PROTEIN PURIFICATION BY USING IMMOBILIZED METAL IONS AFFINITY ZEOLITE ADSORBENT

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ABSTRACT

The applications for purified proteins have grown considerably over recent years due to their biological and nutritional properties. The aim of this research are to develop zeolite as inorganic stationary substrate in immobilized metal ion affinity (IMA) for protein purification and to study the optimum condition to purify protein in order to maximize yield and purity of protein by applying the principle of adsorption process. The effect of contact time, different types of adsorbent and various pH values were studied in a batch experiment by using Bovine Serum Albumin (BSA) as protein sample. The sorption equilibrium was reached within 10 minutes. Fe^{3+} ion showed the best performance for BSA and has the suitable interaction with both HY and HBeta zeolite framework and the protein as compared to Pb^{2+}, Ni^{2+} ions. The adsorption capacity for BSA was found to be the highest at pH 4.5 that is close to isoelectric point (pI) with Fe-HBeta used as adsorbent. The adsorbed amount increases as the pH increases and reduces as the pH exceeds the pI value. This result proves that adsorption capacity of Bovine Serum Albumin depends on the types of adsorbent used, which means the adsorption stoichiometries depends on physical characteristic of adsorbent that could increase and reduce the adsorption capacity of the protein as well as the pH of protein. The adsorption isotherm data of BSA is well fitted to the Langmuir isotherm model.

ABSTRACT

Aplikasi penulenan protein telah berkembang dengan pesat sejak beberapa tahun kebelakangan.ini berdasarkan ciri-ciri biologi dan khasiat pemakanan yang terkandung di dalamnya. Tujuan penyelidikan ini adalah untuk membentuk zeolite sebagai bahan pegun bukan organic di dalam ion logam tarikan (IMA) untuk proses penulenan protein dan juga mengenal pasti keadaan yang terbaik bagi penulenan protein dengan tujuan untuk menghasilkan protein yang mempunyai tahap penghasilan dan ketulenan yang tinggi dengan cara mengaplikasikan prinsip proses penjerapan. Kesan masa tindak balas, jenis bahan penjerap yang berbeza dan nilai pH yang berbeza telah dikaji di dalam eksperimen ini secara 'batch' di mana Albumin Serum Bovin (BSA) digunakan sebagai sampel protein. Jerapan keseimbangan telah dicapai dalam masa 10 minit. Ion Fe^{3+} memberi nilai jerapan yang paling tinggi di dalam BSA dan mempunyai tindak balas yang terbaik dengan zeolite dan protein berbanding dengan Pb^{2+}, Ni^{2+} ion. Keupayaan jerapan yang tertinggi di dalam BSA adalah pada pH 4.5 iaitu menghampiri titik isoelektrik (pI) ketika Fe-HBeta digunakan sebagai bahan penjerap. Jumlah jerapan meningkat apabila nilai pH meningkat dan mengurang apabila nilai pH melebihi nilai pI. Keputusan daripada hasil kajian menunjukkan bahawa keupayaan jerapan Bovine Serum Albumin bergantung kepada jenis-jenis bahan penjerap yang digunakan, iaitu penjerapan bergantung kepada ciri-ciri fizikal bahan penjerap dan pH protein yang mana ia boleh meningkatkan dan mengurangkan keupayaan jerapan sesuatu protein. Data keputusan penjerapan BSA yang diperolehi mematuhi konsep ideal Langmuir.

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LIST OF ABBREVIATIONS

BSA	-	Bovine Serum Albumin
BOD		Biologal Oxygan Demand
DNA	-	Deoxyribonucleic acid
HEMA		Hydroxyethyl methacrylate
MAH		N-methacryloyl-$_L$-histidine methyl ester
IMAC		Immobilized Metal Ions Affinity Chromatography
RNA	-	Ribonycleic acid
Da	-	Dalton
kDa	-	kiloDalton
SDS	-	Sodium Dodecyl Sulfate-
SDS-PAGE		Sodium dodecyl sulfate polyacrylamide gel electrophoresis
pI	-	Isoelectric point
RPM	-	Revolutions per minute

LIST OF SYMBOL

x	-	Quantity adsorbed
m	-	Mass
P	-	Pressure of adsorbent
k, n	-	Empirical constant
θ	-	Theta
α	-	Alpha constant
q	-	Solute concentration
q_m	-	Langmuir isotherm parameter
C	-	Equilibrium concentration
K_d	-	Langmuir adsorption parameter
R^2	-	Regression val

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Effective separation and purification of proteins has been an important issue in the biomedical and pharmaceutical industries. A novel protein adsorption has been developed in biotechnology to achieve highly efficient and economical separation processes. In many cases, proteins which have similar physical and chemical properties need to be separated, and thus highly selective adsorbents are desired (Lei *et al*., 2004).

Proteins are relatively large biomolecules and have a tendency to accumulate at the interface between solutions and solid surface. The adsorption of proteins at interfaces is a widespread phenomenon in both natural and man-made systems. Protein adsorption plays an important role in many disciplines, including biomedical engineering, biotechnology and environmental science. Whether protein adsorption is desirable or not, knowledge of the underlying principles is required in order to control the interaction between proteins and interfaces (Kopac *et al*., 2008).

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. There are various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious

aspect of protein purification. Separation steps exploit differences in protein size, physico-chemical properties and binding affinity.

There are principally three methods available for separating proteins: chemical fractionation, methods using membranes and methods using chromatography. Membrane methods consist, mainly, in separating a fraction of proteins in a specified molecular weight range from other proteins whose molecular weight is greater than the pores of the membrane, although in membrane techniques such as ultrafiltration the sieving and other important mechanisms may play an important role, making the membrane processes complex. The problem of these methods is that incompletely purified proteins are obtained, i.e. simply a mixture of proteins with similar molecular weight. The principal method used to separate pure proteins is chromatography. In these percolation methods, the interaction between the proteins in the mixture and a solid sorbent that fills the column is used to achieve the separation of the different proteins. The interactions may be ion exchange, adsorption, size exclusion, etc. and many chromatographic columns have been manufactured based on these interaction phenomena (E. Are´valo *et al*., 2000).

Recently, as an important and novel application of such materials, the purification of nucleic acids or proteins micron-sized, zeolite sorbents has been reported on the basis of electrostatic or hydrophobic interaction between the aimed biopolymers and zeolite substrates. Therefore, zeolites were expected to be novel chromatographic carriers for biomolecule separation. Nowadays, hierarchical structured zeolitic materials are attracting considerable research enthusiasm in the field of chemistry, biotechnology and advanced materials due to their peculiar hierarchical porous structure, relatively high mechanical strength and external surface area. We have found that, after immobilizing transitional metal ions in zeolite crystals through ion exchange process, the transitional-metal-ion immobilized zeolites would selectively adsorb the histidine-rich domains in the targeted protein molecules, which makes this hierarchical material a promising packing sorbent in IMAC for protein separation (Tang *et al*., 2004).

In this study, the protein adsorption on immobilized metal ion affinity (IMA) zeolite at various pHs and times was carried out using Bovine Serum Albumin as model protein. The interaction between protein and (IMA) zeolite surface was studied using UV-Vis.

1.2 Problem Statement

Nowadays protein purification development has been an important pre-requisite for many of the advancements made in biotechnology. This purification becomes increasingly important because of the increased value of the purified product. Basically the performance of purification method was accessed by their level of purity, separation cost including the capital and maintenance cost, and also the advantage and disadvantage of that kind of separation method. There are many kind of separation method has been use for protein purification. Currently IMAC or Immobilized metal ion affinity chromatography has been widely use in industry.

The traditional stationary phase for IMAC are based on soft gel matrices such as agarose or cross-linked dextral (Hemdan *et al.*, 1985) which are deemed as biologically compatible and highly active sorbents. However, the serious drawbacks of weak mechanical strength for this kind of material limit its further applications to some degree, especially under high pressure (Gaberc *et al.*, 2001). To overcome these defects and further apply IMAC under high pressure, some inorganic adsorbents such as silica-based particles have been attempted as stationary phase for high-through output separation because of their excellent mechanical resistance and modifiability (Groman *et al.*, 1987). So, extra research needs to be done in order to determine the relevant of immobilized ion metal affinity zeolite as an adsorbent in purification of protein.

1.3 Objective of Study

The proposed research was studied to achieve the following objectives:

1. To develop zeolite as inorganic stationary substrate in immobilized metal ion affinity for protein purification by applying the principle of adsorption process
2. To study the optimum condition to purify protein in order to maximize yield and purity of protein.

1.4 Scope of Study

In order to achieve the objectives, the following scopes have been identified:

1. Effect of different contact time
2. Effect of different type of adsorbent
3. Effect of different pH value

In this research, bovine serum albumin (BSA) was used in order to study the protein adsorption selectivity and capacity of the immobilized metal ion affinity zeolites. For the first parameter, the effect of time on adsorption of protein purification was considered at 6 different times in the range of 5 to 30 min. For the effect of pH on the process of protein purification at three different pHs namely 4, 4.5, and 5 were considered. For the last parameter, similar experiments were carried out by using different types of adsorbent which are Pb-HY, Fe-HY, Ni-HY, Pb-HBeta, Fe-HBeta, and Ni-HBeta in order to monitor the effect of different type of adsorbent used.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein

The word 'protein' is defined as any of a group of complex high-molecular-weight organic compounds, consisting essentially of combinations of amino acids in peptide linkages, that contain carbon, hydrogen, oxygen, nitrogen, and usually, sulfur. Proteins are one of the classes of bio-macromolecules (like polysaccharides, lipids, and nucleic acids), that make up the primary constituents of living things (Behar, 2006). A protein molecule that consists of combinations of amino acids in peptide linkages, but a single polypeptide chain is said to be monomeric. Proteins made up of more than one polypeptide chain, as many of the large ones are called oligomeric.

Based upon chemical composition, proteins are divided into two major classes: simple proteins, which are composed of only amino acids, and conjugated proteins, which are composed of amino acids and additional organic and inorganic groupings, certain of which are called prosthetic groups. Conjugated proteins include glycoproteins, which contain carbohydrates; lipoproteins, which contain lipids; and nucleoproteins, which contain nucleic acids.

Classified by biological function, proteins include the enzymes, which are responsible for catalyzing the thousands of chemical reactions of the living cell; keratin, elastin, and collagen, which are important types of structural, or support, proteins; hemoglobin and other gas transport proteins; ovalbumin, casein, and other

nutrient molecules; antibodies, which are molecules of the immune system protein hormones, which regulate metabolism; and proteins that perform mechanical work, such as actin and myosin, the contractile muscle proteins.

2.1.1 Structure of Protein

The subunits of a protein are amino acids or to be precise amino acid residues. An amino acid consists of a central carbon atom (the alpha Carbon C_{alpha}) and an amino group (NH_2), a hydrogen atom (H), a carboxy group (COOH) and a side chain (R) which is bound to the C_{alpha}. Different side chains (R_i) make up different amino acids with different physico-chemical properties. There are 20 different amino acids that make up essentially all proteins on earth. A peptide bond is formed via covalent binding of the Carbon atom of the Carboxy group of one amino acid to the nitrogen atom of the amino group of another amino acid by dehydration (Stevenson, 2002).

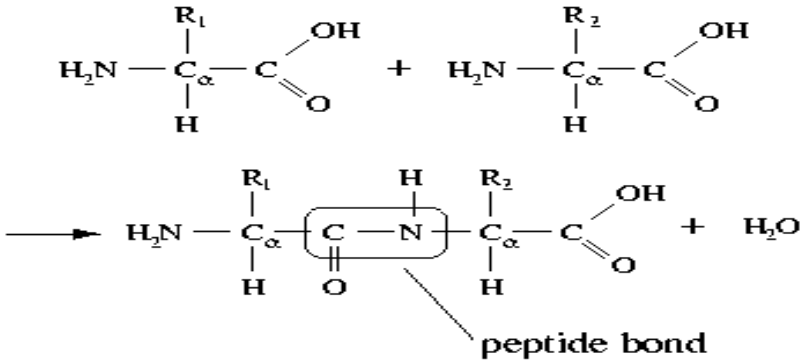


Figure 2.1*:* Peptide bond linking two amino acids (Helmenstine, 2010)

Structural features of proteins are usually described at four levels of complexity which are primary structure, secondary structure, tertiary structure and quaternary structure. The primary structure of peptides and proteins refers to the linear number and order of the amino acids present.

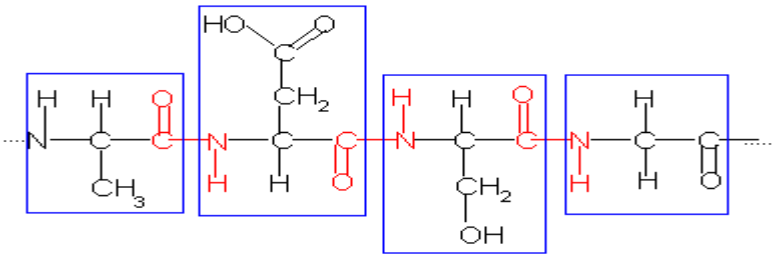


Figure 2.2: The primary structure of a protein (Helmenstine, 2010)

The blue boxes in the figure 2.2 surround individual amino acids. While the red text shows the position of the peptide bonds (peptide or amide linkages) joining the amino acids together. The convention for the designation of the order of amino acids is that the N-terminal end for example the end bearing the residue with the free α-amino group is to the left and the number 1 amino acid while the C-terminal end for example the end with the residue containing a free α-carboxyl group is to the right.

The ordered arrays of amino acids in a protein confer regular conformational forms upon that protein. These conformations constitute the secondary structures of a protein. In general proteins fold into two broad classes of structure termed, globular proteins or fibrous proteins. Globular proteins are compactly folded and coiled, whereas, fibrous proteins are more filamentous or elongated. It is the partial double-bond character of the peptide bond that defines the conformations a polypeptide chain may assume. Within a single protein different regions of the polypeptide chain may assume different conformations determined by the primary sequence of the amino acids.

The tertiary structure of proteins is the result of further bonding between side chains within the protein and with any water that may be present around the protein. Polar amino acids move to the outside of the shape and non-polar amino acids move to the inside when placed in a polar solution. Bonds that are considered part of the tertiary structure include bonds formed between non-polar side chains, disulfide bonds formed between sulfur atoms in cysteine side chains, ionic bonds formed

between acidic and basic side chains, and hydrogen bonds formed between carbonyl groups and hydroxyl or amino groups (Stevenson, 2002).

Quaternary structure is non-covalent interactions that bind multiple polypeptides into a single, larger protein. Hemoglobin has quaternary structure due to association of two alpha globin and two beta globin polyproteins.

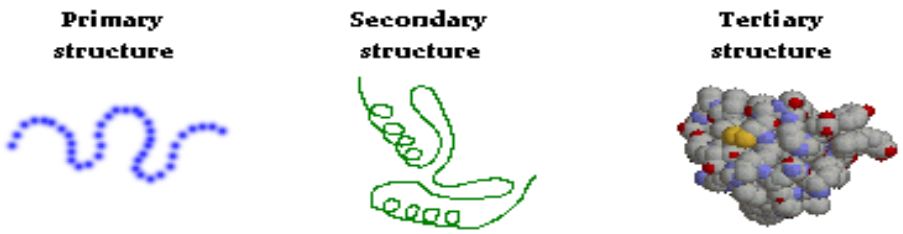


Figure 2.3: Structural features of proteins (Helmenstine, 2010)

To be biologically active, proteins must adopt specific folded three-dimensional, tertiary structures. Yet the genetic information for the protein specifies only the primary structure. Many purified proteins can spontaneously refold in vitro after being completely unfolded, so the three-dimensional structure must be determined by the primary structure. Different conformations of a protein differ only in the angle of rotation about the bonds of the backbone and amino acid side-chains. It may, therefore, appear surprising that a protein folds into a single unique conformation from all the possible rotational conformations available around single bonds in the primary structure of a protein. The polypeptide whilst folding may become trapped in the local energy well and cannot fold to the global energy minimum (Davies and Shaffer, 1979).

2.1.2 Types of Protein

Proteins are responsible for many different functions in the living cell. It is possible to classify proteins on the basis of their functions. Very often, proteins can carry few functions and such proteins can be placed into different groups, but despite this, it is possible to assign main group for each protein.

Table 2.1: Classification by protein functions

Types	**Examples**	**Function**
Enzymes	• DNA- and RNA-polymerases • dehydrogenases	• Catalyze chemical and biochemical reactions within living cell and outside • responsible for all metabolic reactions in the living cells
Hormones	• insulin • grows factor • lipotropin • prolactin	• responsible for the regulation of many processes in organisms • Increase some group of proteins by adding of all protein venoms.
Transport proteins	• cytochrome C • haemoglobin and myoglobin • albumin	• electron transport • oxygen transport • fatty acid transport in the blood stream
Immunoglobulin or Antibodies	• fibrin • thrombin	• neutralize large foreign molecules • can act as enzymes • protective proteins
Structural proteins	• Bacterial proteoglycans • virus coating proteins	• maintain structures of other biological components
Motor proteins	• Actin • myosin	• convert chemical energy into mechanical energy
Receptors	• rhodopsin	• signal detection • translation into other type of signal
Storage proteins	• Egg ovalbumin • milk casein	• contain energy • as a source of energy • building material by other organisms.

2.1.3 Purification of Protein

The applications for purified proteins have grown considerably over recent years due to their biological and nutritional properties especially in the fields of biochemistry and medicine. Many industrial waste products contain an appreciable amount of proteins suitable for recovery. Among these waste products, for instance, is whey from the dairy industry or blood from slaughterhouses. These products contain mixtures of several proteins and methods need to be developed to separate and purify each protein from the mixture. (Are´valo *et al.*, 2000).

Protein purification is the first step of almost all in vitro protein studies. High quality of purified protein could be critical with respect to their activity and crystallization ability. Almost all proteins lose their activity during any manipulations. Even stored at 4°C protein can lose activity and crystallization ability (Sedelnikova., 2006). That is why it is important to purify protein as quick as possible. Although yield and purity are the hardest attributes to predict, they cannot be neglected in the scale-up program. Any change that affects yield or purity will, in the majority of cases involving biotechnology, be far more important than the capital and productivity saved by optimizing the physical variables of the separation. The reason for this is the high unit cost and emphasis on quality characteristic of therapeutic protein.

To purify a protein one must begin with the starting material and fractionate it using any one of a large number of physical or biochemical approaches. The initial material can be separated into fractions using centrifugation, precipitation with salt, binding to ionic columns such as Ion-exchange chromatography, velocity sedimentation, equilibrium density sedimentation, binding to affinity columns, separation by sizing columns, fractionation by isoelectric focusing, and its ability to associate with known ligands or binding partners, or any other method available to the experimentalist (Harrison, 1994).

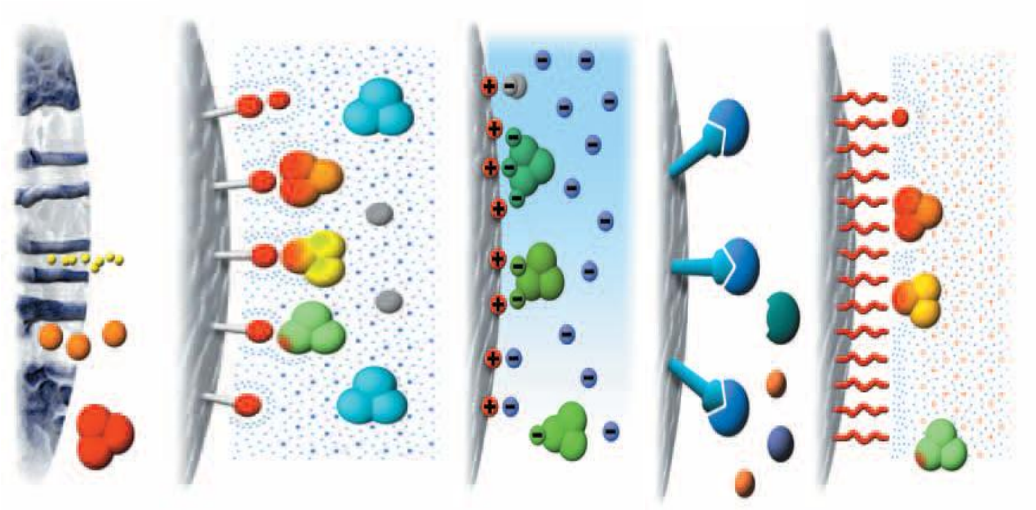
Velocity sedimentation is a method of separating components of a cellular homogenate or mixture. The homogenate is layered as a narrow band on top of a dilute salt solution that fills a centrifuge tube. When centrifuged, sub cellular components sediment at different speeds according to their size and frictional coefficient which depends on their shape and their density which creates a driving force. To stabilize the sedimenting bands against convective mixing caused by small differences in temperature or solute concentration, the tube contains a continuous shallow gradient of sucrose that increases in concentration toward the bottom of the tube typically from 5%-10% or 10-30% sucrose. Following centrifugation, the different components can be collected individually, most simply by puncturing the plastic centrifuge tube and collecting drops from the bottom. When done under appropriate conditions and properly calibrated it can be used to measure sedimentation coefficient. Note the contrast with equilibrium sedimentation.

Equilibrium density sedimentation is a method of separating cellular components on the basis of their buoyant density, which, unlike sedimentation velocity, is independent of their size and shape. The sample is usually sediment through a density gradient that contains a very high concentration of sucrose or cesium chloride. Each cellular component begins to move down the gradient, but eventually reaches a position where the density of the solution is equal to its own density. A series of distinct bands is thereby produced in the centrifuge tube, with the bands closest to the bottom of the tube containing the components of highest buoyant density. This method is so sensitive that is capable of separating macromolecules that have incorporated heavy isotopes, such as 13C or 15N, from the same macromolecules that have not. If layered at the bottom of a density gradient, light components can, of course sediment up which is they float.

The other technique, is by treating protein with SDS, a strong detergent, they are denatured. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility which is a function of length of polypeptide chain or molecular weight as well as higher order

protein folding, posttranslational modifications and other factors. The SDS gel electrophoresis of samples having identical charge to mass ratios results in fractionation by size and is probably the world's most widely used biochemical method. The bigger the macromolecule, the more SDS is bound, so that all macromolecules treated with SDS have the same ratio of charge to mass.

Affinity chromatography is a powerful protein separation method that is based on the specific interaction between immobilized ligands and target proteins (Lee, 2004). Affinity chromatography is a very useful technique for "polishing" or completing the protein purification process. This method generally gives the purest results and highest specific activity compared to other techniques.



Gel filtration hydrophobic interaction Ion exchange Affinity Reversed phase

Figure 2.4: Separation principles in chromatographic purification (Harrison, 2004)

Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure. Purification that would otherwise be time-consuming, difficult or even impossible using other techniques can often be easily achieved with affinity chromatography. The technique can be used to separate active biomolecules from denatured or functionally different forms, to

isolate pure substances present at low concentration in large volumes of crude sample and also to remove specific contaminants (Harrison, 2004).

2.2 Adsorption Process

The use of solids for removing substances from either gaseous or liquid solutions has been widely used since biblical times. This process, known as adsorption, involves nothing more than the preferential partitioning of substances from the gaseous or liquid phase onto the surface of a solid substrate. Adsorption at various interfaces has concerned scientists since the beginning of this century. This phenomenon underlies a number of extremely important processes of utilitarian significance.

Adsorption is member of the family of the removal or separation techniques like stripping and extraction. Adsorption is no filtration technique. There are 2 methods of adsorption: physisorption and chemisorption. Both methods take place when the molecules in the liquid or gas phase become attached to the surface of the solid as a result of the attractive forces at the solid surface (adsorbent), overcoming the kinetic energy of the liquid contaminant (adsorbate) molecules.

Physisorption occurs when, as a result of energy differences and/or electrical attractive forces (weak van der Waals forces), the adsorbate molecules become physically fastened to the adsorbent molecules. This type of adsorption is multilayered and reversible (regeneration by desorption). This is normally what happens with activated carbon. Chemisorption is when a chemical compound is produced by the reaction between the adsorbed molecule and the adsorbent, one molecule thick and irreversible.

In the bulk, molecules experience forces of attraction from all sides. So, the forces are mutually balanced (Jozsef, 2002). At the surface, molecules are attracted only from below and sides so imbalanced forces occur and as a result the net

attraction downwards and unbalanced forces and unbalanced valencies are present at the surface as shown in figure 2.5. These forces lead to adsorption at the surface.

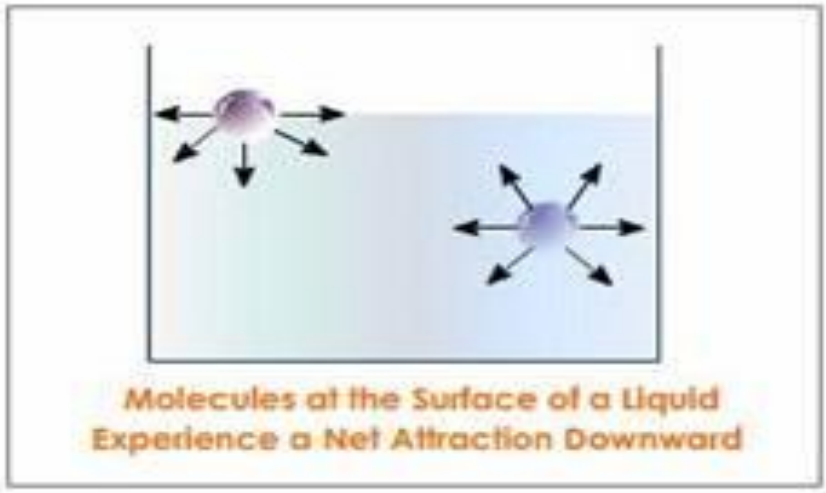


Figure 2.5: Molecules at the surface of a liquid experience a net attraction downward.

There are several factors affecting adsorption including nature of the adsorbate, nature and surface area of the adsorbent, specificity of the reaction, characteristics of the reaction, specific area of the adsorbent, activation of adsorbent, and enthalpy of adsorption. Solids with porous structure are better adsorbents and large surface area is more adsorption (Adamczyk, 2006). Activity of catalyst increases by increasing surface area by powdering. When pressure increases, more of substance adsorbed by physisorption & chemisorption. Physisorption decreases with increase in temperature, chemisorption.increases with increase in temperature. In general, the adsorbability of a compound increases with:

1) Increasing molecular weight
2) A higher number of functional groups such as double bonds or halogen compounds
3) Increasing polarisability of the molecule. (This is related to electron clouds of the molecule.)
4) Increasing critical temperature. Therefore ease of liquification to increase.