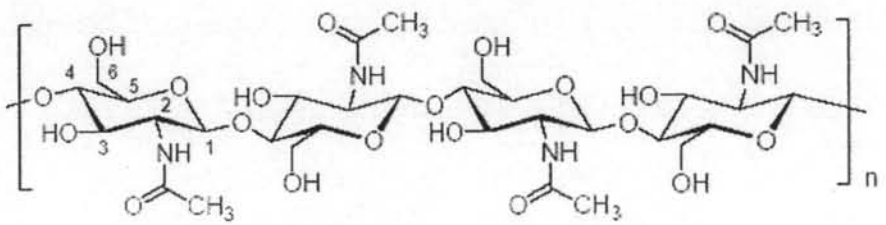
CHAPTER II

THEORY AND LITERATURE REVIEW

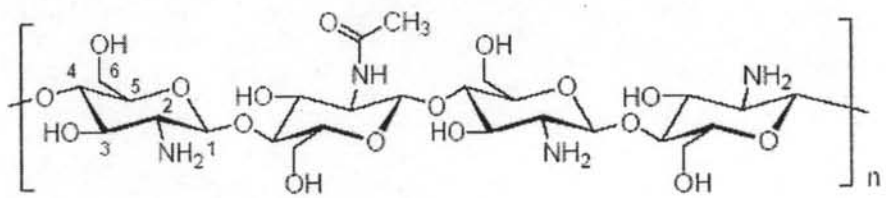
2.1 Chitosan

Chitin is the second most abundant polysaccharide in nature, second only to cellulose; and is primarily present in the exoskeletons of crustaceans (such as crab, shrimp etc.). In addition to crustaceans, it is also found in various insects, fungi and mushrooms.

Chemical structure of chitin consists of linear repeating units of 2-acetamido-2-deoxy-D-glucopyranose attached through β-(1-4) linkages (Figure 2.1a).



(a)



(b)

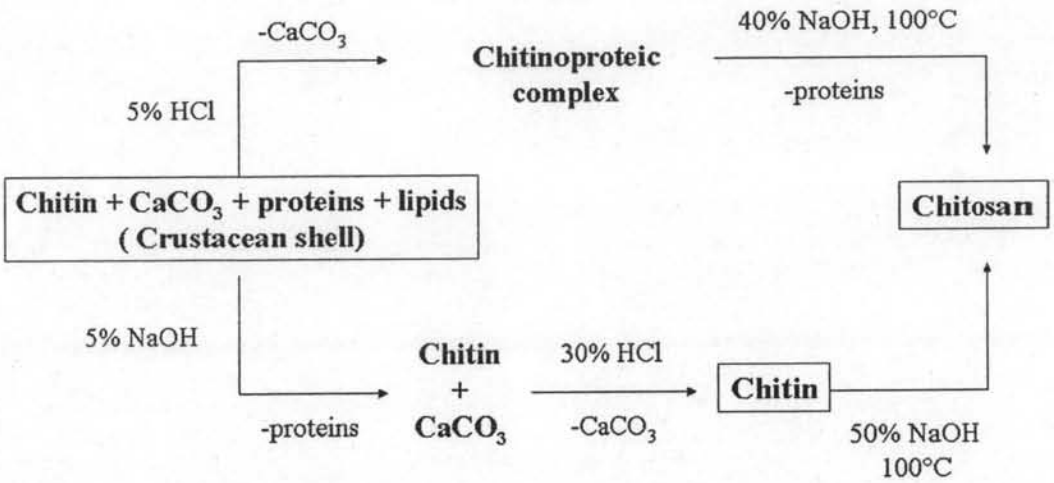
Figure 2.1 Chemical structures of chitin (a) and chitosan (b)

Chitosan (Figure 2.1b) is deacetylated chitin; a structural modification of chitin often performed by alkaline hydrolysis. The actual process involved during the hydrolysis governs the extent of deacetylation in chitin, and accordingly, commercially available samples of chitosan contain anywhere from 70% to 100% deacetylation. In addition to the degree of deacetylation for a given chitosan sample, it

is also characterized by the molecular weight of the macromolecule, and is often available within the range of 150,000 – 600,000. Chitin and chitosan are of high commercial interest due to their high nitrogen content (6.89%), which was first exploited in chelating agents. Additionally, both chitin and chitosan are biodegradable, biocompatible, non-toxic, nonallergenic, renewable biomaterials and find applications in fields such as medicine, perfume and cosmetics, food industry and agriculture. Chitin is highly hydrophobic and insoluble in water, acids, bases and common organic solvents. Some of the organic solvents in which it is soluble are hexafluoroisopropanol, hexafluoroacetone, and chloroalcohols in the presence of aqueous mineral acids. Chitosan, on the other hand, due to the presence of primary amino group in most of glucosamine units that make up the polymer backbone, dissolves in dilute organic acids, but is insoluble in water at or above pH 6-7 and in commonly used organic solvents.

2.2 Isolation of Chitin and Synthesis of Chitosan

Within its natural resources of commercial interest, chitin exists not as a stand-alone biopolymer, but rather in conglomeration with other biomaterials, mainly proteins, lipids, and inorganic salts. The isolation process of chitin starts at the sea-food industry (Scheme 2.1) [1]. One of the by-products of this industry, *viz*, shells from crab, shrimp etc. are first crushed into a pulverous powder to help make a greater surface area available for the heterogeneous processes to follow. An initial treatment of the shell with 5% sodium hydroxide dissolves various proteins, leaving behind chitin, lipids and calcium salts (mainly as $CaCO_3$). Treatment with 30% hydrochloric acid hydrolyzes lipids; dissolves calcium salts (demineralization) and other minor inorganic constituents. Chitin thus obtained can be hydrolyzed using 50% sodium hydroxide at high temperature to provide chitosan. Alternatively, if isolation of chitin is not desired, the acid-base sequence may be reversed to directly produce chitosan. In this method, crushed shells are first treated with 5% hydrochloric acid to remove calcium salts. This is then followed by protein and lipids removal by the treatment with 40% sodium hydroxide at higher temperature. During the base treatment a concomitant hydrolysis of acetamido groups in chitin takes place, resulting in the formation of chitosan.



Scheme 2.1 Isolation of chitin and synthesis route of chitosan

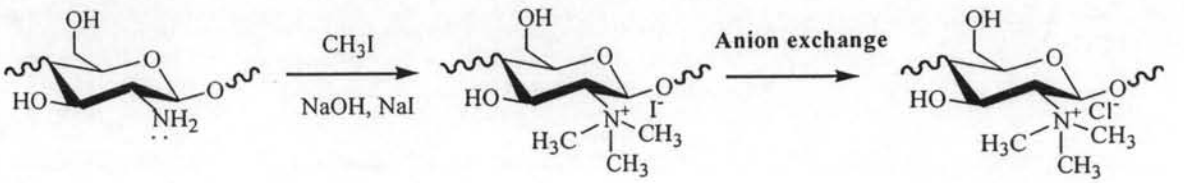
Physical properties of chitosan are governed, at large, by two factors; the degree of deacetylation and the molecular weight. Various techniques were used for determination of percentage of degree of deacetylation (%DD) such as IR, NMR and metachromatic titration and molecular weight such as light scattering spectroscopy, viscometry and gel permeation chromatography.

2.3 Water-soluble Derivatives of Chitosan

Most of the real life applications for any chemical substance, whether natural or synthetic in origin, require the chemical to be processible. In this regard, chitosan, a white flaky solid, is difficult to manipulate with because of the solubility problems in neutral water, bases, and commonly used organic solvents. The pKa value of the primary amino groups in chitosan is determined to be around 6.5. As a result, even though chitosan and its derivatives are soluble in pH values of lower than 6.0, many of its applications in neutral or basic medium, including those of physiological relevance, may not be realized, for the pH under such situations will trigger an immediate precipitation. On the other hand, acidic solutions, in which chitosan is fairly soluble, may not be desirable in many of its applications, especially those in medicine, cosmetics, and food. The documented in literature towards improving the solubility of chitosan at neutral pH is chemically derivatized chitosan [2,3].

Chitosan carries a number of hydroxyl (-OH) and amino ($-NH_2$) groups. This prompts many researchers to find ways of modifying its properties either by physical blending or chemical reactions. The nonbonding pair of electrons on the primary amine groups also makes chitosan a potent nucleophile, reacting readily with most electrophiles.

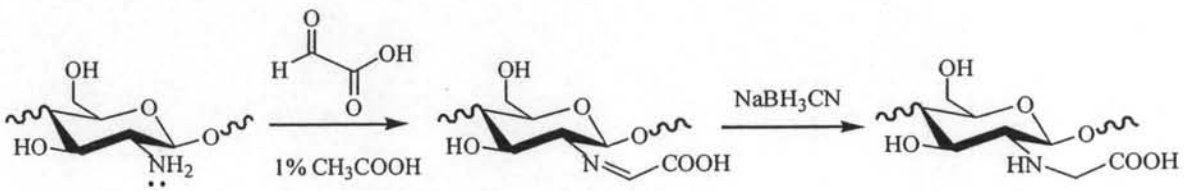
Methylation at the amino groups was also explored by many researchers. The resulting product is quaternary ammonium chitosan (QAC) (Scheme 2.2). [4] The presence of positive charge along the polymer chain helps increase its solubility in water.



Quaternary ammonium chitosan

Scheme 2.2 Methylation of chitosan by methyl iodide

N-carboxymethyl chitosan is another watersoluble derivative of chitosan that has been of particular interest for many researchers. It can be prepared by reductive *N*-alkylation using carboxyl-terminated aldehyde (Scheme 2.3). The product shows water solubility at pH 7. [5] The reaction is carried out under homogeneous condition, provided that the aldehyde used is water soluble, and this allows for a greater degree of substitution which, in principle, should be well distributed along the polymer chain.

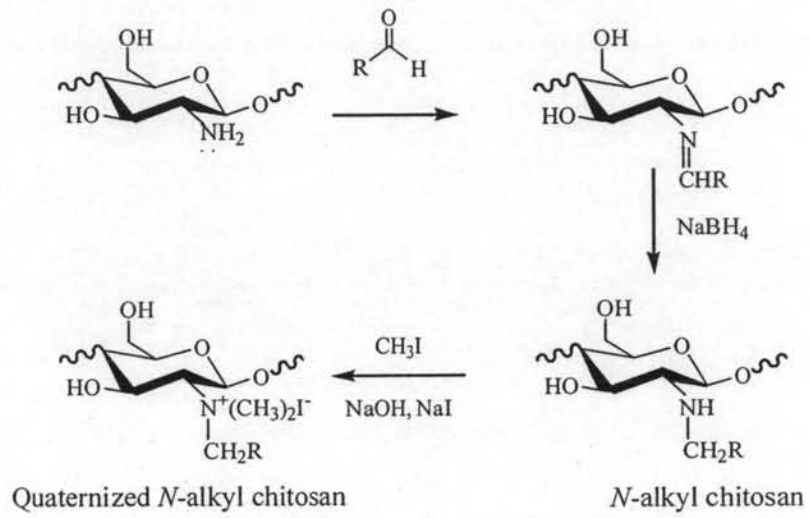


Schiff base

N-carboxymethyl chitosan

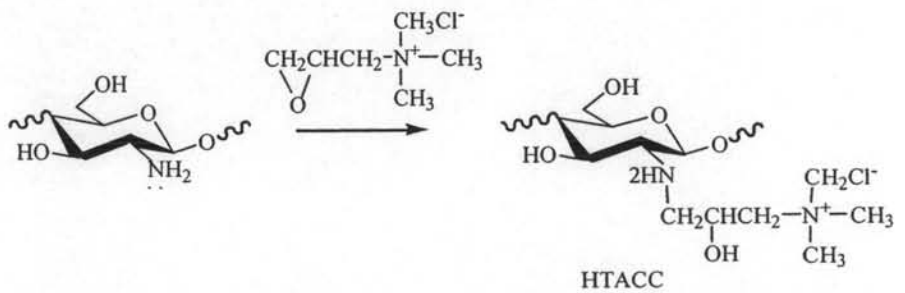
Scheme 2.3 Synthesis of *N*-carboxymethyl chitosan

In 2001, Jia, *et al.* [6] prepared chitosan derivatives with quaternary ammonium group using 96% deacetylated chitosan of various molecular weights. Amino groups on chitosan react with aldehydes to form a Schiff base intermediate. Quaternized chitosan were obtained by a reaction with methyl iodide. The presence of positive charge makes the product soluble in water (Scheme 2.4).



Scheme 2.4 Synthesis of quaternary ammonium salt of *N*-alkyl chitosan

In 1999, Seong, *et al.* [7] synthesized the quaternary ammonium salt using glycidyl trimethylammonium chloride as the quaternizing agent. The product which is called *N*-[(2-Hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) shows excellent solubility in water (Scheme 2.5).

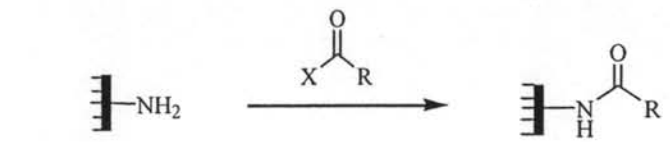


Scheme 2.5 Synthesis of HTACC

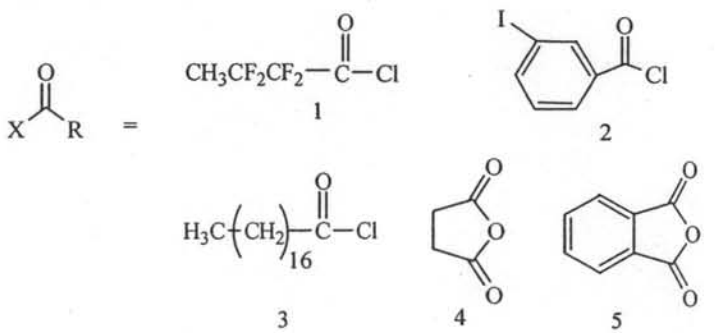
2.4 Surface Modification of Chitosan

Surface modifications of polymer are promising approaches to confer new and improved properties to the existing ones. A number of surface modification techniques were employed to alter the chemical composition and thus the surface properties of chitosan, which included surface crosslinking [8], plasma-treatment techniques [9], radiation-induced grafting polymerization [10], adding surface active components into the polymer casting solution [11], and surface chemical modification [12]. Here, a few related publications using chemical treatment have been mentioned.

In 2003, Tangpasuthadol, *et al.* [13] modified the surface of chitosan films using acid chloride and acid anhydride (Scheme 2.6). ATR-IR and XPS measurements confirmed the occurrence of chemical reaction on the modified chitosan films. Bovine serum albumin (BSA) and lysozyme, two globular proteins varying in size and charge, were selected for the protein adsorption study. Bicinchoninic acid (BCA) assay was used to determine the amount of adsorbed protein on the selected modified chitosan film. The results show that both proteins adsorbed slightly more to the films that were reacted with the chitosan film functionalized with the stearoyl group. Reactions with anhydride derivatives yielded chitosan surface which is more hydrophilic. As a result, the surface-modified chitosan films tended to reduce the adsorption of BSA, but increase the adsorption of lysozyme.

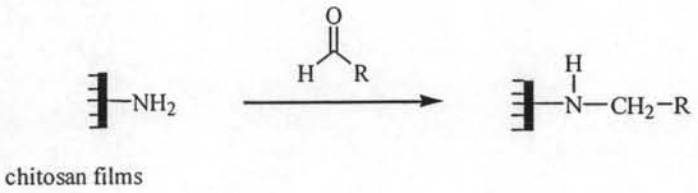


chitosan films

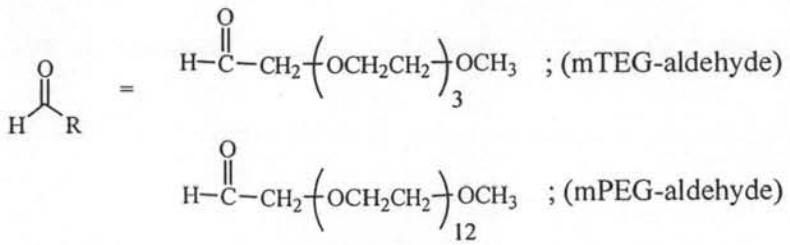


Scheme 2.6 Attachment of carboxylic acid derivatives onto the surface of chitosan

In 2004, Amornchai, *et al.* [14] reported the grafting of monomethoxy ethylene glycol oligomers (mPEG) on the surface of chitosan films (Scheme 2.7). The chemical reactions were performed by immersing the films in organic solvent containing aldehyde derivative of mPEG. ATR-IR and NMR analyses confirmed the presence of ethylene glycol moieties on the modified chitosan films. The modified chitosan films were also subjected to protein adsorption study. Results show the presence of ethylene glycol units reduced the adsorption of proteins (albumin and lysozyme) on the films.

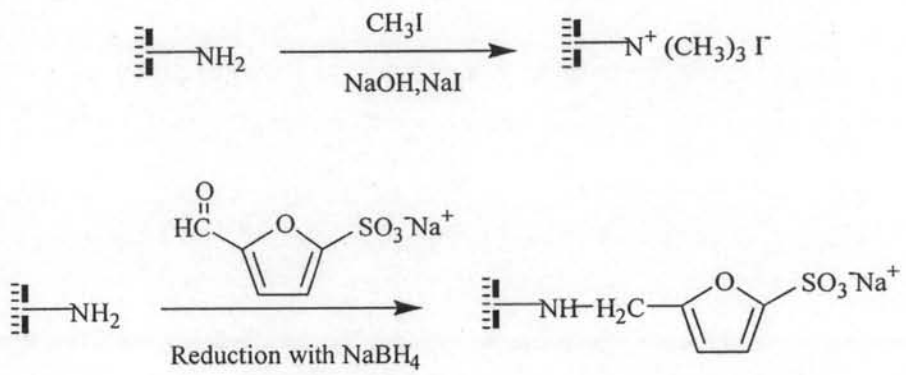


chitosan films



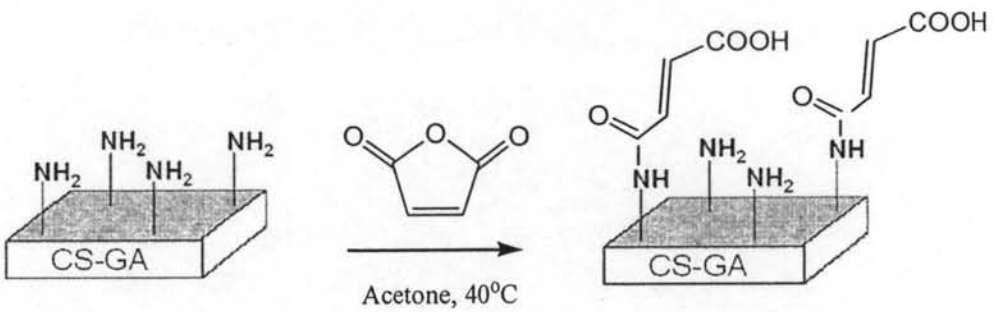
Scheme 2.7 Grafting of aldehyde derivatives onto the surface of chitosan.

In 2007, Hoven. V. P., *et al.* [15] reported the introduction of charged derivatives onto the surface of chitosan film (Scheme 2.8). Quaternary ammonium group and *N*-sulfofurfuryl group were introduced to chitosan surface via methylation using methyl iodide (MeI) and reductive alkylation using 5-formyl-2-furan sulfonic acid (FFSA), respectively. Results from ATR-IR, XPS analyses and zeta potential measurement confirmed the presence of the desired functional group on the surface-modified chitosan films. Negatively charged chitosan film exhibited selective protein adsorption against both negatively charged proteins (albumin and fibrinogen) and positively charged proteins (ribonuclease and lysozyme). In contrast, the protein adsorption of the positively charged chitosan film was anomalous due in large part to excellent swelling causing extensive protein diffusion into the film regardless of the charge characteristics of the protein.



Scheme 2.8 Introduction of positive and negative charges on the surface of chitosan

In 2007, Zhang, *et al.* [16] reported an introduction of carboxyl groups to the surface of chitosan membrane (CS), which was crosslinked with glutaraldehyde (GA), to improve its pervaparation performance. The results showed that the crosslinked CS membranes whose surface was modified by maleic anhydride (MA) exhibited much higher selectivity and flux as compared to the unmodified ones (Scheme 2.9).



Scheme 2.9 Surface modification of CS-GA membrane

2.5 Bacteria [17, 18]

Bacteria are a successful and ancient form of life, quite different from the eukaryotes which include the fungi, plants and animals. They are often maligned as the causes of human and animal disease and they are small cell, found in the environment as either individual cell or aggregated together as clumps, and their intracellular structure is far simpler than eukaryotes. Bacteria have a single circular DNA chromosome that is found within the cytoplasm of the cell as they do not have a nucleus.

2.5.1 Cell Morphology

Perhaps the most elemental structural property of bacteria is cell morphology. Typical examples include:

- coccus (spherical)
- bacillus (rod-like)
- spirillum (spiral)
- filamentous

Cell shape is generally characteristic of a given bacterial species, but can vary depending on growth conditions. Some bacteria have complex life cycles involving the production of stalks and appendages and some produce elaborate structures bearing reproductive spores. Bacteria generally form distinctive cell morphologies when examined by light microscopy and distinct colony morphologies when grown on Petri plates. These are often the first characteristics observed by a microbiologist to determine the identity of an unknown bacterial culture.

2.5.2 The Importance of Cell Size

Perhaps the most obvious structural characteristic of bacteria is (with some exceptions) their small size. For example, *Escherichia coli*, an "average" sized bacterium with average cell length of ca. 1 μm has a cell volume of approximately 1 - 2 μm^3. This corresponds to a wet mass of ca. 1 pg, assuming that the cell consists mostly of water. The dry mass of a single cell can be estimated as 20 % of the wet mass, amounting to 0.2 pg. About half of the dry mass of a bacterial cell consists of carbon, and also about half of it can be attributed to proteins. Therefore, a typical fully

grown 1-liter culture of *Escherichia coli* (at an optical density of 1.0, corresponding to ca. 10^9 cells/ml) yields ca. 1 g wet cell mass. Small size is extremely important because it allows for a large surface area-to-volume ratio which allows for rapid uptake and intracellular distribution of nutrients and excretion of wastes. At low surface area-to-volume ratios the diffusion of nutrients and waste products across the bacterial cell membrane limits the rate at which microbial metabolism can occur, making the cell less evolutionarily fit. The reason for the existence of large cells is unknown, although it is speculated that the increased cell volume is used primarily for storage of excess nutrients.

2.5.3 The Bacterial Cell Wall

As in other organisms, the bacterial cell wall provides structural integrity to the cell. In prokaryotes, the primary function of the cell wall is to protect the cell from internal turgor pressure caused by much higher concentrations of proteins and other molecules inside the cell compared to its external environment. The bacterial cell wall differs from that of all other organisms by the presence of peptidoglycan (poly-*N*-acetylglucosamine and *N*-acetylmuramic acid), which is located immediately outside of the cytoplasmic membrane. Peptidoglycan is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape. It is relatively porous and is not considered to be a permeability barrier for small substrates. While all bacterial cell walls (with a few exception e.g. intracellular parasites such as *Mycoplasma*) contain peptidoglycan, not all cell wall have the same overall structure. There are two main type of bacterial cell wall, Gram positive and Gram negative, which are differentiated by their Gram staining characteristics

The Gram Positive Cell Wall

The Gram positive cell wall is characterized by the presence of a very thick peptidoglycan layer, which is responsible for the retention of the crystal violet dyes during the Gram staining procedure. It is found exclusively in organisms belonging to the Actinobacteria (or high %G+C Gram positive organisms) and the Firmicutes (or low %G+C Gram positive organisms). Bacteria within the Deinococcus-Thermus group may also exhibit Gram positive staining behavior but contain some cell wall

structures typical of Gram negative organisms. Imbedded in the Gram positive cell wall are polyalcohols called teichoic acids, some of which are lipid-linked to form lipoteichoic acids. Because lipoteichoic acids are covalently linked to lipids within the cytoplasmic membrane. Teichoic acids give the Gram positive cell wall, an overall negative charge due to the presence of phosphodiester bonds between teichoic acid monomers. (See Figure 2.2)

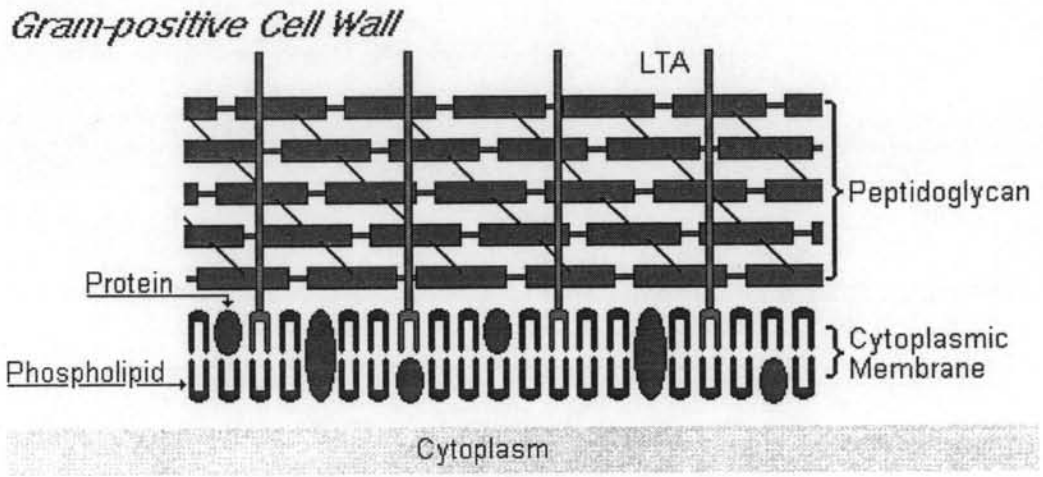


Figure 2.2 Gram-positive bacteria cell wall

The Gram Negative Cell Wall

Unlike the Gram positive cell wall, the Gram negative cell wall contains a thin peptidoglycan layer adjacent to the cytoplasmic membrane, which is responsible for the cell wall's inability to retain the crystal violet stain upon decolourisation with ethanol during Gram staining. In addition to the peptidoglycan layer, the Gram negative cell wall also contains an additional outer membrane composed by phospholipids and lipopolysaccharides which face into the external environment. The highly charged nature of lipopolysaccharides confers an overall negative charge to the Gram negative cell wall. The chemical structure of the outer membrane lipopolysaccharides is often unique to specific bacterial strains (i.e. sub-species) and is responsible for many of the antigenic properties of these strains. As a phospholipid bilayer, the lipid portion of the outer membrane is largely impermeable to all charged molecules. However, channels called porins are present in the outer membrane that

allow for passive transport of many ions, sugars and amino acids across the outer membrane. These molecules are therefore present in the periplasm, the region between the cytoplasmic and outer membranes. The periplasm contains the peptidoglycan layer and many proteins responsible for substrate binding or hydrolysis and reception of extracellular signals. The periplasm it is thought to exist as a gel-like state rather than a liquid due to the high concentration of proteins and peptidoglycan found within it. Because of its location between the cytoplasmic and outer membranes, signals received and substrates bound are available to be transported across the cytoplasmic membrane using transport and signalling proteins imbedded there. (See Figure 2.3)

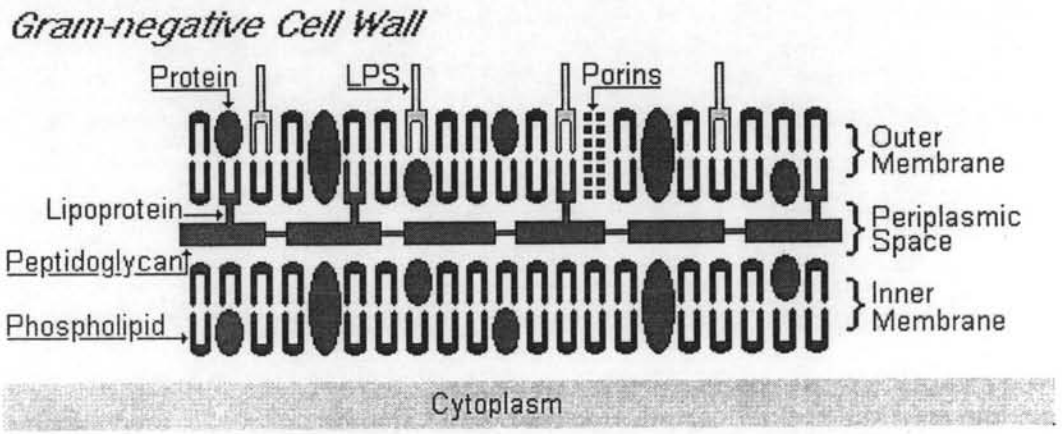


Figure 2.3 Gram-negative bacteria cell wall

2.6 Antibacterial Activity

The antimicrobial activity of chitosan has been observed against a wide variety of microorganisms including fungi, algae and bacteria. (Table 2.1) However, the antimicrobial action is greatly influenced by many factors such as the source of chitosan, degrees of deacetylation and polymerization, the microorganism, pH of the growth medium and environmental conditions (such as moisture content, natural nutrient availability to the microorganism etc.). The antimicrobial activity of native chitosan is higher at around pH 6 (compared to at pH 7.5), when most amino groups remain protonated (pKa value of chitosan= 6.5). Chitosan has been utilized in soil

amendment, seed treatment and the foliar treatment to control fungi growth. In addition, chitosan has been successfully used as food wraps. Chitosan films are tough, elastic and durable; comparable to many of the medium-strength commercial polymers. When applied as a coating over fruits and product, the film controls the influx of moisture and oxygen and also reduces transpiration loss and delays ripening process while preserving the food stock. Biodegradation of chitosan produces known chemical intermediates that are known to be harmless to humans and the environment. Chitosan and its derivatives have several advantages over other types of disinfectants because of higher antibacterial activity, broader spectrum of activity, a higher killing rate and lower toxicity. The activity is wide spectrum and includes bacteria of both cell wall types; Gram positive and Gram negative. However, given the differences in activities due to various chitosan derivatives for different microorganisms, it is rather difficult to predict the specific chemical modifications in chitosan necessary to deliver the desired antimicrobial activity. Additionally, the activity thus seems to be associated with the chain length of the polymer and suggests a cooperative effect of glucosamine units. In comparison, chitin does not show any antimicrobial activity. The antibacterial property of chitosan is particularly useful in the field of medicine where it can be used to make surgical accessories such as, gloves, bandages etc. It has also been used in the removal of waterborne pathogens in waste water and as a food preservative by applying a coat on the exterior of vegetable and fruit product.

Table 2.1 Antibacterial and antifungal activity of chitosan

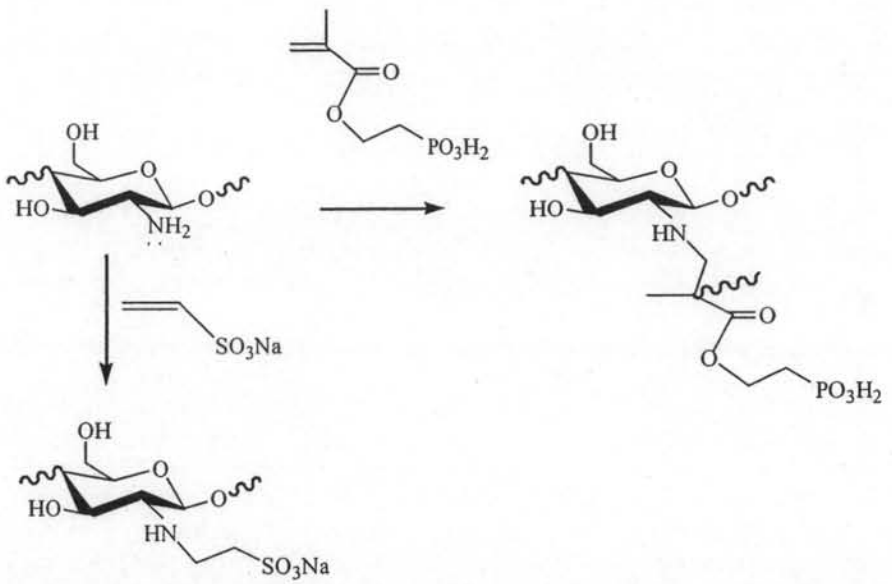
Bacteria	**MIC(ppm)**	***Fungi***	**MIC(ppm)**
Agrobacterium tumefaciens	100	*Drechstera sorokian*	100
Erwinia carotovara ssp	200	*Rhizoctonia solani*	1000
Pseudomonas fluorescens	500	*Trichophyton equinum*	2500
Staphylococcus aureus	20	*Botrytis cinerea*	10
Micrococcus luteus	20	*Fusarium oxysporum*	10

2.6.1 Antibacterial Activities of Chitosan and Chitosan Derivatives

After the discovery of the antibacterial activity of chitosan, many researchers have continued studies in this field. However, the water-insolubility of chitosan is disadvantageous for its wide application as an antibacterial agent.

In 1997, Kim, *et al.* [19] reported antimicrobial activity of quaternary ammonium derivatives of chitosan. They synthesized *N*-alkyl chitosan derivatives by introducing alkyl groups into the amine groups of chitosan via Schiff's base intermediates then quaternized these derivatives by reaction with methyl iodide (Scheme 2.4). Their antibacterial activities against *S. aureus* were explored by the viable cell counting method. The antibacterial activities of chitosan derivatives of chitosan derivatives with quaternary ammonium group increased with increase in the chain length of alkyl substituent. The best reducing agent of Schiff's base for the *N*-alkylation step is sodium cyanoborohydride as reported by Desbrieres and coworkers [20].

In 1999, Jung, *et al.* [21] prepared anionic side chain-grafted, water-soluble chitosan derivatives having zwitterionic properties. These derivatives, mono(2-methacryloyl oxyethyl) acid phosphate and vinylsulfonic acid sodium salt were grafted onto chitosan (Scheme 2.10). Antimicrobial activity against *Candida albicans* (Ca), *Trichophyton rubrum* (Tr), and *Trichophyton violaceum* (Tv) depended largely on the amount and type of grafted chains as well as changes of pH. The highest activity was shown at pH 5.75 against Ca and Tv, due to the difference in affinity between cell walls of fungi and the chitosan derivatives.



Scheme 2.10 Preparation of amphiphilic water-soluble chitosan derivatives

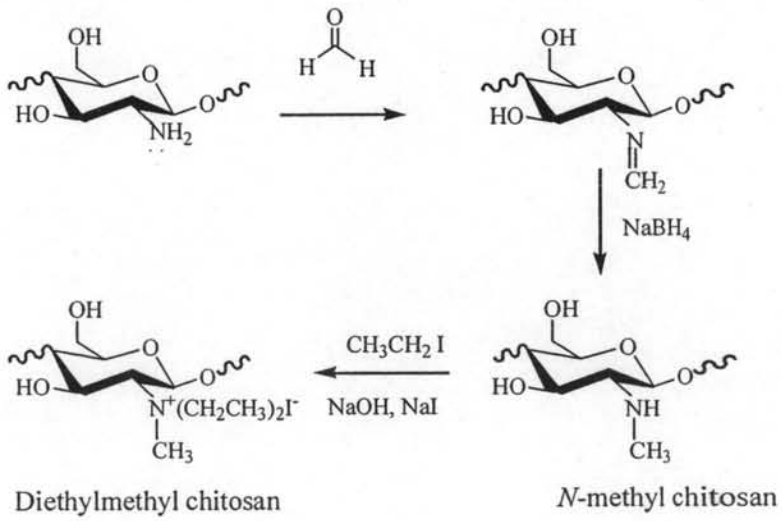
In 2002, Hong, *et al.* [22] determined the antibacterial activities of six chitosan derivatives and six chitosan oligomers with different molecular weights against four gram-negative and seven gram-positive bacteria. The high molecular weight chitosan showed a higher antibacterial activity than chitosan oligomers. QAC exhibit antibacterial activity on both gram-positive and gram-negative bacteria. The exact mechanism of the antimicrobial action of chitosan and their derivatives is still unknown, but different mechanism has been proposed [23].

- Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents.
- It acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth.
- It activates several defense processes in the host tissue, acts as a water binding agent and inhibits various enzymes.
- Binding of chitosan with DNA and inhibition of mRNA synthesis occurs via chitosan penetrating the nuclei of the micro-organisms and interfering with the synthesis of mRNA and proteins.

Further, the studies have been focused on the development of antibacterial surfaces to attain high functionality and high-value product. Poly(ethylene terephthalate) (PET) is basic material in textile and plastics industries. Accordingly, the improvement of the antibacterial properties of PET is important for wide range of industrial application.

In 2001, Huh, *et al.* [24] prepared chitosan-grafted PET (C-PET) and quaternized chitosan-grafted PET (QC-PET). Against *S. aureus*, C-PET and QC-PET showed high growth inhibition in range of 75-86% and still retained 48-58% bacterial growth inhibition after laundering.

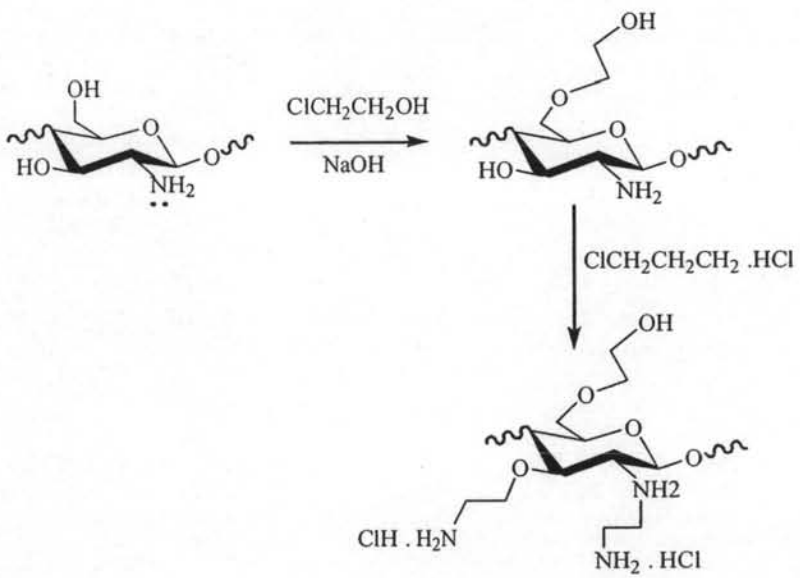
In 2004, Avadi, *et al.* [25] synthesized *N*-methyl chitosan derivatives by introducing methyl groups from formaldehyde into the amine groups of chitosan via Schiff's base intermediates then quaternized these derivatives by reacting with ethyl iodide (Scheme 2.11). Results show the antibacterial activities of quaternized chitosan against *E. c*oli is stronger than that of chitosan. The antibacterial activities of quaternized chitosan is higher than that of chitosan in acetic acid medium, the broth compounds are pH dependent and an increase in concentration of acetic acid results in a significant increase in antibacterial activities.



Scheme 2.11 Synthesis of *N*, *N*-diethyl-*N*-methyl chitosan

In 2006, Sun, *et al.* [26] prepared quaternized carboxymethyl chitosan (QCMC) by introducing *N*-quaternary ammonium group by the reaction of CMC with 2,3-epoxypropyl trimethylammonium. Antibacterial activities of QCMC were evaluated against *S. aureus* and *E. coli*. In comparison with CMC and quaternary chitosan (QC) having the same degree of substitution, it was found that QCMC has stronger antibacterial activity.

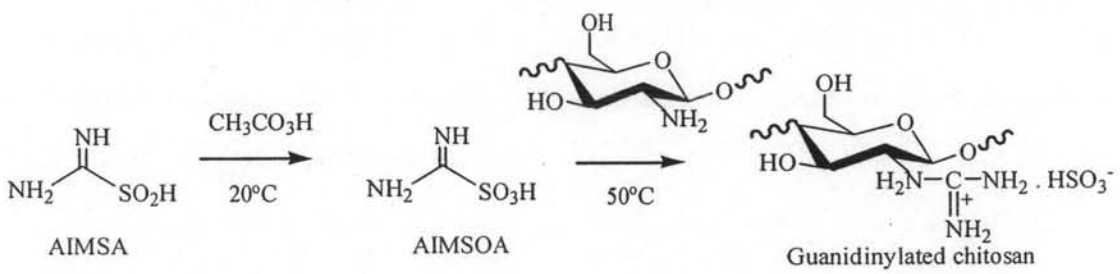
In 2007, Xie, *et al.* [27] synthesized ethylamine hydroxyethyl chitosan (EHCs) from chitosan and chloroethylamine hydrochloride under alkali condition by treating hydroxyethyl chitosan (HECs) with chloroethylamine hydrochloride in sodium hydroxide solution (Scheme 2.12). The good water solubility of EHCs was observed in a wide range of molecular weights. As determined by the optical density method, EHCs showed good antibacterial activities against *E. coli*.



Scheme 2.12 Synthesis of ethylamine hydroxyethyl chitosan (EHCS)

In 2007, Hu, *et al.* [28] synthesized guanidinylated chitosan derivatives with different molecular weights by the guanidinylation reaction of chitosan with aminoiminomethanesulfonic acid (AIMOSOA) (Scheme 2.13). Antibacterial activity of guanidinium derivatives was evaluated against *Staphylococcus aureus, Bacillus*

subtilis, Escherichia coli and *Pseudomonas aeruginosa.* As compared with chitosan, guanidinylated chitosan had much better antibacterial activity. The MIC in aqueous hydrochloric acid (pH 5.4) was 4 times lower than those of chitosan. Interestingly, guanidinylated chitosan inhibited the growth of *S. aureus* and *B. subtilis* at pH 6.6



Scheme 2.13 Synthesis of guanidinylated chitosan

2.6.2 Mode of Action

Chitosan exhibits much more pronounced activity compared to chitin. This is usually described as due to the greater availability of primary amino groups in the former. Under mildly acidic conditions (≈ pH 6), the amino groups acquire a positive charge which is usually associated with the demonstrated activity. The exact mode of interaction between chitosan, its derivatives and the microorganism is still unknown, but different mechanisms have been proposed in the explanation of antimicrobial activity. It is believed that the polycationic nature of chitosan initiates binding with the cell membrane by means of electrostatic attraction with negatively charged microbial cell membrane.

In 1999, Tsai, *et al.* [29], using TEM analyses, also revealed that the cytoplasmic membrane of bacteria was detached from the inner part of the cell wall after chitosan treatment. Once bound to the cell surface, chitosan is thought to affect membrane permeability which results into the leakage of proteinaceous material and other intracellular constituents of the microbial cell causing death due to the loss of essential fluids.

In 1991, Cuero, *et al.* [30] proposed that chitosan can bind with DNA and inhibit mRNA and protein synthesis upon penetration into the nuclei of fungi.

Modes of activity proposing interactions with the intracellular components (and nuclear components) require that chitosan should be hydrolyzed in order to produce fragments of low MW for effective penetration and transport across the cell membrane of the microorganism. However, chitosan interaction with the nuclear components during the course of its activity is still debated. Antibacterial action of chitosan was proposed based on this finding due to chitosan is mainly governed by the suppression of the metabolic activity of the bacteria by blocking nutrient permeation through the cell wall rather than the inhibition of nucleic constituents. Often the antimicrobial effect due to chitosan is realized due to a combination of various triggers. However, it is important to note that the activity of chitosan is highly dependent upon the pH of the local environment.

2.6.3 Factors Affecting Antimicrobial Activity

Several intrinsic (associated with the constitution of the macromolecule) and external parameters govern and control the antimicrobial activity due to chitosan and its derivatives. The unit monomer of chitosan, 2-amino-2-deoxy-D-glucopyranose as a hydrochloride salt does not exhibit any inhibition against several bacteria, including *E. coli* and *S. aureus*. [31] This suggests that antibacterial action of chitosan could be a cooperative effect of several units acting against the bacteria. However, it seems that high MW chitosan (>500,000) shows reduced activity compared to lower MW material which is usually attributed to the difficulties in diffusion for highly viscous solutions of high MW chitosan during the tests. [32] Further, the activity seems to depend on the microorganism assessed. For example, medium and high MW chitosan exhibited higher activities against *Bacillus circulans* compared to chito-oligosaccharides. [33] However the activity reversed when tests were performed on *E. coli*. When comparing the trend against *E. coli*, the activity seems to increase with the MW, until it reaches a certain value (MW= 30,000), after which the activity starts declining. [34]

Unlike the lack of any observable trend between the MW of chitosan and its activity against the microorganisms, the degree of deacetylation (DD) in chitosan shows a direct relationship with the activity exhibited. [35] This is due to a greater availability of potentially cationic sites along the polymer backbone with the increase

in DD. In a related situation, chitosan antimicrobial activity was found greater at lower pH values than those at higher. Effect of temperature on the antibacterial activity indicated that chitosan shows greater inhibition at 37°C.

In this work, antibacterial activity of modified chitosan surfaces was studied using *Staphylococcus aureus* (gram positive bacteria) and *Escherichia coli* (gram negative bacteria). Antibacterial activity of chitosan surface before and after modification was compared. Interaction forces is summarized in Figure 2.4

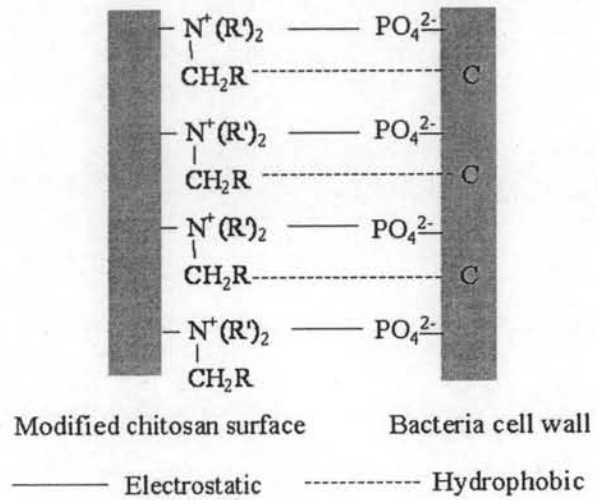


Figure 2.4 Two interaction forces involved in the antibacterial activity

2.7 Characterization Techniques

2.7.1 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The IR beam from the spectrometer is focused onto the beveled edge of an internal reflection element (IRE) where the sample is placed in close contact with the beam is then reflected through the IRE crystal, and directed to a detector (Figure 2.5).

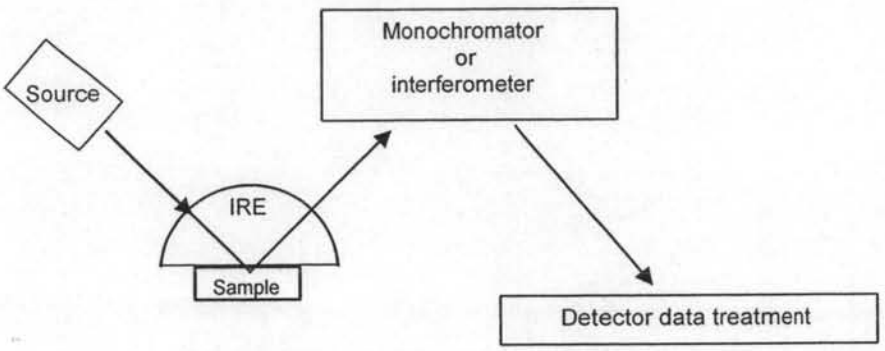


Figure 2.5 Diagram of ATR-FTIR

Typical materials used for ATR prism are Ge, Si and ZnSe. The infrared radiation can penetrate a short distance into the sample, thus interacts with any functionalities existing within that depth in the same manner as in conventional infrared spectroscopy. The depth of penetration (d_p, defined as the distance from the IRE-sample interface where the intensity of the evanescent wave decays to 1/e of its original value) can be calculated using the formula in the following equation:

$$d_p = \frac{\lambda}{2\pi n_p(\sin^2\theta - n_{sp}^2)^{1/2}} \qquad \text{.....(2.1)}$$

where λ = wavelength of the radiation in the IRE, θ = angle of incidence, n_{sp} = ratio of the refractive indices of the sample vs. IRE, and n_p = refractive index of the IRE. Sampling depth of characterization is 1-2 μm.

2.7.2 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 2.6)

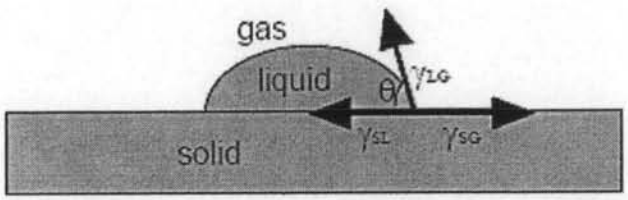


Figure 2.6 Schematic representation of the Young's equation

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

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 2.7.

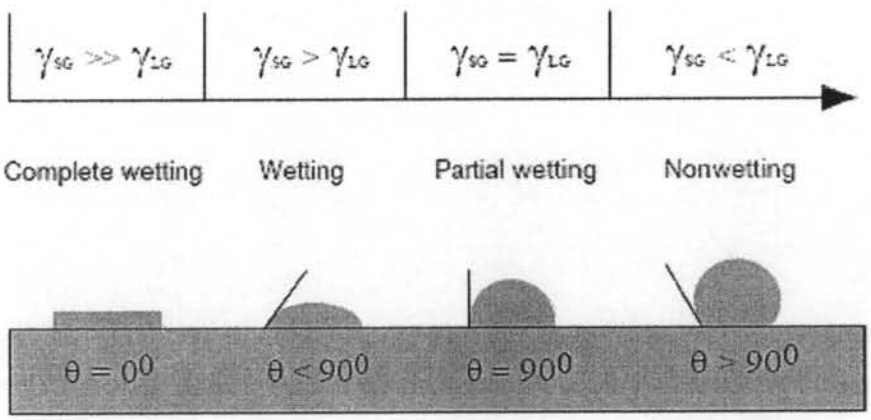


Figure 2.7 Schematic representation of wettability

2.7.3 Zeta potential (ζ -potential)

Zeta potential is defined as the potential at the surface of shear, because any relative movement of the surface with respect to the solution will cause some of the counter ions to be shared off resulting in only partial compensation of the surface charge. One of the most effective methods is to apply an electric field to the suspension and to measure how fast the particles move as a result. That process is called *electrophoresis.* It is that potential which is measured, when one measures the velocity of the particles in a D.C electric field. Laser light with a 632.8 nm wavelength from a 10 mW He-Ne laser was used as the incident beam. The measurements were performed at the scattering angle of 5° with an electric field of 32.5-34.4 V/cm. Because of the Doppler Effect, the frequency of the scattered laser light is different from the frequency of the incident laser beam. This frequency shift is related to the particle velocity. The relationship between the frequency shift and electrophoretic mobility is expressed by the equation.

$$u = (v_d\lambda)[2En\sin(\theta/2)] \qquad \text{.....(2.3)}$$

where v_d is the Doppler frequency, u is the electrophoretic mobility, E is the electrical field, n is the refractive index, λ is the wavelength of the incident laser beam, and θ is the scattering angle. From the obtained electrophoretic mobility, the ζ potential was calculated using the Smoluchowski equation as

$$\zeta = 4\pi\eta u/\varepsilon \qquad \text{.....(2.4)}$$

where u is the electrophoretic mobility, and η and ε are the solution viscosity and electrical permittivity of the solution, respectively. All measurements were performed at 25±1 °C. The experiments were carried out five times for each experimental condition, and the mean value (± standard deviation) of each experimental point is indicated.

The net charge at the surface of material in contact with a polar medium is governed by three processes via ionization/dissociation of surface chemical groups, adsorption of ionic species and dissolution of ions from the material into solution. The electric field pulls the surface charge in one direction but it will also be pulling the

counter ions in the opposite direction. Some of the counter ions will move with the surface charges so the measured charge will be a net charge taking that effect into account. The electrostatic potential near the particle surface is shown in Figure 2.8. It changes very quickly and linearly from its value at the surface through the first layer of counter ions and then changes more or less exponentially through the diffuse layer. The junction between the bound charges and the diffuse layer is again marked by the broken line. That interface separates the bound charge from the diffuse charge around the surface when an external field is applied and. It is called the *surface of shear* or the *slip surface*.

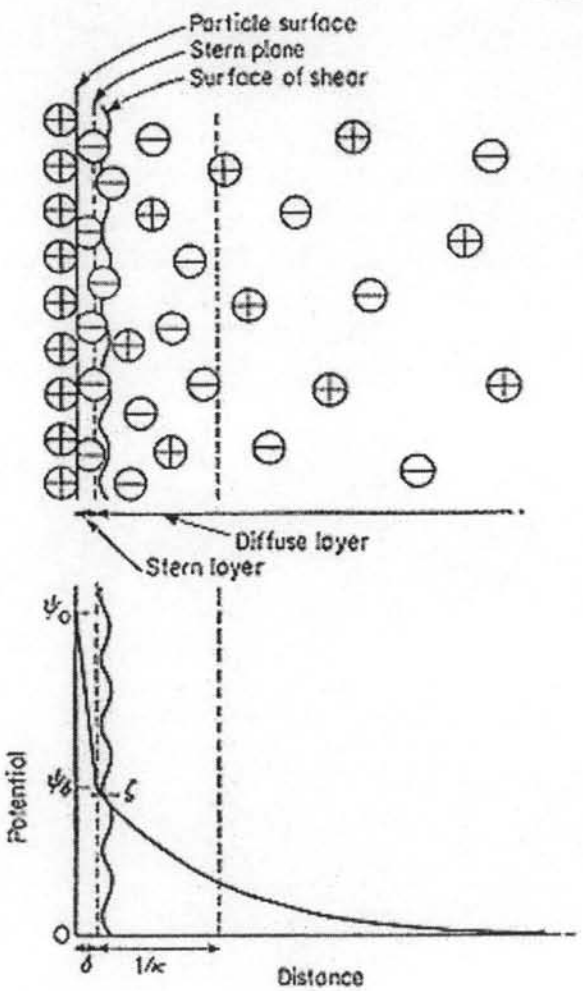


Figure 2.8 Stern model of the electrochemical double layer [36].