CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Luminous Vibriosis in Shrimp [1-11]

Black tiger shrimp, *Penaeus monodon* (Fabricius), is widely cultured in Thailand. The commodity commands high value because of the demand for it in both local and foreign markets. Disease is an important factor in reducing shrimp number in natural populations. Disease problems are considered important to successful production in shrimp aquaculture. Because high-density, confined rearing in unnatural produces stress, some shrimp-associated organism will become prominent. Special measures are required to offset their detrimental effect. Disease may be caused by non-infectious and infectious.

Shrimp diseases due to viral and bacterial infections have caused major economic losses in Thai shrimp farming during the past decade. Bacterial diseases due mainly to *Vibrio* species are often associated with low survival rates in hatchery or growout condition in shrimp aquaculture.The genus *Vibrio* consists of Gram-negative straight or slightly curve rods, 0.5-0.3 x 1.4-2.6 μm. They are non spore-facultative anaerobes and chemoorganotrophs and most are oxidase positive. Most species grow well in media with a sea water base. The common, generally accepted named of the organism *Vibrio* species disease agent is vibriosis or bacterial disease, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis, red-leg disease, sien dum in Thai which translates to black splint, sea gull syndrome or Sindroma de gaviota in Latin America.

Vibriosis is a disease caused by an infection with bacteria of the *Vibrio* genus, a general term referring to an infection by any member of the large group of *Vibrio* bacteria which is caused by eating seafood contaminated with *Vibrio parahemolyticus* or *Vibrio vulnificus*. These bacteria damage the inner wall of the intestine, which causes diarrhea and related symptoms. Vibriosis can be diagnosed and treated by an infectious disease specialist. It is diagnosed when *Vibrio* bacteria are grown from

samples of stool, blood, or blister fluid. The symptoms and a recent history of eating raw seafood are very important clues for diagnosis. Contamination with *Vibrio* bacteria does not change the look, smell, or taste of the seafood. Vibriosis can be prevented by avoiding raw or undercooked shellfish, keeping raw shellfish and its juices away from cooked foods, and avoiding contact of wounded skin with seawater or raw seafood.

Vibrio harveyi (V. harveyi) is commonly present in various marine and brackish habitats, i.e., warm marine waters, surfaces of marine animals, light organs of certain marine fish and cephalopods, and intestine of aquatic animals. *V. harveyi* is a Gram negative straight curved rods with size about 0.5-0.3 x 1.4-2.6 μm, motile, oxidase and catalase positive, and produced green colonies on Thiosulfate citrate bile salt (TCBS) agar.

Bioluminescence refers to the visible light emission in living organisms that accompanies the oxidation of organic compound (luciferins) mediated by an enzyme catalyst (luciferase). Luminescent organisms, which include bacteria, fungi, fish, insects, algae and squid have been found in marine, freshwater and terrestrial habitats, with bacteria being the most widespread and abundant luminescent organism in nature. Although their primary habitat is in the ocean in free-living, symbiotic, saprophytic or parasitic relationships, some luminescent bacteria are found in terrestrial or freshwater habitats. The enzymes involved in the luminescent (*lux*) system, including luciferase, as well as the corresponding *lux* genes, have been most extensively studied from the marine bacteria in the *Vibrio* and *Photobacterium* genera and from terrestrial bacteria in the *Xenorhabdus* genus; in particular the *Vibrio harveyi, Vibrio fischeri, Photobacterium phosphoreum, Photobacterium leiognathi*, and *Xenorhabdus luminescens* species. It has been found that the light-emitting reactions are quite distinct for different organisms, with the only common component being molecular oxygen. Therefore, significant differences have been found between the structures of the luciferases and the corresponding genes from one luminescent organism to another. The expression of luminescence in many bacteria has been found to be hightly dependent on cell density. That is, bacteria found living free in the ocean do not give off light, whereas luminescence is observed from bacteria that are found at the high densities, such as in the confined environments of the light organs of fish

or squid. When *V. harveyi* live freely in seawater, its concentration is less than 10^2 cell mL^{-1}, resulting in no observable luminescence. However, at the high cell densities luminescence was found in the light organ of certain marine fishes and squids (10^{10} to 10^{11}cells/mL), autoinducer can accumulate and reach a critical concentration (5 to 10 nM) required for activation of luminescence gene transcription triggering the synthesis of specific luminescence enzymes.

The bacterial luminescence reaction, which is catalyzed by luciferase, involves the oxidation of a long-chain aliphatic aldehyde and reduced flavin mononucleotide ($FMNH_2$) with the liberation of excess free energy in the form of a blue-green light at 490 nm. At least two chemicals are required. The one which produces the light is generically called a "luciferin" and the one that drives or catalyzes the reaction is called a "luciferase."

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light (490nm)}$$

The basic reaction follows the sequence illustrated above:

- The luciferase catalyzes the oxidation of luciferin
- Resulting in light and an inactive "oxyluciferin"
- In most cases, fresh luciferin must be brought into the system, either through the diet or by internal synthesis.

To the best of our knowledge, there are only two published work that is related to the detection of *Vibrio harveyi*

In 2005, Phianphak et al. [4] produced monoclonal antibodies (MAbs) against *Vibrio harveyi* from mice immunized with heat-killed and SDS-mercaptoethanol-treated highly virulent *V. harveyi* 639. Fifteen MAbs were selected and sorted into 6 groups according to their specificity to various proteins of apparent molecular weight ranging from 8 to 49 kDa. Some antibodies were used for detection of *V. harveyi* at concentrations as low as 10^4 CFU mL^{-1} using immunodot blots. Most of the selected MAbs did not show cross-reactivity to other *Vibrio* species and other gram-negative bacteria tested. Only 1 MAb (VH39-4E) showed slight cross-reactivity to Aeromonas hydrophila. Another MAb (VH24-8H) bound lightly to *V. harveyi* 1526 but strongly

to V. *harveyi* 639, allowing rapid differentiation. Two of the MAb groups were used to localize *V. harveyi* in tissues of infected black tiger shrimp *Penaeus monodon* by immunohistochemistry.

In 1998, Robertson and coworkers[5] developed enzyme-linked immunosorbent assays (ELISA) for the rapid detection of *Vibrio harveyi* from penaeid shrimp and water. The ELISA, which incorporated a polyclonal antiserum produced in a female New Zealand 5 white rabbit, detected 10 cells of *V. harveyi*/mL. Also, the systems detected *V. harveyi* in water from Chinese shrimp hatcheries. The systems permitted the recognition of a wide range of *V. harveyi* isolates. Western blot analysis of bacterial outer membrane proteins (OMP) indicated that a wide epitope was recognised, with many immunoreactive bands in common between isolates of *V. harveyi*.

2.2 Biosensors

The quest for microbiosensors has brought the need for highly selective and highly sensitive organic layers, with tailored biological property that can be incorporated into electronic, optical, or electrochemical devices. The use of Langmuir-Blodgett (LB) and self-assembled (SA) films in such devices stems from the fact that artificial membranes can be assembled easily at the water-air interface and transferred to different substrates, or assembled at the solution-substrate interface. The molecular dimensions of the LB and SA films make the use of even highly expensive molecules economically attractive, and therefore, the development of sophisticated detection systems is possible.

The operation of biosensor can be devided into three main events. In the first, the molecule to be detected is recognized by the sensor. This recognition should be both specific, i.e., it is able to recognize the molecule, and selective, i.e., the recognition of the molecule can be done in the presence of other bioactive molecules. The second is perturbation, i.e., once the molecule is attached to the recognition site, a detectable physical change is triggered. The last event is transduction, which is the ability to recognize the triggered physical change by an instrumental arrangement. The signal is then analyzed and date is provided. This series of steps is illustrated in Figure 2.1.

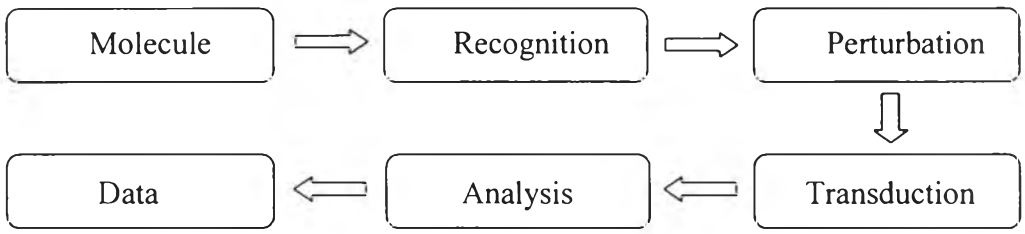


Figure 2.1 Recognition-perturbation-transduction scheme followed by analysis. [12]

The first step, biorecognition, for example: *(a) Enzyme-substrate* — where the enzyme is immobilized on the biosensor. This enzyme will catalyze a chemical reaction, and the biosensing is done by analyzing either a starting material or a product in this reaction. *(b) Antibody-antigen* — where the antigen is immobilized on the biosensor and the formation of an adduct with the antibody is detected. The second step in the biosensing process, transduction, is summarized in Table 1.1, where a list of transduction modes used in biosensor development is presented.

Biocomponents, which function as biochemical transducers can be enzymes, tissues, bacteria, yeast, antibodies/ antigens, liposomes, organelles. Within a biosensor, the recognition biomolecule incorporated possesses an exquisite level of selectivity but is vulnerable to extreme conditions such as temperature, pH and ionic strength. Most of the biological molecules such as enzymes, receptors, antibodies, cells etc. have very short lifetime in solution phase. Thus they have to be fixed in a suitable matrix. The immobilization of the biological component against the environmental conditions results in decreased enzyme activity. The activity of immobilized molecules depends upon surface area, porosity, hydrophilic character of immobilizing matrix, reaction conditions and the methodology chosen for immobilization.

Table 1.1 Transduction modes in biosensors. [12]

Transduction Mode and Device	Observed Output
Optical	
Fiber optics and planar devices utilizing absorption, scatter, polarization, reflectivity, and interference of light	Changes in wavelength, intensity, emission profile, reflectivity, fringe patterns, polarization state, and reflective index
Electrochemical	
Potentiometric devices, amperometric devices (electrode), conductometric devices (chemiresistor)	Changes in voltage, current, impedance, and/or resistance
Galvanometer	
Piezoelectric microbalances, surface acoustical wave (SAW)	Change in mass which produce shifts in frequency or phase of resonance vibration
Thermal	
Thermistor devices	Changes in temperature which produce shifts in electrical out put

2.2.1 Quartz Crystal Microbalance (QCM) [13-16]

Quartz crystal microbalance (QCM) can monitor slight changes in the amount of material on its surface. Most QCMs are single-channel devices, which spatially average the response. A QCM consists of a quartz disk with electrode plated on either side. Because the devices are piezoelectric, an oscillating electric field applied across the device induces an acoustic wave to propagate through it. The frequency of this acoustic wave depends on the thickness of the device and changes with the effective mass-per-unit area of the device. The mass of a thin layer deposited on the crystal can be calculated from a measured change in the resonant frequency of the device.

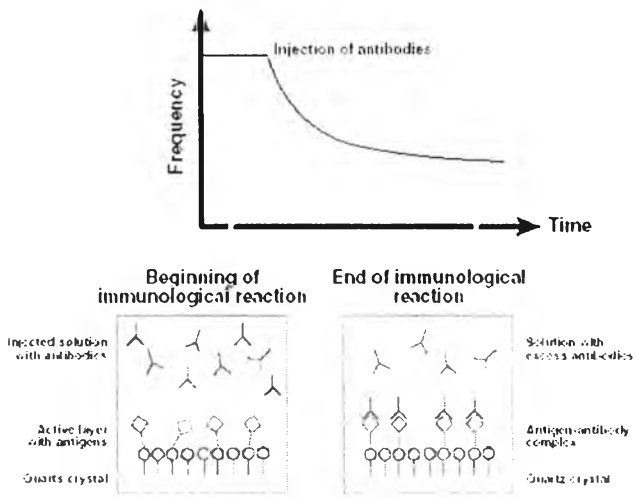


Figure 2.2 General concept of quartz crystal microbalance.

The following literatures describe the detection of bacteria by QCM technique.

In 2001, Fung and Wong [15] developed a new procedure based on the self-assembled monolayers (SAM) of alkanethiols to immobilize antibodies onto gold electrodes of a quartz crystal microbalance (QCM) for detecting *Salmonella paratyphi* A. The procedure includes (1) chemisorption of 3-mercaptopropionic acid (MPA) at electrode surfaces, (2) activation by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydrosuccinimide (NHS), and (3) condensation of antibodies after aminolysis of the NHS adduct. With 50 min incubation under continuous monitoring, working ranges from 10^2 to 10^5 cells/mL with repeatability <10% RSD were obtained.

In 2004, Su and Li [16] developed a method for rapid detection of *Escherichia coli* O157:H7. It was based on the immobilization of affinity-purified antibodies onto a monolayer of 16-mercaptohexadecanoic acid (MHDA), self-assembled on an AT-cut quartz crystal's Au electrode surface with *N*-hydroxysuccinimide (NHS) ester as a reactive intermediate. The stepwise assembly of the immunosensor was characterized by quartz crystal microbalance. The immunosensor could detect the target bacteria in a range of 10^3–10^8 CFU/mL within 30–50 min, and the sensor-to-sensor reproducibility obtained at 10^3 and 10^5 colony-forming units (CFU)/mL was 18 and 11% R.S.D., respectively.

2.3 Self-assembled Monolayer [12-30]

The interactions between molecules and surfaces are some of the most exciting and widely studied aspects of modern surface science. The strengths of the interactions between molecules and substrates are highly dependent upon their chemical natures, ranging from very weak (e.g., n-alkanes adsorbed on gold or graphite) to strong enough to break chemical bonds within the molecule (e.g., ethylene on platinum). One of the most remarkable molecule–substrate interactions is the spontaneous self organization of atoms and molecules on surfaces into well-ordered arrays; the supramolecular assemblies that form often possess both short- and long-range order.

Self-assembly is a phenomenon in which a number of independent molecules suspended in an isotropic state come together to form an ordered aggregate. This phenomenon is ubiquitous in nature, as seen in the formation of micelles by surfactants, bilayers by lipids, or biological cells by living organisms. The principle driving force for formation of this film is specific interactions between the surfactant head group and the substrate surface. Self-assembled films are formed when molecules organize themselves in a 2D arrangement on the surface of a substrate as shown in Figure 2.3.

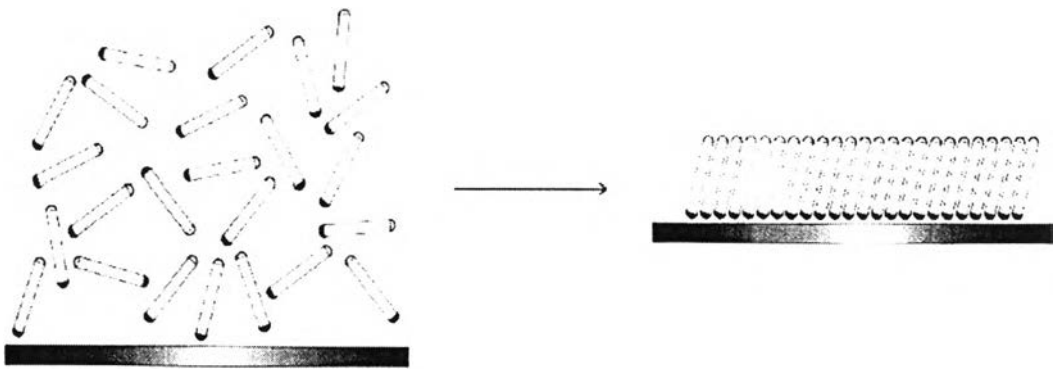


Figure 2.3 Self-assembly of amphiphilic adsorbates onto a solid surface.

The concept of molecules organized into higher order structures is not new. Examples of the ordering of atoms and molecules have been shown throughout biology, chemistry, and physics. In the context of surface chemistry, however, it is important to mention that amphiphilic molecules spontaneously organizing into

assemblies on metal surfaces are only a subset of self-assembled films that have been reported and characterized. Molecules that are amphiphilic can organize themselves at a variety of interfaces (liquid–liquid, air–liquid, solid–liquid, and solid–air interfaces); of particular relevance to this type of surface chemistry are Langmuir– Blodgett (L–B) films, in which amphiphilic molecules organize at one interface (typically air–solution) and are transferred to another (air–solid).

Many different chemistry for the adsorption of amphiphiles on surfaces has been studied over the past twenty years, encompassing a range of substrates and a greater variety of adsorbates. Self-assembled monolayers (SAMs) are typically formed from the exposure of a surface to molecules with chemical groups that possess strong affinities for the substrate or a material patterned on it. How well these assemblies order is a function of the nature of the chemical interaction between substrate and adsorbate, as well as the type and strengths of intermolecular interactions between the adsorbates that are necessary to hold the assembly together. Molecules ''binding to'' surfaces are either described in terms of physisorption, in which the enthalpies of interactions are rather low (considered to be DH < 10 kcal/mol, typically from van der Waals forces), or in terms of chemisorption with DH > 10 kcal/mol. Strengthening interactions between molecules and substrates and between molecules themselves include phenomena such as hydrogen bonding, donor–acceptor and/or ion pairing, and the formation of covalent bonds, rendering the assemblies more stable than their physisorbed counterparts. Other studies have focused upon directly ''grafting'' molecules to surfaces, such as the attachment of aryl-functionalized molecules to silicon, alkyl-functionalized molecules to germanium via Grignard reactions, and molecules to metal surfaces through diazonium salts, all of which indicate the formation of surface–carbon bonds.

Chemisorbing systems have included the assembly of trialkyl-, trichloro-, or trialkoxysilanes on silicon dioxide surfaces, carboxylic acids adsorbing onto aluminum oxide and silver surfaces, and n-alkanethiols chemisorbing to gold surfaces, to name merely a few. As noted above, particularly well-studied SAMs are those formed on transition metal surfaces (e.g., Au, Ag) and surfactants with electron-rich headgroups (e.g., S, O, N) and n-alkyl tails. The affinities between the surfaces and headgroups are strong enough to form either polar covalent or ionic bonds, and

favorable lateral interactions between adjacent molecules are sufficient to draw and to hold the assembly together.

2.3.1 The n-Alkanethiolate SAM

Much research has been focused on the self-assembly of n-alkanethiolate and related molecules on gold substrates. Thiol-based SAMs are attractive structures for several reasons. Well-ordered SAMs can be formed from a variety of sulfur-containing species (i.e., thiols, sulfides, disulfides), yet experiments show that thiol molecules kinetically outcompete the disulfide molecules for available surface sites when the two species are coadsorbed from solution. The gold surface is relatively chemically inert; it does not readily form a surface oxide nor keep a strong hold of adventitiously adsorbed material, and therefore SAMs can easily be prepared in ambient conditions. SAMs render an ordinarily conductive metal surface to be relatively insulating, yet electrons can be moved controllably through the film through applied potentials when integrated into electrochemical cells (vide infra). Additionally, the molecules are stable once adsorbed on the surface, yet they can be affixed to the gold such that they can be selectively processed after adsorption. Many different chemical functional groups have been incorporated into n-alkanethiolate SAMs. Much research has focused on the incorporation of differing terminal groups into SAMs, for interfacial properties such as hydrophobicity and reactivity to be manipulated and controlled. Recognition factors can be incorporated into the exposed interface of a SAM for either cell adhesion, selective protein interactions, or for inhibiting the non-specific binding of proteins. The structures of SAMs as well as the dynamics of their formation will be discussed presently, as alkanethiolate SAMs have often provided the foundation for increasingly complex and functional nanoscale architectures. It is through such fundamental studies of SAMs that the most efficient and effective applied systems can be created and developed.

The surface structures formed by the adsorption of n-alkanethiols on gold surfaces are generally well ordered and crystalline. Upon exposure of a gold substrate to such a thiol in solution or in the gas phase, a bond between gold and sulfur (~44 kcal/mol) forms rapidly, typically within seconds to minutes. Following over the next few hours, contributing a significant amount of order to the assembly, is the close-

packing of the hydrocarbon tails into a primarily all-trans configuration. The adsorption of the molecules extends laterally to accessible substrate, if the exposed thiol is in high enough concentration (with the occupation of ~10^{15} molecules/cm^2). However, the film is restricted from growth normal to the surface because of the molecule's unreactive, methyl-terminated tail, resulting in a chemically passivating film of monomolecular thickness. At low surface coverage, the alkanethiolate molecules lie flat with their hydrocarbon backbones parallel to the gold surface; at higher surface coverage, the molecules begin to stand up, with the hydrocarbon tails tilting approximately 30° from the surface normal and nominally in the all-*trans* configuration so as to maximize van der Waals interactions.

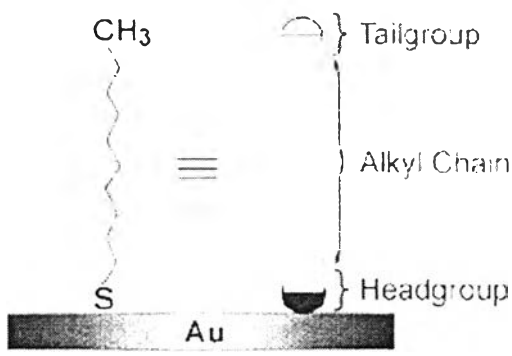
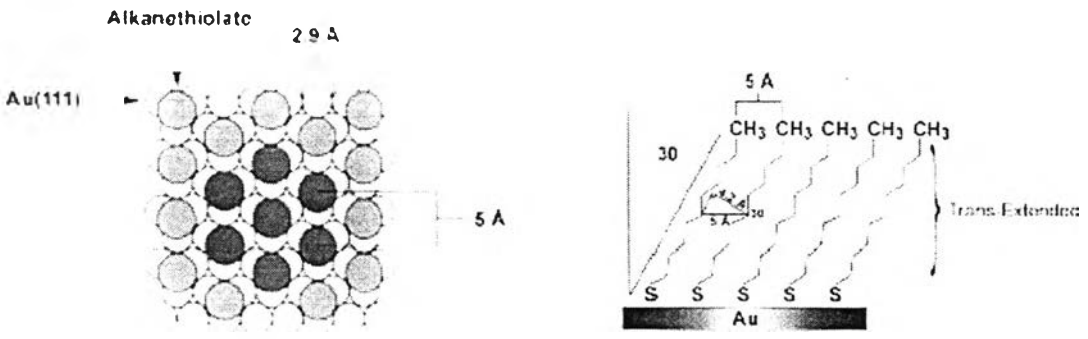


Figure 2.4 Schematic view of the forces in a self-assembled monolayer.

2.3.2 Characteristic

SAMs have been thoroughly characterized using a large number of surface analytical tools. Among the most frequently used techniques are infrared spectroscopy, ellipsometry, studies of wetting by different liquids, x-ray photoelectron spectroscopy, electrochemistry, and scanning probe measurements. It has been clearly shown that SAMs with an alkane chain length of 12 or more methylene units form well-ordered and dense monolayers on Au(111) surfaces. The thiols are believed to attach primarily to the threefold hollow sites of the gold surface, losing the proton in the process and forming a ($\sqrt{3}$x$\sqrt{3}$)R30° overlayer structure (shown in Figure 2.5). The distance between pinning sites in this geometry is 5.0 Å, resulting in an available area for each molecule of 21.4 $Å^2$. Since the van der Waals diameter of the alkane chain is

somewhat too small (4.6 Å) for the chain to completely occupy that area, the chains will tilt, forming an angle of approximately 30° with the surface normal. Depending on chain length and chain-terminating group, various superlattice structures are superimposed on the (√3 x √3)R30° overlayer structure. The most commonly seen superlattice is the c(4×2) reconstruction, where the four alkanethiolate molecules of a unit cell display slightly different orientations when compared with each other.



(a) (b)

Figure. 2.5 Structure of SAMs of alkanethiols on Au(111) from two different perspectives : (a) top view, where the open circles represent gold atoms in a hexagonal close-packed arrangement, and the shaded circles represent alkanethiolate adsorbates (the darker shaded circles highlight the hexagonal (√3 x √3)R30° overlayer structure), and (b) side view, where the adsorbates are packed 5A I apart with their alkyl chains tilted 30° from the surface normal in a trans-extended conformation.

The Au-thiolate bond is strong - homolytic bond strength 44 kcal/mol - and contributes to the stability of the SAMs together with the van der Waals forces between adjacent methylene groups, which amount to 1.4-1.8 kcal/mol. The latter forces add up to significant strength for alkyl chains of 10-20 methylenes and play an important role in aligning the alkyl chains parallel to each other in a nearly all-trans configuration. At low temperatures, typically 100 K, the order is nearly perfect, but even at room temperature there are only few gauche defects, concentrated to the outermost alkyl units.

The estimation of film thickness usually is the first characterization method. Ellipsometry is the common optical technique for the determination of the thickness and reflective index of thin homogeneous films. When a plane-polarized light

interacts with a surface at some angle, it is resolved into its parallel and perpendicular components. These components are reflected from the surface in a different way. When the *s*- and *p*-polarized reflected light beams are combined, the result is elliptically polarized light. Thickness measurements using ellipsometry yield SAM thicknesses that are in good agreement with the 30° chain tilt mentioned above. For example, reported ellipsometric thicknesses of hexadecanethiolate SAMs lie in the 21.1 Å range, to compare with the 21.2 Å that results in a fully extended hexadecanethiol molecule of 24.5 Å length is tilted 30°.

Reflection-absorption (RA) or grazing angle spectroscopy is a very useful technique that gives information about the direction of transition dipoles in a sample. Theoretical consideration of the IR spectroscopy of monolayer absorbed on a metal surface showed that the reflection-absorption spectrum is measured most efficiently of high angles of incidence, and that only the component of incident light that is parallel to the plane of incidence gives measurable absorption.

Contact angle measurements further confirm that alkanethiolate SAMs are very dense and that the contacting liquid only interacts with the topmost chemical groups. Reported advancing water contact angles range from 111° to 115° for hexadecanethiolate SAMs. At the other end of the wettability scale, there are hydrophilic monolayers, e.g., SAMs of 16-mercaptohexadecanol ($HS(CH_2)_{16}OH$), that display water contact angles of <10°. These two extremes are only possible to achieve if the SAM surfaces are uniform and expose only the chain-terminating group at the interface. Mixed SAMs of CH_3- and OH-terminated thiols can be tailor-made with any wettability (in terms of contact angle) between these limiting values.

2.4 Antibody-antigen [31-34]

An antibody is a protein used by the immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target. Antibodies are immune system-related proteins called immunoglobulins. Each antibody consists of four polypeptides joined to form a "Y" shaped molecule that consists of two identical heavy chains and two identical light chains connected by disulfide bonds. The basic unit of each antibody is a monomer. An antibody can be monomeric, dimeric, trimeric, tetrameric, pentameric, etc. The

amino acid sequence in the tips of the "Y" varies greatly among different antibodies. This variable region, composed of 110-130 amino acids, give the antibody its specificity for binding antigen. The variable region includes the ends of the light and heavy chains.

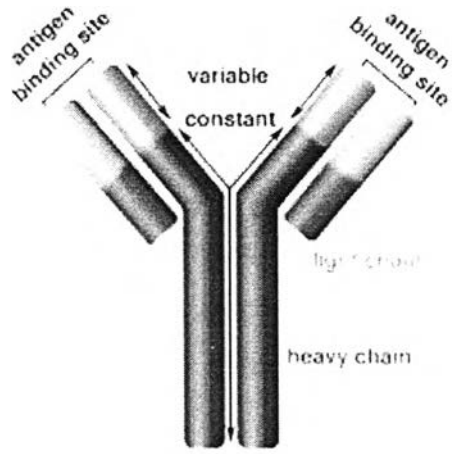


Figure 2.6 The basis structure of antibody. [31]

There are five types of heavy chain: γ, δ, α, μ and ε that define classes of immunoglobulins. Heavy chains α and γ have approximately 450 amino acids, while μ and ε have approximately 550 amino acids. Each heavy chain has a constant region, which is the same by all immunoglobulins of the same class, and a variable region, which differs between immunoglobulins of different B cells, but is the same for all immunoglobulins produced by the same B cell. Heavy chains γ, α and δ have the constant region composed of three domains but have a hinge region; the constant region of heavy chains μ and ε is composed of four domains. The variable domain of any heavy chain is composed of one domain. These domains are about 110 amino acids long. There are also some amino acids between constant domains. There are only two types of light chain: λ and κ. In humans, they are similar, but only one type is present in each antibody. Each light chain has two successive domains: one constant and one variable domain. The approximate length of a light chain is from 211 to 217 amino acids.

The monomer is composed of two heavy and two light chains. Together this gives six to eight constant domains and four variable domains. If it is cleaved with

enzymes papain, two Fab (fragment antigen binding) fragments and an Fc (fragment crystallizable) fragment are obtained, whereas pepsin cleaves below hinge region, so a f(ab)2 fragment and a fc fragment is formed.

Each half of the forked end of the "Y"-shape monomer is called the Fab fragment. It is composed of one constant and one variable domain of each the heavy and the light chain, which together shape the antigen binding site at the amino terminal end of the monomer. The ability to bind a wide variety of foreign antigens arises from events known as somatic recombination. The main reason that the human immune system is capable of binding so many antigens is the variable region of the heavy chain. Different classes of immunoglobulins—IgG, IgA, IgM, IgD and IgE—differ in the assembly of their chains and domains. IgG usually have two heavy chains each containing four domains, and two light chains each containing two domains.

When immunologists describe the properties of antibodies as proteins, most would include a description of the capacity of these molecules to precipitate antigens from solution, even though antibody precipitation is seldom used any more to isolate or detect antigens experimentally and even though antibodies probably rarely precipitate antigens in vivo, except in some autoimmune diseases.

Antibodies have two very useful characteristics. First, they are extremely specific; that is, each antibody binds to and attacks one particular antigen. Second, some antibodies, once activated by the occurrence of a disease, continue to confer resistance against that disease; classic examples are the antibodies to the childhood diseases chickenpox and measles.

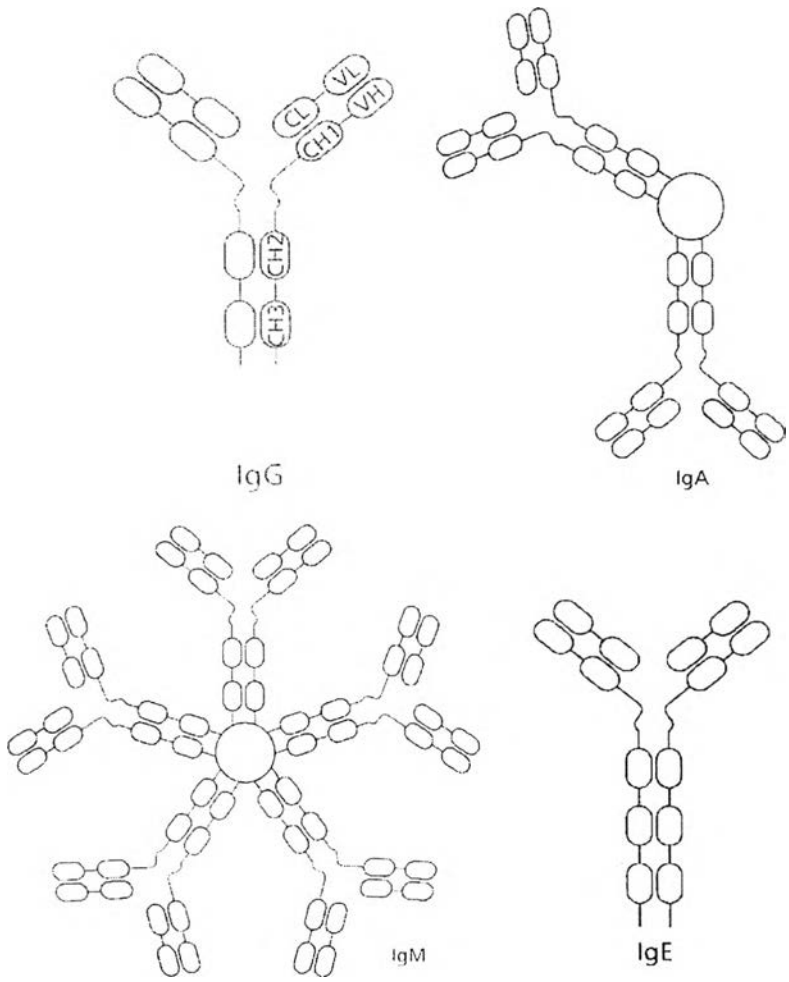


Figure 2.7 Most antibody molecules contain light and heavy chains. Each comprises one variable domain and different numbers of constant domains. The combination of 2 light + 2 heavy chains is a higher order building block. Immunoglobulins of different classes show different states of oligomerization, with additional chains where necessary serving as linkers. [34]

2.4.1 Monoclonal Antibodies [31-34]

Monoclonal antibodies (mAb) are antibodies that are identical because they were produced by one type of immune cell, all clones of a single parent cell. Given (almost) any substance, it is possible to create monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance. This has become an important tool in biochemistry, molecular biology and medicine.

To produce monoclonal antibodies, one removes B-cells from the spleen of an animal that has been challenged with the antigen. These B-cells are then fused with

myeloma tumor cells that can grow indefinitely in culture (myeloma is a B-cell cancer). This fusion is done by making the cell membranes more permeable. The fused hybrid cells (called hybridomas), being cancer cells, will multiply rapidly and indefinitely and will produce large amounts of antibodies. The hybridomas are sufficiently diluted and grown, so that one obtains a number of different colonies, each producing only one type of antibody. The antibodies from the different colonies are then tested for their ability to bind to the antigen (for example with a test such as ELISA), and the most effective one is picked out. Monoclonal antibodies can be produced in cell culture or in animals. When the hybridoma cells are injected in mice (in the peritoneal cavity, the gut), they produce tumors containing an antibody-rich fluid called ascites fluid.

Once monoclonal antibodies for a given substance have been produced, they can be used to detect the presence and quantity of this substance, for instance in a Western blot test (to detect a substance in a solution) or an immunofluorescence test (to detect a substance in a whole cell). Monoclonal antibodies can also be used to purify a substance with techniques called immunoprecipitation and affinity chromatography.

In medicinal treatments, the small variation (if any) in recognizing the antigen helps to reduce side effects. However, there are drawbacks to using monoclonal antibodies as opposed to polyclonals. Each B-lymphocyte produces antibodies that are specific not to an antigen, but to an epitope of that antigen. An epitope is a small piece of the antigen to which the antibody binds. Polyclonal antibodies bind to many epitopes of a given antigen, while monoclonals bind to a single epitope. In the processing of antibodies, certain binding capabilities are degraded. If the monoclonal antibody is susceptible to such degradation, it is useless. Polyclonals will still be useful even if certain epitope-binding species are degraded.

2.4.2 Advantages of Monoclonal Antibodies

- Homogeneity: Monoclonal antibody represents a single antibody molecule that binds to antigens with the same affinity and promotes the same effector functions.

- Specificity: The product of a single hybridoma reacts with the same epitope on antigens.
- Immunizing Antigen: Need not to be pure or characterized and is ultimately not needed to produce large quantities of antibody.
- Selection: It is possible to select for specific epitope specificities and generate antibodies against a wider range of antigenic determinants.

2.5 Identification of Components of a Mixture of Proteins [33-35]

First the components are separated by electrophoresis, then the isolated proteins are digested by trypsin to produce peptide fragments with an r.m.m. of about 800-4000. Trypsin cleaves protein after Lys and Arg residues. Given a typical amino acid composition, a protein of 500 residues yields about 50 tryptic fragments. The spectrometer measures the masses of the fragments with very high accuracy. The list of fragment masses, called the *peptide mass fingerprint,* characterize the protein. Searching a database of fragment masses identifies the unknown sample.

Construction of database of fragment masses is a simple calculation from the amino acid sequence of known proteins, translation of open reading frames (ORFs) in genomes, or (in a pinch) of segments from EST (expressed sequence tag) libraries. The fragments correspond to segments cut by trypsin at lysine and arginine residues, and the masses of the amino acid are known. It should be noted that trypsin doesn't cleave Lys-Pro peptide bonds, and may also fail to cleave Arg-Pro peptide bonds.

Mass spectrometry is sensitive and fast. Peptide mass fingerprinting can identify proteins in sub-picomole quantities. Measurement of fragment masses to better than 0.1 mass units is quite good enough to resolve isotopic mixtures. It is a high-throughput method, capable of processing 100 spots/day (though sample preparation time is longer). However, there are limitations. Only proteins of known sequence can be identified from peptide mass fingerprints, because only their predicted fragment masses are included in the databases. Also, post-translational modifications interfere with the method because they alter the masses of the fragments.

The operation of the spectrometer involves the following steps:

1. Production of the sample in an ionized form in the vapor phase;

2. Acceleration of the ions in an electric field, each ion emerging with a velocity proportional to its charge/mass ratio;
3. Passage of the ions into a field-free region, where they 'coast';
4. Detection of the time of arrival of the ions, the 'time-of-flight'(TOF) indicating the mass-to-charge ratio of the ions;
5. The result of the measurements is a trace showing the flux as a function of the mass-to-charge ratio of the ions detected.

Proteins being fairly delicate objects, it has been challenging to vaporize and ionize them without damage. Two 'soft-ionization' methods that solve this problem are:

2.5.1 Matrix-assisted laser desorption ionization (MALDI)

The sample is introduced into the spectrometer in dry form, and mixed with a substrate or matrix that moderates the delivery of energy. A laser pulse, absorbed initially by the matrix, vaporizes and ionizes the protein.

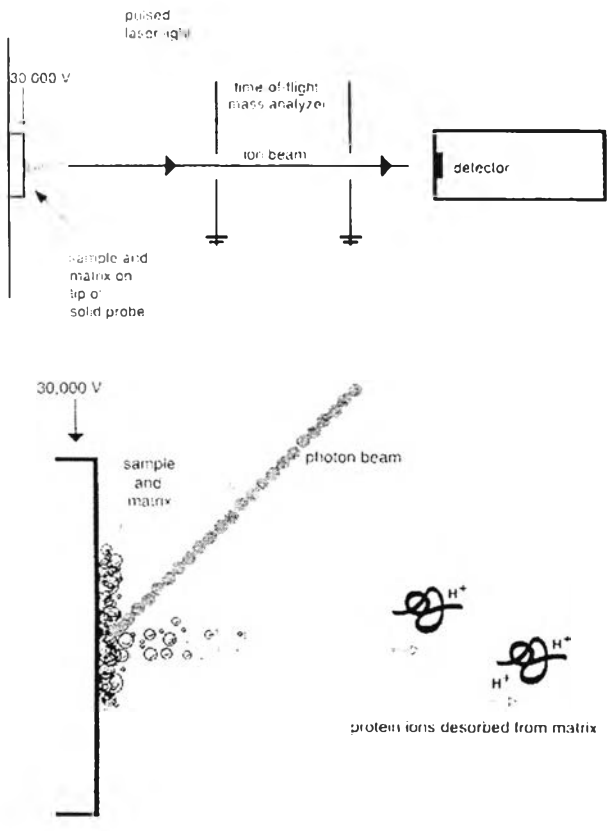


Figure. 2.8 Matrix-assisted laser desorption ionization (MALDI) source.

The mass analyzer separates molecular ions base on their mass-to-change ratio (*m/z*). MALDI is usually combined with a time-of flight (TOF) mass spectrometer. Ions are accelerate to a kinetic energy of approximately 25 keV and subsequently allowed to fly through a fixed distance field-free region of about one meter in length before they are recorded by the electric signal generated upon impact at a detector. The time-of-flight in the field-free region is related to the *m/z* of a given ion. Having identical kinetic energy, small molecular ions are moving faster than large molecular ions and thus arrive earlier at the detector. The *m/z* for an ion can be determined from its time-of-flight by comparison to the time-of-flight of known standard. An advance of the TOF analyzer is that the total ion population is detected in one experiment resulting in very high sensitivity. The mass range of the TOF analyzer is theoretically unlimited. However, large molecular ions move relatively slowly and are therefore not detected as efficiently as small, fast ions which generate a stronger response upon impact at the detector. The practical mass range for MALDI TOF MS is from 500 Da up to 150000Da. Note that high mass analysis is considerably more challenging than low mass analysis.

2.5.2 Electrospray ionization (ESI) method

The method starts with the sample in liquid form being sprayed through a small capillary with an electric field at its tip, creating an aerosol of highly charged droplets. As the solvent evaporates, the droplets contract, bring the charges closer together and increase the repulsive forces between them. Eventually the droplets explode into smaller droplets, each with less total charge. This process repeats, creating ions, (which may be multiply charged) devoid of solvent. These ions are transferred into the high-vacuum region of the mass spectrometer.

2.6 Characterization Techniques

2.6.1 Quartz Crystal Microbalance

Recently, piezoimmunosensors namely quartz crystal microbalance (QCM), based on a combination of highly specific immuno-recognition and ultra-sensitive QCM mass detection, which use a quartz piezoelectric (P/Z) crystal detector as the transducer, have been developed for the assay of biological substances and

microorganisms in the food industry, for the monitoring of environmental pollutants and for clinical diagnostics. Unlike previous enzyme-linked immunoassay procedures, which require an enzyme label, QCM provides a direct label-less method for the assay of bacteria. Generally, primary or capture antibodies are immobilized onto an AT-cut quartz crystal, adsorption of bacteria onto the immobilized antibodies results in an increase in the surface mass loading of the crystal, and this will decrease the sensor's resonant frequency. Piezoelectric crystal detection is based on the principle of the microgravimetric quartz crystal microbalance, as described by the Sauerbrey equation, given as follows

$$\Delta F = -2.3 \times 10^6 F_0^2 \Delta m/A \qquad (2.1)$$

where ΔF is the change in frequency of the crystal (Hz), F_0 is the resonant frequency of the crystal (MHz), Δm is the mass deposited on the electrode surface (g) and A is the area coated (cm^2). QCM is thus capable of measuring a small change in mass because of changes in their resonant frequency upon a weight increase on their surface.

Five megahertz AT-cut quartz crystals were used as the reaction carriers. The Sauerbrey sensitivity of these crystals is 1 Hz = 17.7 ng/cm^2.

2.6.2 Contact Angle Measurements

Contact angle measurements are often used to assess changes in the wetting characteristics of a surface and hence indicate a change in surface energy. The technique is based on the three-phase boundary equilibrium described by Young's equation, (Figure 2.9)

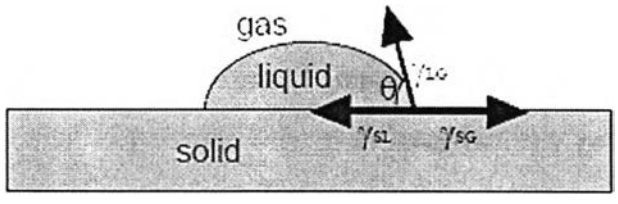


Figure 2.9 Schematic representation of the Young's equation.

$$\gamma_{LG}\cos\theta = \gamma_{SG} - \gamma_{SL} \quad (2.2)$$

where γ_{ij} is the interfacial tension between the phases i and j, with subscripts L, G, S corresponding to liquid, gas, and solid phase respectively and θ refers to the equilibrium contact angle.

The Young's equation applies for a perfectly homogeneous atomically flat and rigid surface and therefore supposes many simplifications. In the case of real surfaces, the contact angle value is affected by surface roughness, heterogeneity, vapor spreading pressure, and chemical contamination of the wetting liquid. Although the technique to measure contact angles is easy, data interpretation is not straightforward and the nature of different contributions to the surface is a matter of discussion. Generally, one can define the complete wetting, wetting, partial wetting, and nonwetting according to Figure 2.10.

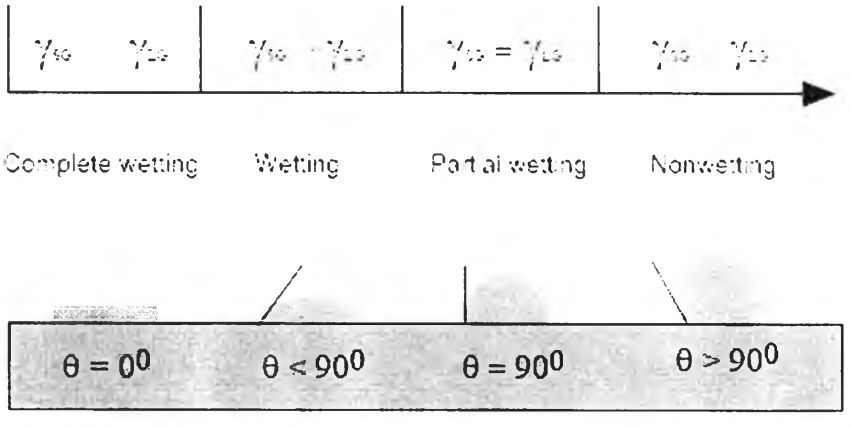


Figure 2.10 Schematic representation of wettability.

2.6.3 Reflection-Absorption Infrared Spectroscopy

Reflection-absorption (RA) or grazing angle spectroscopy is a very useful technique that gives information about the direction of transition dipoles in a sample. Theoretical consideration of the IR spectroscopy of monolayer absorbed on a metal surface showed that the reflection-absorption spectrum is measured most efficiently of high angles of incidence, and that only the component of incident light that is parallel to the plane of incidence gives measurable absorption. Figure 2.11 presents a

schematic description of a monomolecular film on mirror, with the incident light and direction of the polarization.

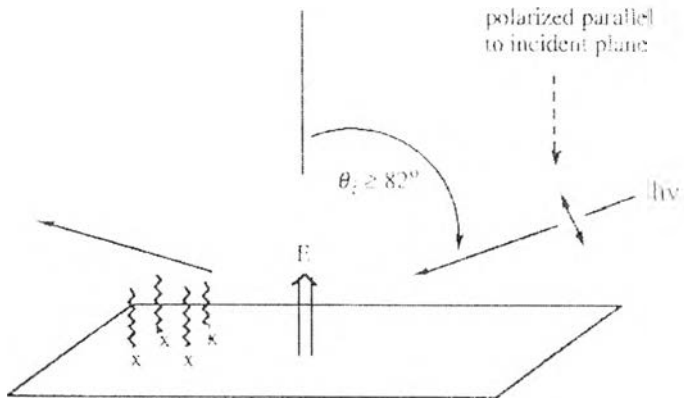


Figure. 2.11 Reflection-absorption infrared spectroscopy.