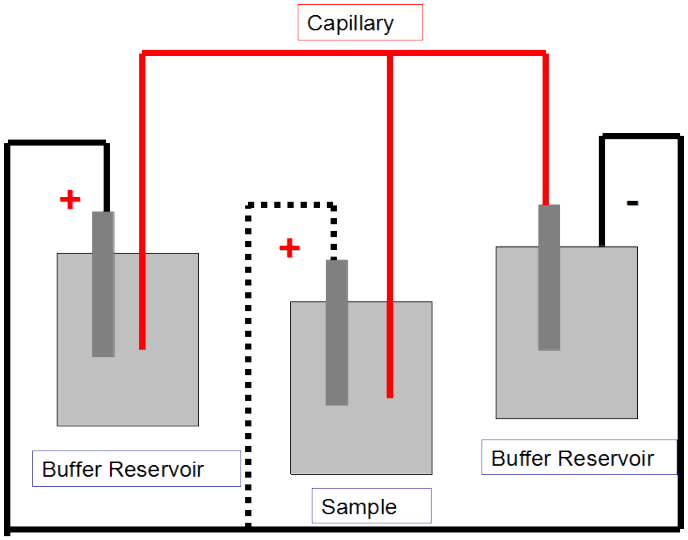


Figure 1-2. The instrumental setup of a CE system[20]

A more widely used equipment is called High-Performance Liquid Chromatography (HPLC). The working principle of HPLC is that different components in the sample will have different interactions with the stationary support, stronger interaction will result in slower movement[22]. Different molecules will be separated and detected by comparing the retention time (the time start at the point when sample is injected and the end point is when the specific compounds travel through the column to the detector and get the maximum peak height [23])to the database in the system.

Ion Chromatography (IC) shares a very similar working principles to HPLC. A considerable part of amines are weak base in water which means the they can provide specific positive ions. These ions flow to a separation column and interact with the fixed ions of negative charge[24]. The process is illustrated in figure 1-3, different ions will slow down to an extent characteristic[24] due to their own properties (According to recent research[25], with longer carbon chain and increased electronegativity of molecule, the slower amines move).

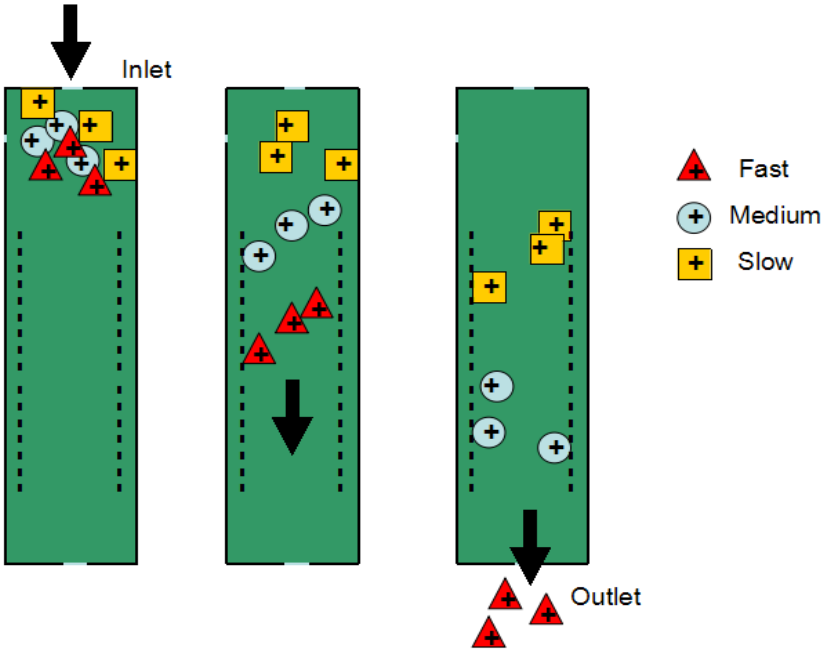


Figure 1-3. The instrumental setup of a CE system[26]

Another possible solution is thin layer chromatography (TLC), it provides a relative easy way of detecting different organic compounds in recent years. The working principle is also very similar to CE. A small drop of sample applied to the TLC plate, the mixture is developing through the plate due to capillary action. Different molecules will reach in different position via characteristic velocity[27]. Therefore a specific component can be detected and separated. The problem of this method is significant: when it turns to have the compounds have the same color in the mixture - even they were separate, it is hard to distinguish in the plate. Also the accuracy and resolution of the method is relative low. However, if experts could find out a better solution to add some color and make the TLC plate a better uniformity, it is still a very promising way for amines detection in the future.

The solution of previous three methods(CE, HPLC and IC) are very sensitive and have a very high accuracy. They were proved to be able to detect very small amount of toxic biogenic amines like cadaverine in drinking water. The biggest problem is that they need process for a very long time, usually it will cost a few days to do, and sometimes for accuracy the same sample need to be test for several times. Thus for the meat available on market, even the result indicates the meat should be throw away but it might be already sold to customers, which will have a risk in food safety.

These equipments are very big, fragile and some are very expensive, the operation is also complicated and dangerous (usage of some poisoning solution and chemicals). That means they are super user-unfriendly. For normal customers, it is nearly impossible for them to use those tools if they just want to test the meat they bought and to see if the meat is already getting bad before expiry date due to the poor clean condition of their fridge.

Consider those possibilities of those situation, developing a fast, sensitive and easy-carry sensor to detect the quality of meat to ensure the food safety is the only way to achieve economic and environmental future for meat market.

1.2. 3. Introduction to Electrochemical Biosensor

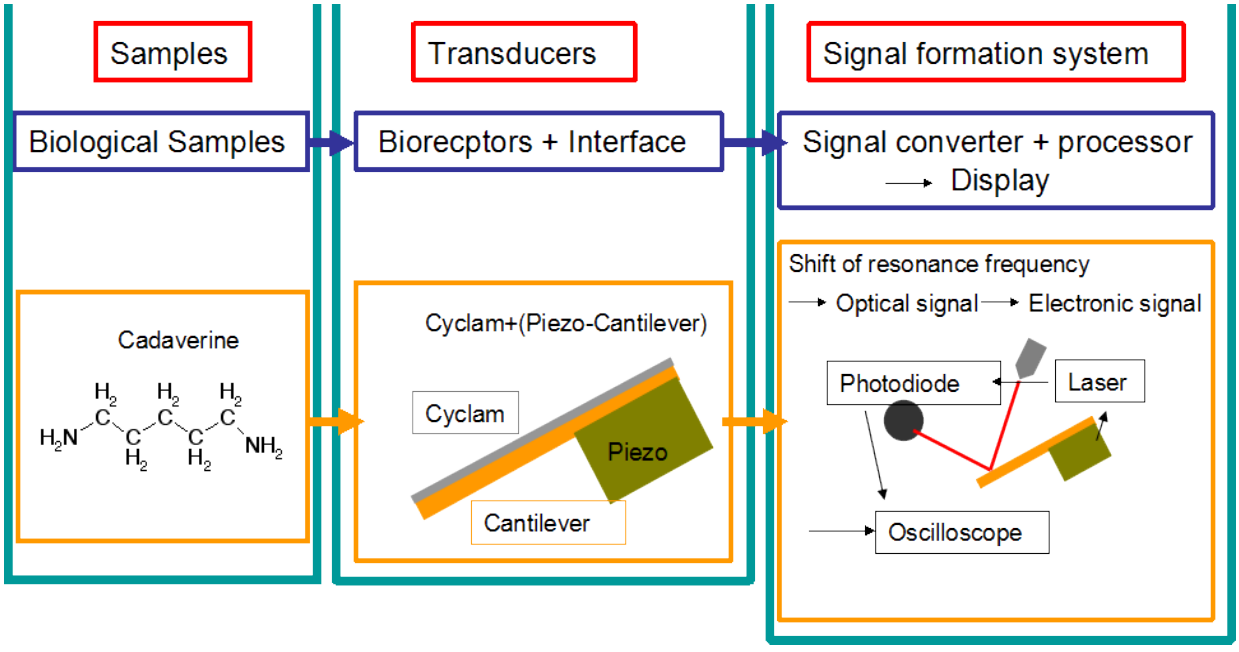


Figure 1-4. Basic elements and working principle of electrochemical biosensor[28]

The study of electrochemical biosensor is a fascinating field attracted a lot scientists' attention because of the advantages of cheap, fast and simplicity. The basic principles of an electrochemical biosensor is explained in figure 1-4. Such an electrochemical biosensor always define the biological material as the sensing element like the biological component or organism itself. The element called transducer in figure 1-4 indicates a conversion element from biological change to signals which is able to be detected by nowadays technology[29]. The last element is electronic system which base on the existing instrument and display the signals.

The biosensor opens a new door for small amount of amines detecting. In this project, the optical set-up is based on such a general solution. Since the testing target is cadaverine with very low concentration, the applying method is to use a micro cantilever transducers. First functionalize the micro cantilever with a specific bioreceptor. The cadaverine produced during meat spoilage will bond naturally to the selected receptor, thus result in a change of equivalent mass and furthermore a shift of resonance frequency. Instead of electrochemical signals but optical signals is used in this project which will be discussed in details in chapter 3.

1.3. Basic principles of Micro Cantilever (MC)

Nano or micro cantilever is a hot topic in recent researches as a biosensor or chemical sensor of small amount. Molecular adsorption onto a cantilever will shift its resonance frequency and its surface forces[30]. The operation of MC can be divided to static bending and dynamic deflection which will be discussed in details in section 1.3.1 and section 1.3.2.

1.3.1. Static mode of MC operation

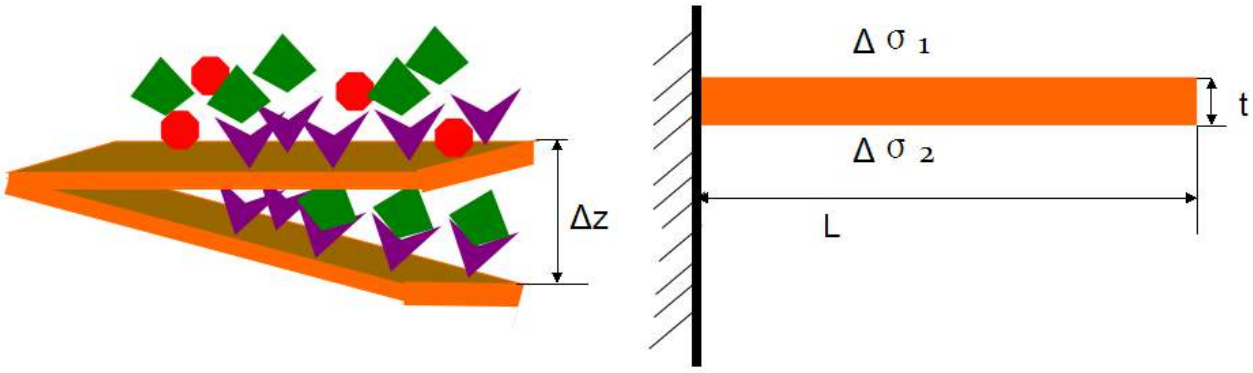


Figure 1-5. Schematic of MC biosensor working principle in static mode

* Cantilever mentioned in this thesis refers to micro cantilever

Static deflection is used to measure small amount of compounds adsorbed onto MC. More material absorption on the surface the more the cantilever will bend, deflect or distort[31][32][33]. If we consider the influence from environment like temperature as a constant and then we can compare the deflection before and after molecules' interaction to detect small amount of material. Such an interaction might

be results of van der Waals forces, surface hydrophobicity or conformational changes of adsorbed materials[34][35]. Although it is not fully understood about in static deflection of MC due to various possibilities of sensing. However a lot of study and research now are based on the model developed by G.G.Stoney in 1909[36]. Figure 1-5 shows the principle of work of the micro cantilever as a biosensor, after functionalizing a cantilever with specific chemical layer and then a solution contain the interest biological or chemical molecules can be applied. The cantilever beam will deflect after bonding due to surface stress change.

The free end displacement Δz of the cantilever after interest molecules bonding is related to the total surface stress change between its top and bottom surface ($\Delta\sigma_1$ - $\Delta\sigma_2$), which is typically reported by equation 1-1[36].

$$\Delta\sigma_1 - \Delta\sigma_2 = \frac{E \cdot t^2 \cdot \Delta z}{3 \times (1-v) \cdot L^2} \qquad (1\text{-}1)$$

Where *E* is Young's elastic modules of cantilever beam material, *L* is the length of beam, *v* is the Poisson's ratio of the material, and *t* here is the thickness of beam (include the functionalized chemical layer for higher accuracy).

The static mode is not widely used in biosensor application due to its accuracy of parameters in equation 1-1. The cantilever is always bond to a thin layer or even several layers on one side to be specific for one compound absorption. Therefore it is a bit tricky to determine thickness of cantilever, also it increases the complexity on calculating *E* and *v* because of some uncommon materials application, especially for some organic selective chemical layer.

1.3.2. Dynamic response of MC operation

In contrast to the static mode of micro cantilever, the dynamic oscillations will not significant change by residue or coating a layer on the medium of the surface[37]. Since it does not need to be functionalized by one side as the resonance frequency only depend on the total change of the mass[36]. Although the relation of the resonance frequency shift and mass change expresses a little differently from recent research, still it can be concluded in equation 1-2.

$$\Delta m = R \cdot k \cdot \left(\frac{1}{{f_1}^2} - \frac{1}{{f_0}^2} \right) \qquad (1\text{-}2)$$

Here *R* is a constant but different depends on the shape of micro cantilever, for example, it is $1/0.96\pi^2$ for rectangular cantilever[36]. And *k* here is the spring/force constant of the cantilever and it is only depend on the materials itself. Equation 1-2 here clearly indicates that the additional mass will reduce the resonance frequency, where f_0 and f_1 are the resonant frequency before and after the chemical bonding (mass change), respectively.

The biggest advantage of using resonance frequency shift of micro cantilever to detect a small amount of compound is very high sensitivity. Since the resonance frequency of such a small cantilever is very high and can reach to several hundreds kilohertz, therefore it could be a very small value in the ex-

pression of ($1/f1^2 - 1/f0^2$) and thus micro cantilever is able to test very small mass change. However shift of resonance frequency might also come from stiffness changes[38], which cause a small inaccuracy in dynamic mode.

The optical method with micro cantilevers for a simple implementation takes advantage of resonance frequency shift we talked above. In this scheme the displacement of micro cantilever free end is detected by measuring the reflected laser beam movement into position-sensitive photodetector (PSD) with mathematical measurements of the change in the optical path position from environment[36]. As we can see from figure 1-4. This project is based on such an idea and focused on functionalizing micro cantilevers and detecting small amount of cadaverine from meat.

2.1. Chemicals Selection

For functionalization of a micro cantilever, the first step is to select the right chemical layer coating on top of it. Such a layer play a role as a mediation buffer for both the metal layer on top of micro cantilever and amines.

2.1.1. Related Knowledge of Cadaverine

Although there is no significant evidence and data support indicates that cadaverine and other amines like putrescine play an important role as accomplice to histamine which can cause serious damages to human organ like stomach, lung and heart. The pure histamine taken by mouth showed a quite high level of poisoning amount to human, even to amount of 180mg does not give obvious symptoms, however by taking bad food/rotten meat will cause food poisoning at the level of 1.5mg/kg body weight[39]. Therefore it can be concluded that other amines could enhance toxicity though the interaction of enhancement is still unclear now. The image and molecular structure of cadaverine is shown in figure 2-1.

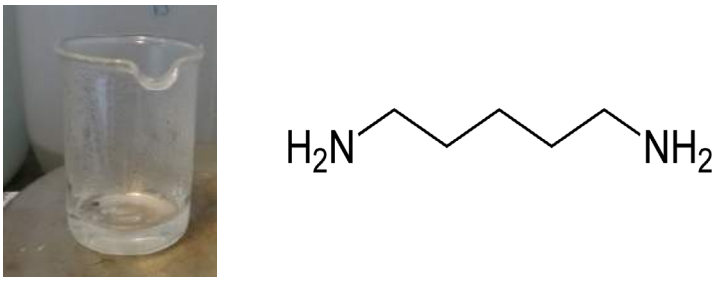


Figure 2-1. Image and molecular structure[40] of cadaverine

As one of the toxic product from protein spoilage, pure cadaverine is an organic light yellow liquid in standard state (temperature at 25°C and pressure at 1×10^5Pa). The boiling point of cadaverine is 179.1°C and the density is 0.873g/ml[40]. According to related research, proposed maximum tolerable level (still they are associated with some uncertainty and do not consider interaction of amines) for cadaverine in seasonings, fermented sausages, cheese, fish and sauerkraut are 1540mg/kg, 1080mg/kg, 540mg/kg, 510mg/kg and 430mg/kg, respectively[41]. This amount are relative high, some can be detectable. When it comes to some processed food with very strong taste like the food mentioned above, the unpleasant smell of cadaverine is not that obvious to be detected, also like cheese which is the main food source people get cadaverine from, people can not really smell the cadaverine due to the strong smell of cheese itself. Also we should notice these food are not the main food and we will not eat large amount per day. But still, it is necessary to be able to sense not only a low concentration, but also a relative high concentration for the sensor develop in this project.

Since we have mentioned in chapter 1 that the maximum of pure cadaverine oral toxicity from rat study should be 180mg/kg. If we consider an adult with average weight of 75kg, then the possible harmful

value of cadaverine he could take is 13.5g. And we know that the recommend daily protein taken is 132g to 165g (it is calculated by the equation that recommend amount of protein is 0.8-1 grams of protein per pound of body weight[42].) for people with weight of 75kg which is 165 pounds. For simplicity, consider the person just eat chicken breast to gain protein, then around 639g to 786g chicken breast need to be consumed per day (per 100 gram chicken breast contain 21 gram protein), therefore the limitation of eatable chicken breast should have the concentration of cadaverine lower than 13.5g/639g to 13.5g/ 786g chicken breast weight. In order to further quantify testing experiment, assume the density of chicken breast is 1g/ml like water (chicken breast contain more than 60% water inside) and then the value can be transfer to 1ml/42ml to 1ml/51ml as amount of cadaverine/water (chicken breast).

2.1.2. Selective Mediation Layer - Cyclam(1,4,8,11-tetraazacyclotetradecane)

The requirements of functionalizing a cantilever with a thin gold layer of 30nm on top are mainly two parts. Firstly, the functional group should be able to bind a transition metal complex and stay stable after binding. The other demand is such a chemical group should have the ability of featuring cadaverine or the amines group produced during meat degradation process.

Meat spoilage occurs at the time of slaughtering since the bacteria is all around. According to the patent[43] of the polymeric food spoilage sensor, it provides a possible solution of such an organic compound that can be used for biogenic amines. And also from reference [44], the chemical called cyclam(1,4,8,11- tetraazacyclotetradecane) became the ideal candidate in this project.

Cyclam is an organic solid and it is white (or light yellow) fiber-like solid under standard condition. The image and molecular structure is given by figure 2-2. It can form very stable complexes with transition metal including gold[44] and can stabilize the complexes by a very rare oxidation[46]. The recommend storage condition should be cool and dry, because the cyclam might has a strong absorption of water from the air.

Figure 2-2. Image and molecular structure[45]of cyclam

2.1.3. Solution for Cadaverine - Water and Glycerol (1,2,3-Trihydroxypropane)

In order to lower down the concentration of cadaverine, a solvent must be chosen for cadaverine. In the first place, cadaverine or other common amines should be easily dissolved in such a solvent without having any chemical reaction. Also this solvent will not effect or effect little on cyclam. Last but importantly, this solvent need to be common in market, therefore it is user-friendly. Additionally, it

would be really nice if it is harmless and not very expensive. Certainly not every requirement can be fulfilled in this case.

The first chosen candidates are ethanol, water and glycerol. These chemicals are selected for their commonly application, and solubility with cadaverine. After comparing testing results (Appendix II, Test 5 and Test 12) the ethanol is out of consideration due to the strong ability of washing away of cyclam - which makes it a promising solution in cleaning.

The advantages of selecting water to be the solvent are quite straightforward. Since water is one of the products from spoilage and also meat itself contains a lot of water, it is very easy to make such an solution for testing and does not need any preparation for users in the future. Most amines including cadaverine are soluble in water in various concentration under standard condition. Of course water is very safe and without any odor and color. The main problem of it is that cyclam is also soluble in water - which makes the measurement is a little hard to direct the conclusion, more specifically, it is hard to tell the change of surface feature is caused by amines or by water itself. Usually apply a small liquid drop of the mixture of water and cyclam to a functionalized surface, later the water will evaporate and leave most cadaverine on the surface.

Glycerol is mainly applied for gas phase due to its very high boiling point 290°C (this will be discussed in details in section 2.2.2). It has the advantages of colorless, odorless, very low toxicity (since it applies to food industry as a drier, but not very big amount) and cheap. Since it has some properties of miscible in water and ethanol, and from figure 2-3, we can see it has symmetrical structures and contains three hydroxy - that should make itself a strong polar solvent for most amines (polar molecules with amino) in theory. The main concern here is about its viscosity and not that easy to achieve as water for most users.

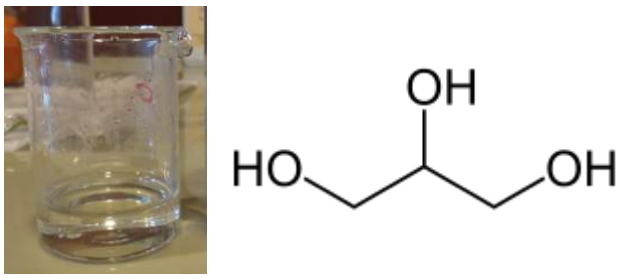


Figure 2-3. Image and molecular structure[47] of glycerol

2.2. Functionalizing Method and Measurement

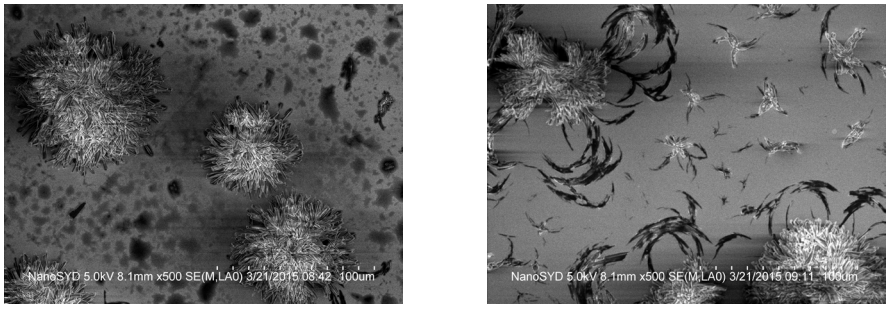
The first recipe of functionalization is based on a general assumption and developed by Roana Melina de Oliveira Hansen. The cyclam is dissolved in 2-ethoxyethanol and will bond to gold surface after some processing time. Dry it to a relative stable phase and then apply cadaverine solution. The cadaverine is diluted in water or glycerol in various concentration and bond to cyclam on gold layer by heating to gas phase or just apply a drop of liquid mixture. The sample we used is silicon with a thin layer of gold on top. The equipment we use here for measurement is optical microscope, SEM (Scanning Electron Microscope) and AFM (Atomic Force microscope).

2.2.1. Optimized Functionalization Process Parameters

The first recipe (See Appendix II: Test 1) gives a successful result with solution of 2-ethoxyethanol and further investigation is based on this recipe by varying the concentration of cyclam, processing time, baking temperature, surface cleanness (See Appendix II: Test 3, Test 4 and Test 6).

Due to the lack of relevant research of this field, large amount of samples are processed and tested in this project. Usually cyclam form a cluster or fiber-like structure on the gold layer. There are a few explanations for cyclam different behavior when it under different functionalization conditions.

Firstly, surface cleanness will result in a different structure of cycalm under the same functionalization condition. For the one with simple cleaning showed in figure 2-4 (a), we can see big round clusters (diameter within 1500um) and small black spot might be mixture of water and some other contaminants. However when observing the one with plasma cleaning process given by figure 2-4 (b), the black spot is disappeared, and the formation of cyclam changed to the combination of big (diameter within 1500um) and small (diameter within 800um) cluster with fiber around. The explanation for this surface feature could be during plasma cleaning, not only the contaminants but also some gold atom are removed and therefore cause some surface roughness and finally make cyclam spread unevenly.



(a) (b)

Figure 2-4. SEM images of comparison of surface cleanness (without baking after functionalization), 0.05mg cyclam+2.5ml 2-ethoxyethanol, 24 hours processing time, with magnification of 500: (a). Sample without plasma cleaning; (b). Sample with plasma cleaning.

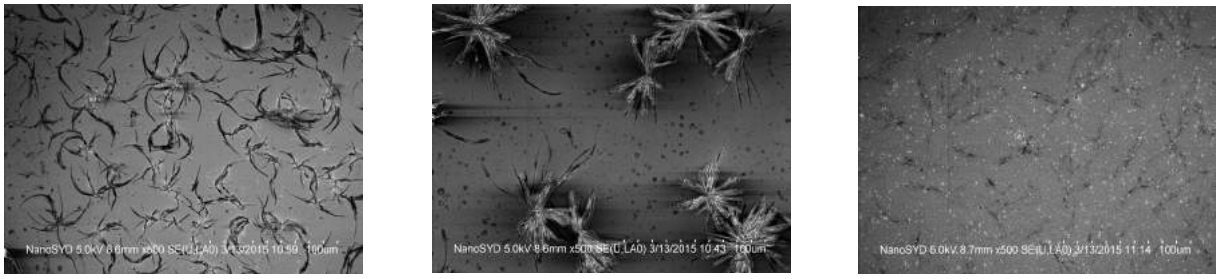
As for another parameter - baking temperature is a process to help evaporate 2-ethoxyethanol after functionalization. As shown in figure 2-5, what can be tell from it is that baking at low temperature will help to gather cyclam to form cluster, however with high temperature it seems evaporate with 2-ethoxyethanol for good. The possible reason here is that the bonding between cyclam and gold is not that stable and oxidation process might not happen because gold are noble metal. The cyclam prefers to stay in 2-ethoxyethanol instead of bonding to or oxidation on the gold layer. As we know that the diffusion coefficient of substance is temperature dependent. Here the substance is cyclam. For low temperature, cyclam is more spread in the solvent and less of them will diffuse to sample surface due to low energy, forming small structures after evaporation of 2-ethoxyethanol.

However if a determined temperature is applied, cyclam will have more energy to diffuse and more cyclam will transport to sample surface, also the process of evaporation will speed up and make the cyclam inside to gather - it is like when we boiling water, the water in the bottom of container will be heated to gas phase and form bubbles, and these water bubbles will push each other and try to come

out from the water surface to the air. Cyclam is a material with really low molecular weight and therefore can be easily pushed like this way.

According to Le Chatelier's principle, we know that most solid solute become more soluble in solvent as the temperature increase because most of dissolution like this is an endothermic process. In our case, at a very high baking temperature, which means the cyclam might also be more dissolved and then just volatilize with solvent 'bubbles' together.

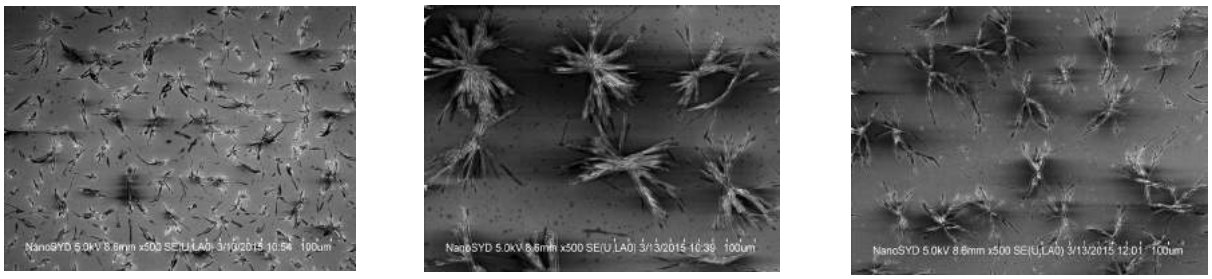
One thing need to be noticed here is that although use the same solution and condition, the difference between sample in figure 2-4(a) and figure 2-5(a) is caused by the inappropriate storage condition of cyclam as we mentioned before. The one in figure 2-5 is first made and the fresh cyclam is white spread fiber-like solid, however after some time the cyclam absorbed water (or some other things) in the air and stick to each other and form a light yellow cluster and make it harder to dissolve in 2-ethoxyethanol. This can be solved by waiting more time to let it dissolved or simply melting the cyclam first and then cool down to get more pure one.



(a) (b) (c)

Figure 2-5. SEM images of comparison of baking temperature after functionalization, 0.05mg cyclam+2.5ml 2-ethoxyethanol, 24 hours processing time, with magnification of 500: (a). Sample without baking, dry at room temperature; (b). Sample baked at 60°C for 2 hours; (c). Sample baked at 100°C for 2 hours

Processing time somehow influence the size of cluster and the spacing between them. As shown in figure 2-6, with shorter processing time, the cyclam is more spread and form more a dense and small fiber-like structure on the surface. While with longer processing time, the cluster and their spacing will be bigger. However interestingly, such a big cluster will break down if we prolong processing time to 42 hours.

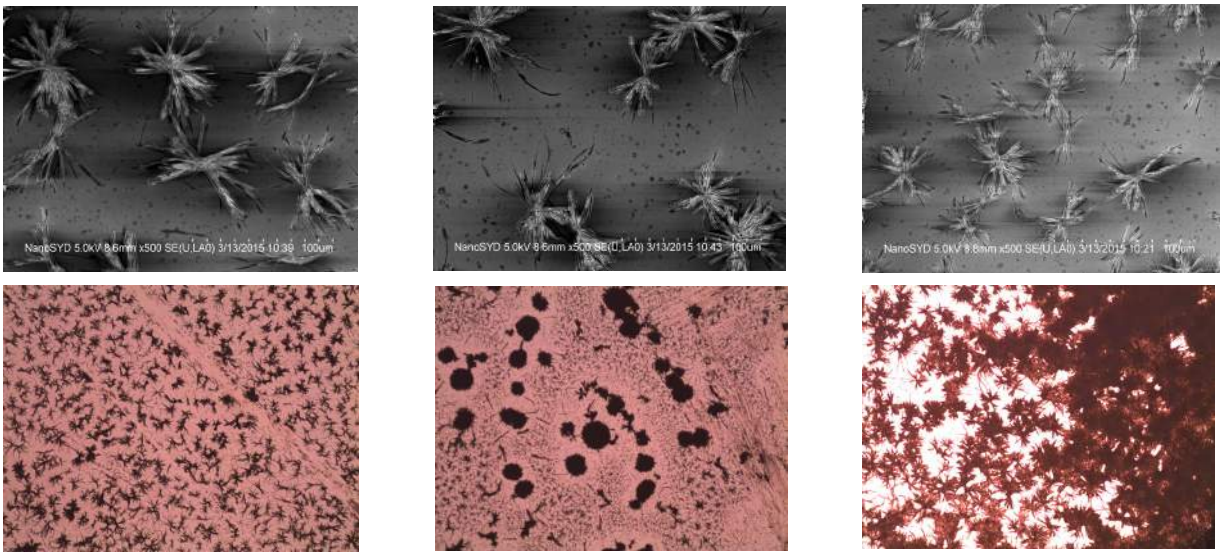


(a) (b) (c)

Figure 2-6. SEM images of comparison of processing time, 0.025mg cyclam+2.5ml 2-ethoxyethanol, baked at 60°C for 2 hours after functionalization, with magnification of 500: (a). Sample with 16 hours processing time; (b). Sample with 24 hours processing time; (c). Sample with 42 hours processing time

The assumption for such phenomenon is cyclam will attract and gather to each other in 2-ethoxyethanol when left it without heating or stirring, therefore longer processing time will result in bigger clusters. However the size of cluster has a threshold - like building block, to some point, the balance will be destroyed due to additional one on top. Then the cluster of cyclam in the solvent collapse once it reach the its own tolerance. As we can see the spacing with processing time of 16 hours and 24 hours is relatively uniform while the uniformity is kind of lost with 42 hours.

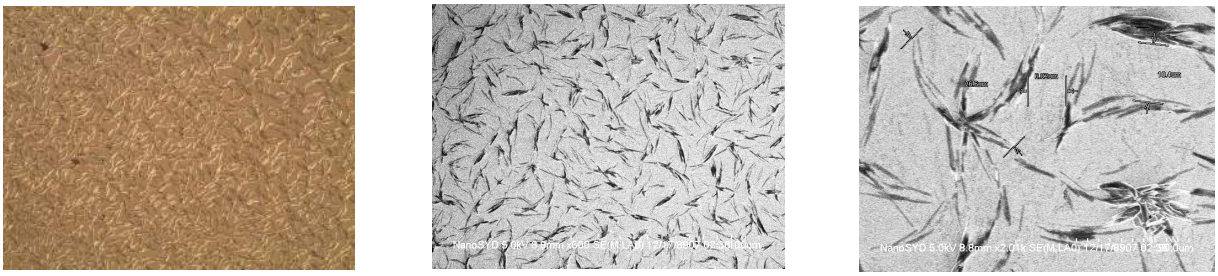
The concentration also influence on the surface feature after functionalization which can be seen from figure 2-7. It is a bit confusing from the SEM result because of the unevenness of the surface feature. However it can be explained when we also compare their surface feature by optical images with magnifications of 5. Low concentration apparently will give a better result of uniformity while higher concentration will result in some very big cluster and cover the gold surface make it really hard to be detected by SEM.



(a) (b) (c)

Figure 2-7. Cyclam concentration comparison, process for 24 hours, baked at 60°C to dry for 2 hours. Up images = optical images with magnification of 5; bottom images = SEM images with magnification of 500: (a). Sample with concentration of 0.025mg cyclam+2.5ml 2-ethoxyethanol; (b). Sample with concentration of 0.05mg cyclam+2.5ml 2-ethoxyethanol; (c). Sample with concentration of 0.075mg cyclam+2.5ml 2-ethoxyethanol

Further investigation mainly focus on concentration and processing time to find better solution for cantilever functionalization (Appendix II Test 6). The idea is to form a very small and dense clusters on surface, the reason is the tips of cantilever are very small - the width is about 35um. From the previous comparison, we can conclude that without baking, low concentration and short processing time are preferred in our case. Thus the recommend recipe is preparing clean samples and immerse to solution of 0.01mg cycalm+2.5ml 2-ethoxyethanol for 5min, and then dry it at room temperature. Figure 2-8 gives the result cyclam formation feature.



(a) (b) (c)

Figure 2-8. Optimized solution of functionalization, 0.01mg cyclam+2.5ml 2-ethoxyethanol for 5min, and dry at room temperature: (a). Optical image with magnification of 20; (b). SEM image with magnification of 500; (c). SEM image with magnification of 2K, the length of cyclam fiber indicate here is 26.6um, spacings are within 15um.

Notice that sufficient stirring during process will help to form a more uniform surface pattern. The whole process just cost around 30 minutes. The average length and spacing is obviously smaller than 40um and most of them are even less than 30um.

2.2.2. Comparison of Different Cadaverine Bonding Methods

After functionalization of samples, a method need to be found to bond cadaverine molecules on gold layer. There are two ways of doing bonding process. One way is to use the liquid phase with low concentration of cadaverine solved in water, the other way is to use gas phase of cadaverine by heating it to gas from glycerol which has much higher boiling point. Cadaverine in these two solvent are showed in figure 2-9. Interestingly although both water and glycerol are colorless liquid, after mixing with cadaverine (light yellow oily liquid), the mixture of water and cadaverine becomes white while the mixture of glycerol and cadaverine change to yellow. With sufficient stirring, it is easily to see a lot of white tiny particles (cadaverine) floating up and down under sunshine in both solvent.

Figure 2-9. Image of cadaverine mixture with different solvent. Left beaker: 10ml water+0.25ml cadaverien; right beaker: 10ml glycerol+0.25ml cadaverine

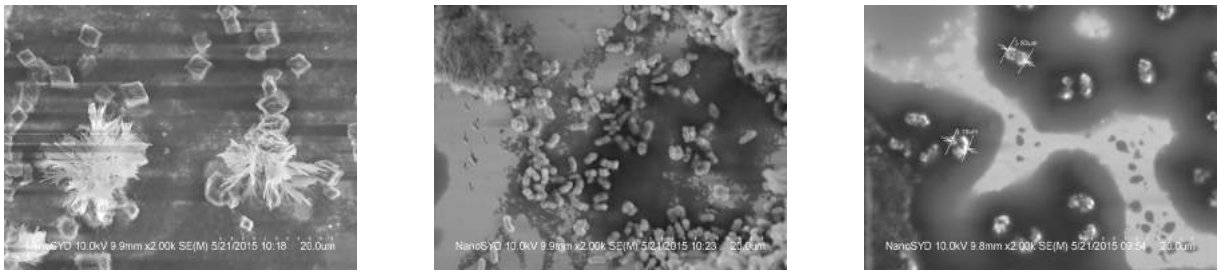
2.2.2.1. Liquid Phase of Cadaverine in Low Concentration Solution

Detailed testing process can be found in Appendix II Test 2, Test 4 and Test 6. The method is applying a drop of the mixture of water and cadaverine in various concentration. This method is quite fine with very high concentration of cadaverine as shown in figure 2-10(a). However when come to low concentration, the results are not very satisfied due to water is easily wash away the cyclam functionalized on the sample as we can see from figure 2-10(b).

(a) (b)

Figure 2-10. Optical images of comparison of different concentration of cadaverine liquid bonding, functionalized by solution of 0.05mg cyclam+2.5ml 2-ethoxyethanol, process for 24h, baked at 60°C for 2 hours, with magnification of 5: (a). Sample applied pure cadaverine (95%); (b). Sample applied mixture of 2ml water+0.5ml cadaverine (95%)

The idea to solve such a problem is to use a syringe with small needle to apply very small drops evenly on the sample(Appendix II Test 10) - this can be fulfilled by microfluidics in future improvement. Also high concentration of cyclam will improve bonding results. Figure 2-11 shows three concentrations as representatives based on the calculation we did in section 2.1: 1/56 (0.25ml cadaverine + 14ml water); 1/40 (0.25ml cadaverine + 10ml water); 1/24 (0.25ml cadaverine + 6ml water). Higher concentration like 1/16 (0.25ml cadaverine + 4ml water) is smelly enough to be sensed by human nose. As we can see the white tiny particles (cadaverine) were bonding to cyclam. Obviously their feature are very different. From figure 2-11(a) and (c) we can see that with higher concentration, more particles spread on the surface. However the figure 2-11(b) is bit confusing here, the particles are much smaller and spreading closer to each other. Many factors could cause such a result, for example like better stirring or better solution apply by needle, however these issue can not well controlled by hand now - but these condition could be easily controlled by specific equipment in future application.



(a) (b) (c)

Figure 2-11. SEM images of comparison of processing time, pure cyclam gas process for 10s, use needle to apply liquid phase of cadaverine in water in different concentration, with magnification of 2K: (a). Sample applied with solution of 0.25ml cadaverine+6ml water; (b). Sample applied with solution of 0.25ml cadaverine+10ml water; (c). Sample applied with solution of 0.25ml cadaverine+14ml water. The size of the white particles in the range of 3um-4um.

2.2.2.2. Gas Phase of Cadaverine in Low Concentration Solution

Such a solution is made for simulating a piece of meat and testing if cadaverine is binding to cyclam. The gas phase was first applied by mixture of water and cadaverine. Since we use very low concentration and cadaverine is soluble in water, so we should get a mixture of cadaverine totally solved in water. However the boiling point of this mixture range from 100°C (boiling point of water) to 179.1°C (boiling point of cadaverine). As we can see in figure 2-12, which is based on the theory of two components liquid system according to [48]. However 150°C we used before has bad sides: the change of surface is mainly caused by water vapor instead of cadaverine. Since the most gas heated from the mixed solution is water vapor.

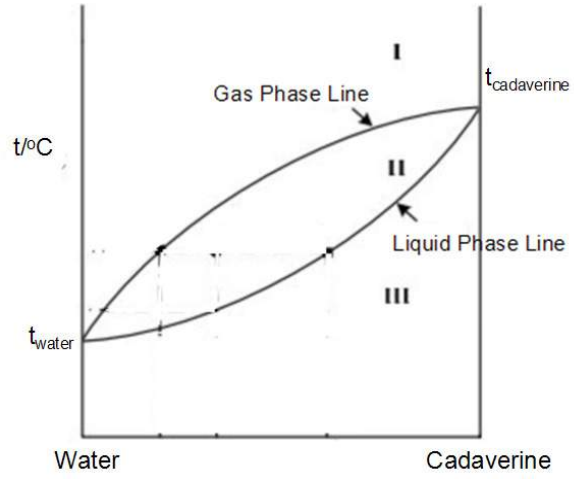
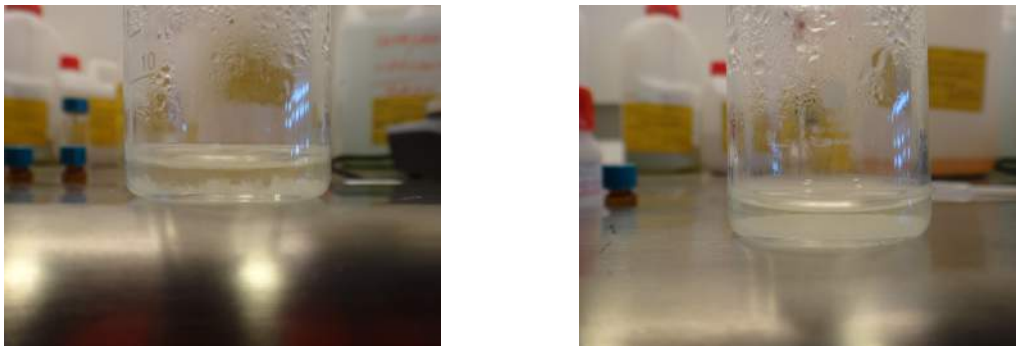


Figure 2-12. Schematic diagram of principles for cadaverine and water liquid system[48]

Also from the figure 2-13(a) that cadaverine will be pushed by water bubbles and gather together to form a white foam-like cluster floating under water surface. Thus it is easy to deduce that most cadave-rine is not coming out from water. Certainly cadaverine will be dissolved again when the mixed solution cools down, which can be seen from figure 2-13(b).



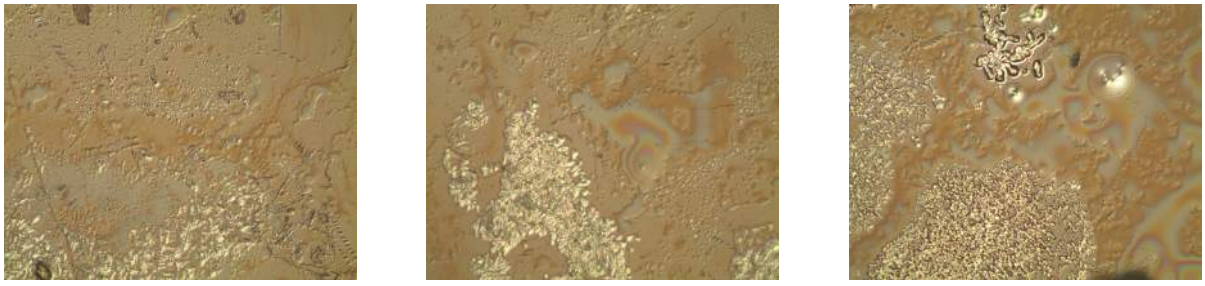
(a) (b)

Figure 2-13. Images of mixed solution of water and cadaverine: (a). Mixed solution after heating to 150°C, cadaverine form a white foam-like cluster floating in water; (b). Mixed solution after cooling down, cadaverine is dissolved again in water

Glycerol is chosen here due to its high boiling point (290°C) and therefore could ensure that cadaverine will be boiled to gas phase and come out from glycerol when we heat to 200°C (higher than the boiling point of cadaverine to ensure it boiled and gasified) and process for 5min.

Glycerol's density is 1.26g/cm^3 which is higher than water, but for simplicity, still three concentrations were tested: 1/56 (0.25ml cadaverine + 14ml glycerol); 1/40 (0.25ml cadaverine + 10ml glycerol); 1/24 (0.25ml cadaverine + 6ml glycerol). One thing need to be noticed that in order to make ensure under the same condition, after sufficient stirring we take out the same amount of the mixed solution in beaker and heat for the same time period to compare the concentration effect (more details can be seen in Appendix II Test 10).

As we know that in the mixed solution the solvent with higher boiling point still will speed up its evaporation due to high temperature. And also some amount of glycerol will be also evaporated to bond to sample surface. The biggest disadvantage of glycerol solution is its viscosity and very high boiling point, once it bond to the sample surface, it is very hard to dry it without influence cadaverine bonding. Even the sample leave for very long time at room temperature it is still hard to dry. This glycerol 'cover' make it hard to observe by SEM, and also it may cause some complexity in accurate measurement for future application. The optical images of these bonding results are showed in figure 2-14. Although not very obvious, still we could distinguish that with higher concentration, the spreading of these tiny particles are more densely formed together.



(a) (b) (c)

Figure 2-14. Optical images of mixed solution of glycerol and cadaverine, processing for 5min, with magnification of 20: (a). Sample applied gas solution of 14ml glycerol+0.25ml cadaverine(95%); (b). Sample applied gas solution of 10ml glycerol+0.25ml cadaverine(95%); (c). Sample applied gas solution of 6ml glycerol+0.25ml cadaverine(95%)

In order to reduce such an influence from glycerol, processing time was shortened (Appendix II Test 14), however this effect is not significantly eliminate. Certainly shorten processing time is not only for reducing glycerol influence on sample surface, but also for tiny surface of micro cantilevers the bonding process need to be short, otherwise it may result in destroying or removing functionalized cyclam either by cadaverine gas or glycerol gas.

The comparison of different cadaverine bonding time can be seen from figure 2-15, functionalization solution is 0.05mg cyclam+2.5ml 2-ethoxyethanol for 5min, leave to dry at room temperature, bonding processing time vary from 5 seconds to 30 seconds. The formation of surface feature is hard to explain which can be influenced by imprecise control of process condition, but we can still distinguish these bright small particles on these samples. It is hard to tell from the figure 2-15 though, in principle with

longer processing time, more cadaverine will bond. However there is a limit that the cadaverine is almost gasified and also the bonding with glycerol should be reduced. Therefore further optimization of bonding time need to be investigated in the future.

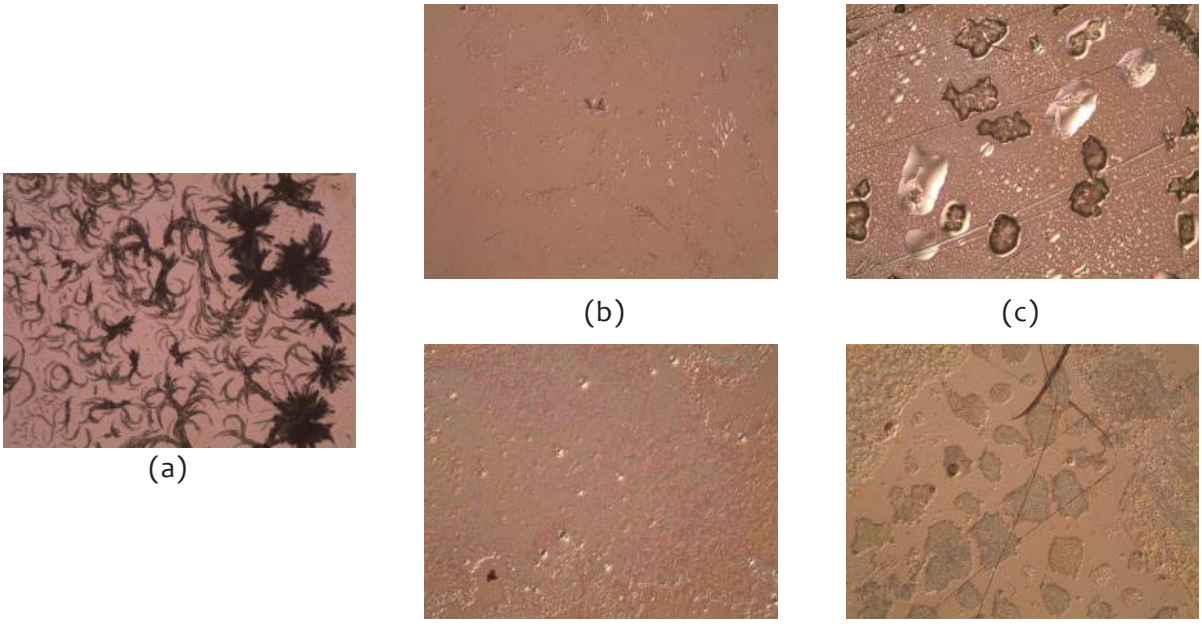
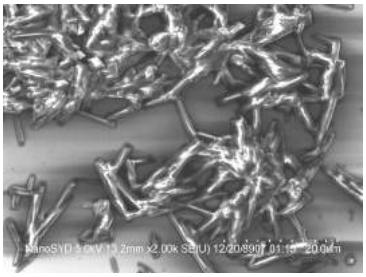


Figure 2-15. Optical images with magnification of 20 , mixed solution of 10ml glycerol+0.25ml cadaverine: (a). Reference sample before cadaverine bonding; (b). Sample process cadaverine bonding for 5s; (c). Sample process cadaverine bonding for 10s; (d). Sample process cadaverine bonding for 20s; (e). Sample process cadaverine bonding for 30s

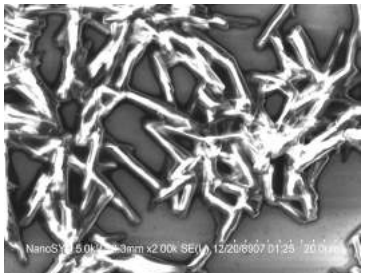
2.2.3. Meat Detection

For more practical future application real meat used on tests. Chicken breast is the first choice in the list because it contain high protein and little fat, in this way we can ensure the high purity of products-biogenic amines including cadaverine.

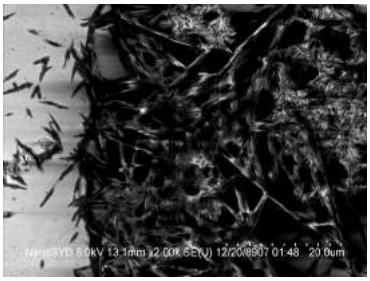
The samples were first functionalized with 0.01mg cyclam+ 2.5ml 2-ethoxyethanol for 5min, leave to dry at room temperature. The surface feature is showed in figure 2-8. More details can be found in Appendix Test 11. Here part of the results are showed in figure 2-16. Four samples were processing for an hour after 24 hours, 48 hours, 60 hours and 72 hours, respectively. The spoilage condition of chicken breast is at room temperature in a unfixed box (open to air), the chicken was becoming stink after 48 hours, and after 72 hours it was really stink to human nose. Water and cadaverine (perhaps other biogenic amines) are slowly evaporate to the sample surface and absorbed by cyclam. As we can see the cyclam is getting close to each other and even form a big cluster, also we can see there are some tiny white particles were bonding to cyclam especially the one after 72 hours.



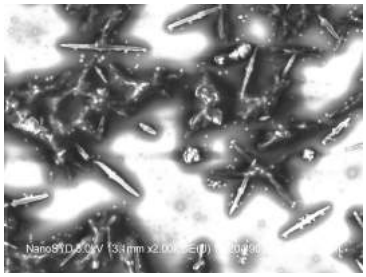
(a)



(b)



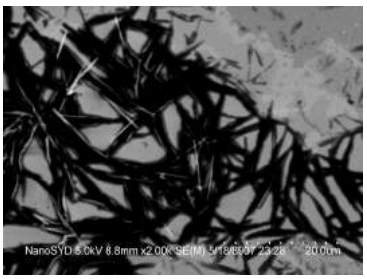
(c)



(d)

Figure 2-16. SEM images of comparison of different process period of cadaverine bonding from chicken breast, 1 hour process time, with magnification of 2K: (a). Sample process after 24 hours; (b). Sample process after 48 hours; (c). Sample process after 60 hours; (d). Sample process after 72 hours

In order to see the different effect between water evaporation and cadaverine evaporation, a bit further test with pure water evaporation and mixed solution of water and cadaverine evaporation were done and the results could be seen from figure 2-17.



(a)

(b)

Figure 2-17. SEM images with magnification of 2K: (a). Sample with pure water evaporation for 1 hour; (b). Sample with mixed solution (10ml water+0.25ml cadaverine) evaporation for 1 hour

Comparing figure 2-16 and figure 2-17, the effect from water vapor on cyclam seems to assemble these fibers and form them to be a big cluster - but it will not influence the sigle fiber shape and size much. However as we can clearly see that cadaverine molecules could reshape these cyclam fibers (form smaller fibers) due to interacting and bonding. These differences can be seen from figure 2-18 more clearly. Water vapor help to form a very big cluster while cadaverine seems to reshape those cluster or slow down water vapor to form big cluster. If we compare figure 2-18 (a) and (c), with cadaverine and other molecules bonding, a thin white film formed on the cyclam cluster and made the edge of cluster more smooth compare to pure water bonding.

(a) (b) (c)

Figure 2-18. SEM images of different evaporated solution with magnification of 500: (a). Sample with 1 hour water evaporation; (b). Sample with 1 hour mixed solution (10ml water + 0.25ml cadaverine) evaporation; (c). Sample with 1 hour evaporation from chicken breast after 60 hours

2.3. Testing Result and Discussion

After a large amount of sample testing, micro cantilevers were functionalized used the recipe developed in the section 2.1. Due to its vey small surface size and perhaps different surface energy, the bonding results are not entirely satisfactory. Also higher concentration and longer processing time were tried to make the functionalization working, details can be seen from Appendix II Test 8.

2.3.1. Two Assumptions for the Failure Results of MC Functionalization

Having considered the failure results of functionalization to micro cantilevers, two assumptions distinguished by different concentration and processing time could be drived here to explain such a phenomenon. Firstly, as we know that high concentration and long processing time will result in very big cluster with diameters much bigger than the width of cantilever, therefore it is really hard for micro cantilevers to bond such an big cluster. More specifically, if we take a closer look of the cyclam cluster in figure 2-19, we can see that cyclam cluster formed on the gold layer is island-like and the cyclam layer bond directly to the sample has biggest diameter compare to other assembled cyclam layers later. Therefore such a cyclam cluster will not be attracted so easily on samll tips but rather dissolved in 2-ethoxyethanol when we take it out from the solution.

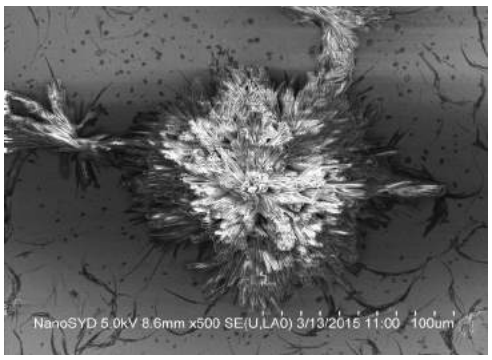


Figure 2-19. SEM images with magnification of 500, functionalized by 2.5ml 2-ethoxyethanol+0.05mg cyclam solution for 24 hours, leave to dry at room temperature

When it comes to low concentration and short process time, although the cyclam cluster or fiber is much smaller and within 35um, however it still results in very poor bonding results might due to weak surface energy between small cantilever surface and cyclam. Although the small cyclam cluster showed in figure 2-20 might be able to stick on the tip due to its small size, it still has high chance that cyclam is more disolved in the solvent because of low concentration. With larger surface, cyclam can spread and form itself, but for small scale the difficulties of bonding increase dramatically.

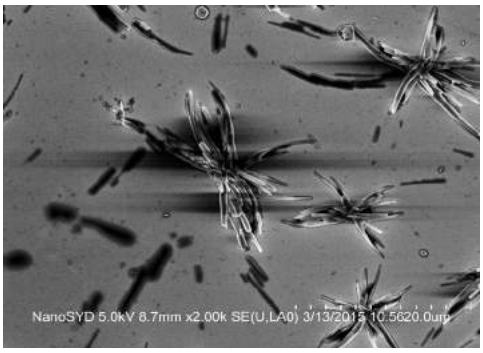


Figure 2-20. SEM images with magnification of 2K, functionalized by 2.5ml 2-ethoxyethanol+0.025mg cyclam solution for 16 hours, bake to dry at 60°C for 2 hours

To make these assumptions more vividly, for example, if we consider cyclam in 2-ethoxyethanol as pasta in ketchup, and regard sample (5mmx5mm) as a big folk or spoon while cantilevers as small chopsticks (Certainly there is only one chopstick or several chopsticks but can not clamp together). Apparently we all know that if we want to take the pasta from ketchup, it is much easier to use fork or spoon instead of a thin chopstick.

2.3.2. Optimized Functionalization for MC

The succeed solution for functionalizing micro cantilever is to use nanoplotter 2.1 from Gesim to spot the solution onto the cantilever. It is very accurate way with precise spot volume control sytem, another big advantage is that it is non-contacting technology[49] and therefore the fragile cantilever can be well protected. The concern for the method is the complexity for future assembly and operation, also the higer cost on fabrication. But in general, this way is quite straightforward and functionalized result can been seen from figure 2-21.

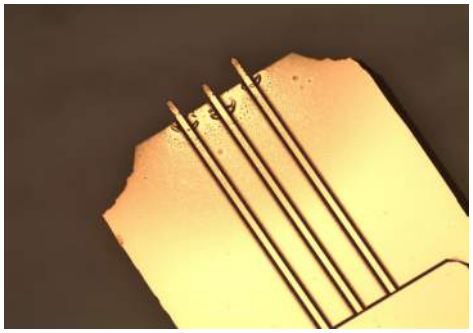
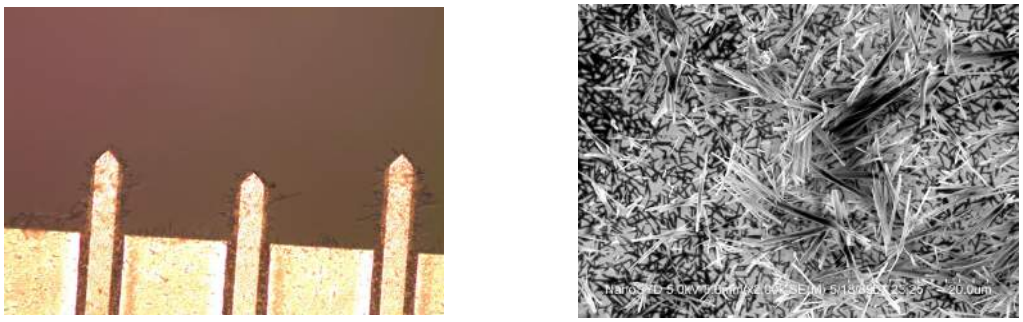


Figure 2-21. Optical images with magnification of 5, functionalized by 2.5ml 2-ethoxyethanol+0.05mg cyclam solution by nanoplotter 2.1

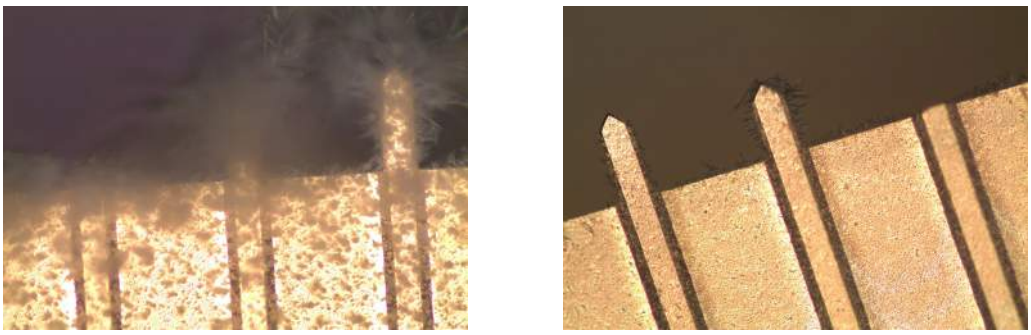
Although it is a possible way to functionalize a cantilever, but the disadvantages we just discussed are quite obvious. Thus it is neccessary to develop another possible solution. The idea is to make cyclam as small sa possible and with high concentration at the same time. A simple idea based on such requirements come out after some consideration - melting and boiling the cyclam to gas phase (200°C in our case) and then let them cool down and stick to the cantilevers (Appendix II Test 9). The functionalized cantilevers by this method can be seen from figure 2-22.



(a) (b)

Figure 2-22. Functionalized by heating cyclam to gas phase at 200°C and bonding processing for 5s, distance between cantilever and cyclam is in the range of 0.5cm-1cm: (a). Optical image of cantilevers with magnification of 20; (b). SEM image of sample with magnification of 2K

This method is quite simple and very easy to apply. Only two things need to be noticed here: processing time of cyclam gas and placement of cantilever. Too long process time with very hot cyclam gas will result in broken cantilevers (As we can see from figure 2-23(a)) and also it will cause some mechanical failure (bond too much cyclam) on cantilever for future application. Also the placement of cantilever should be controlled well, if we place the cantilever too close to the melted and boiled cyclam, it will result in very heavy bonding and cause mechanical failure, as showed in figure 2-23(b), the right broken cantilever is closer to cyclam, and also we can see that cyclam bonding is heavier on the middle cantilever compare to the left one. If the cantilever is too far away from cyclam there will be no bonding because cyclam will transform back to solid back very fast, therefore cantilever should be placed within 1cm from cyclam (amount is around 0.01mg).

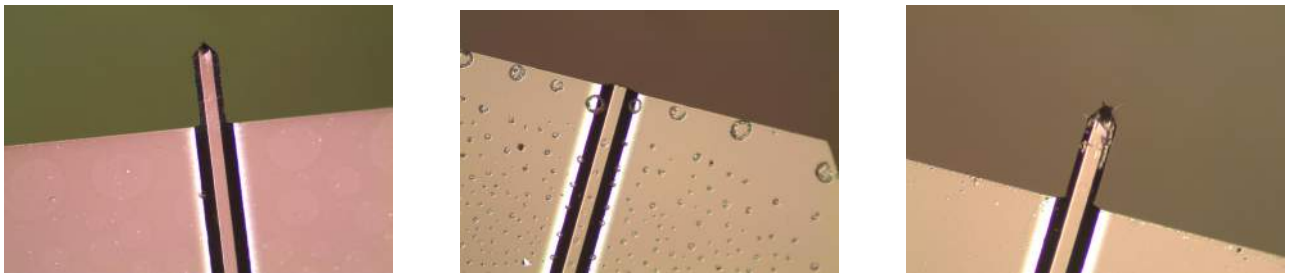


(a) (b)

Figure 2-23. Optical images with magnification of 20, functionalized by heating cyclam at 200°C to gas phase and bond to cantilever: (a). Cantilevers functionalized for 15s, distance between cantilever and cyclam is in the range of 0.5cm-1cm; (b). Cantilevers placed vertically, process for 5s, distance between the edge of cantilever and cycalm is within 5mm.

However the problems of this method are quite obvious. Firstly, the formation of functionalization can be different from each other by a even very tiny change, or in other words, it is hard to control precisely. Another thing is that such a functionalization will result in a heavy bonding on cantilever and easily break cantilever or cause mechanical failure. The last but importantly, the hazard of cyclam will increase (like to eyes and to trachea)[50] when it turns to gas phase.

To lower down the concentration of cyclam to get better functionalization, a further investigation was done in Appendix II Test 13 and Test 14. The improvement could be just simply apply a few seconds of solvent gas either before or after cyclam gas processing. Possible solvent is 2-ethoxyethanol and acetone. The promising results were showed in figure 2-24. However, such a method contain some uncertainties and more samples and cantilevers tests is needed to optimize the recipe of functionalization.



(a) (b) (c)

Figure 2-24. Optical images with magnification of 20, functionalized by combining solvent gas solution and cyclam gas solution: (a). Cantilever functionalized with 10s 2-ethoxyethanol gas then 20s pure cyclam gas solution; (b). Cantilever functionalized with 20s pure cyclam gas then 3s 2-ethoxyethanol gas solution; (c). Cantilever functionalized with 20s pure cyclam gas then 5s acetone gas solution

In addition, during testing, a new promising solution were developed, as we can see from figure 2-25, using mixed solution of cyclam and acetone(0.04mg cyclam+2ml acetone in our case), process for 1 hour, which will give some bonding of cyclam to cantilevers. And also this method need some further optimization in the future, but compare to the melting method, this way is safer, easier and can also reduce some cost and complexity attributed to no heating process.

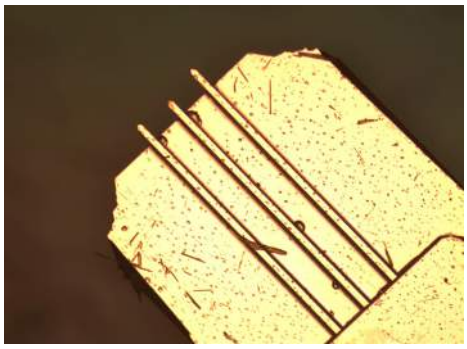


Figure 2-25. Optical images with magnification of 5, functionalized by solution of 0.04mg cyclam+2ml acetone, process for 1h, leave to dry at room teperature

One note need to be kept in mind for future application is that cyclam has some properties of absorbing water (or perhaps some small particles) in the air even it was functionalized on the cantilever. Figure 2-26 compare the results of cantilever with pure cyclam gas functionalization. The image (figure

2-26(a)) of cantilever just after functionalization and the image (figure 2-26(b)) of the same one after 24 hours after functionalization, we can clearly see that the cyclam bonding feature is changed and since it may contain some water molecules or particles, the resonance frequency will have a small shift and cause some inaccuracies in the measurement. Therefore, it is better to do the cadaverine bonding as soon as possible after testing the resonance frequency of functionalized cantilevers to improve the accuracy, or we can optimize an encapsulation method in the future.

(a) (b)

Figure 2-26. Optical images with magnification of 5, functionalized by heating cyclam at 200°C to gas phase and bond to cantilever: (a). Cantilever measured immediately after functionalization; (b). Cantilever measured 24 hours after functionalization (the cantilever was stored in a fixed sample box and kept in cleanroom)

2.3.3. Optimized Cadaverine Bonding Method

Cadaverine bonding can be applied by liquid solution or gas solution. For the liquid solution, we use mixture of water and cadaverine, this method gives quite nice result as showed in figure 2-11. But this method is not applied in this project due to the very small functionalized surface of the cantilever, a drop and even a small drop from needle tip will be too much and the water could easily wash away the cyclam bonded on cantilevers, as we can see from figure 2-27. The liquid phase of cadaverine bonding can be achieved by using nanoplotter or microfluidics system in the future.

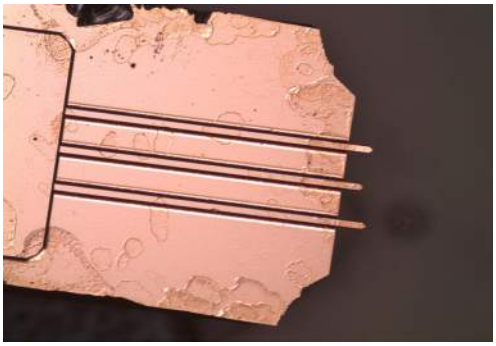
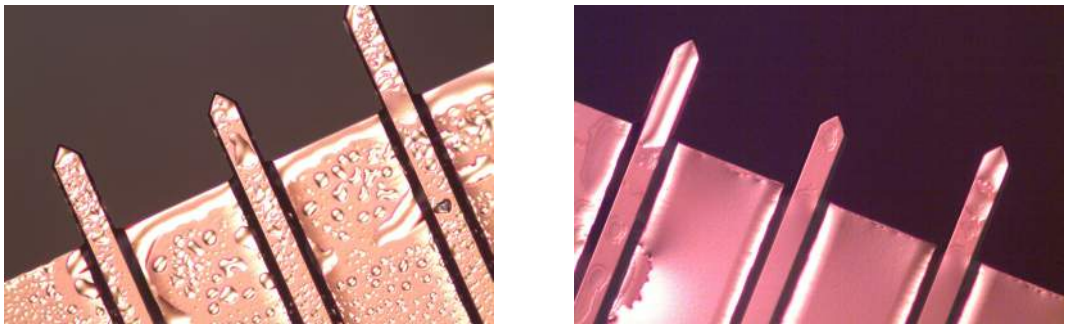


Figure 2-27. Optical images with magnification of 5, functionalized by pure cyclam gas bonding for 5s, distance between tip to cyclam is in the range of 0.5cm-1cm. Liquid drops of mixed solution of 6ml water and 0.25ml cadaverine

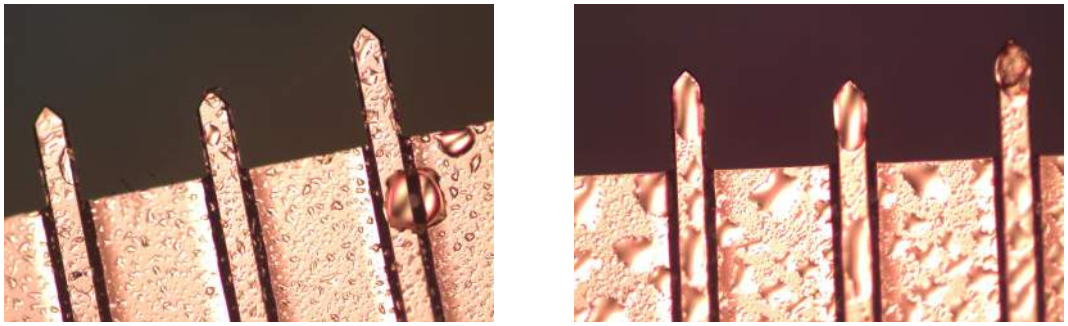
For gas phase of cadaverine bonding, it is quite achievable. Three cantilevers with different functionalization method were tested and results were showed in figure 2-28 to figure 2-30, the detailed pro-

cedures can be found in Appendix II Test 15.



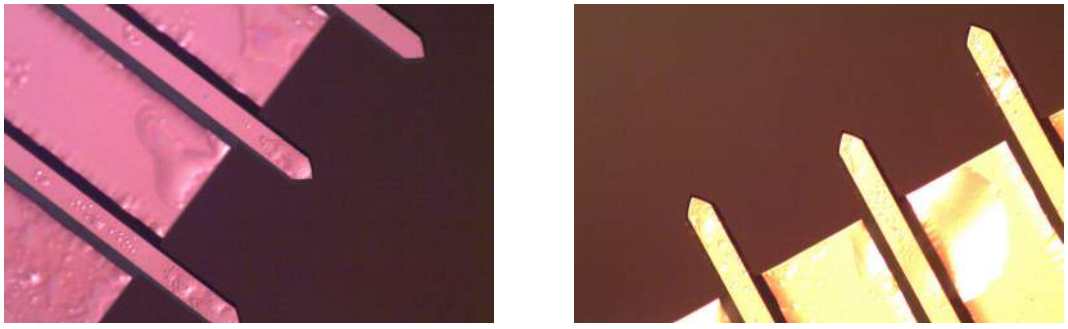
(a) (b)

Figure 2-28. Optical images with magnification of 20, functionalized by solution 0.05mg cyclam+2.5ml 2-ethoxyethanol by nanoplotter 2.1, gas phase bonding from mixed solution of glycerol and cadaverine for 10s (a). Cantilever with solution of 0.25ml cadaverine+6ml glycerol; (b). Cantilever with solution of 0.25ml cadaverine+14ml glycerol



(a) (b)

Figure 2-29. Optical images with magnification of 20, functionalized by pure cyclam gas bonding for 5s, gas phase bonding from mixed solution of glycerol and cadaverine for 10s: (a). Cantilever with solution of 0.25ml cadaverine+6ml glycerol; (b). Cantilever with solution of 0.25ml cadaverine+14ml glycerol



(a) (b)

Figure 2-28. Optical images with magnification of 20, functionalized by solution 0.04mg cyclam+2ml acetone process for 1h, dry at room temperature, gas phase bonding from mixed solution of glycerol and cadaverine for 10s (a). Cantilever with solution of 0.25ml cadaverine+6ml glycerol; (b). Cantilever with solution of 0.25ml cadaverine+14ml glycerol

What we can tell from these figures is that with relative higher concentration of cadaverine, the bonding results looks better while with lower concentration the cyclam seems washed away by glycerol,

since the glycerol has a very high boiling point and viscosity, they will stick to the cantilever and very hard to get rid of especially on small cantilevers. Therefore shorter time might be a better solution to avoid such a thing or try to find a better solvent instead of glycerol in the future application.

2.4. Solution for Reusability

In order to make the future application more economical and practical, an equipment can be used for many times is preferred. Thus a solution can clean the cantilever after functionalization and cadaverine bonding need to be found.

Actually the ideal case is to find a solution that will clean cadaverine without influencing cyclam, but it is really difficult now due to unstable bonding between cyclam and cantilevers. Therefore we are now focusing on a solution will easily wash away cyclam and cadaverine together. The requirement of such solution should be easy to find and not very expensive, cyclam and cadaverine shuld be very soluble in this solution and therefore easily be washed away. From Appendix II Test 12, the possible solution we have found here is ethanol and PBS(Phosphate-Buffered Saline) buffer. Where we can see the cleaning effect from figure 2-31, the reference sample was divided into several pieces, different clean solution was applied later.

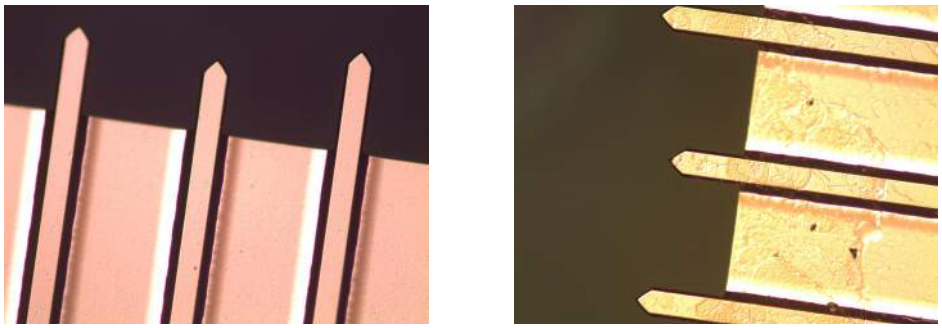
(a) (b) (c)

Figure 2-31. Optical images with magnification of 5: (a). Reference sample after functionalization by solution 0.05mg cyclam+2.5ml 2-ethoxyethanol for 24hours, leave to dry at room temperature; pure cadaverine liquid drop applying; (b). Sample after ethanol liquid rinse for 20s and then water rinse for 10s; (c). Sample after PBS buffer rinse for 20s and then water rinse for 10s

Ethanol is very common solvent for most known chemicals and with many application in both industry and medical field. It is colorless liquid with a strong odor[51]. After testing, cyclam shows strong solubility in ethanol, and also cadaverine. The cleaning result from figure 2-31(b) also proved this point. And water rinse here is to remove the residue still on the surface and reduce the effect of ethanol which might influence next round testing. PBS buffer is a water-based salt solution that commonly used in biological research[52]. Most biological molecules are soluble in this buffer and at the beginning we guess this buffer could just clean cadaverine without influencing cyclam, but the clean result in figure 2-31(c) is not that fine though we can see more residue on the surface. The buffer used in this project is prepared by Jong Wook Noh.

The residue still on the surface in figure 2-31 can be further cleaned by repeating solution one more

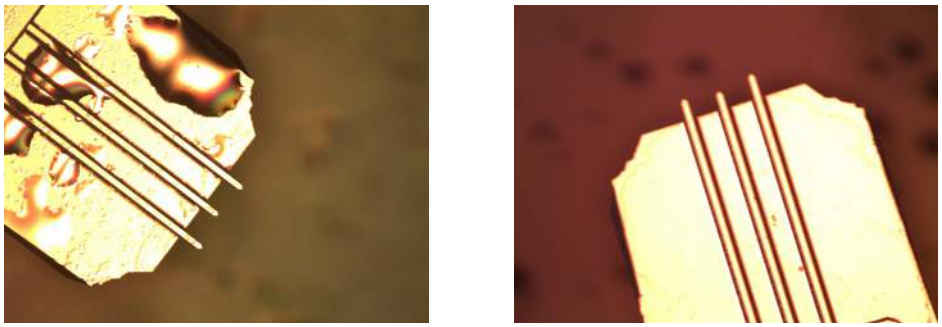
time. However, the clean work is much easier for cantilever due to its small surface. As we can see from figure 2-32, both solution works nicely. And apparently the one with PBS buffer leaves some residues perhaps cyclam in the surface, but more tests are needed for verification. And also for future application, this clean method could be assembled with microfluidics system.



(a) (b)

Figure 2-32. Optical images of cantilevers after functionalization of pure cyclam gas bonding, gas phase of cadaverine bonding (concentration of 0.25ml/6ml in glycerol for (a) and 0.25ml/14ml in glycerol for (b)), with magnification of 20: (a). Cantilever cleaned by ethanol rinse for 20s and water rinse for 10s; (b). Cantilevers cleaned by ethanol rinse for 20s and water rinse for 10s.

Certainly gas phase of ethanol could be applied to clean the functionalized surface - especially for the cantilevers, as showed in figure 2-33. In this way the cantilever will have less risk of breaking by liquid solution, but it needs another round of heating process which also increase the cost.



(a) (b)

Figure 2-33. Optical images with magnification of 5: (a). Reference cantilever after functionalization by solution of 0.04mg cyclam+2ml acetone for 1 hour processing, gas phase of cadaverine bonding by heating mixed solution with 6ml glycerol+0.25ml cadaverine at 200°C; (b). Cantilevers after ethanol gas phase cleaning for 30s and then water vopor cleaning for 10s

3.1. AFM Instrumentation

AFM is an equipment that applies to measure the surface feature of different samples. The optical setup developed for this project is based on the principle of part of AFM instrumentation which is able to measure resonance frequency of a cantilever.

3.1.1. Basic Principles in AFM

AFM (Atomic force microscopy) is a tool that allow us to investigate the surface structure and characteristics in nano-scale. The main instrumentation is illustrated in figure 3-1. The principle of image formation in AFM is the weak force between tip integrated to the end of cantilever and atoms of the surface. As the tip moves along the surface, the atomic force will bend the cantilever and therefore the cantilever will deflect up or down[53].The deflection of cantilever will be detected by the reflected laser beam to the photodiode and the topographic feature is thus transfer to the software application in the PC. A 3D image can be constructed by the feedback signal from the motion of cantilever in the Z-direction as a function of sample's X and Y position[53].

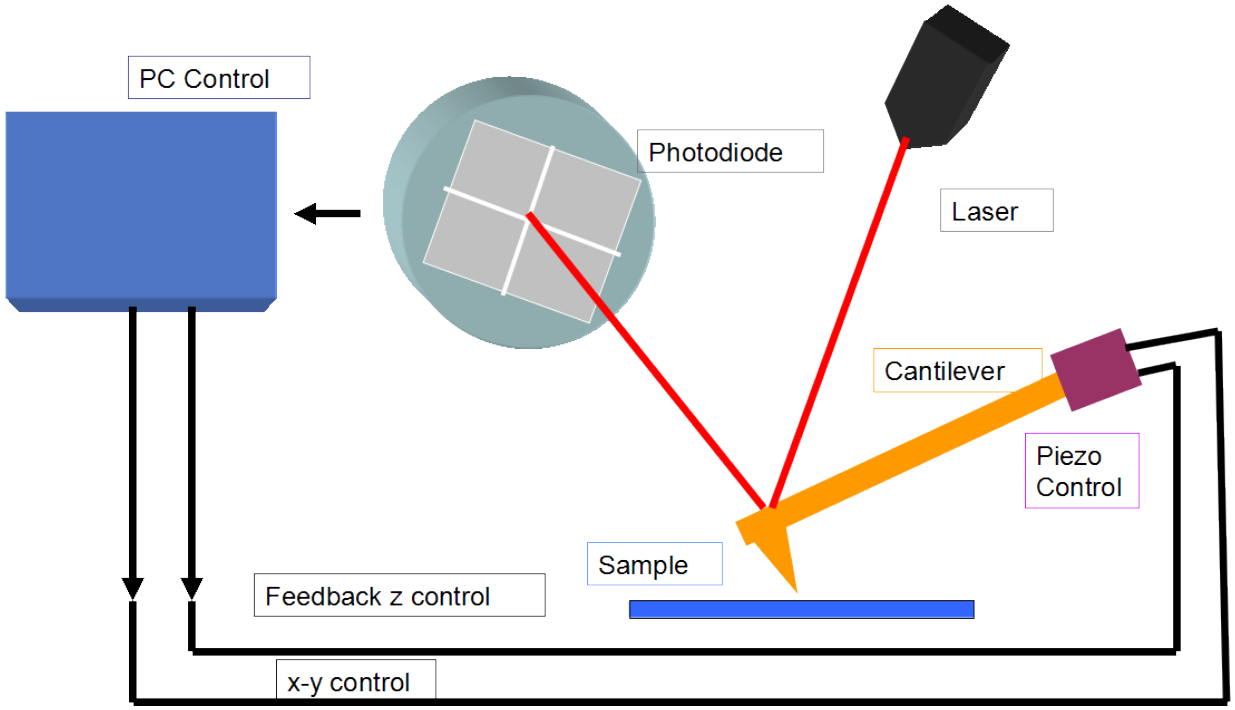


Figure 3-1. A schematic illustration of an atomic force microscope connected to a computer[54]

Three concepts are used in AFM, which are piezoelectric scanner, force sensor and feedback control[3]. The piezoelectric scanner is the device based on the principle of piezoelectric effect which illustrated in figure 3-2. Piezoelectric effect occurs only in non-conductive solid which describe a relation between

an electrical voltage in solids and mechanical stress[56]. In AFM, it applies the property of converting electric power to mechanical motion. Typically this will be used to control the movement of tip to scan the surface of sample due to its ability to control tiny motion accurately which is down to nanometers. The force sensor is the sensor that will construct the readable signal from the small deflection of cantilever. There are several ways of fulfilling the requirement, which will be further discussed in section 3.1.2. The feedback control refers to the control system will drive the piezoelectrics to maintain the tip-sample distance after it receive the signal from the force sensor, therefore ensure AFM a very sensitive tool[55].

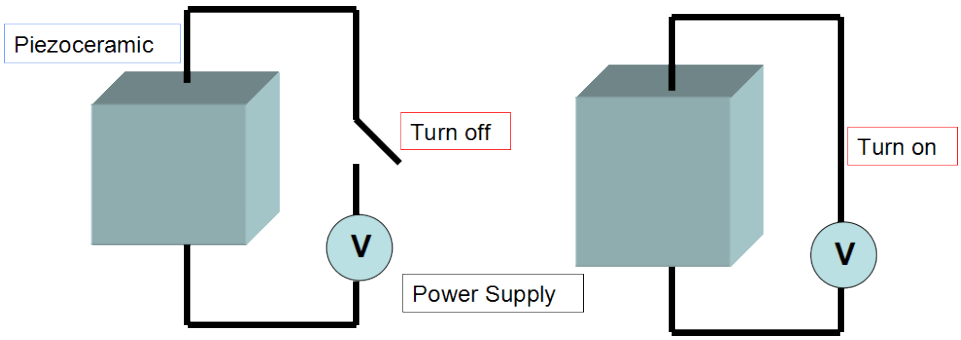


Figure 2. A piezoelectric device will expand when a voltage potential is applied to the top and bottom electrodes[56]

3.1.2. Applied Solution of Force Sensor

The most challenging part of AFM is the force sensor (which is also called force transducer). As we have discussed in the previous section, the sensor is used to transform the signal of the atomic force between probe and sample to readable signals and then transport to the readout system. Therefore a way of detecting of such a small force and transfer it to a readable signal becomes the existing problem need to be solved in the first place.

During several decades' development of AFM, there are several ways to fulfill such a force transducer, the ideas were based on scanning tunneling microscopy (STM), interferometer, crystal oscillator, piezo-resistive cantilever[55] and optical system, which can be seen from figure 3-3 to figure 3-7, those are based on the reference[55].

The original AFM use the technique of scanning tunneling microscopy. The application takes advantage of the STM electronics and thus the motion of cantilever is measured by the STM tip. The method is quite straightforward. A metal tip applied with certain voltage is scanning over the back of cantilever, the metal tip is fixed to a piezoelectric drive. The tunneling current of STM tip is very sensitive to the gap width between STM tip and AFM tip, soon the signal of tunneling current will be amplified and processed by PC and then give the feedback control voltage to reform piezoelectric device to remain constant gap width (tunneling current)[57].

In principle, the deflection of cantilever will change by different width of the gaps between the STM tip, AFM probe and therefore a changing signal will be sent to data processing and display system. Figure 3-3 gives the schematic diagram of this idea.

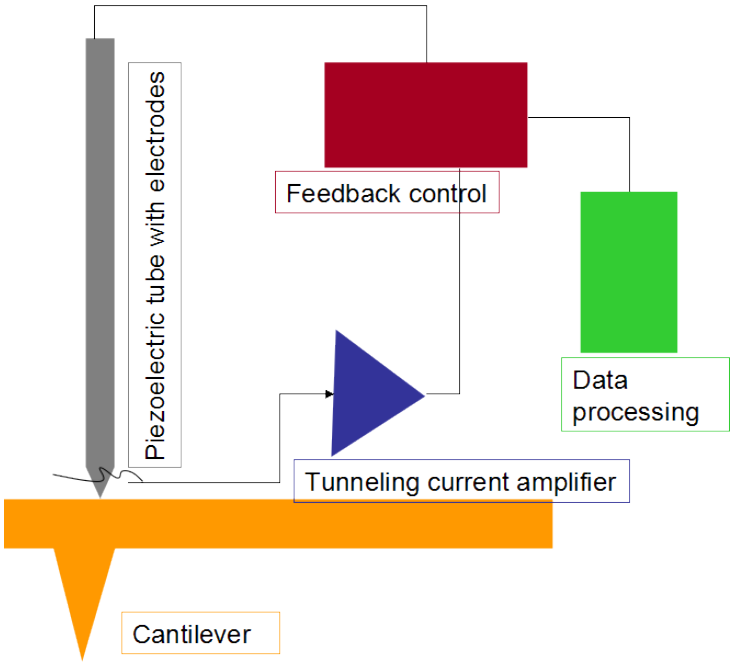


Figure 3-3. STM as a force sensor employed in AFM designs

Soon a new method developed which is based on the principle of Michelson interferometer. As illustrated in figure 3-4, the laser beam is split into two beams with the same frequency and amplitude. Although the beam goes right just pass through the splitter plate once while the beam goes up pass through the plate three times, there is no need to add a compensation plate since we use laser as light source here.

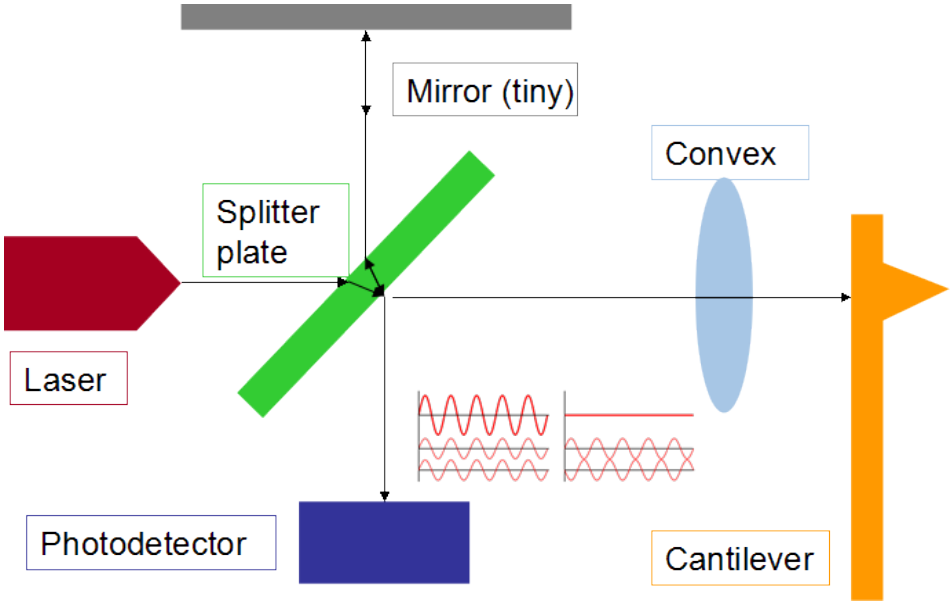


Figure 3-4. Michelson interferometer as a force sensor employed in AFM designs

As we all know that two light waves from the same light source incident will result in an effect called interference. The superposition of waves will result in either constructive interference or destructive interference depend on the phase difference, the former occurs when the light waves are in phase while the destructive one happens when they are in half a cycle out of phase[58]. There will be a significant change in amplitude of the signal to photodetector when the cantilever shift a distance of half of the wavelength - therefore it is a super sensitive method.

The method used to be applied in AFM is the technique called Crystal Oscillator. As the name indicates, a piezoelectric crystal is applied to measure the force between a probe and sample[59]. As we talked in the previous section, the piezoelectric material has the ability to convert mechanical motion to electric signals, and vice versa, thus the interaction between AFM probe and sample surface will result in a change of vibration which is proportional to the force[55]. Figure 3-5 gives the illustration of this technique.

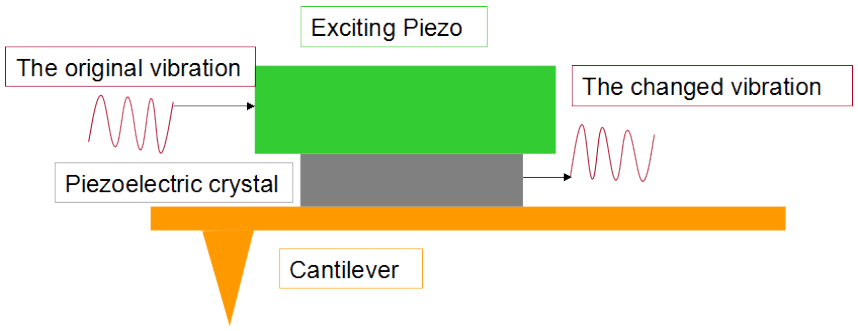


Figure 3-5. Crystal oscillator as a force sensor employed in AFM designs

A more advanced technique used in AFM based on the principle of piezoresistive sensor but with simplified version. Instead of mounting but a layer of piezoresitive sensor is fixed on the cantilever. The technique of so-called Piezoresistive cantilevers will change the resistance in the top layer of piezoelectric materials when the cantilever vibrate[60][61]. The motion of cantilever will immediately transform to the mechanical change of the piezoresistive sensor and therefore the bias between two electrodes of the device. The idea is shown in the figure 3-6.

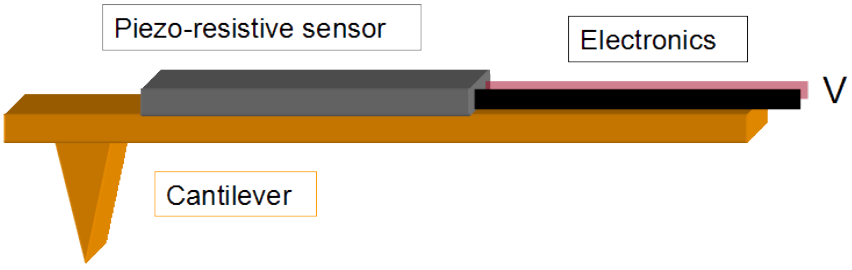


Figure 3-6. Piezo-resistive cantilever as a force sensor employed in AFM designs

A widely used force sensor applied in most AFM nowadays is a set of optical system. As illustrated in figure 3-7, the system includes a laser source and focusing optics to form a focused laser beam spot on

the back of cantilever, then the reflected beam will hit to a photodiode where measure the position of incident. Therefore a small movement of cantilever is easily measured and transferred to big signal change by photodiode, and this system is not difficult to assemble in AFM[64][65][66].

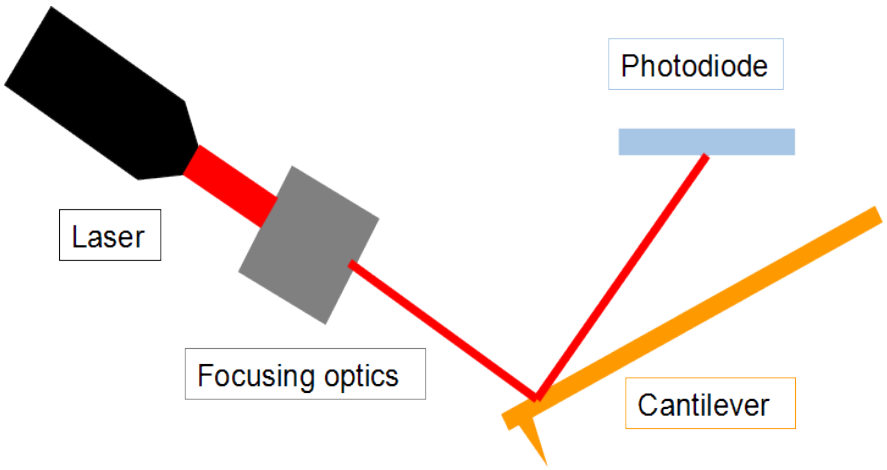


Figure 3-7. Optical system as a force sensor employed in AFM designs

3.1.3. Comparison of the Applied Solutions of Force Sensor

Since there are some techniques could be applied in AFM, however every method has its own advantages and disadvantages. The first AFM was built in 1985 assembled a very reliable and developed technical support: scanning tunneling microscopy, however soon people realized that it is not that easy to mount STM tip and AFM tip together in a proper way. As we can speculate that these tips are very tiny and fragile, one is already tricky to mount perfectly, fixing two of them is more complicated - certainly increase the difficulty of implementation and operation greatly[55].

The idea of interferometer is a method with high resolution, since the small movement of cantilever can be detected - with half wavelength scale of the laser beam from light source. Unfortunately, it is not always such an ideal case when people were trying to implement the technique due to hopping of cantilever during scanning process, which will influence the result of interference fringes to the read-out display[55].

The scanning process is illustrated in figure 3-8, when the tip is scanning from (a) to (b), the cantilever will bend due to the atomic force, and thus the reflection light will change the interference fringe pattern and the electronic system will record '1' (which indicate the height of the small spot is $\lambda/2$-λ). When the tip scan from (c) to (d) the interference fringe will change twice and system will record '2' (which indicate the height of this spot is λ-$3\lambda/2$), in this way the AFM is able read the surface characteristics, however the biggest problem of this method is the vibration of cantilever itself when it is scanning- as we all have the experience that when we apply a force to a spring and then we set it free, it will vibrate for a while itself. For the case (a) to (b) it might be fine because the amplitude of vibration less than $\lambda/2$, however it is really hard to distinguish the signals either from the surface structure or the vibration of cantilever itself for the later case.

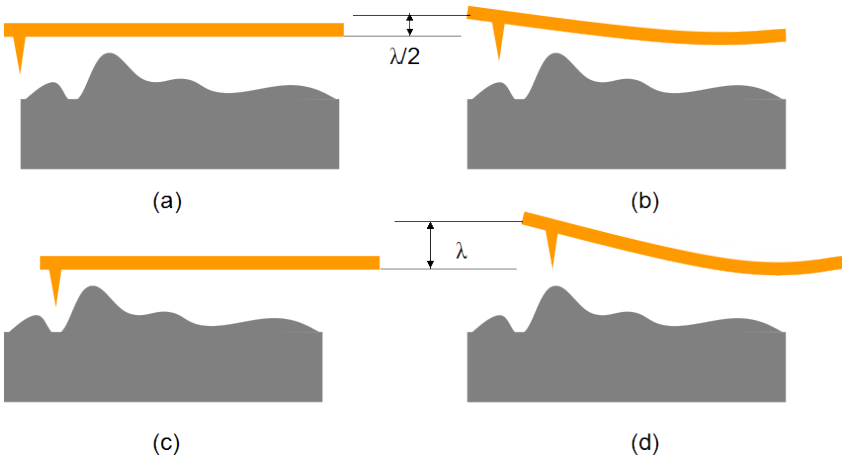


Figure 2-8. Illustration of tip scanning of a sample surface

Fabricating a cantilever with a piezoresistive part is quite a nice way, however such a cantilever is relative difficult to fabricate. Although some companies already has such a technology, it is still a big challenge for experts to reduce cost and complexity of fabrication. But for the idea of crystal oscillator and optical system, they are relative simple and reliable for our project.

3.2. Optical Setup for Detecting Small Amount of Cadaverine

In this master project, resonance frequency need to be tested and thus to measure a very small amount of cadaverine. In order to develop a readily available, easily built and cost effective setup, from the force sensor developed in AFM these years, a simplified optical setup was built and developed for this project.

3.2.1. Possible Optical Solution - Inspiration from AFM

Cadaverine is an organic compounds with strong unpleasant smell, however the smell is undetectable for human's nose when the concentration is lower than 50mg/ml (which is the mixture of 0.5ml cadaverine with concentration of 95% and 8ml water) in water. The data 50mg/ml here is a rough estimate since it is based on several people's smell sense. Due to the amount is so little and that means the sensor have to be very sensitive.

As we discussed in chapter 1, micro cantilever is becoming a new and promising biosensor these years. The small tip is very sensitive to tiny change in molecular weight scale, this property have gained quite a lot of attention. The idea could be either testing the bending or shift of resonance frequency of cantilever before and after cadaverine bonding. Due to the properties of auto vibration after the interaction of some molecules and the complexity of electrics part, testing the shift of resonance frequency is apparently a better direction to implement.

Section 3.1.2 and section 3.1.3 introduce and compare different solutions of force sensor for AFM.

Certainly the first solution is out of our consideration due to the mechanical complexity, also the last solution is not our preference because we are aiming low-cost while such a piezo-resistive cantilever is relative expensive. Therefore the solutions of interference, crystal oscillator and optical system are our candidates, however consider the simplicity and stability of implementation, apparently the idea of crystal oscillator and optical system are the better solution that we could focus on and develop in our setup.

3.2.2. Piezoelectric drive optical setup

The implementation of the optical setup is mainly based on the idea of optical system applied in AFM and drawn on some inspiration from crystal oscillator. The schematic of this setup is illustrated in figure 3-9.

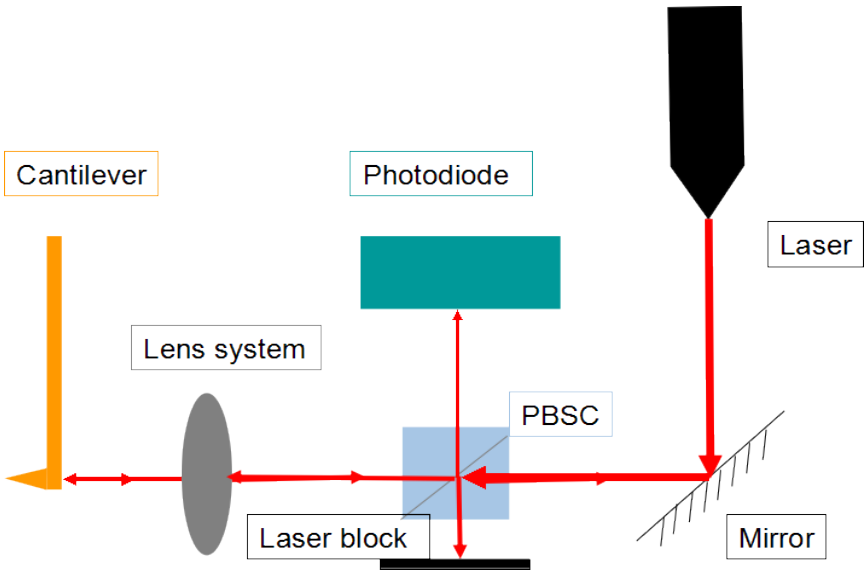


Figure 3-9. Schematic of optical setup based on the principle of piezoelectric for small amount of chemical test

Here a laser source is used to give the laser beam which will be reflected by the mirror, thus the beam will be directed to a polarizing beam splitter cube(PBSC) and divided into two polarized lights. One polarized light will pass through the cube and be guided to a lens system which help to focus the laser spot while the other will be trapped or blocked. The focused laser beam will finally hit the end of cantilever and the reflected laser from it will back to the PBSC, where the reflected signal will be immediately be guided to and detected by the photodiode.

This idea is implemented in the figure 3-10. Once piezo start driving the resonance frequency of cantilever, a more violent vibration happens on the cantilever and results in a bigger reflection signal which will be detected by the photodiode. In this way resonance frequency is measured. Comparing the resonance frequency of cantilever before cadaverine bonding and after cadaverine bonding, it is a easier and faster way to find out the small amount of cadaverine and therefore for meat quality testing in future application.

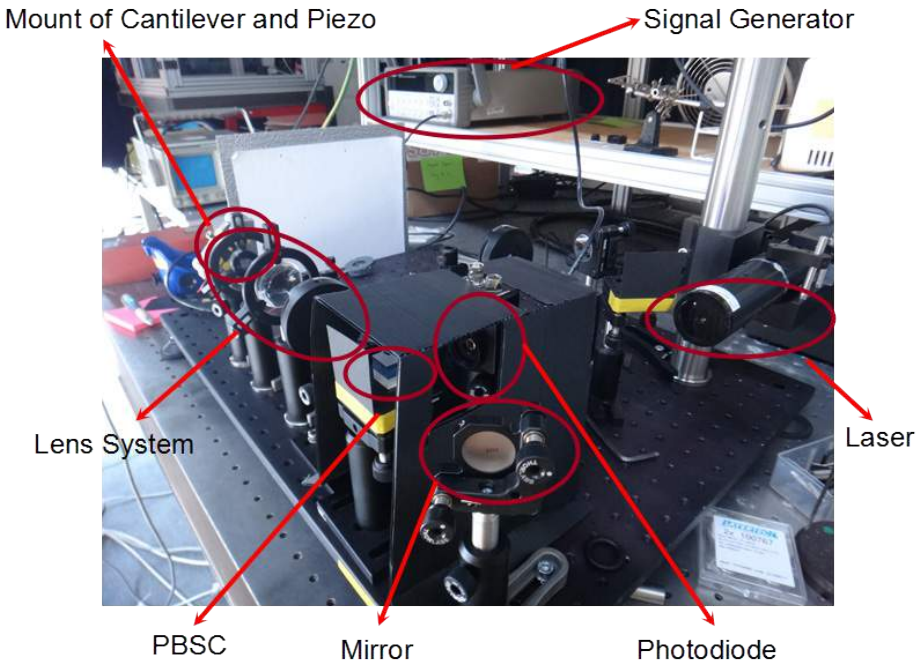


Figure 3-10. Implementation of the optical setup based on the principle of piezoelectric for small amount of chemical test

The laser source is GL S5372 from NEC Corporation with red light of wavelength around 635nm. This laser source is a composed laser diode with optical focus and the diameter of output laser spot is within 2mm. Such a laser source is chosen for several reasons. First of all, there is no requirement of high laser power or intensity because of extremely sensitive photodiode in our optical setup. Secondly, red laser is observable for human eyes and also red is an alarm color, thus it is safer and easier to use during experiment phase. Certainly it is relative cheaper compare to most other laser source, and it is more widely used in today's world.

The function of mirror here is mainly changing the direction of laser beam. As we can see from figure 3-10 that the laser source is big while the space of platform is limited, thus a better arrangement is needed and mirror here plays a role of director for laser beam. In addition, the mirror can be used as an adjustment to align and also to scan the laser to cantilever.

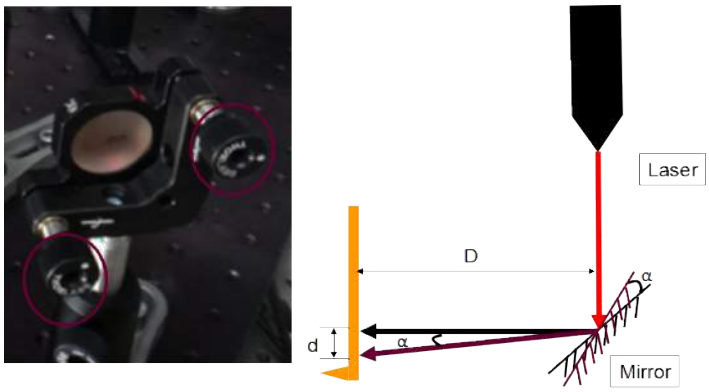


Figure 10. The principle of adjustment and scan of mirror

By adjusting with the knobs of the mirror and an small angle is tilted - therefore the laser is guided to

another direction, like the simple example illustrated in figure 3-11, after tilting a small angle α, the black mirror will come to the position of the brown one, and certainly the two reflected light will result in the same angle between themselves. The distance between two laser spot *d* is proportional to the angle α and the distance *D* between mirror and cantilever.

The PBSC is the abbreviation of polarizing beam splitter cube. It is an optics that split an unpolarized light into two polarized beams. In figure 2-12 demonstrates a beam splitter that set 'p' type polarized light as transmitted radiation and 's' type polarized light as reflected beam. P-polarized indicates electric field intensity vector is parallel with the plane of incidence and s-polarized means the vector is perpendicular to incidence plane[62]. Usually the entrance side and exit side are both coated with a thin anti-reflection layer[63], such a design allow people to collect the reflected light signal with higher accuracy. The optical splitter cube is commercially composed of two cemented prisms and one of them with a thin film package on the hypotenuse[62]. The film package inside the cube is always optimized by changing refractive index (which mainly depends on the materials) and thickness of different layer to make efficient function of polarization.

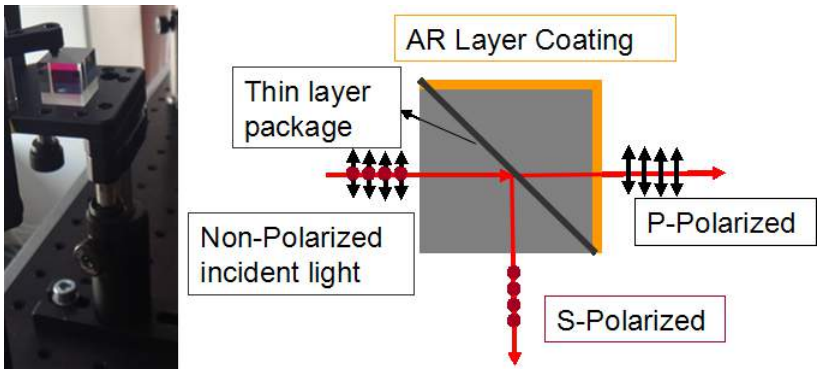


Figure 3-12. Working principle of a PBSC, based on reference [63]

The lens system includes several plano-convex lenses and a quarter waveplate. This part was changing all the time to make the best focus of laser beam on cantilever. This experiment requires quite high precise alignment and focusing due to the tiny size of probes. The polarized light pass through the quarter waveplate first to achieve elliptically polarized light which will be more stable in the atmosphere even with some small noises disturbances. Also the waveplate help to reduce the influence of the scatter from beam splitter. Since the width of tips on cantilever is 35um and the distance between two tips is around 150um - which means the diameter of the laser spot must be focused into the range smaller than 0.2mm. Therefore several lenses are applied here to focus the incident laser beam as much as possible.

The mounting and adjustment of the piezo and cantilever has shown in figure 3-13. As described in the previous section, when a high voltage is applied to the piezo, the piezo will have a small displacement within 2um. The higher voltage, the more displacement. Certainly such a sensitive piezo will oscillate after input a signal from signal generator and once it reaches the resonance frequency of cantilever, the cantilever will vibrate violently and result in a much bigger signal of reflection, which will be immediately detected by photodiode. The mounting is developed for different types and more detail can be found in Appendix I.

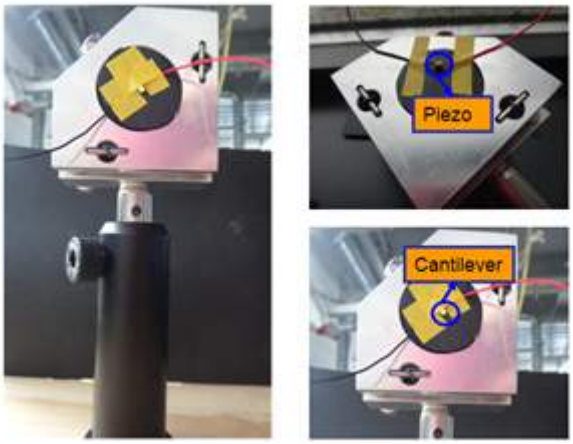


Figure 3-13. The mounting and adjustment of piezo and cantilever

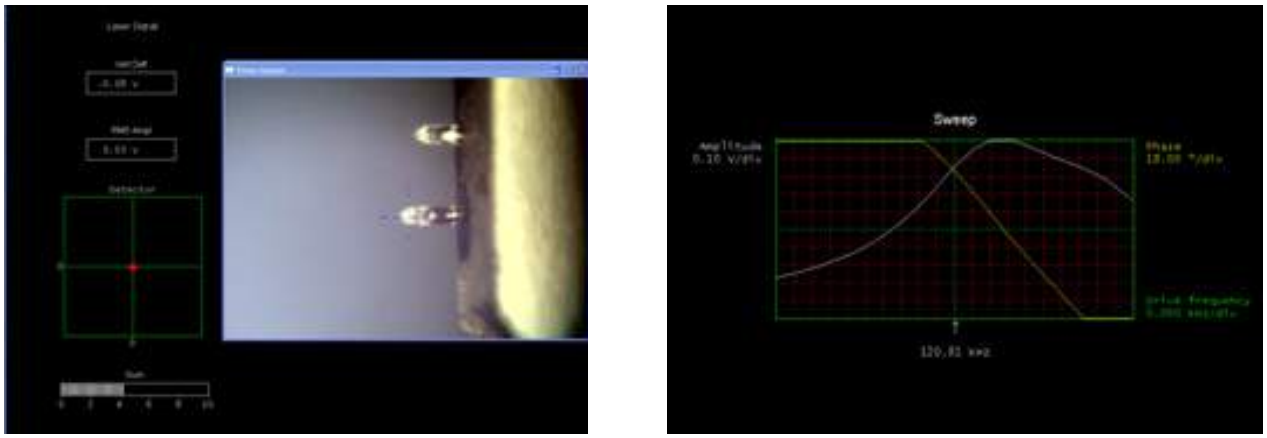
The photodiode used here is PDA36A-EC from THORLABS, accompanied with an oscilloscope (HEWLETT PACKARD 546450, MIXED SIGNAL OSCILLOSCOPE), when a photon with sufficient energy hit the diode and an electron will be excited. In this way current is formed and signals can be sent to oscilloscope. The readout is highly dependent on the light in or out of the matrix of photodiode. Since the reflected signal from cantilever is very weak, and also there is a high possibility that most reflection is coming from the sample instead of micro cantilever, thus it is better to hit the laser beam to the edge of matrix and ensure to receive wanted frequency result.

4.1. Measurement by AFM

AFM is an existing tool for testing cantilever' resonance frequency as we have mentioned in the previous chapter. Measurements made by AFM is of high accuracy, and the difference of testing results from several measurements for cantilevers without functionalization is within 0.1KHz.

4.1.1. Testing Method with AFM

The measurements were successfully made by AFM. Replacing the tip in AFM to the cantilever after functionalization. As shown in the figure 4-1, it gives resonance frequency of the longest cantilever(with the length of 130um, width of 35um). And the resonance frequency of this cantilever showed in the graph is around 120.81KHz.



(a) (b)

Figure 4-1. Testing of the resonance frequency of functionalized cantilever: (a). Alignment to the longest cantilever; (b). Measured resonance frequency

The 'sum' in the figure 4-1(a) illustrates the laser spot alignment to the cantilever. Which will be further explained in figure 4-2, only with sufficient sum signal of the reflection from the cantilever is acceptable in AFM, which means the laser beam is hitting on the middle of the back of cantilever (usually without functionalization) and therefore the right signals can be reflected and guided to the photodetector.

Also the reflected beam should be focused on the center of the photodetector in order to set the original position, as we can see the red spot in the 'Detector' frame in figure 4-1(a). In this way, when we apply cantilever with a range of frequency, and only at the resonance frquency the position shift of laser beam is significant bigger than at other frequencies. Therefore the resonance frequency of a cantilever is found.

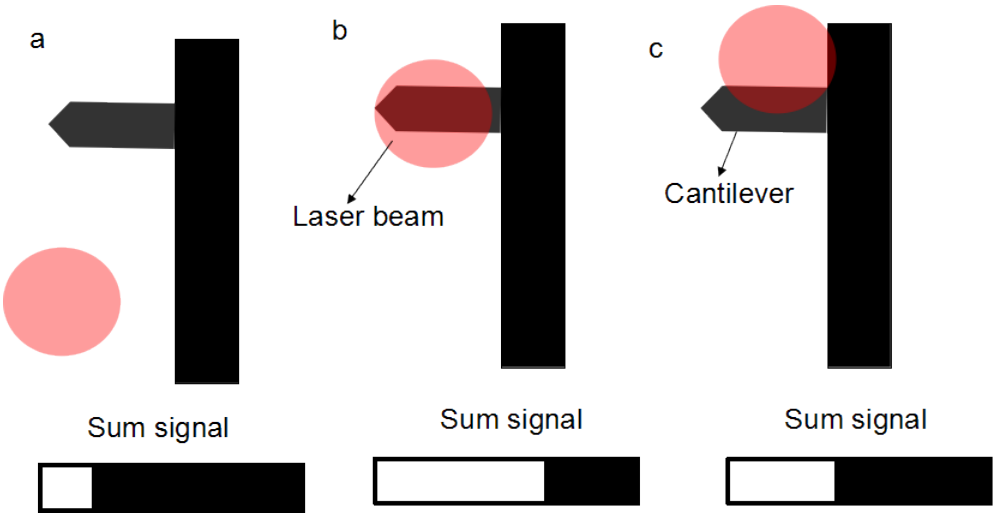


Figure 4-2. Laser alignment to the cantilever[67]

4.1.2. Test Results by AFM

The cantilevers without any functionalization is measured by AFM firstly and the results are given in table 4-1.

Table 4-1. The measured resonance frequencies for cantilevers with different lengths (without functionalization)

Length of cantilever(um)	Resonance Frequency of Cantilever F(KHz)
130	120.44KHz
90	244.23KHz
110	166.82KHz

Later the resonance frequencies of two chips with six functionalized cantilevers are given in table 4-2, these two chips applied with solution of 0.05mg cyclam and 2.5ml 2-ethoxyethanol by nanoplotter 2.1, these datas are the average value of three measurements. Also the resonance frequencies of the cantilevers functionalized by pure cyclam gas phase bonding is tested, as we can see the results from table 4-3.

Table 4-2. The measured resonance frequencies for cantilevers with different lengths (functionalized by nanoplooter 2.1 with solution of 0.05mg cyclam+2.5ml 2-ethoxyethanol)

Length of cantilever (um)	Resonance Frequency of Cantilever A (KHz)	Resonance Frequency of Cantilever B (KHz)
130	121.55	120.84
90	244.65	244.82
110	166.97	166.93

Table 4-3. The measured resonance frequencies for cantilevers with different lengths (functionalized by pure cyclam gas bonding for 5s)

Length of cantilever (um)	Resonance Frequency of Cantilever C (KHz)	Resonance Frequency of Cantilever D (KHz)
130	132.42	122.73
90	258.83	231.06
110	183.21	unmeasurable

*Notice: For simplicity, Cantilever A to Cantilever D here indicate the Cantilever 5, Cantilever 6, Cantilever 7 and Cantilever 10 in Appendix II Test 9, respectively.

However, the resonance frequency of cantilevers with functionalization of pure cyclam gas result in very broad resonance frequency peak. And also we need to supply a very high driving voltage (can reach to almost 6000mV while for cantilever without functionalization is usually within 1000mV). This phenomenon might caused by very high concentration of cyclam bonding which result in a very heavy cantilever end, thus a much higher power supply need to vibrate the heavy cantilever. As we can see from figure 2-23, the cyclam fiber bond to the edge of the cantilever will scatter the laser beam and thus influence the reflected signals. Therefore the measured resonance frequencies might not be the real ones of these cantilevers.

If we just look the data from the table 4-2, we can see the resonance frequency of the cantilevers with the same length shares a very similar results - it could be a coincidence, but more likely that these cantilevers are from the same fabrication wafer and shares a very similar resonance frequency, and the way of functionalization by nanoplotter is quite accurate and average, and thus result in a very similar resonance frequency shift. The maximum difference is around 0.7KHz.

With a very small amount of cadaverine molecules, the shift of resonance frequency will be relative small, too. Since the cantilevers with pure cyclam functionalization have a really unstable resonance frequencies and the uncertainty range is quite big - a few kiloherz, and thus only the cantilever functionalized with solution of 0.05mg+2.5ml 2-ethoxyeothanol by nanoplooter should be tested after cadaverine bonding.

4.1.3. Test Results Discussion

Unfortunately, the AFM is not working and further verification can not be done. If possible, the measurements will be done before the defense and will be present on the exam. But still, with the data we have now, we can make some discussion about the idea in theory.

As we can compare the data from table 4-1 and table 4-2, the resonance frequency is incresed a few hundred Heltz after functionalization. From the equation 1-1 we have mentioned in chapter 1, such a small shift of resonance frequency means that there might be some small additional mass on the ends of these small tips.

For simplicity, if we define the sensitivity as the ratio of resonance frequency shift (Hz) and mass

change (kg), we could get an expression shown in equation 4-1[70].

$$\frac{\Delta f}{\Delta m} = \pi \sqrt{\frac{k}{m^3}} \quad (4\text{-}1)$$

Where k (N/m) is the spring constant and m (kg) is the efficient mass.

From the datasheets of cantilever in Appendix III, take the one with length of 130um for example, we can easily calculate the mass m here is around 2.13×10^{-11}kg, due to k here is ranging from 1.7 to 14, therefore the sensitivity is finally result in the range of 4.17×10^{16} and 1.20×10^{17}. Here we could simply take a value of 8×10^{16} in this range.

From table 4-1 and table 4-2 we can see these shifts of the resonance frequency is 1110Hz and 400Hz (assume all the cantilever with the same length share the same resonance frequency before functionalization), if we take the value of 1100Hz, then we can calculate the mass change is around 13.75pg. Assume the solution (0.05mg cyclam+2.5ml 2-ethoxyethanol) we made is very homogeneous, then we can roughly estimate the applied mixed solution on this cantilever is about 7×10^{-7}ml, which actually makes some sense if we consider the mixed solution on top of cantilever form a thin layer as illustrated in figure 4-3. If the thickness of this thin liquid film is a few hundred micrometers or even smaller, then the mixed solution really functionalized on the cantilever is within 1×10^{-6}ml.

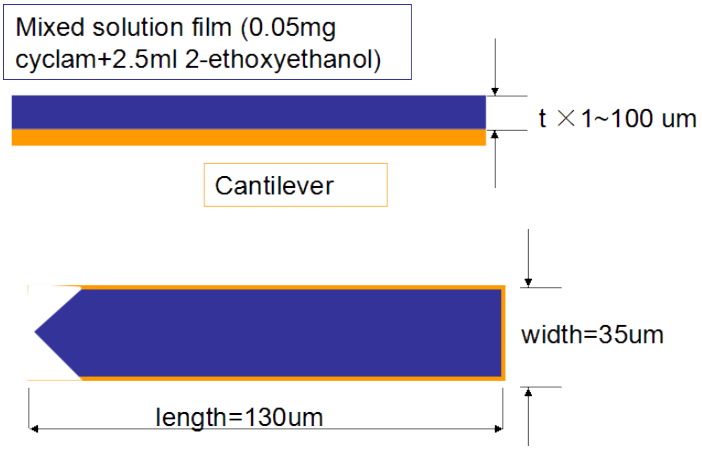


Figure 4-3. Illustration for volume estimation of mixed solution applied on the cantilever (130um length)

The above calculation is based on some assumption and estimation, it is trying to approve that it is possible to use a cantilever to test small mass change by measuring resonance frequency shift. However as we can see from Appendix III, the range of these frequency are very big, the difference between possible maximum resonance frequency and minimum one can reach to 245KHz. Therefore it is very important to use the same cantilevers to test the shift of resonance frequency.

The concern is that the bonding tests may result in bonding some cadaverine molecules, but glycerol is also bonding to the cantilever and thus influence the result. But these effects could be possibly solved by build a relative stable ad controllable system for functionalization and a reliable database after hundreds or even thousands of sample tests in the future.

4.2. Measurement by Optical Setup

The optical setup was built and developed for many times but still result in a measurement failure. The reason can be vary from the limitation of power from signal generator to the unsuitable photodiode. The possible correction and improvement in the near future is to assemble right devices.

4.2.1. Optical Setup Testing Results

As discussed in the previous chapter, the optical setup is based on the similar principle to AFM. However a lot of difficulties came out and resulted in a measurement failure. And the result signal from photodiode is shown in the figure 4-4. The calculation of frequency can be expressed by:

$$f = \frac{1}{t} \quad (4\text{-}1)$$

Where t is the time period between two peaks or two valleys in signal display.

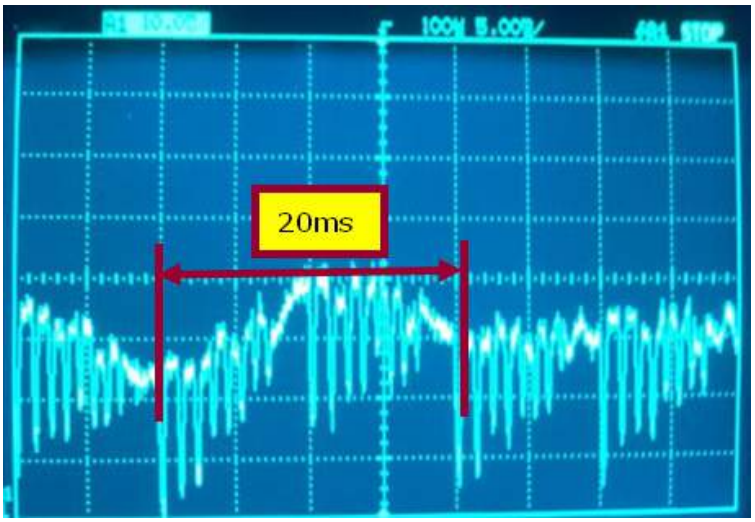


Figure 4-4.Testing result from photodiode: the frequency might from photodiode itself

The testing result can be read from figure 4-4, the signal output is combined with two waves and the bigger one is relatively stable in a range from 0.05KHz to 0.1KHz. This frequency is too low compare to our input signal, thus this relative stable frequency probably is just the noise from the photodiode itself.

Ignoring the small frequency and measure the signals with bigger frequency which is indicated in figure 4-5, and soon we find out that this frequency is within 1KHz, which is not the frequency we are looking for either. There is a high possibility that this frequency is from the environment since it is changeable during different testing period.

Even the laser source is turned off, these two signals are still displaying on the oscilloscope which connect to photodiode, that is further approved that these readable signals with very low frequency are not from the cantilever.

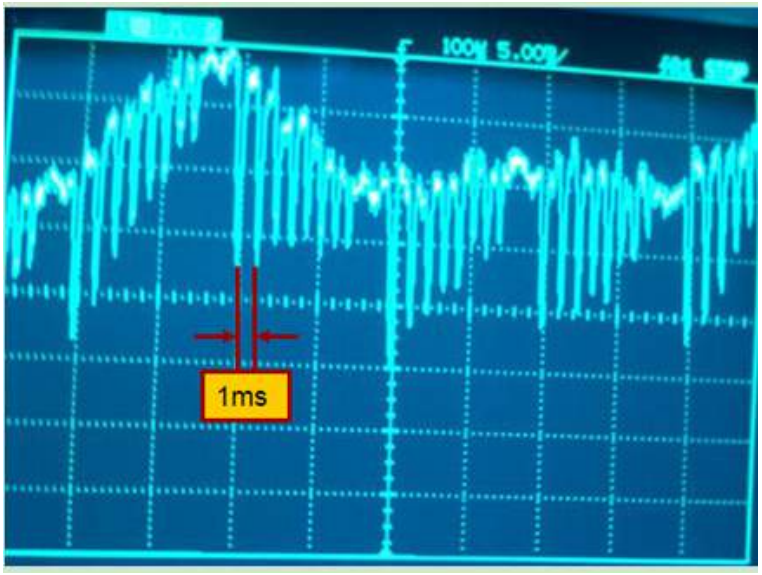
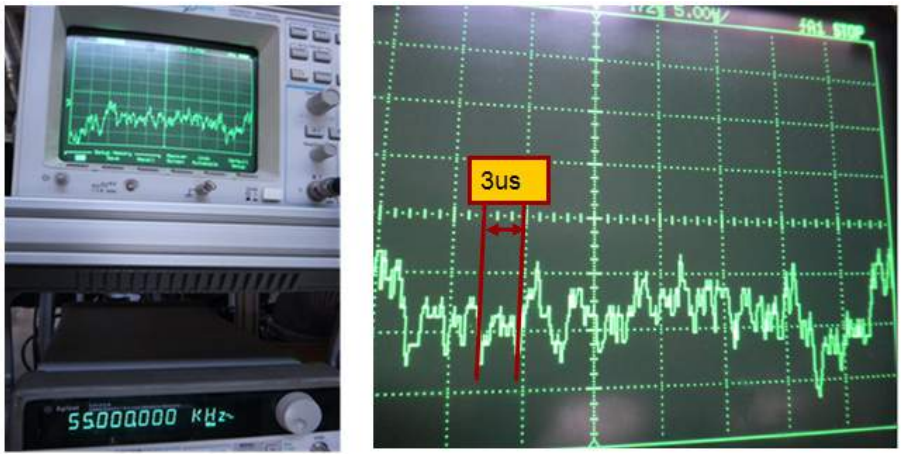
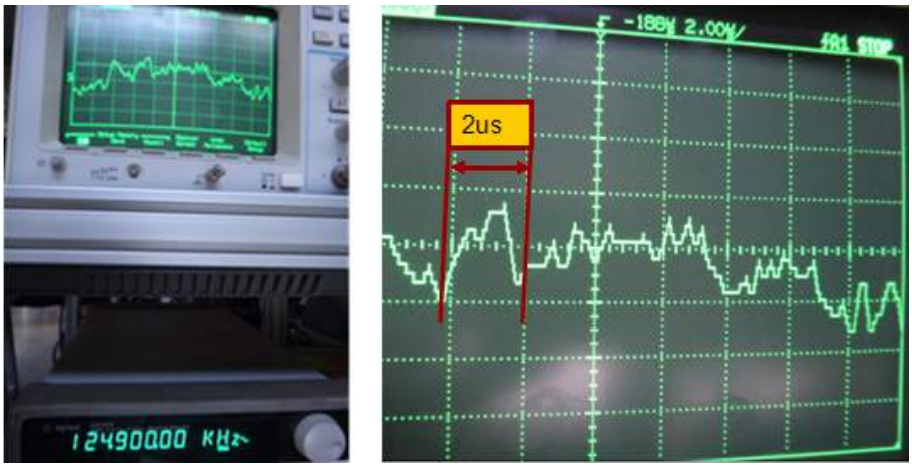


Figure 4-5. Testing result from photodiode: the frequency from background noise

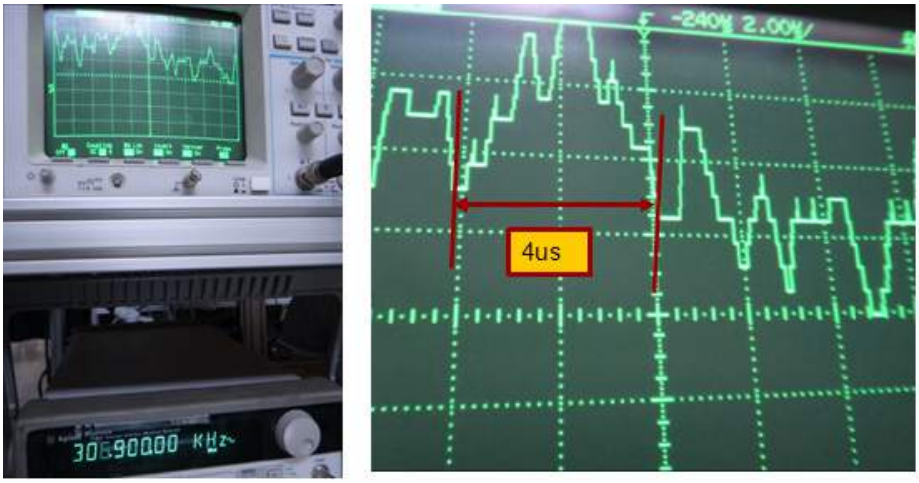
Having considered the reflected light could be very weak and therefore result in a tiny change compare to the noises. By reducing the time period and the results from the photodiode are shown in figure 4-6, and apparently these frequencies are very unstable and does not fit the input signal at all. The time period indicates in figure 4-6 (a) to (c) just give an approximately average period.



(a)



(b)



(c)

Figure 4-6. Readout from photodiode: (a). Input signal: 55KHz, Output readout: ~333KHz; (b). Input signal: 125KHz, Output readout: ~500KHz; (c). Input signal:307KHz, Output readout: ~250KHz

Although there are some possibilities that the cantilever will not vibrate immediately after the frequency signal sent from the signal generator. Also the exciting piezo may have some inaccuracy or delay in transferring the frequency to cantilever. However in such case, there are still should be some relations between the input signal and output readout - which we could not see from the tests and therefore the optical setup we built for this project is not working appropriately.

4.2.2. Explanations for Optical Setup Testing Failure

From the previous section, although a lot efforts and ideas were applied on the optical setup, the testing results are very depressing. Basically, three parts need to be taken into consideration in analysing experimental failure: input, transmission and output. Thus, several reasons were found here and listed in this section.

First of all, the input should be the reflection from micro cantilever - or more exactly, should be the right vibration of cantilever. After carefully examing the signal generator - Agilent 33120A 15 MHz Function/Arbitrary Waveform Generator, we noticed that the default offset voltage is 100mV and apparently the piezo will not be functional at such a low power supply. And the maximum output voltage of this Generator is less than 20V, as we can see from figure 4-7, still the displacement of piezo is too less to transfer correctly to micro cantilever.

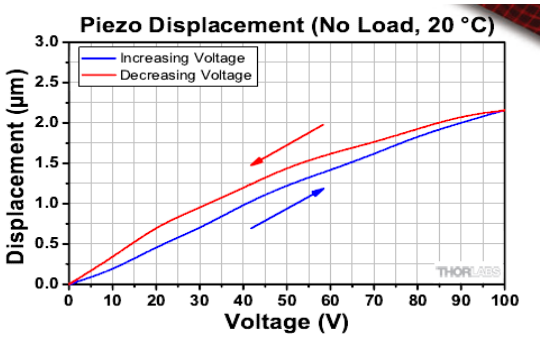
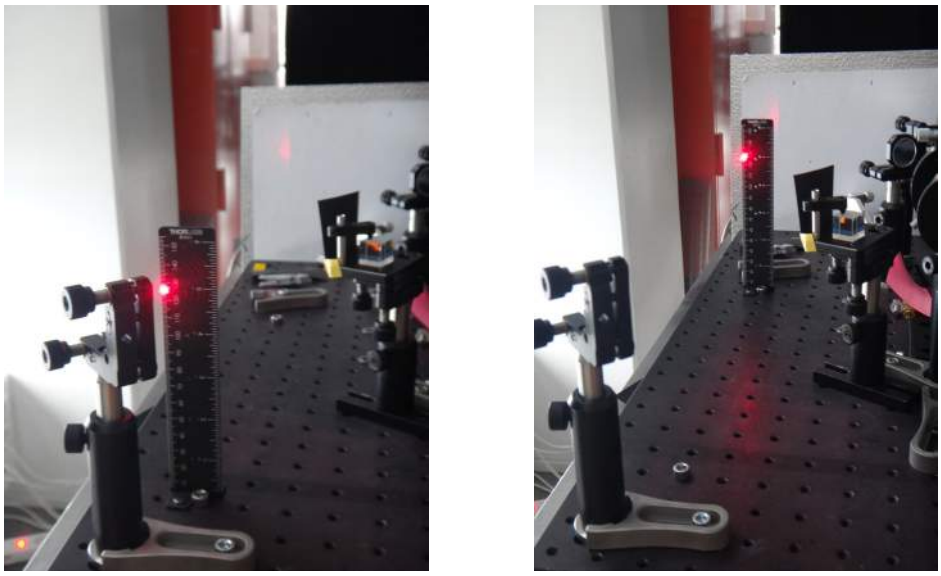


Figure 4-7. The relasion between piezo displacement and applying voltage(Appendix IV)

In addition, the mounting used to fix piezo and cantilever is not very precisely - such a small displacement tranfer from piezo to cantilever means they have to be fixed in a relative tight way, if there are a gap larger than 2um or even 0.5um in our case, the vibration of piezo will not transfer to the cantilever. Both piezo and cantilever are very small and thus increase the difficulty in mounting them together tightly without breaking cantilevers at the same time.

What's more, even though the piezo and cantilever are mounted together and fixed tightly to each other by clamps, we still can not make sure that the mechanical movements of piezo will transfer to the cantilevers' vibration correctly. The tough part of this mounting is there is no simple feasible way to check if this transmission is successful or not. The maximum displacement of piezo is 2um and even with such a movement, it is still hard to tell either the piezo works correctly or the cantilever get the frequency correctly from piezo. And apparently no matter which one mentioned above is not working appropriately will result in testing failure.

The transmission in this setup is fulfilled by laser beam. The alignment is not a big problem, as we can see from figure 4-8 (a) to (b). A ruler here used to make sure the laser beam is at a straight line, tilted laser beam could be adjusted by mirror on the corner as we discussed in chapter 3.



(a) (b)

Figure 4-8. Laser beam alignment

However the focusing of laser beam is quite tricky to achieve during measurement. The lens system needs to be placed and adjusted appropriately to make the focus laser spot as small as possible. But due to the diffraction of lens (It can be reduced by focusing the light in the center of lens or use several lenses) itself and cause a slightly scatter from the optical path, thus the diameter of laser spot is hard to control within 200um. Therefore the laser spot was controlled to focused on the edge to ensure the reflection is at least partly from the cantilever, which is illustrated in figure 4-9. Certainly it is an ideal case and still there could be a big risk of misalignment because we are not allowed to look directly from the laser spot.

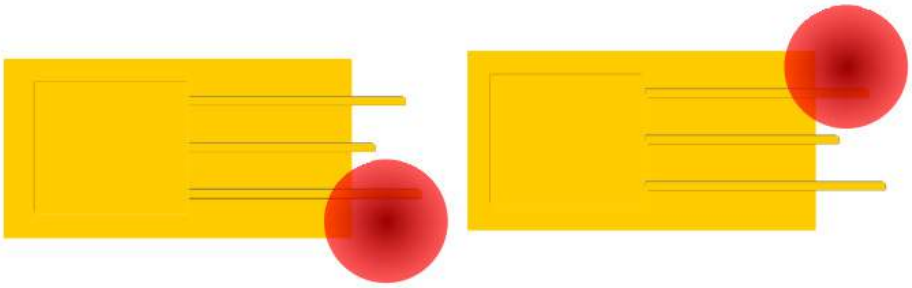


Figure 4-9. Schematic of the edge focusing of a micro cantilever

More importantly, output readout may work in a poor way - actually refers to the photodiode in this experiment might not be the best choice since it only works perfectly with signal in or out the matrix, as showed in figure 4-10. While the vibration of micro cantilever is quite small and the reflected beam may not so easily cross the edge of matrix. And also in this project, the signal is very weak and micro cantilever movements is kind of unstable due to the poor mounting. The weak and unstable reflected beam will result in a wrong input to photodiode even though it is extremely sensitive, and certainly the output readout from oscilloscope is not correct either.

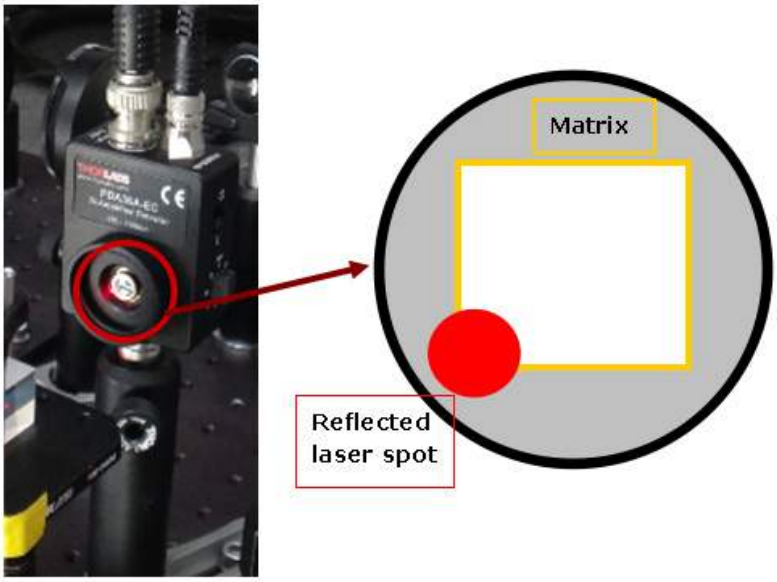


Figure 4-10. Working principles of photodiode

In addition, the background noises and light influence are also a big issue. As just discussed before, the signal we want is too weak to ignore all kinds of noises, and these noises is not easy to avoid during experimental phase in the lab.

4.2.3. Correction and Improvement in the Near Future

Several ways could be applied to the correct optical setup in the near future. For the piezo we could change to one with lower drive voltage and bigger contact surface which will be much easier to fix

cantilever on top. There are huge amount of different piezo in the market[69], and for future application the recommend piezo plate shoud have a resonance frequency no lower than 300KHz (because the resonance frequency of cantilevers in this project can reach to 250KHz or even higher, but this requirement is negotiable if different cantilevers are chosen for future testing). Or we could simply change a signal generator to the one with high output voltage, but this way is obviously more expensive.

A quadrant photodiode can be used in the setup. Since such a device consists of 4 separated photosensitive surface, and when the laser beam hit on the center and spread all four areas, the X output (left and right quadrants) and Y output (top and bottom quadrants) can be adjusted to an initial value in the beginning, later even a small deviation can be sensed and transferred to the readout system[68]. Therefore the beam just need to hit on the center of the matrix which will make it a easier application and get better feedback of the reflected signals.

The alignment and focusing of laser beam could be improved by a more complicated lens system. Or we could simply replace the cantilevers with three ends to just one end, thus the diameter of laser spot does not need to be so small.

Chapter 5 Conclusion and Outlook

5.1. Conclusion

Micro cantilever is a new approach as a biosensor for small amount chemical or biological content detection. In this master project, micro cantilever is applied in a similar way to measure the low concentration of cadaverine, and it aimed to become a sensitive, fast and cheap sensor for meat spoilage in the future.

In this thesis, we made huge amount of samples in functionalization part in order to achieve a possible method for cantilever to be selective to cadaverine (perhaps other toxic biogenic amines). Cyclam (1,4,8,11-tetraazacyclotetradecane) is nicely bonding to the gold surface and shows some ability in absorbing water and cadaverine. The fiber-like white solide cyclam usually form a cluster on metal surface (varies in diameters depends by different functionalization condition) and therefore trap the cadaverine molecules inside.

A few possible ways of cantilever functionalization are mentioned in this thesis, with mixed solution of cyclam and 2-ethoxyethanol by nanoplotter 2.1 applying; heat pure cyclam solid to gas phase and then let it cool down and reform solid on cantilever (followed by applying gas phase of 2-ethoxyethanol or acetone can lower the concentration and reduce the cyclam 'grow' on the end of cantilever); mixed solution of cyclam and acetone. Different methods has its own advantages and disadvantages, and the later two method still need some optimization.

Cadaverine bonding can be either by applying liquid solution of mixture of water and cadaverine or gas bonding by heating mixed solution of glycerol and cadaverine. Either of the way is relative reliable and gives nice results as we can see from SEM images or optical images. Also a real meat testing with chicken breast has done which approve the idea that cyclam is able to absorb or trap cadaverine molecules to some extent. Only gas solution was applied on functionalized cantilever because it is easier compare to liquid solution - since the cyclam is soluble in the water, when applying a liquid solution drop (with very low concentration of cadaverine) will result in the water washing away the cyclam functionalized on cantilever instead of cadaverine bonding.

The optical setup based on the principles of AFM was built to measure the resonance frequency shift. However it needs some more work or further investigation, thus part of the frequencies were measured by AFM. The resonance frequency result in a few hundred hertz to a few kilohertz shift after functionalization. These results in this thesis indicates it is a possible even a promissing way to assemble a sensor with micro cantilever for detecting meat freshness in the future.

5.2. Outlook

Since it is at the really beginning of testing a new idea, there are a lot work can be done to further optimize and improve the project.

In the first place, the functionalization method could be optimized to a more reliable recipe after more samples and cantilevers testing. Furthermore, a microfluidics system could be built for liquid solution of cadaverine bonding to cantilever - this way is actually more user-friendly and cost less energy (without heating to a very high temperature).

Next, the optical setup could be improved by changing several parts like piezo plate and photodiode, and the mounting design could be later optimized to fix cantilever and piezo easily and perfectly. And try to assemble the whole system in a proper way.

What is more, huge amount of cantilevers need to be tested to see if there are a reliable relation between the concentration of cadaverine and the resonance frequency shift of cantilever before and after cadaverine(with this concentration) bonding. Not only to test different concentration of cadaverine, but also to test the same concentration for multiple times to see if it results in a very similar frequency shift.

Finally, if everything works well, these cantilevers after optimized functionalization can be used in real meat testing and later a formula could developed, in the future a software could be developed and assembled to the whole setup.

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I. Mountings of Cantilever and Piezoelectric Chip

Three mountings are designed or found to fix piezo and cantilever to ensure the mechanical displacement will transfer to cantilever vibration and to find out the resonance frequency of cantilever correctly.

The first mounting of piezo and cantilever was designed by James Hoyland as showed in figure I-1. It is really hard to fix cantilever and peizo together in this mounting by hand since they are very small and especially the cantilever is too small and easily to be broken down.

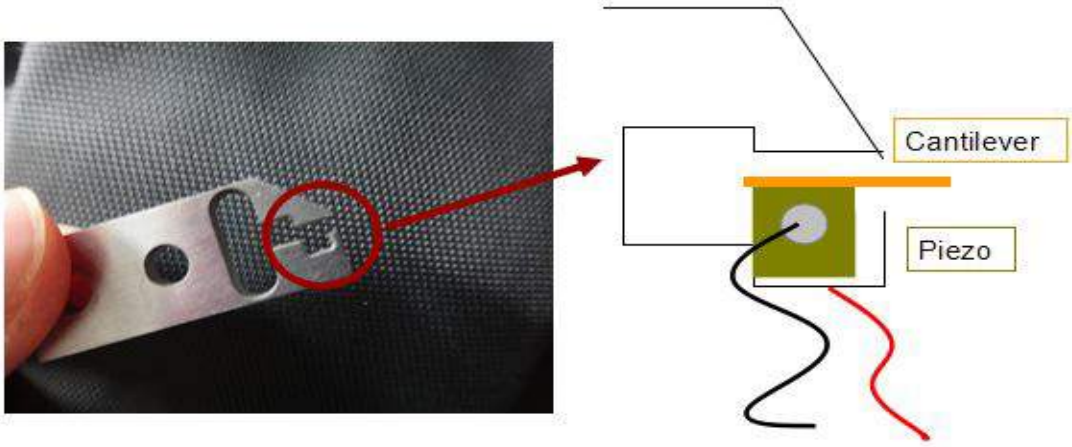


Figure I-1. Illustration and image of the first mount of cantilever and peizo

Soon a new design is developed which is inspired from clamps from daily life. It is very easy to fix the cantilever and piezo together. As we can see the design details and images of it in figure I-2. However the problem of this mounting is instability when it is fixing piezo and cantilever together, and position adjustment of cantilever is hard to do, therefore it is not very easy to achieve and guide the right reflection from cantilever.

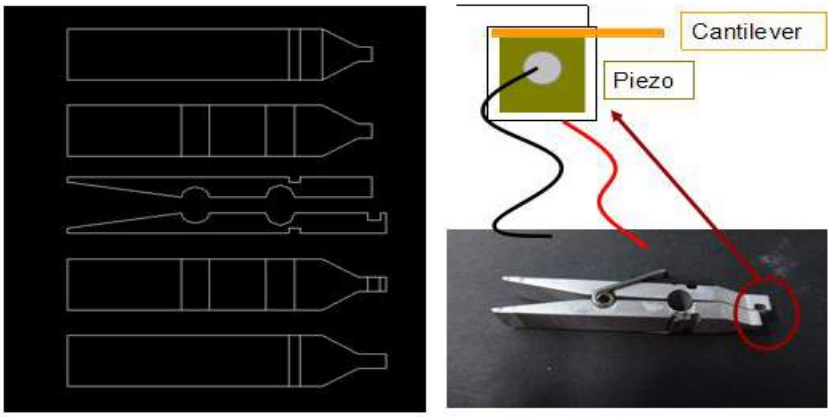


Figure 2. Illustration and design of the second mount of cantilever and peizo

The mount used in the optical setup is showed in figure II-3, and in this way we could easily fix peizo and cantilever together and also able to do some alignment like to find the right position. In addition the black paper will not reflect any laser back and therefore ensure the reflection is mostly from the cantilever.

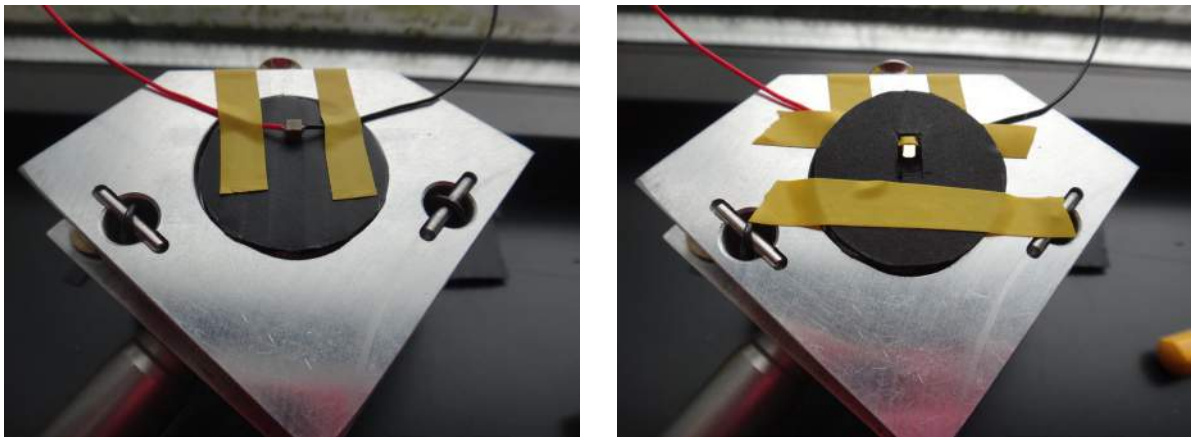


Figure I-3. Illustration of the third mount of cantilever and peizo

II. Functionalization Details

Test 1. Better Solvent for Cyclam to Bond to Gold Surface

- **Variables:** Solvent (water, 2-Ethoxyethanol)
- **Number of samples:** 2
- **Experimental details:**
  - Mix the solvent and solute and shake the mixture slightly
  - Put the sample with clean gold layer to the mixture and leave them for 24 hours
  - Take the sample out and bake at 60°C for 2 hours

Table II-1

Sample	Solvent	Solute	Processing time	Baking temperature	Baking time
1	2.5ml water	0.05mg cyclam	24 hours	60°C, hot plate	2 hours
2	2.5ml ethoxyethanol				

- **Result:**

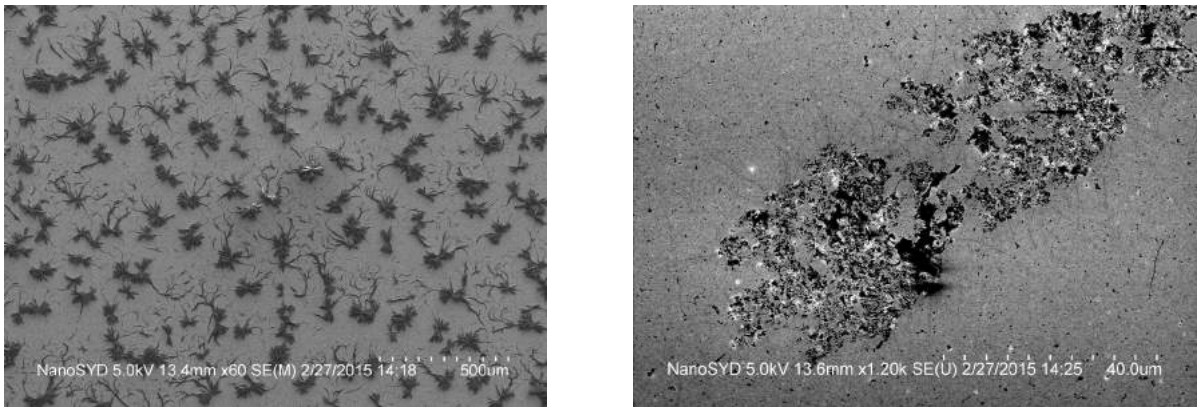
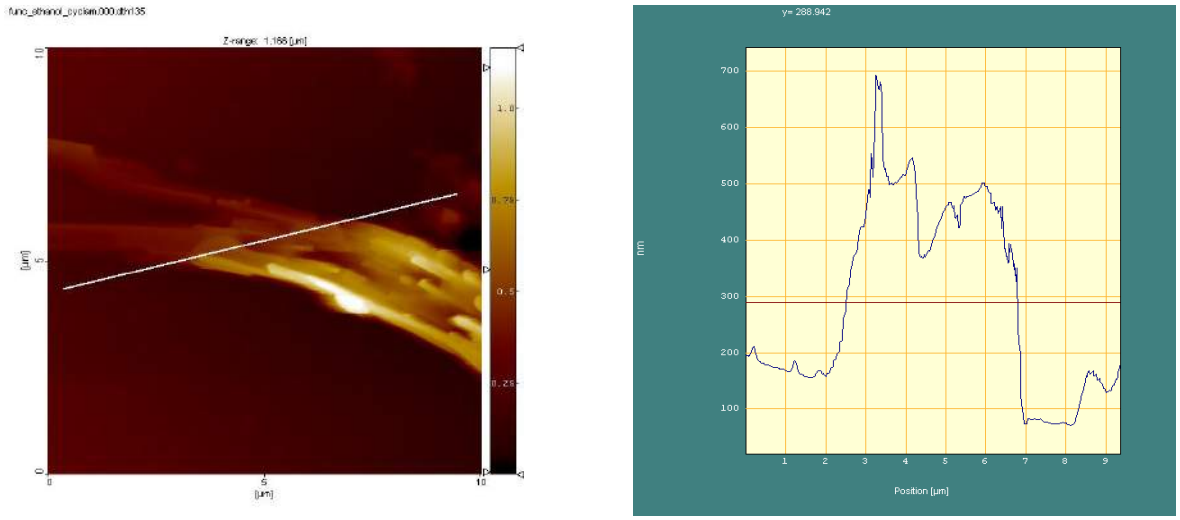
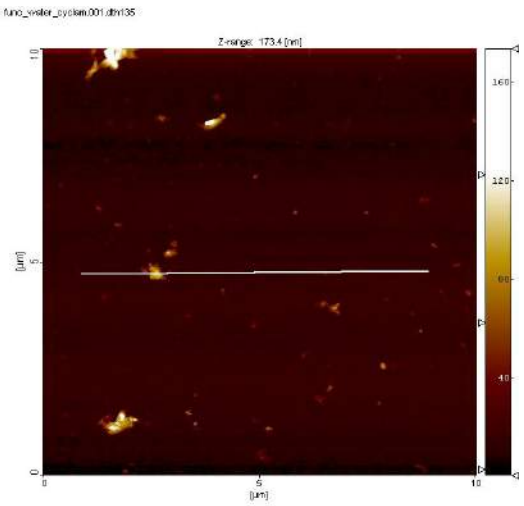


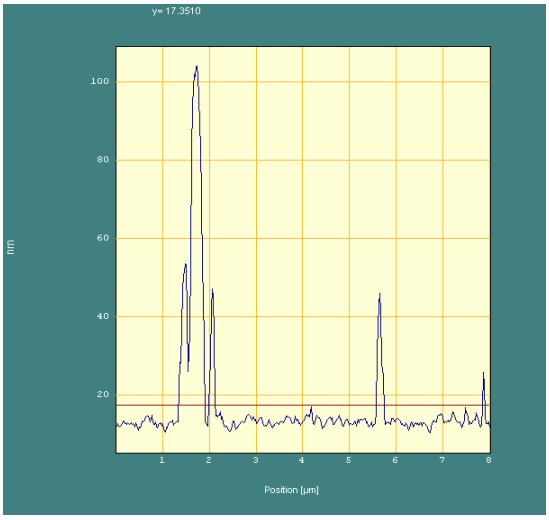
Figure II-1. SEM images: (a). Sample 1: functionalization with solvent of 2-ethoxyethanol, magnification of 50; (b). Sample 2: functionalization with water, magnification of 1.2K



(a) (b)



(c)



(d)

Figure II-2. AFM graphs, the thickness of cyclam on gold: (a) (b). Sample 1: functionalization with 2-ethoxyethanol (100nm-700nm); (c) (d). Sample 2: functionalization with water(less than 100nm)

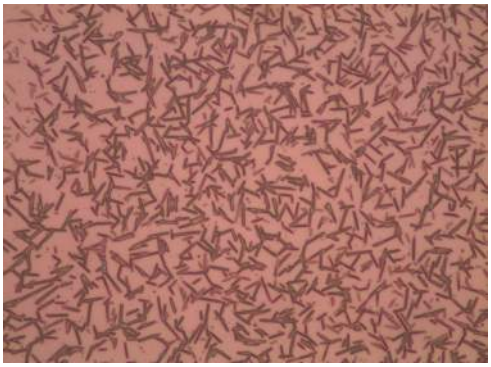
Test 2. Cadaverine Bonding Test: Liquid Phase and Gas Phase

- **Variables**: Bonding phase of cadaverine (liquid, gas)
- **Number of samples**: 2
- **Experimental details**:
  - Functionalize samples as described in Test 1
  - Mix the 0.5ml cadaverine (95%) and 2ml water and shake it slightly
  - A drop of mixture to sample 3 and leave it to dry
  - Heat the mixture at 150°C and let the evaporated gas to the sample 4 for 3min

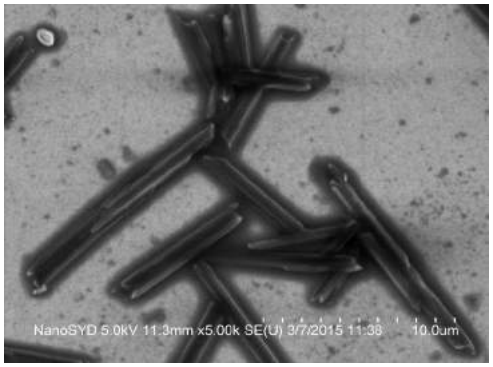
Table II-2

Sample	Functionalization details	Cadaverine preparation	Cadaverine bonding method
3	2.5ml 2-ethoxyethanol +0.05mg cyclam, process for 24h, bake at 60°C for 2h	0.5ml cadaverine (95%) + 2ml water	Liquid drop
4			Gas evaporation

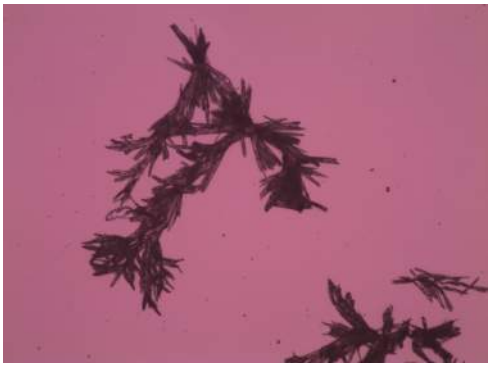
- **Result**:



(a)



(b)



(c)

(d)

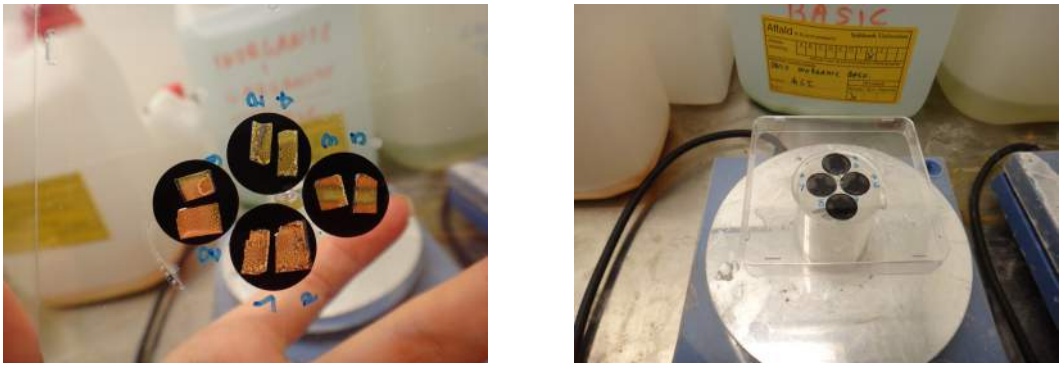
Figure II-3. Sample 3, bond to the gas phase of cadaverine (water); Sample 4, bond to the liquid phase of cadaverine (water): (a). Sample 3, Optical image with 50 magnification; (b). Sample 3, SEM image with 5K magnification; (c). Sample 4, Optical image with 50 magnification, (d). Sample 4, SEM image with 5K magnification

Test 3. Functionalization Parameters Optimization

- **Variables**: Concentration of cycalm, processing time, baking temperature
- **Numbers of samples**: 8
- **Experimental details**:
  - Functionalize sample 5 to sample 11 with the parameters showed in the table II-3
  - Stick 8 samples to a cover, as showed in figure II-4(a)
  - Heat the mixture of 0.5ml cadaverine (95%) and 2ml water to 150°C
  - Place the cover on top of the mixture and wait for 3min, as showed in the figure II-4(b)

Table II-3

Sample	Solvent (2-ethoxyethanol)	Solute (cyclam)	Processing time	Baking temperature	Baking time
5	2.5ml	0.025mg	24 hours	60°C	2 hours
6		0.025mg	5 minutes	60°C	
7		0.025mg	42 hours	60°C	
8		0.05mg	24 hours	room temperature	
9		0.05mg	24 hours	60°C	
10		0.05mg	24 hours	100°C	
11		0.075mg	24 hours	60°C	
12	\	\	\	\	\



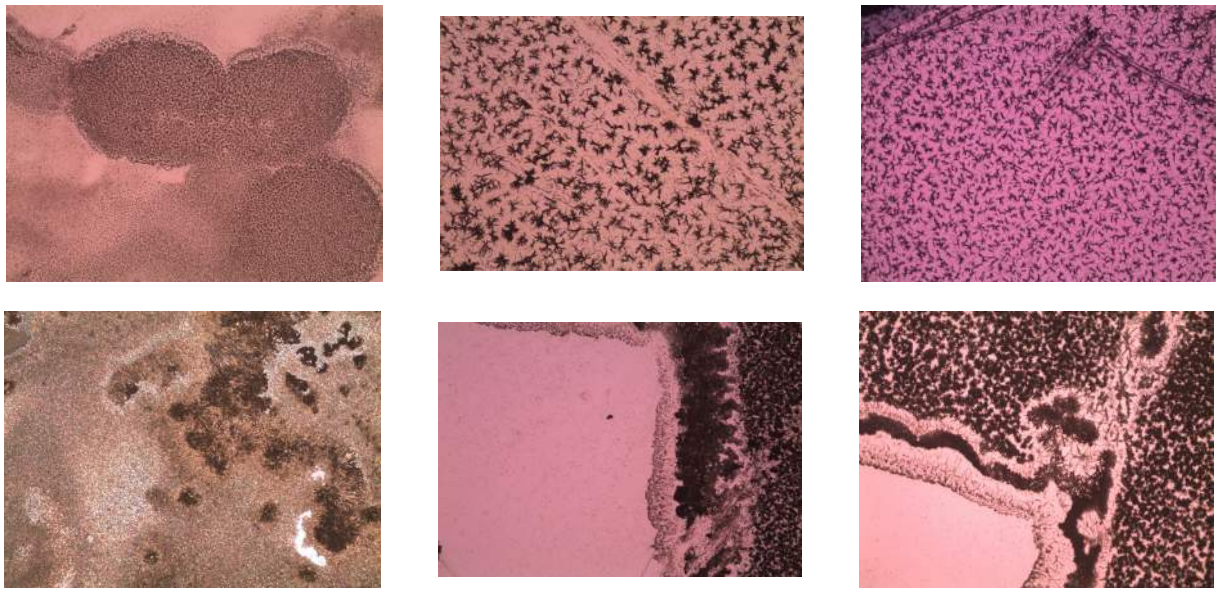
(a) (b)

Figure II-4. Images of gas phase cadaverine bonding to functionalized sample

- Result:

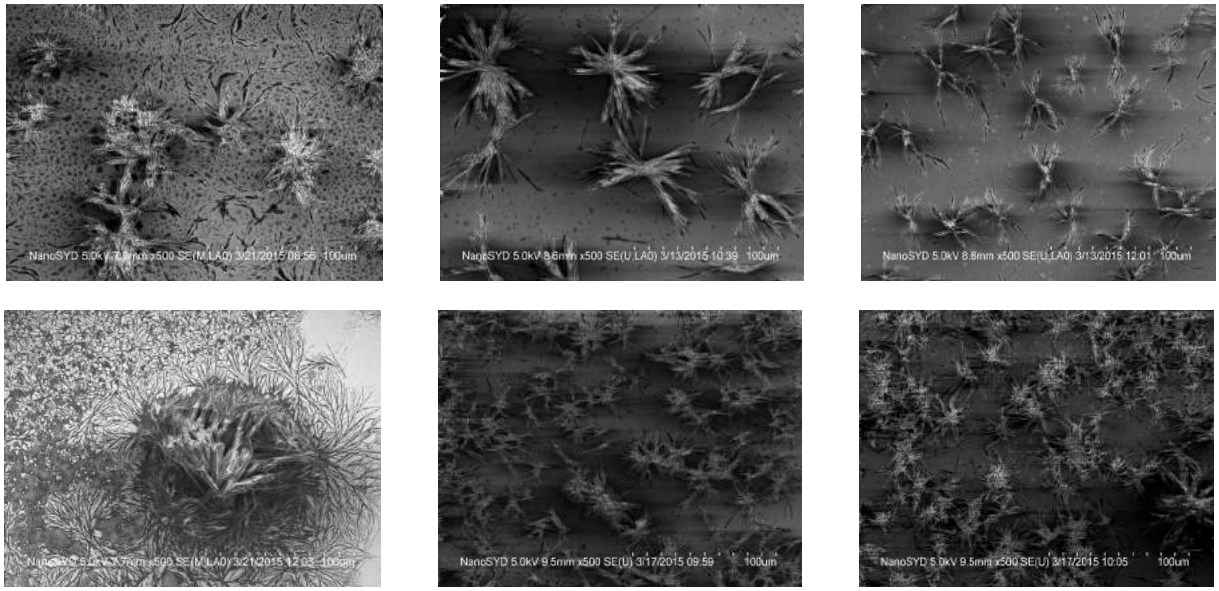
*Up images: after functionalization with cyclam before cadaverine bonding; Bottom images: after cadaverine bonding

- Processing time (5min, 24h, 42h)



(a) (b) (c)

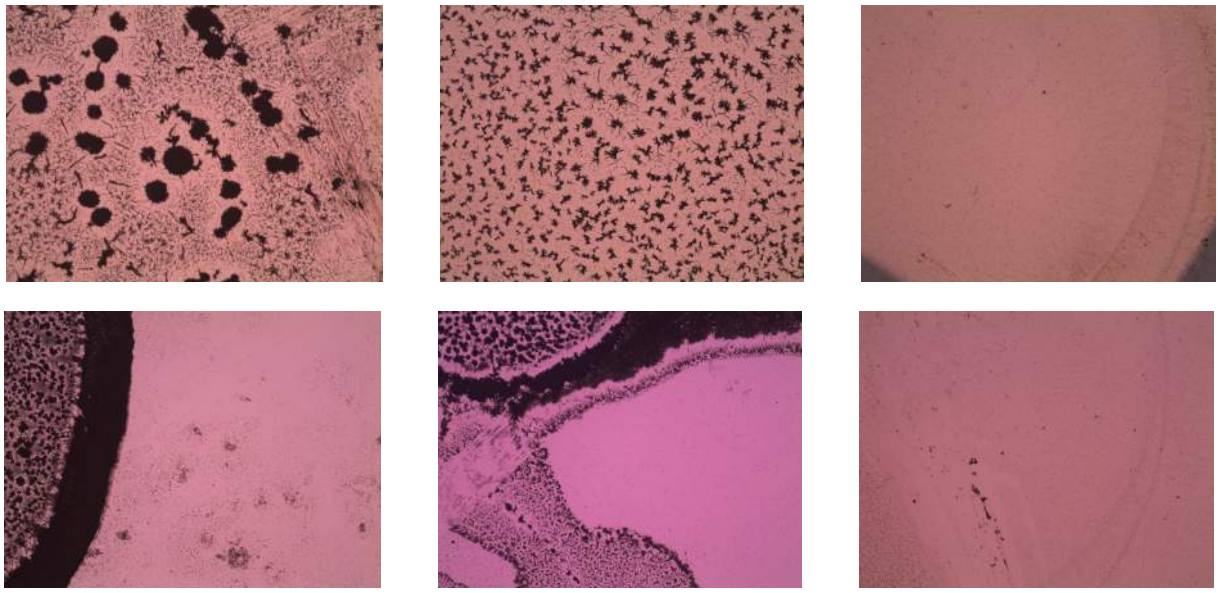
Figure II-5. Optical images for comparison of different processing time with magnification of 5: (a). Sample 6, 5 minutes, (b). Sample 5, 24 hours: (c). Sample 7, 42 hours



(a) (b) (c)

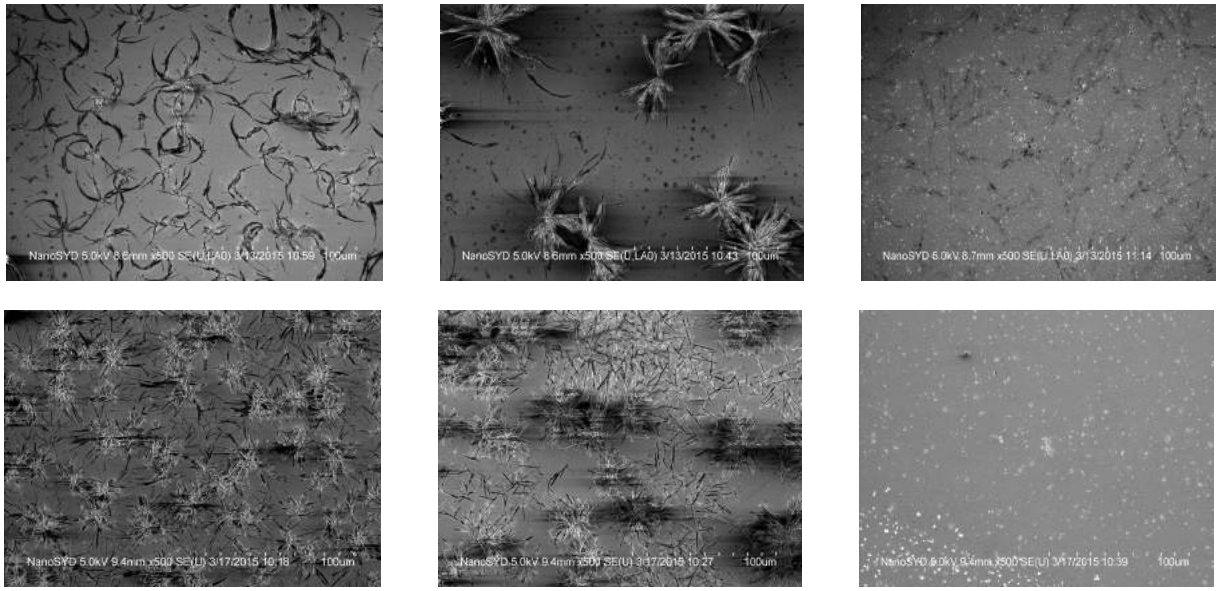
Figure II-6. SEM images for comparison of different processing time with magnification of 500: (a). Sample 6, 5 minutes, (b). Sample 5, 24 hours: (c). Sample 7, 42 hours

- Baking temperature (Room Temperature, 60°C, 100°C)



(a) (b) (c)

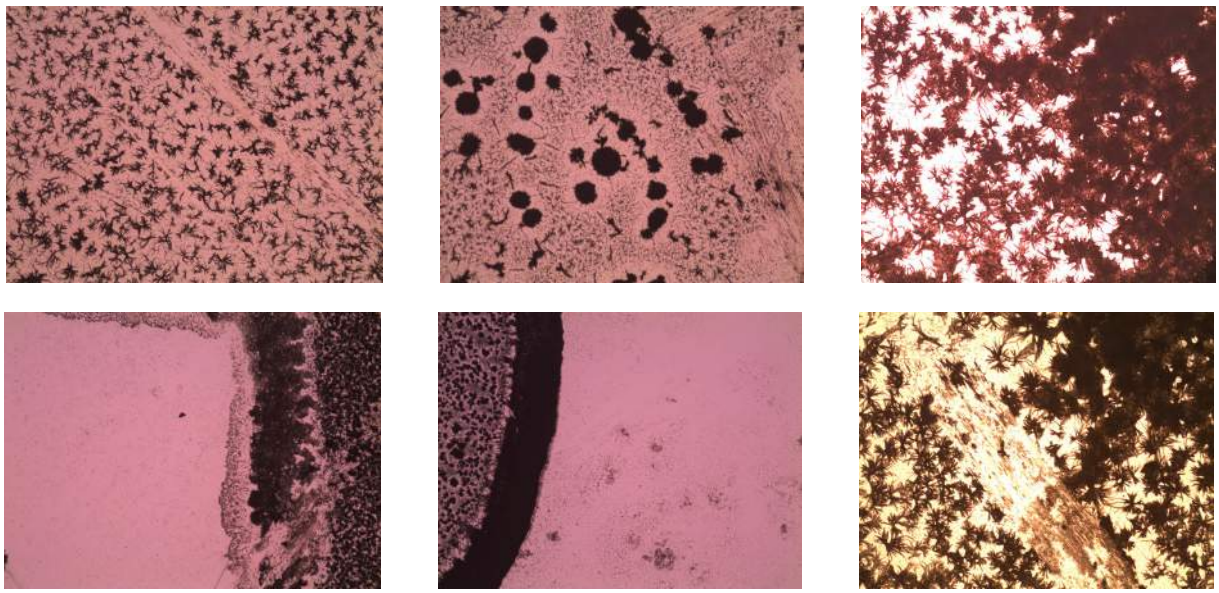
Figure II-7. Optical images for comparison of different baking temperature with magnification of 5: (a). Sample 8, Room Temperature; (b). Sample 9, 60°C; (c). Sample 10, 100°C



(a) (b) (c)

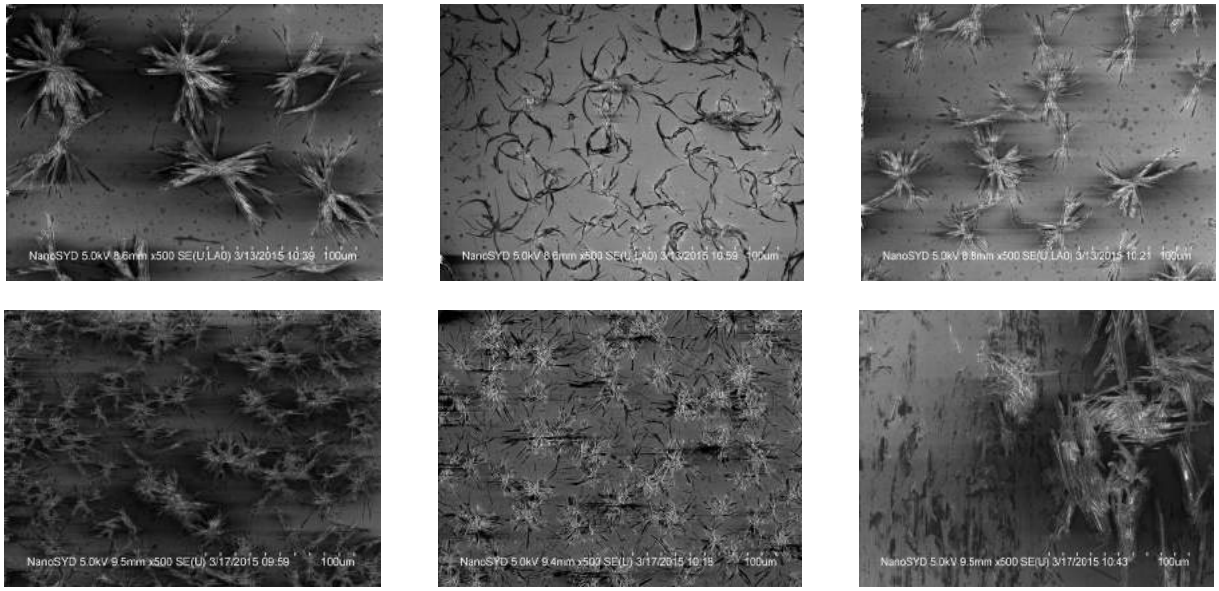
Figure II-8. SEM images for comparison of different baking temperature with magnification of 500: (a). Sample 8, Room Temperature; (b). Sample 9, 60°C; (c). Sample 10, 100°C

- Concentration of cycalm (0.025mg, 0.05mg, 0.075mg)



(a) (b) (c)

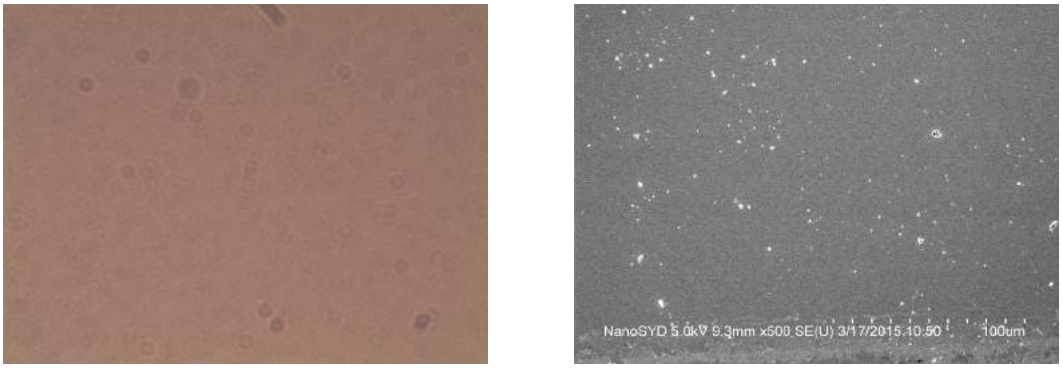
Figure II-9. Optical images for comparison of different concentration of cyclam with magnification of 5: (a). Sample 5, 0.025mg cyclam + 2.5ml ethoxyethonal; (b). Sample 9, 0.05mg cyclam + 2.5ml ethoxyethonal; (c). Sample 11, 0.075mg cyclam + 2.5ml ethoxyethonal



(a) (b) (c)

Figure II-10. SEM images for comparison of different concentration of cyclam with magnification of 500: (a). Sample 5, 0.025mg cyclam + 2.5ml ethoxyethonal; (b). Sample 9, 0.05mg cyclam + 2.5ml ethoxyethonal; (c). Sample 11, 0.075mg cyclam + 2.5ml ethoxyethonal

- *Cadaverine bonding to sample without functionalization



(a) (b)

Figure II-11. (a). Sample 12, optical image with magnification of 5, after gas phase of cadaverine bonding; (b). Sample 12, SEM image with magnification of 500, after gas phase of cadaverine bonding

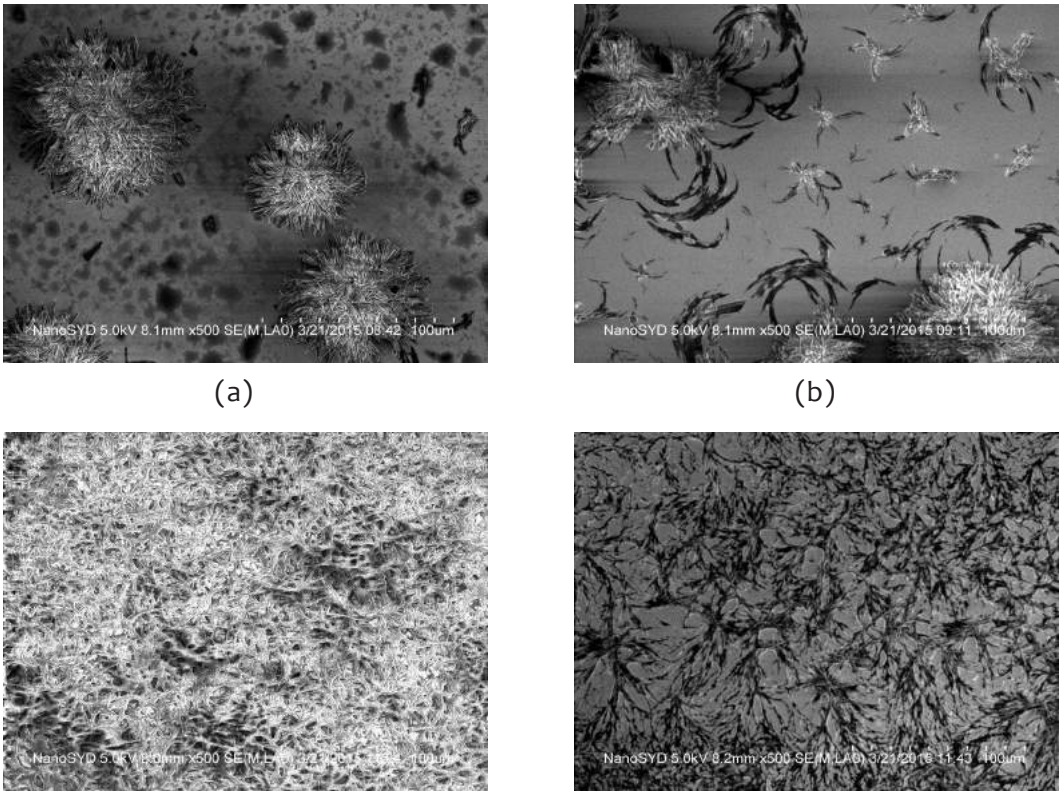
Test 4. The Effect of Surface Cleanness/ Surface Energy

- **Variables**: the cleanness of gold surface (plasma cleaning, simple cleaning)
- **Number of samples**: 3
- **Experimental details**:
  - Simple cleaning: rinse with acetone in ultrasonic for 5min, clean with isopropanol and water
  - Plasma cleaning: simple cleaning first, then use oxygen plasma with low flow to clean surface for 30s with power of 200W
  - Functionalization and cadaverine bonding methods are the same as the previous tests

Table II-4

Sample	Functionalization details	Cadaverine bonding	Surface cleaning
13	0.05mg cyclam + 2.5ml 2-ethoxyethanol, process for 24h, dry at RT	0.5ml cadaverine(95%) + 2ml water: gas evaporation	Simple cleaning
14			Plasma cleaning
15		Liquid drop: cadaverine(95%)	Simple cleaning

- **Result:**
  - **Different surface cleaness comparison**

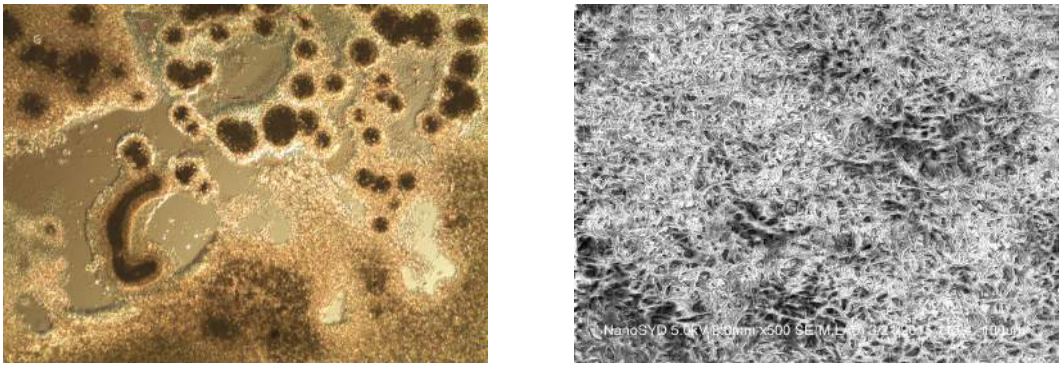


(a) (b)

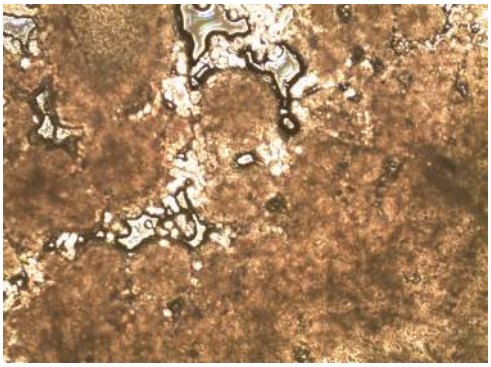
(c) (d)

Figure II-12. SEM images with magnification of 500, sample 13: without plasma cleaning, sample 14: with plasma cleaning: (a). Sample 13, after functionalization with cycalm, before cadaverine bonding; (b). Sample 14, after functionalization with cycalm, before cadaverine bonding; (c). Sample 13, after cadaverine bonding; (d). Sample 14, after cadaverine bonding

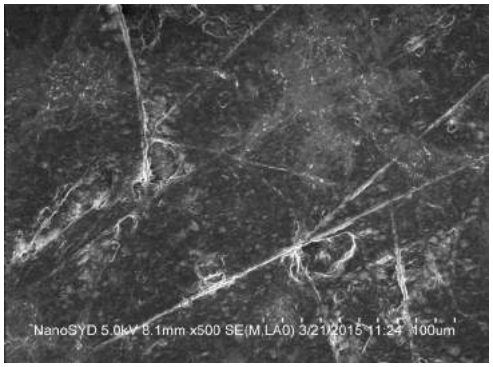
- ***Different condition of cadaverine bonding**



(a) (b)



(c)



(d)

Figure II-13. Both samples without plasma cleaning, sample 13: after gas phase of cadaverine bonding; sample 15: after liquid phase of pure cadaverine bonding: (a). Sample 13, Optical image with magnification of 5, (b). Sample 13, SEM image with magnification of 500; (c). Sample 15, Optical image with magnification of 5, (d). Sample 15, SEM image with magnification of 500

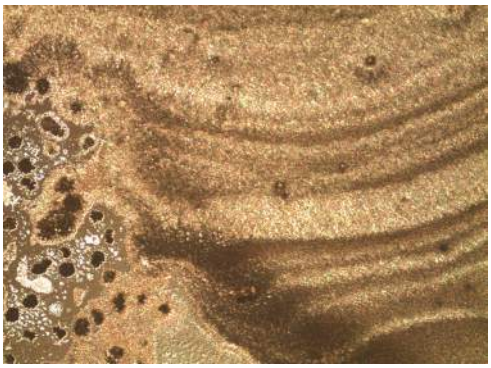
Test 5. Different Gas Phase of Cadaverine Bonding

- **Variables**: Solution of cadaverine (water, ethanol); the influence of solvent (water, ethanol)
- **Number of samples**: 4
- **Experimental details**:
  - Functionalization and cadaverine bonding method are the same as previous tests and parameters are given in table II-5.

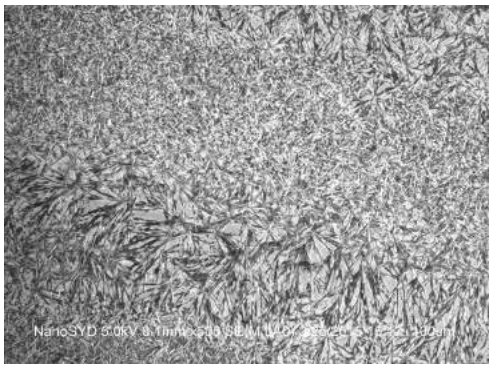
Table II-5

Sample	Functionalization details	Cadaverine preparation	Cadaverine bonding
16	0.05mg cyclam + 2.5ml 2-ethoxyethanol, process for 24h, dry at RT	0.5ml cadaverine(95%)+4ml water	Heat the mixture to 150°C and let gas bond to samples
17		0.5ml cadaverine(95%)+4ml ethanol	
18		4ml water	
19		4ml ethanol	

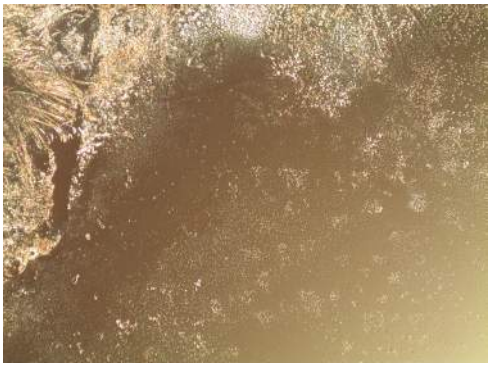
- **Result**:



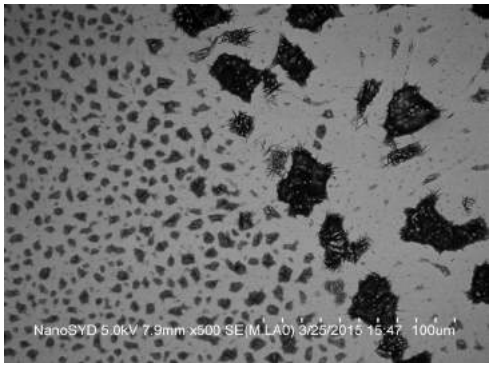
(a)



(b)

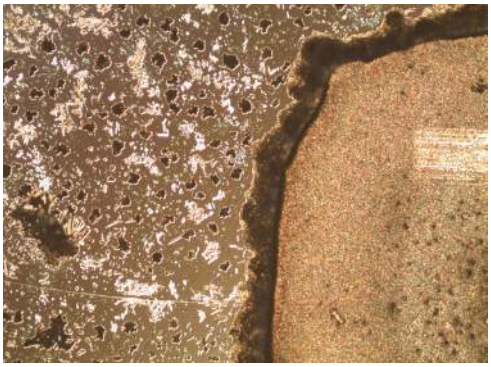


(c)

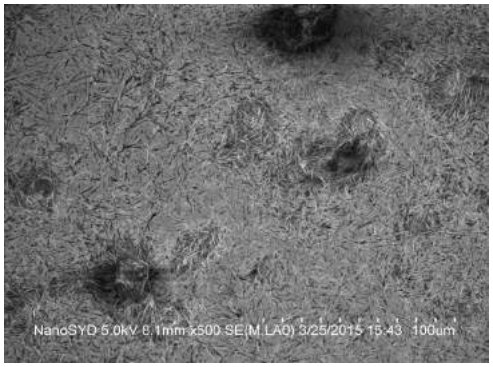


(d)

Figure II-14. Sample 16: after gas phase (0.25ml cadaverine + 4ml water) bonding; sample 17: after gas phase (0.25ml cadaverine + 4ml ethonal) bonding: (a). Sample 16, optical image with magnification of 5, (b). Sample 16, SEM image with magnification of 500; (c). Sample 17, optical image with magnification of 5, (d). Sample 17, SEM image with magnification of 500



(a)



(b)

(c)

(d)

Figure II-15. Sample 18: after gas phase (pure water) bonding; sample 19: after gas phase (pure ethonal) bonding: (a). Sample 18, optical image with magnification of 5, (b). Sample 18, SEM image with magnification of 500; (c). Sample 19, optical image with magnification of 5, (d). Sample 19, SEM image with magnification of 500

Test 6. Further Functionalization Parameter Optimization

- **Variables**: the concentration of cycalm, processing time, different phase of cadaverine bonding
- **Number of samples**: 9
- **Experimental details:**

- Simple cleaning
- Functionalize sample 20 to sample 28 with parameters showed in table II-6, break each sample after functionalization into two pieces
- (1). Gas solution: heat to 150°C, and let the gas to bond surface for 3min
- (2). Liquid solution: a drop of mixture to the sample and leave it till dry

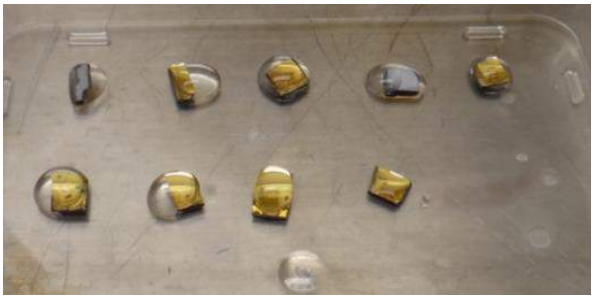


Figure II-16. Liquid solution of cadaverine bonding

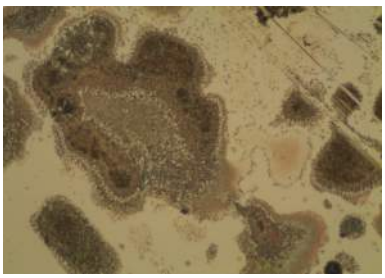
Table II-6

Sample	Solvent (2-ethoxyethanol)	Solute (cyclam)	Processing time	Dry process	Cadaverine bonding
20	2.5ml	0.01mg	5min	At RT (around 30-45min)	0.5ml cadaverine(95%) +4ml water: (1). Gas phase (2). Liquid solution
21		0.02mg	5min		
22		0.03mg	5min		
23		0.04mg	5min		
24		0.05mg	5min		
25		0.05mg	10min		
26		0,05mg	15min		
27		0.05mg	20min		
28		0.05mg	30min		

- **Result:**
  - **Part 1: Concentration of cycalm(0.01mg/0.02mg/0.03mg/0.04mg/0.05mg)**

1.(a)

(b).

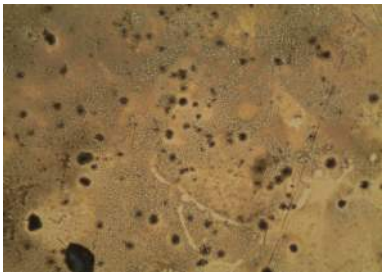


(c).

Figure II-17. Optical images with magnification of 20: (a). Sample 20, before cadaverine bonding; (b). Sample 20(1), gas phase of cadaverine bonding; (c). Sample 20(2), liquid solution of cadaverine bonding

2.(a)

(b).



(c).

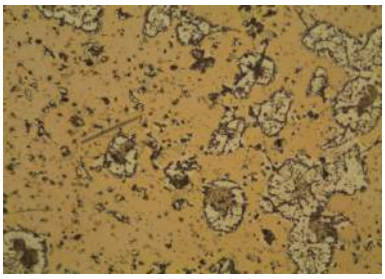
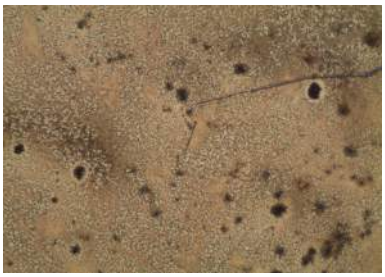


Figure II-18. Optical images with magnification of 20: (a). Sample 21, before cadaverine bonding; (b). Sample 21(1), gas phase of cadaverine bonding; (c). Sample 21(2), liquid solution of cadaverine bonding

3.(a)

(b).



(c).

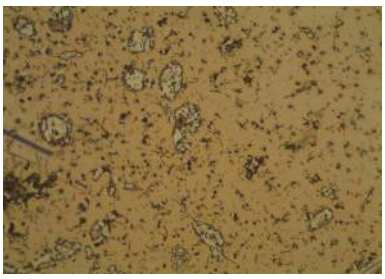
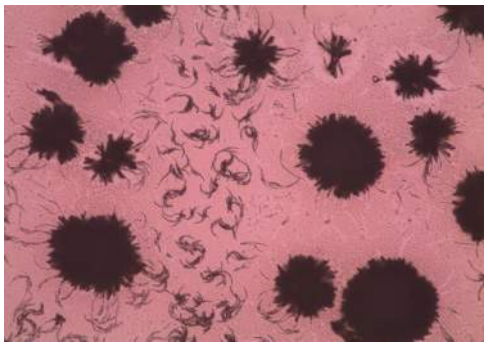
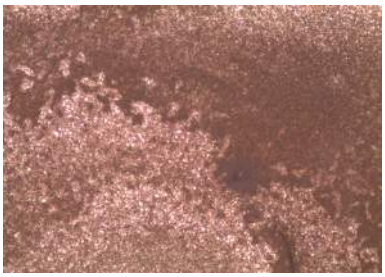


Figure II-19. Optical images with magnification of 20: (a). Sample 22, before cadaverine bonding; (b). Sample 22(1), gas phase of cadaverine bonding; (c). Sample 22(2), liquid solution of cadaverine bonding

4.(a)



(b).

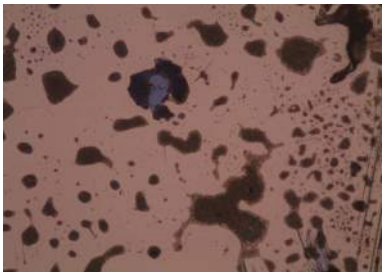


(c).

Figure II-20. Optical images with magnification of 20: (a). Sample 23, before cadaverine bonding; (b). Sample 23(1), gas phase of cadaverine bonding; (c). Sample 23(2), liquid solution of cadaverine bonding

5.(a)

(b).



(c).

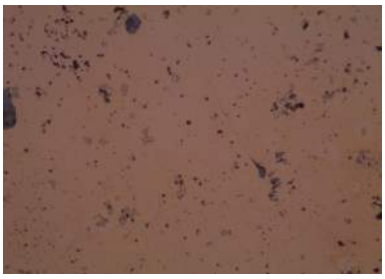
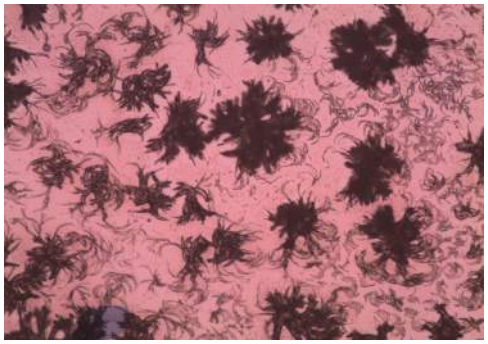


Figure II-21. Optical images with magnification of 20: (a). Sample 24, before cadaverine bonding; (b). Sample 24(1), gas phase of cadaverine bonding; (c). Sample 24(2), liquid solution of cadaverine bonding

- **Part 2: Processing time (5min-show in table 5, 10min, 15min, 20min, 30min)**

6.(a)



(b).

(c).

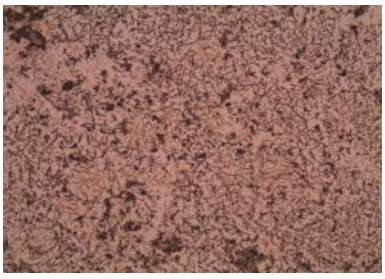


Figure II-22. Optical images with magnification of 20: (a). Sample 25, before cadaverine bonding; (b). Sample 25(1), gas phase of cadaverine bonding; (c). Sample 25(2), liquid solution of cadaverine bonding

7.(a)

(b).

(c).

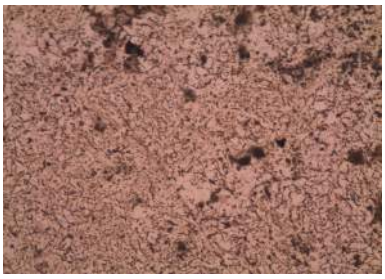
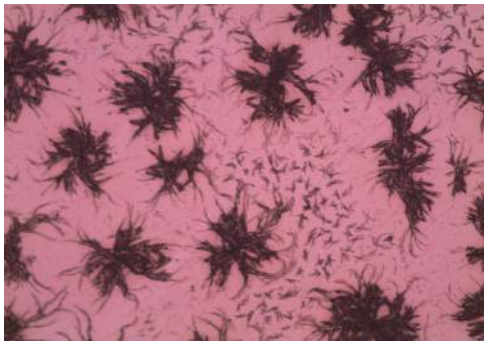
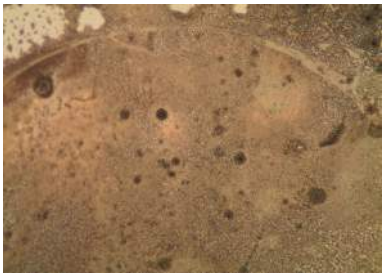


Figure II-23. Optical images with magnification of 20: (a). Sample 26, before cadaverine bonding; (b). Sample 26(1), gas phase of cadaverine bonding; (c). Sample 26(2), liquid solution of cadaverine bonding

8.(a)



(b).



(c).

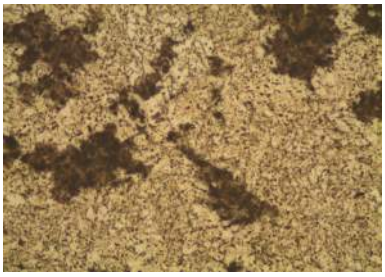
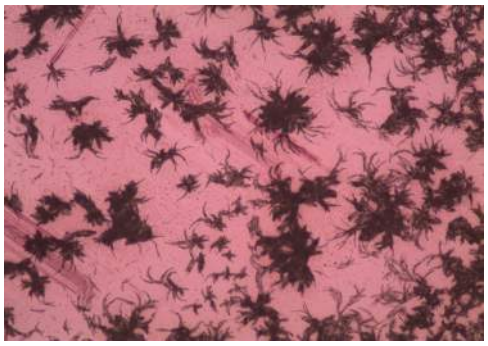
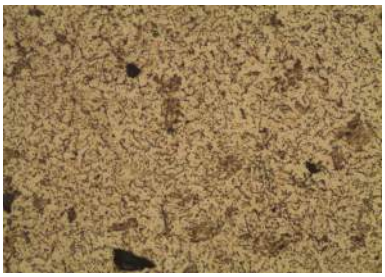


Figure II-24. Optical images with magnification of 20: (a). Sample 27, before cadaverine bonding; (b). Sample 27(1), gas phase of cadaverine bonding; (c). Sample 27(2), liquid solution of cadaverine bonding

9.(a)



(b).



(c).

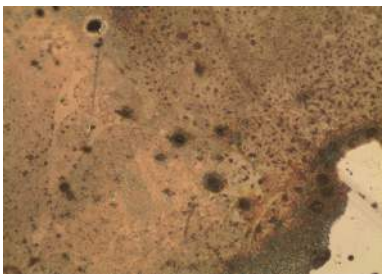


Figure II-25. Optical images with magnification of 20: (a). Sample 28, before cadaverine bonding; (b). Sample 28(1), gas phase of cadaverine bonding; (c). Sample 28(2), liquid solution of cadaverine bonding

Test 7. Different Concentration of Cadaverine by Syringe

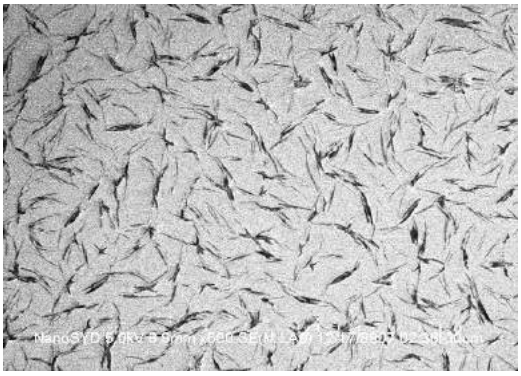
- **Variables**: the concentration of cadaverine bonding
- **Number of sample**: 1
- **Experimental details**:
  - Simple cleaning
  - Functionalize and break the sample after functionalization into three pieces
  - Use syringe apply liquid solution (parameters showed in table II-7) to sample

Table II-7

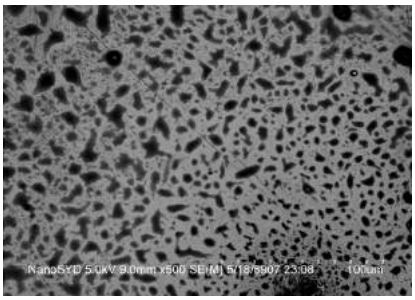
Sample	Functionalization details	Cadaverine preparation
29	2.5ml 2-ethoxyethanol+ 0.01mg cyclam, process for 5min, leave to dry at RT	(1). 6ml water + 0.25ml cadaverine (95%)
		(2). 10ml water + 0.25ml cadaverine (95%)
		(3). 14ml water + 0.25ml cadaverine (95%)

- **Results:**

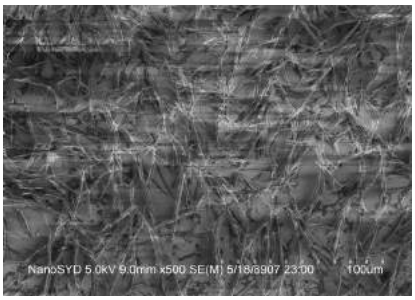
(a)



(b)



(c)



(d)

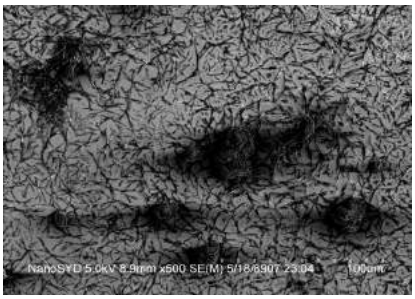


Figure II-26. SEM images with magnification of 500: (a). Sample 29, before cadaverine bonding; (b). Sample 29(1), cadaverine/water: 1/24; (c). Sample 29(2), cadaverine/water: 1/40; (d). Sample 29(3), cadaverine/water: 1/64

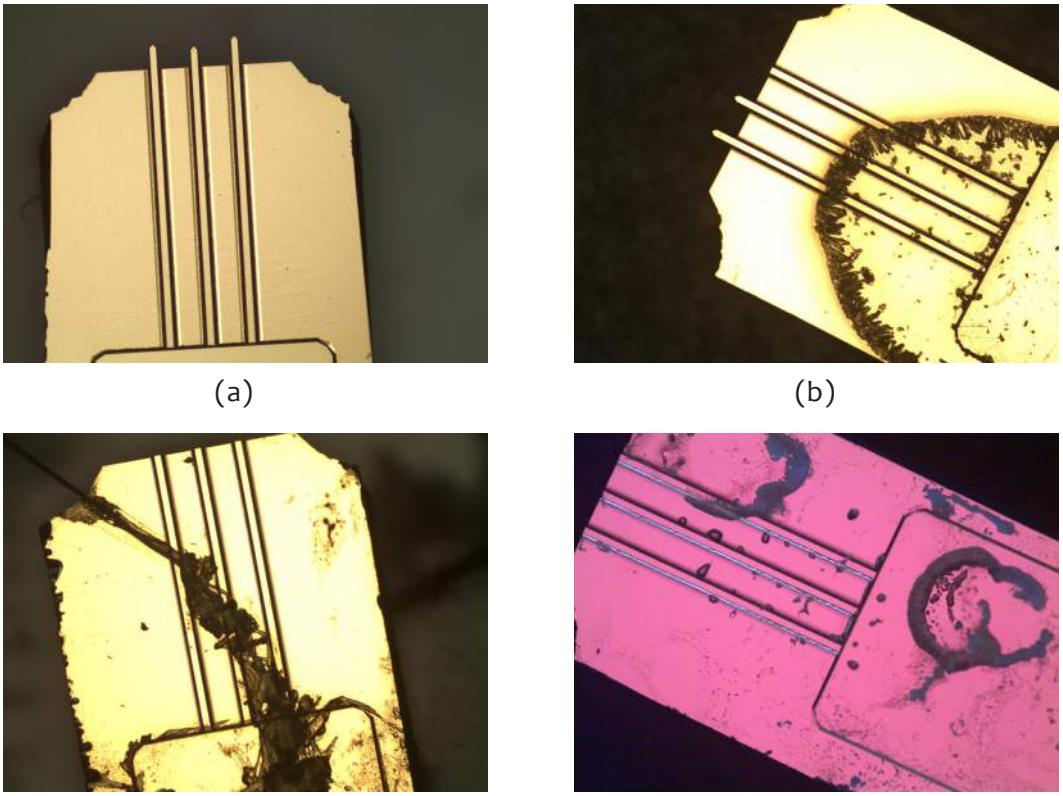
Test 8. Functionalization of Cantilevers - Failure Results

- **Variables**: concentration of cyclam, processing time
- **Number of cantilevers**: 4
- **Experimental details**:
  Functionalize the cantilevers with parameters showed in table II-8, leave at RT to dry

Table II-8

Cantilever	Functionalization solution	Processing time
1	0.01mg cyclam + 2.5ml 2-ethoxyethanol	5min
2	0.05mg cyclam + 2.5ml 2-ethoxyethanol	1 hour
3		24 hours
4		42 hours

- **Results:**



(a) (b)

(c) (d)

Figure II-27. Optical images of cantilevers after functionalization with magnification of 5: (a). Cantilever 1; (b). Cantilever 2; (c). Cantilever 3; (d). Cantilever 4

Test 9. Functionalization of Cantilevers - Succeed Results

- **Variables**: fuctionalization methods
- **Number of cantilevers**: 6
- **Experimental details**:
  - Use Nanoplotter 2.1 in Fraunhofer ISIT in Germany to apply the solution in table II-9 to cantilevers
  - Heat cyclam in beaker to 200°C, evaporate pure cyclam gas to the cantilevers for a certain time

Table II-9-1

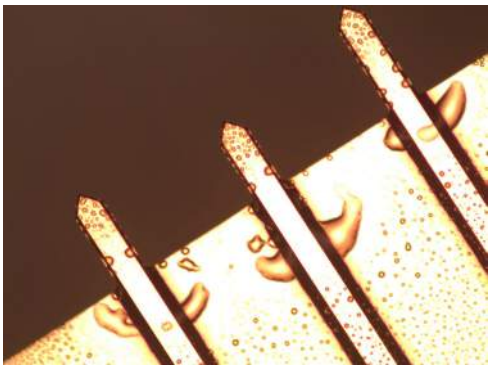
Cantilever	Functionalization solution	Method
5	0.05mg cyclam + 2.5ml 2-ethoxyethanol	Spot to cantilevers by Nanoplotter 2.1
6		

Table II-9-2

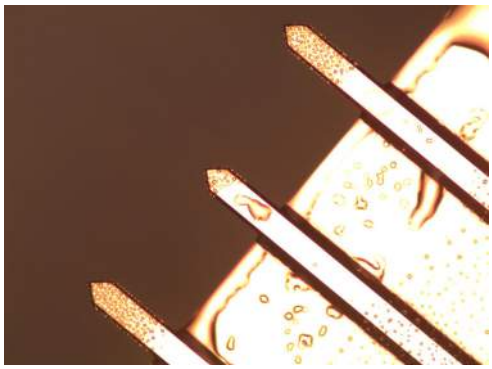
Cantilever	Functionalization solution	Method	Process time	Placement and Position
7	Pure cyclam gas	Heat cylam at 200°C to gas phase, later cool down and bond to cantilevers	5s	parallel, within 1cm from cantilever surface to cyclam
8			5s	vertical, within 0.5cm from the edge of cantilever to cyclam
9			10s	parallel, within 1cm from cantilever surface to cyclam
10			15s	parallel, within 1cm from cantilever surface to cyclam

- **Results:**

*Sample 30 here is used to investigate the surface structure by SEM

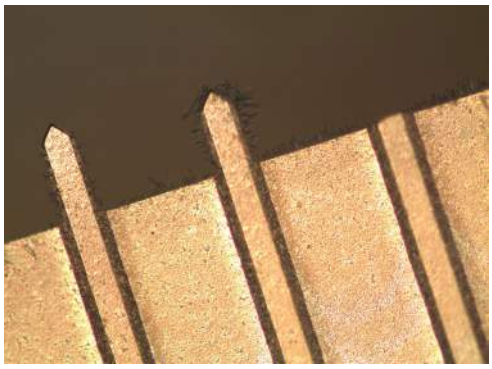


(a)

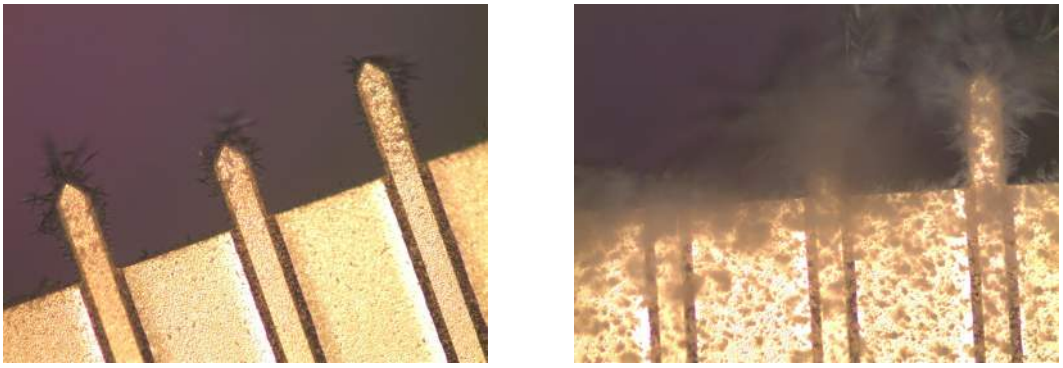


(b)

(c)

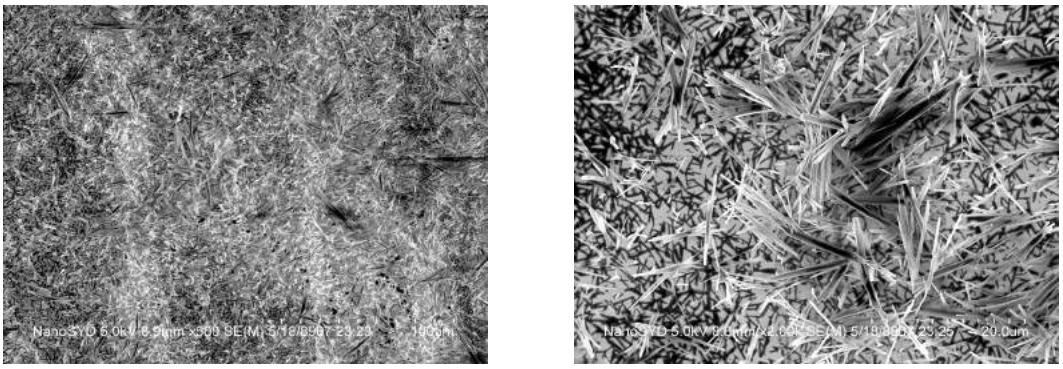


(d)



(e) (f)

Figure II-28. Optical images with of cantilevers after different ways of functionalization with magnification of 20: (a). Cantilever 5; (b). Cantilever 6; (c). Cantilever 7; (d). Cantilever 8; (e). Cantilever 9; (f). Cantilever 10



(a) (b)

Figure II-29. SEM images of functionalized sample 30 after bonding pure cyclam gas: (a). With magnification of 500; (b). With magnification of 2K

Test 10. Cadaverine Bonding Test with Water and Glycerol

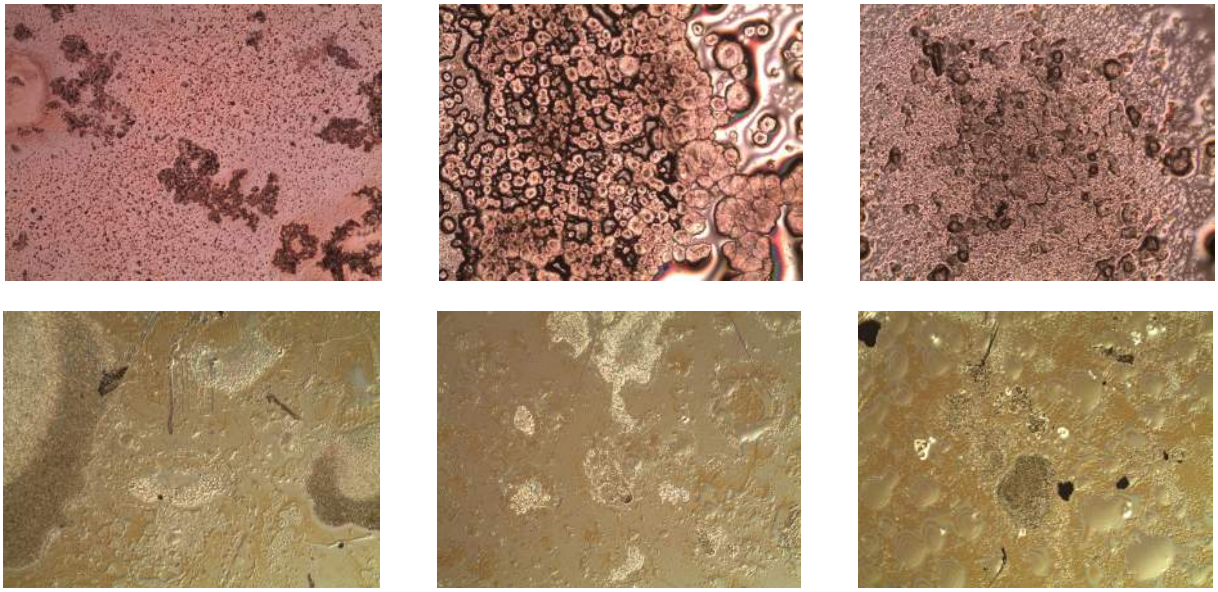
- **Variables**: the concentration of cadaverine, solution for cadaverine (water, glycerol)
- **Number of samples**: 6
- **Experimental details**:
  - Simple cleaning, functionalizing 6 samples by pure cyclam gas bonding (200°C)
  - Liquid phase of cadaverine bonding: sample 31-33, apply the mixed solution by syringe
  - Gas phase of cadaverine bonding: sample 34-36, heat cadaverine to gas phase (200°C) from mixture of glycerol and cadaverine.

*Noticed to use the same amount of these mixed solution with different concentration.

Table II-10

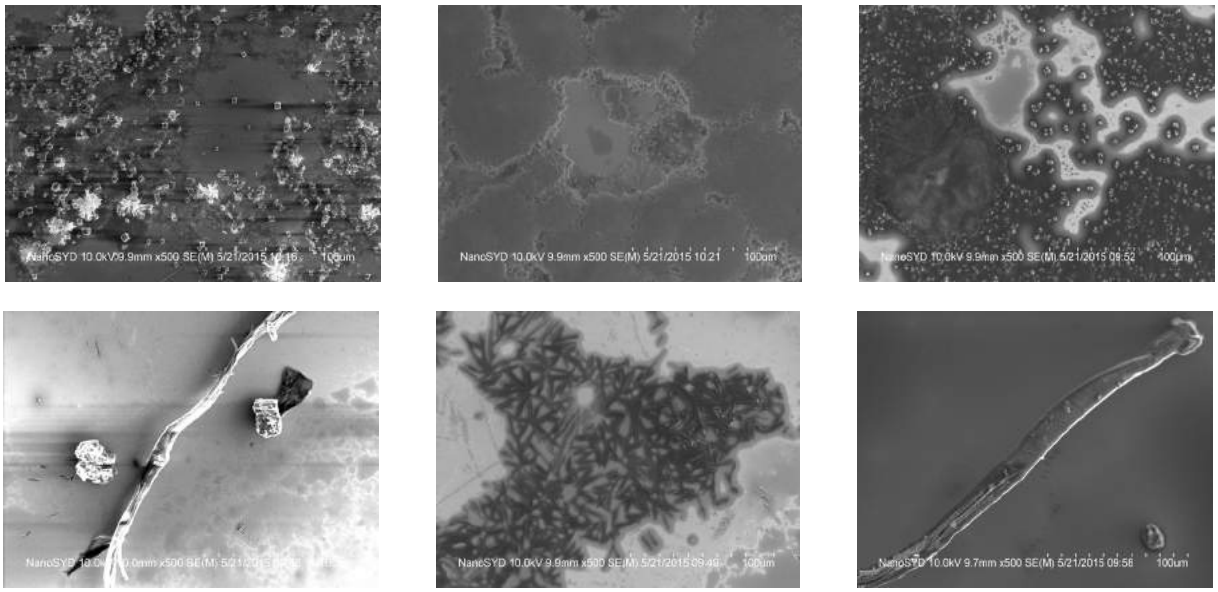
Sample	Cadaverine bonding method	Cadaverine solution
31	Liquid phase: use syringe to apply a small drop of the mixed solution, leave to dry at RT	6ml water + 0.25ml cadaverine (95%)
32		10ml water + 0.25ml cadaverine (95%)
33		14ml water + 0.25ml cadaverine (95%)
34	Gas phase: heat the mixture to 200°C and leave the sample being evaporated to the vapor for 3min	6ml glycerol + 0.25ml cadaverine (95%)
35		10ml glycerol + 0.25ml cadaverine (95%)
36		14ml glycerol + 0.25ml cadaverine (95%)

- **Results:**



(a) (b) (c)

Figure II-30. Optical images with magnification of 5: (a). Sample 31; (b). Sample 32; (c). Sample 33; (e). Sample 34; (d). Sample 35; (e). Sample 36; (f). Sample 37



(a) (b) (c)

Figure II-31. SEM images with magnification of 500: (a). Sample 31; (b). Sample 32; (c). Sample 33; (e). Sample 34; (d). Sample 35; (e). Sample 36; (f). Sample 37

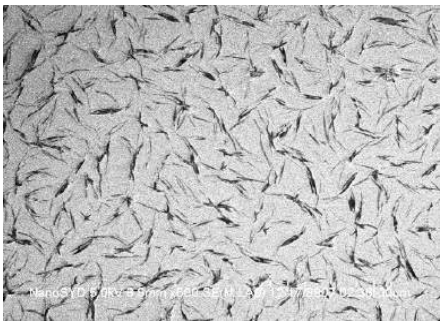
Test 11. Bonding Test of Cadaverine in Chicken Breast

- **Variables**: processing time, different time period
- **Number of samples**: 10
- **Experimental details**:
  - Simple cleaning, functionalizing 8 samples by 0.01mg cyclam+ 2.5 2-ethoxyethanol, process for

5min, and leave them dry at RT

- Sample 37-40 are leave for 24 hours, 48 hours, 60 hours and 72 hours
- Sample 41-44 are leave for 1 hour after 24 hours, 48 hours, 60 hours and 72 hours

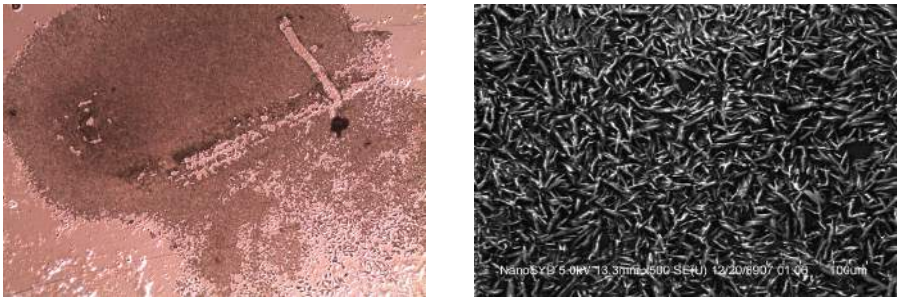
Table II-11

Functionalization result	Sample	Processing time/ period
 Figure II-32. SEM images of functionalized sample 37-46, with magnification of 500	37	24 hours
	38	48 hours
	39	60 hours
	40	72 hours
	41	1 hour after 24 hour
	42	1 hour after 48 hour
	43	1 hour after 60 hour
	44	1hour after 72 hour
	45	1 hour of water evaporation
	46	1 hour of mixed solution of 0.25ml cadaverine (95%) + 10ml evaporation

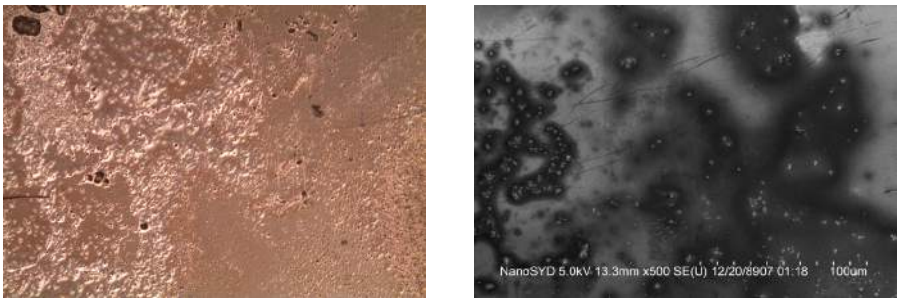
- **Results:**

- *Left images: optical images with magnification of 5; Right images: SEM images with magnification of 500

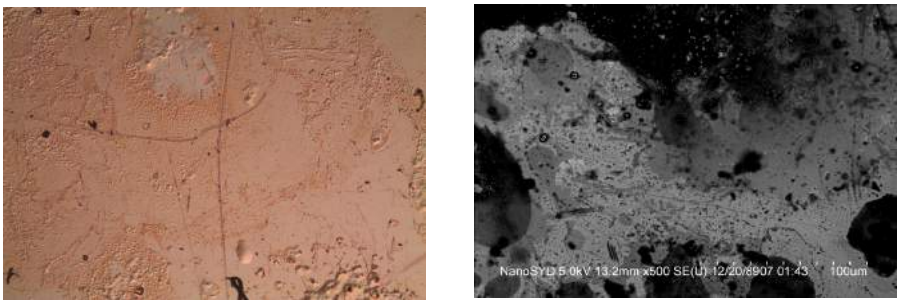
- Part 1. Comparison of different process time (24 hours, 48 hours, 60 hours, 72 hours)



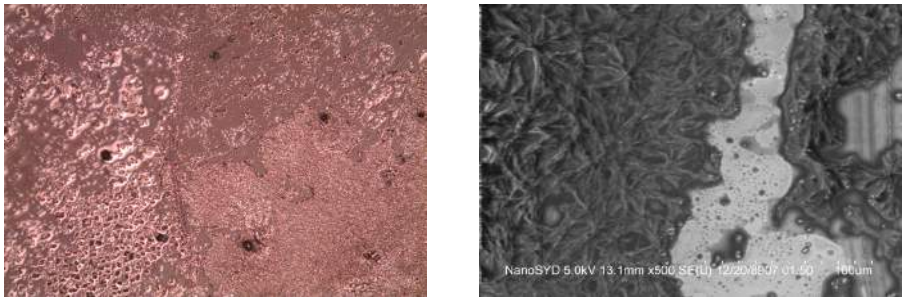
(a)



(b)



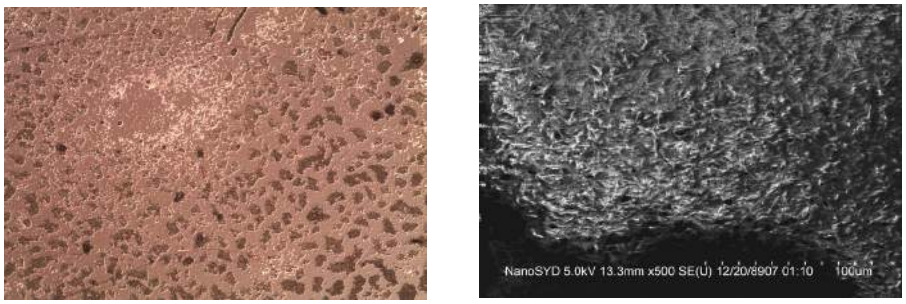
(c)



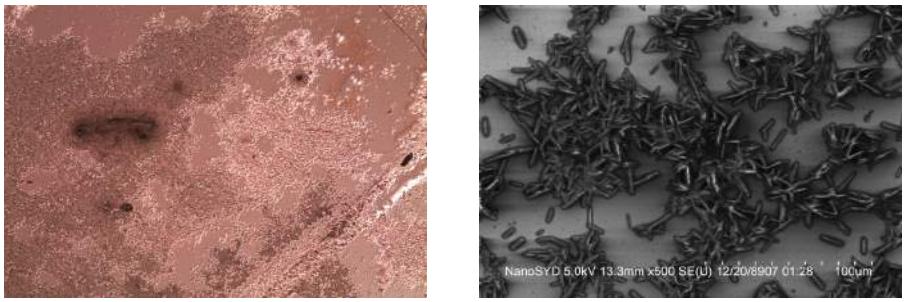
(d)

Figure II-33. (a). Sample 37; (b). Sample 38; (c). Sample 39; (d). Sample 40

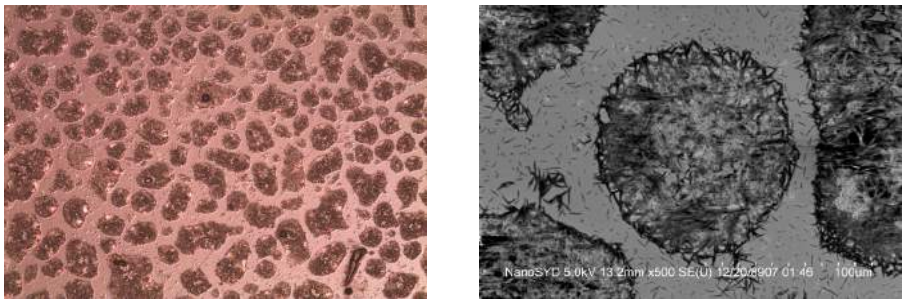
- Part 2. Comparison for different process period (after 24 hours, 48 hours, 60 hours, 72 hours)



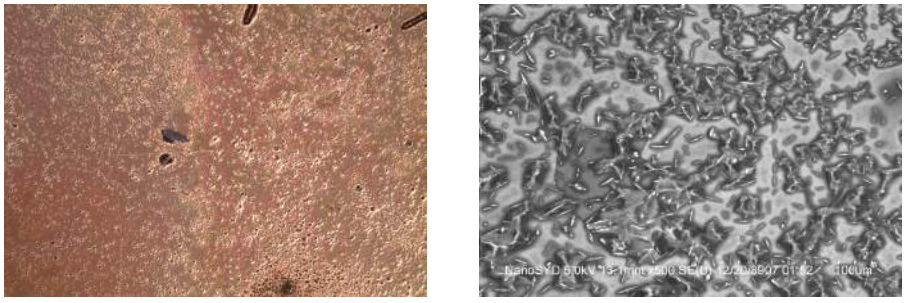
(a)



(b)



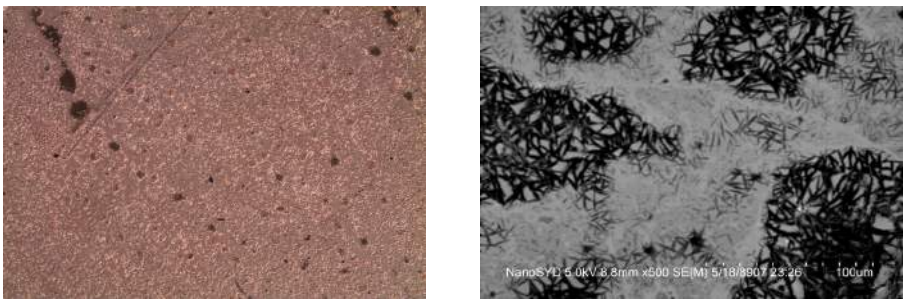
(c)



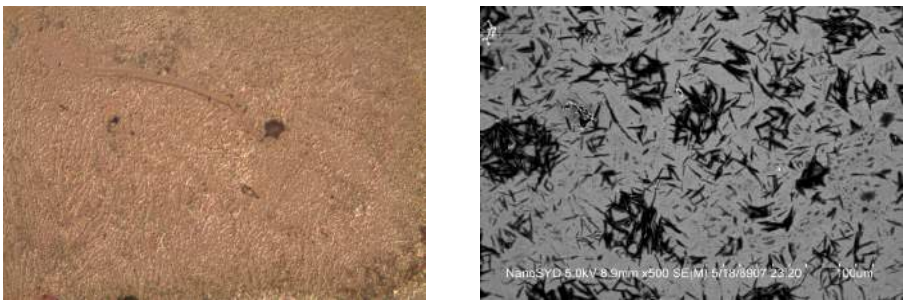
(d)

Figure II-34. (a). Sample 41; (b). Sample 42; (c). Sample 43; (d). Sample 44

- **Part 3. Comparison to water and mixed solution of water and cadaverine**



(a)



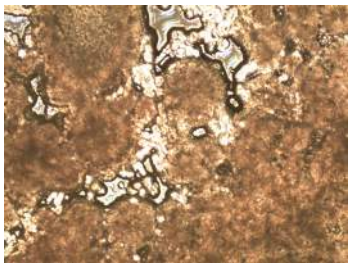
(b)

Figure II-35. (a). Sample 45; (b). Sample 46

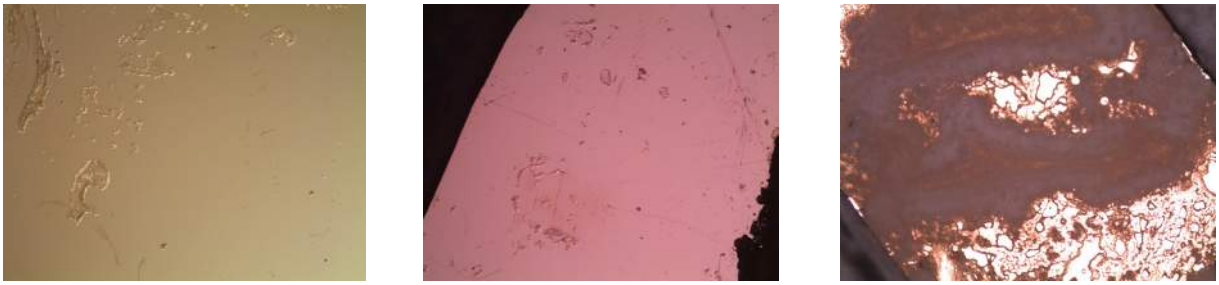
Test 12. Reusability Tests

- **Variables**: solution for cleaning surface: acetone, PBS buffer, ethanol
- **Number of samples**: sample 15 (in Test 4)
- **Experimental details**:
  - Break the sample into three pieces
  - Rinse sample with the specific solution for 20s
  - Clean the solution with water for 10s, leave at RT to dry

Table II-12

Sample 15	Cleaning solution
 Figure II-36. Optical image of Sample 15 with magnification of 5 before cleaning	(1). Ethanol (liquid solution) rinse for 20s + water rinse for 10s
	(2). PBS Buffer (liquid solution) rinse for 20s + water rinse for 10s
	(3). Acetone (liquid solution) rinse for 20s + water rinse for 10s

- **Results:**



(a) (b) (c)

Figure II-37. Optical image with 5 magnification of Sample 15 after cleaning: (a). Sample 15(1), ethanol cleaning; (b). Sample 15(2), PBS buffer cleaning; (c). Sample 15(3), acetone cleaning

Test 13. Functionalization of Cantilever - Further Improvement

- **Variables**: Solution for reducing concentration of melted cyclam on cantilever
  - Part 1: Solvent: acetone, 2-ethoxyethanol, water
  - Part 2: Processing time for solution
- **Number of cantilevers** (silicon surface): 17 (3 for part 1; 12 for part 2) (some were broken)
- **Experimental details**:
  - Gas phase of pure cyclam process at 200°C
  - Heat the specific solution to gas phase (acetone: 60°C; water: 100°C; 2-ethoxyethanol: 140°C)
  - Processing time and order are shown in table II-13-1 for part 1 and table II-13-2 for part 2

Table II-13-1

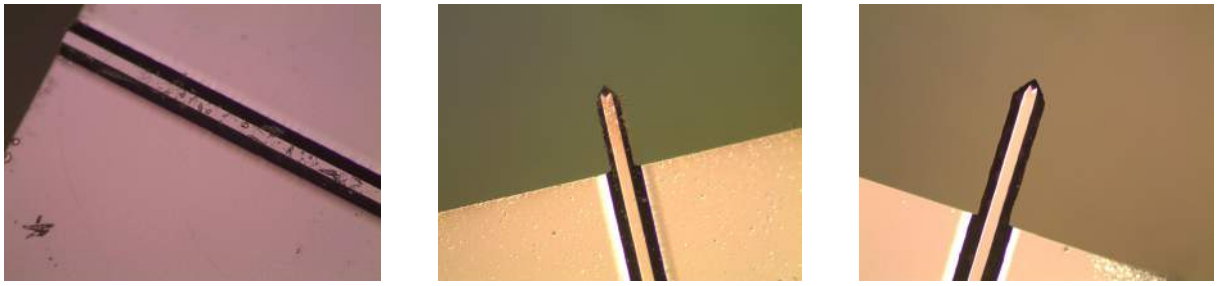
Cantilever	Functionalization first process details	Functionalization second process details
11	10s 2-ethoxyethanol gas processing	10s pure cyclam gas processing
12	10s acetone gas processing	
13	10s water vapor processing	

Table II-13-2

Cantilver	Functionalization first process details	Functionalization second process details
14	10s 2-ethoxyethanol gas processing	30s cyclam gas processing
15		20s cyclam gas processing
16		10s cyclam gas processing
17	20s cyclam gas processing	10s 2-ethoxyethanol gas processing
18		5s 2-ethoxyethanol gas processing
19		3s 2-ethoxyethanol gas processing
20		10s acetone gas processing
21		5s acetone gas processing
22		3s acetone gas processing
23	10s acetone gas processing	20s cyclam gas processing
24		10s cyclam gas processing

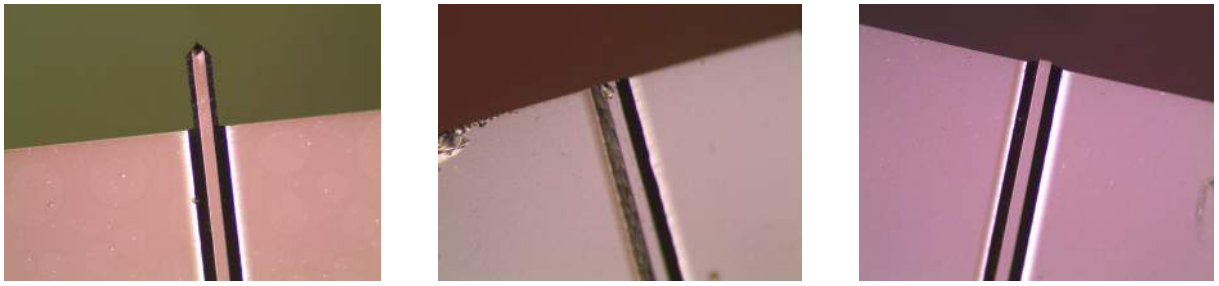
A bit further investigation of cantilever 25 and cantilever 26 with mixture of 0.04mg cyclam + 2ml acetone, process for 1 hour, leave to dry at RT.

- **Results:**



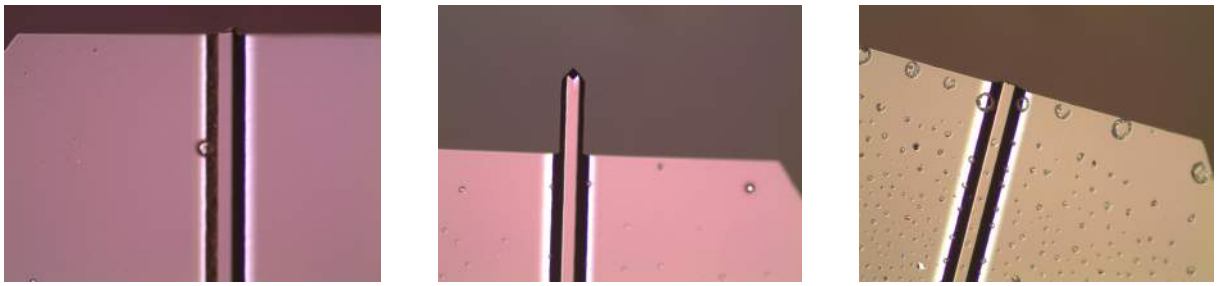
(a) (b) (c)

Figure II-38. Optical images of cantilevers for comprison of different solvent in reducing concentration of cyclam, with magnification of 20: (a). Cantilever 11; (b). Cantilever 12; (c). Cantilever 13



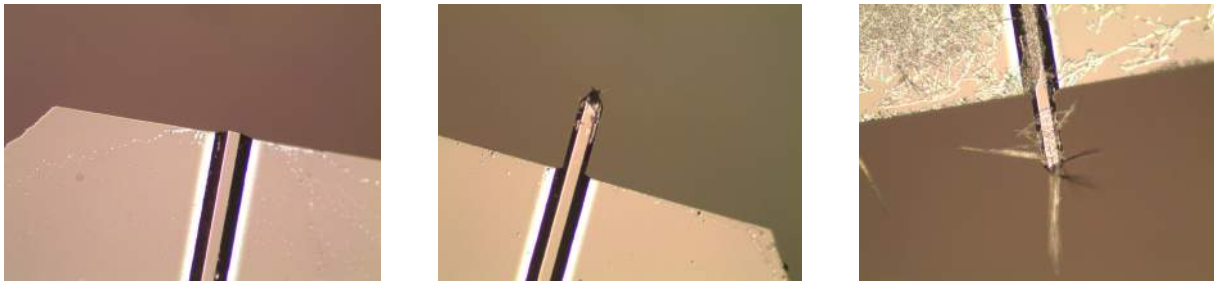
(a) (b) (c)

Figure II-39. Optical images of cantilevers for comparison with different processing time of pure cyclam gas after 10s 2-ethoxyethanol gas processing, with magnification of 20: (a). Cantilever 14; (b). Cantilever 15; (c). Cantilever 16



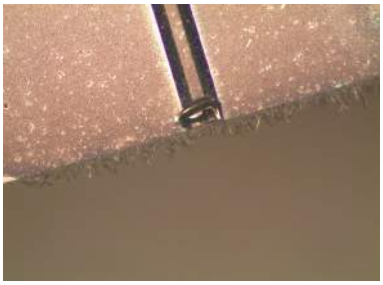
(a) (b) (c)

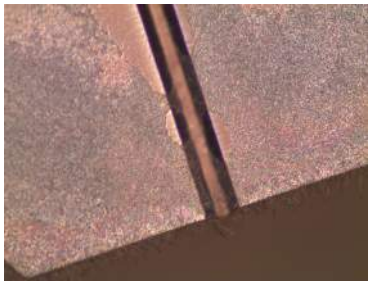
Figure II-40. Optical images of cantilevers for comparison with different processing time of 2-ethoxyethanol gas after 20s processing pure cyclam gas, with magnification of 20 with magnification of 20: (a). Cantilever 17; (b). Cantilever 18; (c). Cantilever 19



(a) (b) (c)

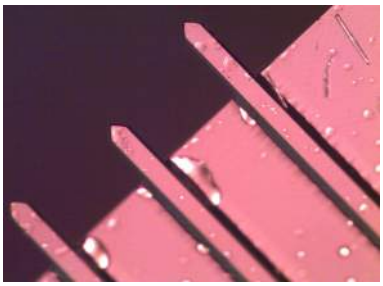
Figure II-41. Optical images of cantilevers for comparison with different processing time of acetone gas after 20s processing pure cyclam gas, with magnification of 20: (a). Cantilever 20; (b). Cantilever 21; (c). Cantilever 22

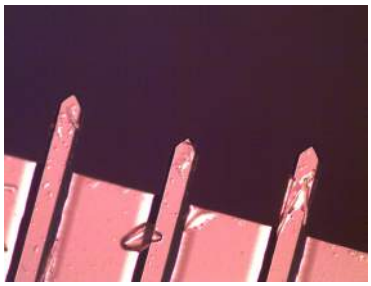




(a) (b)

Figure II-42. Optical images of cantilevers for comparison with different processing time of pure cyclam gas after 10s acetone gas processing, with magnification of 20: (a). Cantilever 23; (b). Cantilever 24





(a) (b)

Figure II-43. Optical images of cantilevers after functionalization with solution of 0.04mg cyclam + 2ml acetone, with magnification of 20: (a). Cantilever 25; (b). Cantilever 26

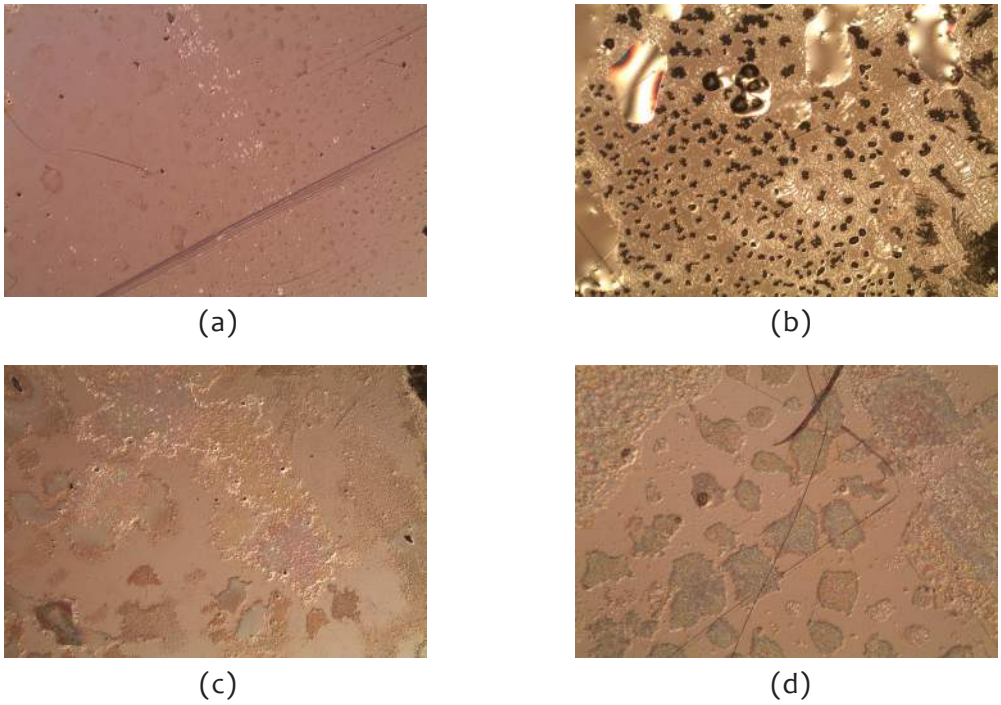
Test 14. Process Time optimization of Cadaverine Bonding with Glycerol

- **Variables**: process time of cadaverine gas bonding
- **Number of samples**: 4
- **Experimental details**:
  - Functionalize 4 samples with solution of 0.05mg cyclam+ 2.5ml 2-ethoxyethanol for 5min, leave to dry at RT
  - Cadaverine bonding process are showed in table II-14

Table II-14

Sample	Bonding method	Cadaverine preparation	Processing time
47	mixture of 10ml glycerol + 0.25ml cadaverine	Heat the mixed solution to 200°C and let the gas bond to the four samples	5s
48			10s
49			15s
50			20s

- **Results:**



(a) (b)

(c) (d)

Figure II-44. Optical images of cantilevers with different cadaverine bonding time: (a). Sample 47, 5s; (b). Sample 48, 10s; (c). Sample 49, 15s; (d). Sample 50, 20s

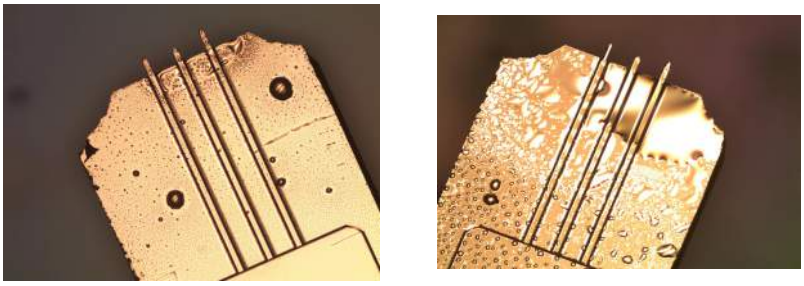
Test 15. Cadaverine Bonding to Cantilevers

- **Variables**: Concentration of cadaverine, different functionalized cantilevers
- **Number of cantilevers**: 6
- **Experimental details**:
  - Functionalize and cadaverine bonding process are showed in table II-15

Table II-15

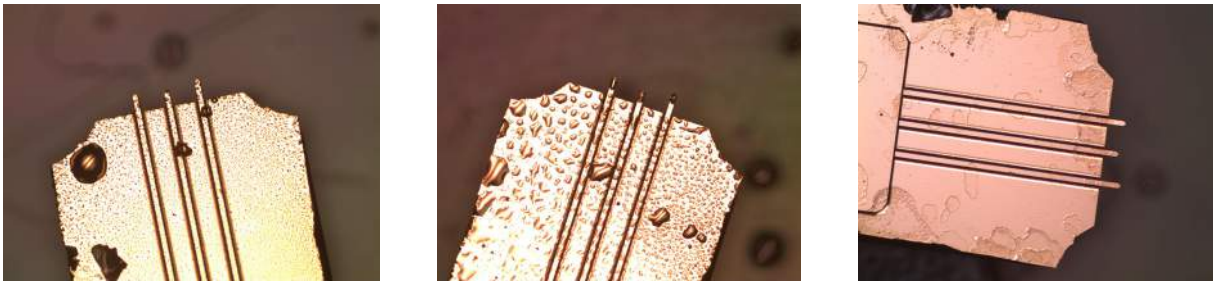
Cantilever	Functionalization details	Cadaverine preparation	Cadaverine bonding method
5’	0.05mg cyclam + 2.5ml 2-ethoxyxthanol, applied by nanoplotter 2.1	6ml glycerol+0.25ml cadaverine	gas phase of cadaverine bonding by heating the mixture(200°C), process for 10s
6’		14ml glycerol+0.25ml cadaverine	
7’	Heat pure cyclm to gas phase and bond to cantilever surface	6ml glycerol+0.25ml cadaverine	
9’		14ml glycerol+0.25ml cadaverine	
27’		6ml water+0.25ml cadaverine	a liquid drop applying by syringe
25’	0.04mg cyclam + 2ml acetone, process for 1h, leave to dry at RT	6ml glycerol+0.25ml cadaverine	gas phase of cadaverine bonding by heating the mixture(200°C), process for 10s
26’		14ml glycerol+0.25ml cadaverine	

- **Results:**



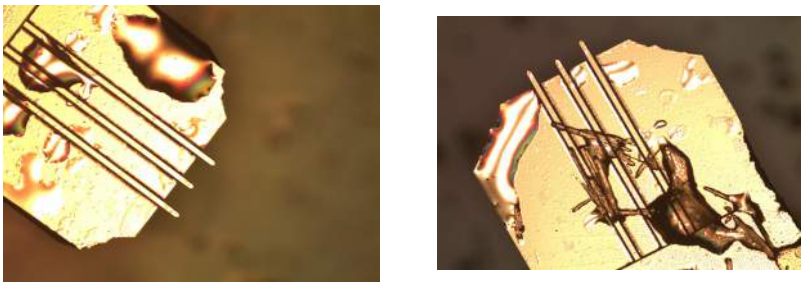
(a) (b)

Figure II-45. Optical images of cantilevers functionalized with solution of 0.05mg cyclam+ 2.5ml 2-ethoxyethanol, after cadaverine bonding, with magnification of 5: (a). Cantilever 5', 6ml glycerol+0.25ml cadaverine; (b). Cantilever 6', 14ml glycerol+0.25ml cadaverine



(a) (b) (c)

Figure II-46. Optical images of cantilevers functionalized with pure cyclam gas, after cadaverine bonding, with magnification of 5: (a). Cantilever 7', 6ml glycerol+0.25ml cadaverine (gas); (b). Cantilever 9', 14ml glycerol+0.25ml cadaverine (gas); (c). Cantilever 8', 6ml water+0.25ml cadaverine (liquid)



(a) (b)

Figure II-47. Optical images of cantilevers functionalized with solution of 0.04mg cyclam+ 2ml acetone, after cadaverine bonding, with magnification of 5: (a). Cantilever 25', 6ml glycerol+0.25ml cadaverine; (b). Cantilever 26', 14ml glycerol+0.25ml cadaverine

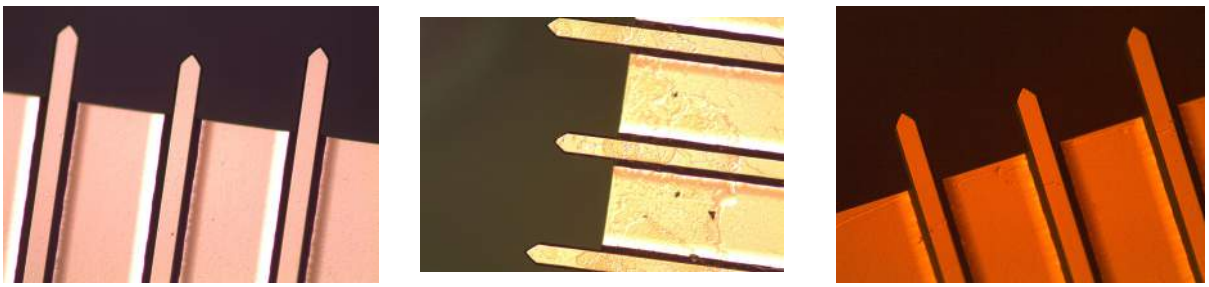
Test 16. Cantilevers cleaning tests

- **Variables**: solution for cleaning: ethanol, PBS buffer
- **Number of cantilevers**: 3
- **Experimental details**:
  - Cleaning details are showed in table II-16

Table II-16

Cantilever	Cleaning solution
7''	Ethanol (liquid solution) rinse for 20s + water rinse for 10s
9''	PBS buffer (liquid solution) rinse for 20s + water rinse for 10s
25''	Ethanol (gas solution) for 30s + water vapor for 10s

- **Results:**



(a) (b) (c)

Figure II-45. Optical images of cantilevers after improved functionalization method with magnification of 20: (a). Cantilever 7'', ethanol liquid cleaning; (b). Cantilever 9'', PBS buffer liquid cleaning; (c). Cantilever 25'', ethanol gas cleaning

III. Data sheets of cantilever

All the information from:
http://www.spmtips.com/afm-tip-hq-nsc35-tipless-cr-au

- HQ:NSC35/TIPLESS/CR-AU

Table III-1

Probe material	N-type silicon
Topside coating	Gold
Detector coating	Gold

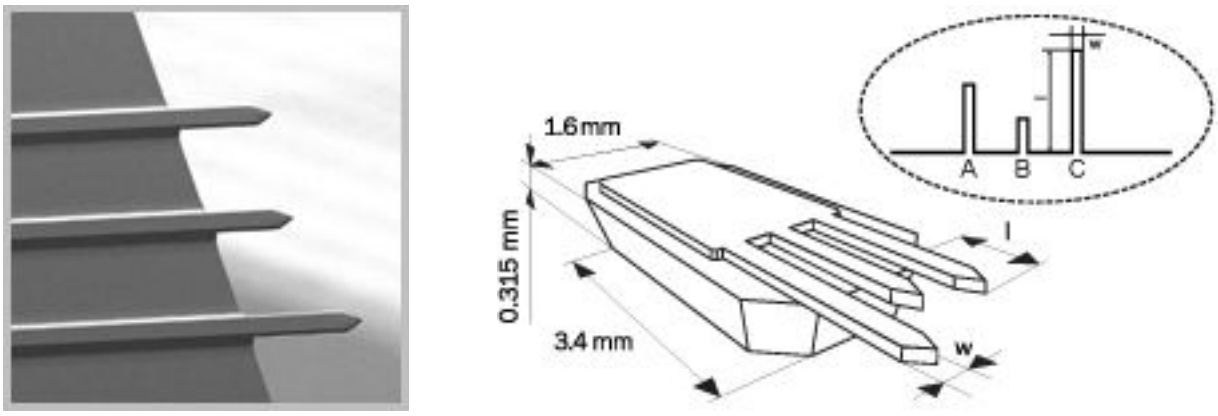


Figure III-1. (a). SEM images of 3 tipless cantilevers on chip (NSC35/36 Series); (b). Schematic drawing of 3 tipless cantilevers on chip

Table III-2

NSC35, Cantilevers	Resonance frequency (KHz)			Force Constant (N/m)			Length (<5um)	Width (<3um)	Thickness (<0.5um)
	min	typ	max	min	typ	max			
B	185	300	430	4.8	16	44	90	35	2.0
C	95	150	205	1.7	5.4	14	130	35	2.0
A	130	205	290	2.7	8.9	24	110	35	2.0

- Tipside coating, nm: Cr 20, Au 30
- Backside coating, nm: Cr 20, Au 30
- Chip dimensions, mm: 3.4×1.6×0.315

IV. Data sheets of piezoelectric chip

All the information from:
https://www.thorlabs.com/newgrouppage9.cfm?objectgroup_id=7563

- PA3CEW Piezoelectric Chip with Wires, 100V, 2.0um Travel

The PA3CEW piezoelectric chip consists of stacked piezoelectric ceramic layers (which are mechanically in series) that are sandwiched between interdigitated electrodes (which are electrically in parallel). It offers a maximum displacement of 2.0um (error within 15%). A red wire is attached to the electrode that should receive positive bias, and a black wire is attached to the electrode that should be grounded.

Figure IV-1. The image of PA3CEW piezoelectric chip

To attach a load to the piezo, we recommend using an epoxy that curse at a temperature lower than 80°C(170°F). Load should only be mounted to the central area of the largest face since the edge do not translate. Mounting a load to the smaller faces may lead to mechanical failure. Operating the device at high frequencies will cause its temperature to rise, which increases the maximum displacement and may necessitate external temperature regulation to stay underneath the maximum operating temperature of 130°C (266°F).

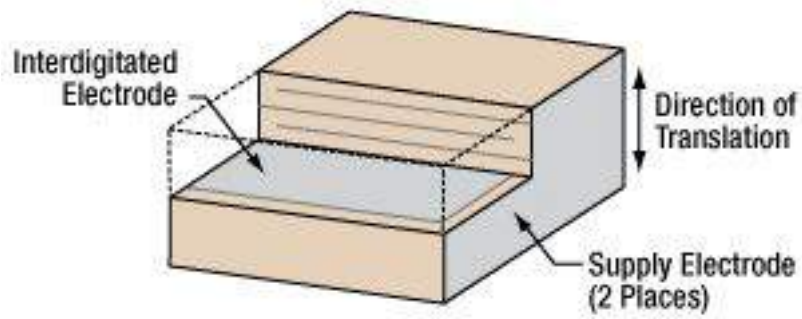


Figure IV-2. Three-Dimensional Cross Section of Multilayer Piezo with Interdigitated Electrodes