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# MECHANISM FOR THE ADSORPTION OF MUCIN ON HYDROXYAPATITE

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## DEDICATION

This work is dedicated to my late parents: Mal Zakariyyah Kuda Kazaure and Mrs. Khadijat Z. Kazaure.

## CERTIFICATION

This Thesis by Sule Zakari KAZAURE (Adm. No. 01311312006) has met the requirements for the award of Doctor of Philosophy of the Usmanu Danfodiyo University, Sokoto and is approved for its contribution to knowledge.

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| Abbreviations | |
|---|---|
| HAP | Hydroxyapatite |
| CHAP | Calcium hydroxyapatite |
| CHP | Calcium hydrogen phosphate |
| PMMA | Poly-methylmethacrylate |
| BSA | Bovine serum albumin |
| LSZ | Lysozyme |
| MGB | Myoglobin |
| ACP | Amorphous calcium phosphate |
| XRD | X-ray diffraction |
| CPCs | Calcium phosphate ceramic |
| CPP | Calcium pyrophosphate |
| OCP | Octa calcium phosphate |
| PHEMA | Poly (2-hydroxy ethylmethacrylate) |
| DCA | Dynamic contact angle |
| PBS | Phosphate buffer solution |
| TCP | Tri calcium phosphate |

## ABSTRACT

The adsorptive ability of mucin onto hydroxyapatite was evaluated based on its adsorptive amount and adsorptive strength. The adsorptive amount was determined by measuring the amount of mucin adsorbed onto hydroxyapatite while adsorptive strength was determined by measuring the amount of mucin remaining adsorbed after rinsing. The amount of mucin adsorbed onto the substrates in all cases followed the order Sigma grade HAP > synthesized HAP > CHP and generally increased with increase in the amount of adsorbent. More than 80% of mucin was adsorbed unto HAP with or without $Ca^{2+}$ ion pretreatment. The adsorption isotherm fitted the Langmuir model and electrostatic attraction mechanism is proposed.

# CHAPTER ONE

# INTRODUCTION

## 1.1 The Origin of Biomaterials

Virtually everyone has a simple biomaterial in the body. Common tooth fillings represent the first generation of biomaterials, but many people also rely on more critical implants including joint replacement and cardiovascular implant. Although these have performed successfully, a new generation of biomaterials is emerging that will last longer and be better adapted to prolong life in the environment of the human body (Williams and Tanner, 1997).

The life expectancy of the current world population is far higher than what it was a century ago. Millions of people receive implants to help maintain their quality of life after illness or accident. Historically, biomaterials employed in medical implant applications have been developed as a consequence of their mechanical or physical properties with limited consideration of their biological properties. However, the performance of medical devices is often limited by post-operative complications, poor tissue integration or the need for revision surgery. In recent years, it has been recognised that many implants fail as a consequence of poor interfacial biocompatibility are the ability of a material to elicit an appropriate host responses for a particular application. This has led to the development of the field of interfacial bioengineering which aims to modify the host response to a biomaterial whilst maintaining the existing mechanical and physical properties. The use of prostheses made of bio-inert materials, led to a revolution in medical care, but with limited device survivability and inability to respond to changes

in environmental factors (Chapman, 1993). The earliest biomaterials were sutures, but significant use of biomaterials or joint replacement implants for prostheses, began in the UK in the late 1950s when John Charnley developed "low friction arthroplasty". This changed hip joint replacement from being an occasional major salvage to an almost routine operation. In fact, about 40,000 hip joint replacement in the UK and half a million in are performed each year in the world (William and Tanner, (1997).

## 1.2 Proteins' Adsorption on Solid Surfaces

Protein adsorption to surfaces of medical implants is an essential aspect of the cascade of biological reactions taking place at the interface between a synthetic material and the biological environment. The types and amounts of adsorbed proteins mediate subsequent adhesion, proliferation and differentiation of cells as well as deposition of mineral phases. Therefore, the nature of this adsorption process becomes of great interest in order to better understand the bio-integration of implant materials, with the long-term objective of synthesising either fully bio-compatible materials or in the ideal case, truly bio-active materials to replace current materials. This would bring the advantage of improved patient recovery and subsequently improved quality of life. With an ageing population, this subject has naturally become one of great academic and industrial interest over recent years. However, synthesising full bio-compatible materials requires more understanding of the fundamental aspects of protein adsorption (Chapman, 1993).

Fixation of implants to the bone is one of the most significant factors in order to obtain satisfactory outcome in applications, such as total hip replacement. Investigations have

been directed at providing direct bonding between implants and bone, using bioactive ceramics to avoid loosening, which is the major problem of artificial joint implantation (Lu *et al;* 1999). Whilst metal oxides are directly used for applications where their extreme hardness is necessary (e.g. femoral head replacement), most metallic biomaterials are themselves covered by a protective, stable oxide film such as titanium oxide on titanium. In these cases proteins only interact with the oxide film and not with the underlying metal. Closer investigations of protein-oxide interface are therefore vital to the field of biomaterials as it strives to make the transition from merely bio-inert to fully bioactive implant materials (Lundstorm; 1985).

The adsorption of proteins on solid surfaces is an important phenomenon taking place as soon as a foreign material is brought into contact with a biological system. It is thus involved in situations of bio and blood compatibility and fouling in the process industry. Furthermore, there are several surface orientated diagnostic methods based on proteins are large and complicated molecules and the adsorption process it therefore far from simple to model (Lundstrom; 1985).

Many adsorption processes are treated thermodynamically in terms of Langmuir type of isotherms where different kinds of interactions between the surface and the molecules and the molecules themselves are incorporated. The adsorption of protein molecules is however, often a highly dynamic phenomenon. The molecules may change orientation and conformation during or after the adsorption. The properties of the surface play an

important role. Protein molecules are normally more influenced by a nonionic or hydrophobic surface than by a polar and hydrophilic surface (Lundstrom, 1985).

Protein adsorption appears to be mainly irreversible. Proteins form generally thin layers on surfaces like gold and platinum and thicker more extended layers on (oxidised) metals like titanium and zirconium (Lundstrom, 1985).

The free energy of the substrate surface determines to some extent, the structure of the adsorbed protein layer. The adsorption isotherms often show a steep or plateau at low protein concentrations, followed by a slow increase in the number of adsorbed molecules over a wide range in protein concentration (Lyklema and Norde, 1979; Soderquist and Walton, 1980; Lyklema, 1980).

If protein is adsorbed on a hydrophobic silicon surface, the amount of adsorbed protein depends on how the protein is added to the solution. If the protein is added in steps at certain time intervals the adsorbed amount becomes smaller than if the protein is added at once. (Soderquist and Walton, 1980). Thus the effect depends both on the nature of the protein and the surface.

When protein molecules are adsorbed on a metal surface, there is a change in the potential, which occurs mainly after the protein molecules have adsorbed on the surface. The potential change often contains a large reversible part, which is observed upon rinsing of the surface, although most of the adsorbed protein molecules stick on the surface (Soderquist and Walton, 1980; Arnebrant *et al.,* (1985).

Hydroxyapatite is widely utilised as a component in dental hygiene agents. The ability to adsorb and remove dental plaque adhering to a tooth's surface is recognised as an important property of hydroxyapatite particles (Mottoo *et al.,* (1999).

## 1.3 Design of Biomaterials

The reaction of implants to the host depends on the chemical and physicochemical properties of the materials, as well as the site ions and type of implantation. When designing biomaterials there is need to have the knowledge of both materials science and the biological interactions between material and the body. To produce successful biomaterials that will survive in the body for a long time, materials need to be developed specifically for clinical applications. The primary requirement is biocompatibility, in which case the material is not toxic and has appropriate mechanical properties in terms of rigidity and strength (Lu *et al.,* 1999). Along with these basic requirements, however, many other factors may need to be included. By choosing the appropriate material, a biological response may be achieved that encourages the surrounding tissue to bond to the implant. It is advantageous to tailor the mechanical properties at the implant to match those of the body component which it is replacing that is produce an analogue (David, 2003).

The biocompatibility of biomaterials is not only the absence of direct or indirect cytotoxicity, but also includes the entire field of biosafety and biofunctionality. The International Standard Organisation (ISO) in directive 10993 (ISO, 1992) described the methods for biological evaluation of medical devices as a basis for the assessment of

biocompatibility. Part 4 describes the selection of tests for interaction with blood and Part 5 the tests for estimation of cytotoxicity (ISO, 1002, 1999). The importance of biomaterials in clinical medicine cannot be over emphasised and indeed, it has been reported that ultimately, almost every human in technologically advanced societies will host a biomaterial (David, 2003). Unfortunately, the design of many clinical biomaterials is inadequate and complications associated with their use have been reported (David, 2003). The major complication associated with medical devices is infection, resulting in considerable morbidity and mortality in patients. The incidence of medical devices-related infection is dependent on the type of implanted device and ranges from 1-2 % for orthopedics and 70-100 % for continuous ambulatory peritoneal dialysis catheters with an associated long dwell period (David, 2003).

## 1.4 **Aim of the work**

The aim of this work is to propose the possible mechanism for the initial adsorption of mucin to hydroxyapatite in vitro by:

(i) studying the adsorption ability of mucin onto hydroxyapatite (HAP) based on its adsorptive amount and adsorptive strength,

(ii) using Langmuir's model to analyse the isotherm fats,

(iii) studying the affinity of mucin to hyroxyapatite,

(iv) studying the effect of the surrounding ionic composition and pH on the adsorption process,

(v) using these results to suggest a probable mechanism for the adsorption of mucin onto hydroxyapatite.

## 1.5 Justification

The importance of the adsorption of proteins onto hydroxyapatite (HA) in a variety of oral biological events cannot be overemphasised. When biomaterials come into contact with various biological fluids (blood, saliva, tears), protein adsorption at the solid-liquid interface is the first phenomenon that occurs. Apatites are biomaterials due to their biocompatibility. HA can form a bond with bone and tissue, biological apatite is the main constituent of the hard tissues by its large molecular weight and high level of O-linked oligosaccharide (Shi, 2000). It is the major constituent of mucus in various parts of the body and covers the surfaces of the buccal cavity and epithelial organs. Mucin has been identified in several additional types of dental biofilms such as salivary pellicle on hydroxyapatite (HAP) (Rolla *et al.,* 1983). It has been reported that the pellicle influences the initial attachment of micro-organisms to the tooth surface and remains interposed between the enamel and dental plaque (Hillman *et al.,* 1970).

A concise understanding of the mechanism of protein adsorption onto HA will contribute immensely to the present trends in caries research, where there is interest in the role proteins are playing as potential inhibitors of the enamel or dentine demineralization. Van Blitterwijk *et al* (1985) reported that bone-implant interfaces comprise a so-called bonding zone composed of a calcium-and phosphorus-rich proteinaceous matrix. The role of mucous glycoproteins as a macromolecular surfactant is of great importance in the science and technology of biomaterials. Biosurfaces such as dentures are placed on a mucosal surface. Several mechanisms for human salivary albumin (HAS) adsorption to biomedical polymers have been reported (Lee *et al.,* 1992; Xie *et al.,* 1991; Gombotz *et*

*al.,* 1991; Keogh *et al.,* 1992; Sevastianov, 1995). However, report on the mechanism of mucin adsorption on these biomedical materials are virtually not available.

# CHAPTER TWO

# REVIEW OF RELATED LITERATURES

## 2.1 Biomedical Applications of Hydroxyapatite

The mineral apatite is a phosphate of calcium with either fluoride, chloride or hydroxyl ions $Ca_5 (PO_4)_3$ (OH, F, C1). Apatite, approximately represented as in one of the most ubiquitous minerals in nature. Werner devised the name apatite from a Greek word meaning to 'deceive' as in was frequently mistaken for beryl and other species.

Apatite has been found to be widely distributed both geographically and petrologically as it occurs in many kinds of rocks including, metamorphic, limestone, granites and even in iron ores. It has been prepared artificially and mined for the manufacture of fertilizer and to a slight extent, jewelry

(Eanes and Posner (1970).

Hydroxyapatite is a component of bone. It is a calcium phosphate mineral that is also found in rocks and sea coral. Plastic surgeons use hydroxyapatite implants made from seal coral that have been treated so that their structure and chemical make-up is almost identical to the hydroxyapatite of human bone. When implanted into the body, the implant is accepted. Its porous nature allows normal tissue integration to take place. Unlike solid hydroxyapatite implants the injectable (or spreadable) paste is non-porous; so bone and soft tissue ingrowth does not occur. The process of producing hydroxyapatite implants from sea coral involves intense heating that removes all the

proteins thus rendering the structure totally non-immunogenic (i.e. it does not provoke allergic reactions) (Carl, 2003).

Calcium hydroxyapatite, (CHAP), is the basic calcium phosphate mineral, $Ca_4$ $(PO_4)_3$ $Ca(OH)_2$ and is the most important member of apatites with biological, geological and economic significance. It constitutes the principal inorganic phase of teeth and bones. It is the most abundant mineral in human tissue and one of the few crystals formed in *vivo* (Sowerby and Emeleus, 1975; Eanes and Posner; 1970). Bioapatite, the main constituent of mineralised tissue in mammalian bones, is a calcium phosphate-based mineral that is similar in structure and composition to hydroxyapatite (Carl, 2003). Bone differs from other connective tissues by its hardness and physical strength: these stem from the extra cellular deposition of calcium phosphate within a soft fibrous organic matrix. A mature bone contains about 65 % calcium phosphate made up of an amorphous calcium phosphate and a crystalline apatite phase (Posner, 1969). Hydroxyapatite is especially suitable for implantation since it is chemically close to the calcium phosphate. This similarity encourages the bone to accept the material as its own with minimal adverse reactions (Constantiono *et al.,* 1992; Wang *et al.*, 1994).

The biological apatites are microcrystalline compared to the macro dimension of mineral apatite of variable composition and therefore heterogeneous within each category and even within the calcified tissue such as enamel, dentine and bone (McConnell, 1973). Biological apatite is however known to be associated with many other elements in both

small and large amounts. For this and similar reasons, biological apatites are referred to as impure hydroxyapatite (Bigi *et al.*, 1984).

Some of the impurities associated with biological apatite include $Sr^{2+}$, $Ba^{2+}$, $Mg^{2+}$, $K^{+}$, $Pb^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Cd^{2+}$ and other cationic trace elements which are able to replace calcium either wholly or partly in the hydroxyapatite lattice. Others include $CO_3^{2-}$, $HPO_4^{2-}$, and $P_2O_7^{4-}$ capable of substituting for the $PO_4^{4-}$ ion and finally, $C1^{-}$, $F^{-}$, $H_2O$ and $-CO_3^{2-}$ which replace the $OH^{-}$ group in the hydroxyapatite (Baud and Very, 1973; Baud *et al.*, 1977; Legeros *et al.*, 1980). The hydroxyapatite structure is subject to isomorphous substitution.

The incorporation of foreign ions affects the crystallinity, morphology and lattice parameters, and as a consequence the stability of the apatite structure (Bigi *et al.*, 1991). The closer coordination of the fluoride as compared to the hydroxyl by the calcium accounts, in part, for the greater chemical stability of fluoride substituted HAP as evidenced by its resistance to dental caries (Posner, 1996). The entry of the carbonate into the crystal structure especially in the phosphate position causes a lattice distortion (Legeros *et al.*, 1978). Furthermore, HAP precipitated in the presence of carbonate tends to be poorly crystallised, showing broad X-ray diffraction patterns and its weakening effects on the bonds in the structure thus increasing the rate of dissolution and solubility.

Hydroxyapatite is the most commonly used biomaterial for repair and reconstruction of damaged or diseased parts of the human bone due to its widely accepted biocompatibility

(Zhitomirsky, 2000; Ma *et al.*, 2003). However, components made solely of HAP were found to lack toughness and therefore could fail catastrophically. As a result, HAP coated titanium components, which combine the advantages of the mechanical strength of titanium metal and the bioactivity of HAP, are developed and reckoned to be one of the most promising group of implants materials in orthopedic and dental fields.

Until the 1980s, the material used in joint replacement came from other engineering applications. The grout or bone cement (poly-methylmethacrylate, PMMA) used by Sir John Charnley was originally developed for making dentures, but is also suitable for fixing prostheses, and is still used in over 80% of hip and over 90% of knee replacements (Williams and Tanner, 1997).

It is noteworthy that, hydroxyapatite is widely utilised as a component in oral hygiene agents. Hydroxyapatite particles could be added to tooth paste as an important component for removing plaque adhered to tooth surface and/or eliminate mouth odour caused by oral disease, (Aoki *et al.*, 1982; Motoo, 1994; Tamura *et al.*, 1995).

## 2.2 Adsorptive Capacity of Calcium Hydroxyapatite

Hydroxyapatite is a form of calcium phosphate that has long been used in the chromatographic separation of proteins and DNA (Tiselius *et al.*, 1956). Hydroxyapatite is best known as a crystalline material but is now available in a range of ceramic derivatives that are vastly superior in terms of flow rate, stability and reproducibility over many cycles of use.

The adsorption of protein to hydroxyapatite is complicated because it involves both anionic and cationic exchange (Gorbunoff, 1990). When using phosphate, acidic proteins are more readily eluted than basic proteins, although the phosphate concentration required to elute any protein can be reduced by raising the pH (Bernardi, 1973). A minute protein bound to hydroxyapatite can be fractionated by a series of phosphate wash steps of increasing pH (Rossano *et al.,* 2001).

However, phosphate is a weak buffer outside the 6.0-7.5 pH range, yet strong buffering is desirable when using hydroxyapatite to avoid the local pH changes that occur in the highly polarised environment at the surface (Scopes, 1993). Analysis of the effect of altering the pH upon protein elution would therefore benefit from improved buffering since the buffering capacity of the phosphate rapidly decreases away from neutrality (Ewald *et al.,* 2002).

The effects of acetonitrile on the adsorption behaviour of bovine serum albumin (BSA) onto calcium hydroxyapatite [$Ca_{10}$ $(PO_4)$ $_6$ $(OH)$ $_2$, HAP] materials by combining the ultraviolet (UV) and circular dichroism (CD) measurements of BSA solution. The structural change of BSA molecules with addition of acetonitrile was investigated by UV and CD spectroscopy measurements prior to study of adsorption behaviour of BSA onto HAP (Kandori *et al.*, 2002). The CD spectra revealed that the fraction of α-helical content of BSA is remarkably decreased at acctonitrile concentrations above 30 vol. %, while β-sheet content is increased. On the other hand, the percentages of random coil and turn contents were decreased only slightly. In addition to this secondary structural

change of BSA, the UV spectra suggested that the tertiary structure of protein molecules was also changed by the addition of large amounts of acetonitrile; BSA molecules associate to form molecular aggregates acetonitrile 40 vol. %. From the adsorption of BSA onto HAP particles (ca.30 nm in the particle length) from a water-acetonitrile mixed solution, it was revealed that the adsorption behaviour of BSA strongly depends on the change of secondary and tertiary structures of BSA by addition of acetonitrile. The contraction of BSA molecules at low acetonitrile concentrations (10-20 vol. %) gave their small cross-sectional area, providing a large amount of adsorption ($n_{BSA}$) although the latter was decreased above 30 vol. % acetonitrile by enlargement of BSA molecules with solvation and unfolding some α-helical domains. The $n_{BSA}$ values of the systems with acetonitrile exhibited a maximum; $n_{BSA}$ was increased at a lower BSA concentration region, although it was decreased at a higher BSA concentration due to self-association (Kandori *et al.*, 2002).

Accompanying the change of $n_{BSA}$ with acetonitrile addition, the maxima of electrophoretic mobility of the HAP particles were observed for the systems with acetonitrile, although the electrophoretic mobility of HAP particles was normally increased and saturated with increase in protein coverage for the native structure on the system without acetonitrile. On the other hand, because the aggregated BSA molecules could be cooperatively bound, the adsorption of BSA onto the HAP particles with large size (108 nm in particle length) was enhanced in the presence of acetonitrile (Kandori *et al.*, 2002).

Competitive and cooperative adsorption of bovine serum albumin (BSA) and lysozyme (LSZ) on synthetic calcium hydroxyapatite (CHAP) were investigated at 15 °C in deionised distilled water of pH 6 using liquid chromatography. The adsorption rate of LSZ was faster than that of BSA due to its larger diffusion coefficient, though the saturated adsorption of LSZ was less than that of BSA. The rates of BSA adsorption onto CHAP did not change by the addition of LSZ, while those of LSZ were considerably reduced by addition of BSA with formation of ($BSA^{-}$ $LSZ^{+}$) agglomerates in the solution. The cooperative adsorption behaviour of LSZ was observed in the presence of lower amounts of BSA due to the preferential adsorption of the larger ($BSA^{-}$ $LSZ^{+}$) agglomerates. However, in the case of higher BSA content, the adsorption of LSZ was inhibited by capturing the LSZ molecules in the ($BSA^{-}$ $LSZ^{+}$) agglomerates. The similar cooperative adsorption behaviour of BSA was also observed on all systems examined in the presence of various amounts of LSZ (Kandori *et al.,* 1999).

The adsorption of myoglobin (MGB) onto various kinds of colloidal synthetic hydroxyapatite [$X_{10}(PO_4)_6$ $(OH)_2$, notably CHAP, SrHAP and CSrHAP for X = Ca, Sr and Ca + Sr, respectively] particles was investigated at 15 °C in a 1 x $10^{-4}$ $moldm^{-3}$ KC1 solution at pH6.0. The adsorption rate of MGB onto CHAP was comparable to that of lysozyme (LSZ) and was faster than that of bovine serum albumin (BSA) (Kandori *et al.,* 2000). The distinction was explained by the difference in the molecular mass of these proteins (MGB: 17800 Da, LSZ: 14600 Da, BSA: 67,200 Da); the diffusion rates of the smaller MGB and LSZ molecules to the CHAP surface are faster than that of the larger BSA. The adsorption isotherms of MGB on these synthetic hydroxyapatites (HAP)

exhibited the Langmuir type and the surface charge of the MBG-covered HAP showed almost a constant negative value and was independent of the MGB concentration for all the HAP. The values of the saturated amounts of adsorbed MGB for CaHAP were independent of the Ca: $PO_4$ molar ratio of the materials, supporting less importance of an electrostatic interaction between MGB and HAP because MGB molecules are electrostatically neutral at pH 6 (Kandori *et al.,* 2000).

Ceramic coatings on metal substrates are used in several applications, with the aim of increasing the corrosion resistance of the metal. In the biomedical field, coatings are used to modify the implant surface and, in some cases, create a new surface with totally different properties with respect to the substrate. Due to the similarity with the inorganic components of the bony structure, synthetic hydroxyapatite [$Ca_{10}$ $(PO_4)_6$ $(OH)_2$] was one of the first materials used to coat metals. The use of HAP as coating is advisable, as this compound does not exhibit good mechanical properties in bulk form. On the other hand, when the prosthesis implant is made of metal, this can undergo corrosion processes, with consequent release of metal ions into the tissue. Hence metal implants are coated by bioactive ceramics, such as hydroxyapatite, that not only prevent corrosion, but also increase the growth rate of tissue within the pores (Balamurugan *et al.,* 2002).

Paul (1980) examined blood compatibility and protein adsorption for hydroxyapatite and hydroxy1-carbonate apatite. Those apatites were synthesised under $CO_2$- containing $N_2$ atmosphere by wet chemical method and subsequent calcining. From infrared (IR) analysis, the carbonate ions substituted both phosphate ion and hydroxyl sites. Blood

clotting properties were evaluated in terms of active partial thromboplastin time, prothrombin time, and amount of fibrinogen the plasma contacted with the apatites, indicating that all the apatites hardly influenced the blood clotting system. The apatites were contacted with a solution containing both bovine serum albumin (BSA) and β2-microglobalin (β2- MG), and the amount of those proteins adsorbed on them was examined. The hydroxyl-carbonate apatites synthesised under $CO_2$- containing $N_2$ atmosphere and calcined below 400 °C had the greatest selectivity in adsorbing β2-MG. The better selectivity for β2-MG adsorption was empirically correlated to the carbonate ions incorporated in the hydroxyapatite lattice.

## 2.3 Mucin

Mucin is a glycoprotein characterized mainly by its class of high molecular weight and high level of O-linked oligosaccharides (Strous and Dekker, 1992). It is the major constituent of mucus which covers the luminal surfaces of epithelial organs and serves as a physical barrier between the extra cellular milieu and the plasma membrane. Mucins are found in two forms, soluble secretory mucins and membrane-bound mucins (Shi, 2000). Secretory mucins constitute viscous gels due to their high molecular weights and often serve to protect the delicate apithelial cells against the extra cellular environment. Essential for gel formation is the capability of secretory mucins to form intermolecular disulfide bridges, resulting in oligomeric structures. Organs with environmental exposure, e.g. the eyes, gastrointestinal tract, trachea, lungs, bladder, pancreatic tract, gallbladder, and reproductive tracts, protect their epithelia by producing secretory mucin (Roussel *et al.,* 1988). The membrane-bound mucins are similar to the secretory mucins

in that there is a multiple tandem repeat of amino acid sequences containing all the attachment sites for O-linked oligosaccharides. However, they differ from the secretory mucins in that they contain a hydrophobic domain anchoring the molecules in the plasma membrane. In addition, they lack intermolecular association through disulfide bridges. It has been found that mucins from the gastric tract, lungs, salivary and sweat glands, breast, and tumour cells are structurally related to high-molecular weight glycoproteins, which are produced by epithelial cells as membrane protein (Strous and Dekker, 1992).

With regard to their respective availability, secretory mucin is more readily obtained than the membrane bound counterpart. The former can be purified from many body fluids such as tears, saliva and other secretions. The O-linked glycans in mucin are quite heterogeneous and vary in length from 1 to 20 residues units (Frostner and Frostner, 1994). Although the O-linked oligosaccharide constitutes the major carbohydrate in many mucins, some N-linked sugar chains are also reported (Khatri and Frostner, 1993). The O-linked glycosidic linkage is found between Thr/Ser and GalNAc, where the GalNAc residue directly attaches to the hydroxyl group of serine or threonine. Five kinds of monosaccharide are commonly found: GalNAc, GlcNAc, galactose, fucose, and sialic acid (Shi, 2000). Sialic acid negatively charges the oligosaccharide. GalNAc may be the only sugar or be terminated with a sialic acid residue. The main chain of oligosaccharide consists of series of Gal β(1-3) and Glc NAc β(1-4) and is terminated by ά-galactose, GalNAc, fucose, sialic acid or sulphated saccharide, (in most cases, sulphate group is linked to galactose, GalNAc, or GlcNAc) (Strous and Dekker, 1992). Branched saccharides also exist. They determine most of the characteristics of mucin as a whole.

Mucin contains a number of oligosaccharide clusters flanked by "naked" stretches of protein backbone (Carlstedt *et al.,* 1985).

## 2.4 Occurrence and Preparation of Calcium Hydroxyapatite

All human bones consist of about 40 vol % (70 wt %) bone mineral hydroxyapatite in a matrix of collagen-a natural composite (William and Tanner, 1997). In the formation of bone material, the amorphous phase is laid down first by some active process of the bone cell. Subsequently some of the phase is stabilised to remain noncrystalline, while a larger portion is transformed via solubilisation to the crystalline form (Eanes and Meyer, 1977).

The description of the in *vivo* (native) transformation of the amorphous calcium phosphate to the crystalline calcium phosphate is inferred from studies on synthetic analogues. The reaction of calcium and dibasic phosphate salts in neutral or basic solution has as its final product, crystalline hydroxyapatite. During the precipitation of the crystalline material a precursor phase is formed which is amorphous to x-ray diffraction (Eanes *et al.,* 1965). This amorphous phase converts in the presence of water to micro crystalline hydroxyapatite. The life time of the metastable amorphous precursor in aqueous solution is a function of the presence of certain macromolecules, interfering ions, pH, viscosity, ionic strength and temperature (Eanes *et al.,* 1965; Termine and Posner, 1970).

In the presence of high concentration of calcium and phosphate (total calcium and total phosphate greater than 10 mM) and pH values greater than 6.8, the precipitation of

hydroxyapatite in bones always proceeds by the formation of an amorphous precursor (Eanes *et al.,* 1965; Boskey *et al.*, 1973; Termine *et al.,* 1976). In the absence of such foreign ions such as $F^-$ and $CO_3^{2-}$HAP is the thermodynamically stable end product of calcium phosphate precipitations carried out at physiological pH, 7.4 and temperature 37 $^oC$. Eanes and Mayer, (1977), and Boskey *et al.,* (1978) found that, in the presence of lower concentration of calcium and phosphate (total Ca and total $PO_4$ each being less than 2 mM), the first precipitate formed at pH 7.4 has x-ray diffraction pattern, morphology and colloidal properties distinct from those of amorphous calcium phosphate (ACP) found in the presence of higher concentrations of reactants. This observation contradicts the result of Termine and Posner (1970) and Eanes and Posner (1970) who concluded that ACP was an obligatory precursor to HAP. Furthermore, it is apparent that the pH and specific concentrations of calcium and inorganic phosphate as well as the ionic strength, temperature and presence of heteronuclei rather than calcium phosphate millimolar products alone are critical in determining the initial phase precipitated in the course of HAP formation (Noncollas and Tomazic, 1974).

Carl *et al* (2003) investigated the crystallographic structure of bioapatite in human fetuses by synchrotron radiation X-ray diffraction (XRD) and microdiffraction (μ-XRD) techniques. Rietveld refinement analysis of XRD and (μ -XRD) data allow for quantitative probing of the structural modification of bioapatite as functions of the materialisation process and gestational age.

Ajibola (1995) prepared HAP by heating 1100 $cm^3$ of 0.19M disodium hydrogen phosphate solution to boiling and 400 $cm^3$ of 1.264 $g/dm^3$ calcium acetate solution of added drop-wise with constant stirring. After precipitation, the content of the flask were filtered, washed with warm water till washings were neutral and dried at 105 $^oC$ for four hours. HAP was precipitated as above using 0.50 M $Ca(OH)_2$ solution and 0.30 M orthophosphoric acid solution. Similarly HAP was precipitated using 0.019 M diammonium hydrogen phosphate solution and 400 $cm^3$ of calcium nitrate solution (containing 0.08 mole of $Ca^{2+}$) as reagents.

Linghong *et al* (2003) synthesised HAP with $Ca(NO_3)_2$ and $(NH_4)_2$ $HPO_4$ by wet – chemical method. The as-received powder was sintered at: 600, 800, 1000 and 1200 $^oC$. Synthetic Calcium Phosphate Ceramic (CPC) can be transformed to a biological apatite through a sequence of reactions which include dissolution, precipitation and ion exchange. By virtue of the reactions being materials dependent, it is important to determine parametric rate effects. The effect of stoichiometry and crystal structure of Calcium Phosphate Ceramic (CPCS) on the dissolution kinetics was focused using monophase, biphase and multiphase CPCS with a Ca/P ratio equal to or greater than 1.5. The experiments were performed in a calcium and phosphate-free tris buffer solution at pH 7.3. The dissolution behaviour of the CPCs increased in the order of stoichiometric hydroxyapatite, calcium deficient hydroxyapatite, oxyhydroxyapatite, beta-tricalcium phosphate, alpha-tricalicum phosphate and tetracalcium phosphate. Dissolution of biphase and multiphase CPCs increased prorated the concentration of more soluble component (Ducheyne *et al.,* 1993).

Balamurugun *et al* (2002) synthesised HAP using the sol-gel technique and the solution was allowed to age up to seven days prior to coating. It was found that, similar to the wet-chemical method of HAP powder synthesis, an ageing time was required to produce a pure form of HAP phase, a methodology which has been successfully used to produce hydroxyapatite thin film coating via sol-gel route. Sol-gel technology offers an alternative technique for producing bioactive surfaces for improved bone attachment. (Balamurugun *et al.*, 2002).

Graham and Brown (1993) studied the low temperature formation of octacalcium phosphate ($Ca_8\ H_2\ (PO_4)_6^-\ 5H_2O$) and the relationship between phase formation, microstructural evolution and variations in solution chemistry. At the highest temperature studied (70 $^{o}$C), initial precipitation of OCP occurred in approximately 1 hour, but its inevitable hydrolysis to a more stable hydroxyapatite (HAP) phase took place over several days. At room temperature nearly three days are required to initiate OCP formation. At pH<7, pure phase OCP is the final product, while HAP form when initial pH values are higher than this.

On the whole, the chemistry of HAP is greatly complicated by its ability to form interlayer mixtures with, OCP. On the other hand, the structural relationship provides a rational basis for understanding physiologically important phenomena such as a mechanism for prevention of dental caries by fluoride in drinking water, the cause of ribbon-like and platy morphologies of biological apatite crystallites, the unusual variability in the stoichiometry of apatite precipitates, a possible mechanism for

incorporation of impurities and defects into dental enamel, a mechanism of growth of biological apatite and a rationale why teeth enamel vary in their susceptibility to caries (Brown *et al.,* 1979).

Composite (biphasic) mixtures of two of the most important inorganic phases of synthetic bone applications namely calcium hydroxyapatite ($Ca_{10}$ $(PO_4)$ $_6$ $(OH)$ $_2$ (HAP) and tricalcium phosphate ($Ca_3$ $(PO_4)_2$ (TCP), were prepared as submicrometer-sized, chemically homogenous, and high purity ceramic powders by using a novel, one-step chemical precipitation technique. Starting materials, calcium nitrate tetrahydrate and diammonium hydrogen phosphate salts that were dissolved in appropriate amounts in distilled water were used during powder precipitation runs. The composite bioceramic powders were prepared with composition of 20 - 90 % HAP (the balance being the TCP phase) with increments of 10 %. The pellets prepared from the composite powders were sintered to almost full density in a dry air atmosphere at a temperature of – 1200 $^{o}$C. Phase evolution characteristics of the composite powders were studied via X-ray diffractometry as a function of temperature in the range of 1000 – 1300 $^{o}$C. The sintering behaviour of the composite bioceramics were observed by using scanning electron microscopy (SEM). Chemical analysis of the composite samples was performed using the inductively coupled plasma – atomic emission spectroscopy technique (Nezahat and Cuneyt, 1998).

## 2.5 Mechanism of Adsorption of Mucin to Hydroxyapatite

Lori and Nok (2003) investigated the role of electrostatic interactions in the adsorption of mucin to titanium in *vitro* and the building profile of mucin to titanium was analysed according to an adsorption isotherm. Mucin was dissolved and the solution suspended with native calcium, magnesium or potassium treated Ti potassium powder, at pH 3.0 and 7.4. The amount of unabsorbed protein in the supernatant fluid was measured. The maximum amount of adsorbed mucin was 0.11 mg/1.0 g of Ti. The mucin-Ti association constant was then estimated. Pretreatment of Ti with calcium or magnesium alone, or combined resulted in increased adsorption of mucin to Ti. No increase in adsorption was recorded following pre-treatment of Ti with potassium. The results indicated the involvement of electrostatic interactions in adsorption of mucin to Ti.

Wassell *et al* (1995), on the other hand, studied the adsorption of bovine serum albumin (BSA) onto hydroxyapatite (HA) as a function of the protein concentration, pH and ionic strength. Isotherm data (adsorption being a reversible process) were analysed using the Langmuir model. The adsorption parameters AT (maximum amount of protein adsorbed, mg $^{-2}$) and K (affinity constant, Lg $^{-1}$) were calculated for each solution condition (except NaF). For the pH dependence of adsorption both AT and K increased with decreasing pH, indicating that both electrostatic and hydration affects were important. For the ionic strength dependence, increasing NaC1 concentrations resulted in a slight increase in AT, but K decreased. With increasing $CaC1_2$ concentrations, the AT and K values increased, the opposite being true for increasing concentrations of $Na_2HPO_4$ and NaF. Both enhanced and inhabited adsorption depending on the concentrations.

The adsorption of bovine serum albumin (BSA) onto titanium powder was studied as a function of protein concentration and pH, and in the presence of calcium and phosphate ions. Isotherm data revealed that the adsorption process did not follow the Langmuir model (Williams *et al.,* 1985; Veerman *et al.,* 1987; Ellingsen, 1991; Williams and Williams, 1989; Sunny and Sharma, 1991).

Pulat (2003) studied the adsorption of bovine serum albumin (BSA) onto poly (2-hydroxyethyl methacrylate) (PHEMA) beads modified by using the pair of hexamethylene diisocyanate-suberic acid bis-N-hyroxy succinimide as a function of protein concentration and adsorption time. The adsorption studies were carried out in phosphate buffer solution (PBS) at pH 7.4. The isotherm data were analysed using the Langmuir model and the adsorption parameters Q0 and b were calculated. It was determined that the degree of adsorption of BSA increased with the increase of the adsorption time and BSA concentration until a certain value. PHEMA beads were characterised by using FTIR spectra and SEM analysis. The adsorption of BSA onto PHEMA beads were clearly observed from SEM micrographs. The swelling tests of the beads were performed at 37 $^{o}$C in PBS.

Soderquist and Walton (1980) and Van and Norde (1983) observed maximum adsorption of BSA onto titanium at 10 min and 15 min for 0.1 and 0.5 $gl^{-1}$ BSA, respectively; after which there was decrease in adsorption to a steady state. Previous studies of albumin adsorption onto other surfaces also recorded kinetic 'overshoot' Van and Norde (1983). These studies therefore, indicate that adsorption after sometime may be the result of

rearrangements in the structure of already adsorbed molecules. Conformational changes in the molecules led to some unfolding, which resulted in an increased number of protein sites contacting the surface. Some protein molecules may become detached in favour of the 'spreading' of other adsorbed molecules.

Adsorption increased with decreasing pH, although the effect was less marked at lower protein concentrations. The maximum adsorption ($Ad_{max}$) at pH 6.8 and pH 5.15 were $1.13 \pm 0.2$ $mgm^{-2}$ (n = 5) and $1.31 \pm 0.2$ $mgm^{-2}$ (n = 4), respectively. Clearly, the isotherms were not continuous (except at pH 7.15), but showed inflection points (Lyklema and Norde, 1973; Hlady and Faredi-Milhofer, 1979).

The adsorption of BSA onto titanium surface was found to increase in the presence of calcium acetate, although this affect decreased with increasing calcium acetate concentrations. Calcium is known to adsorb to BSA hence the zeta-potential values become less negative. The presence of $Na_2HPO_4$ decreased adsorption of BSA onto titanium, but increased the adsorption with increasing phosphate concentrations (Shimabayashi *et al.*, 1991, Healy and Ducheyne (1992).

Healy and Ducheyne (1992) showed that the adsorption of BSA onto hydroxyapatite (HAP) increased with decreasing pH, but the isotherms were continues, being described by the Langmuir model. The degree of adsorption of BSA by hydroxyapatite HAP increased with increase in the concentration of $CaC1_2$ solution due to the bridging effect of $Ca^{2+}$ between adsorbate (BSA) and adsorbent (HAP). On the other hand, adsorption

decreased remarkably with increase in the concentration of $K_2HPO_4$. This was explained in terms of the effects of ionic strength and competitive adsorption between inorganic phosphate anion ($PO_4^{3-}$) and BSA, because BSA is negatively charged over the examined pH. A similar effect was observed in the presence of phosphorylated compounds such as phosphoserine, phytate and phosphorylated polyvinyl alcohol. The inhibiting effect of these compounds was stronger than that of their mother compounds (serine, inositol and polyvinyl alcohol). The result showed that phosphate groups bound to the mother compounds interfered with the adsorption of BSA by HAP in the same manner that $PO_4^{3-}$ does. Although the adsorption of BSA was almost irreversible with respect to dilution with water, desorption occurred when these organic phosporylated compounds were added after the accomplishment of the adsorption of BSA. However, the effective concentration of the phosphorylated compounds for the desorption of BSA was fairly higher than that for the competitive inhibition against the BSA adsorption. Shimabayashi *et al.,* (1991) and Shi *et al.,* (2005) studied the ion-exchange equilibrium and the dependence of the parameters in the steric mass-action (SMA) model on salt concentration and buffer pH around the isoelectric point of a protein. Bovine serum albumin (isoelectric point = 5.4) was used as a model protein and DEAE sepharose FF as an ion exchanger. Finite batch adsorption experiments and isocratic elution chromatography were performed for the determination of the model parameters (i.e. characteristic charge, equilibrium constant and steric factor). The results revealed that pH had significant effects on the parameters. With an increase of pH from 4.5 to 6.5, the characteristic charge increased from 0.9 to 3.0 and leveled off as a plateau at pH and

above 5.5. The charge groups in the contact region of the protein surface were considered to play a crucial role on the characteristic charge.

The decrease of pH and increase of salt concentration lowered the absolute value of the zeta potential of the protein surface and led to a decrease of the equilibrium constant. The steric factors remained unchanged at a value of 31 at pH 5.5 and 6.0 and increased to 44.5 at pH 5.0 and 96.8 at pH 4.5. This was mainly as a result of the lower adsorption capacity of BSA at pH <5.5. Furthermore, the increase of the molecular volume of BSA at pH 4.5 would be an additional reason for the increase of the steric factor. Taking into account the effect of the pH and salt concentration on these parameters, the SMA model described the ion exchange equilibrium of protein more accurately (Shi *et al.,* (2005)

Hajra and Chattoraj (1991) studied the adsorption BSA at the solid-water interface as a function of protein concentration, ionic strength of the medium, pH and temperature using silica, barium sulphate, carbon, alumina, chromium, ion exchange resins and sephadex as solid interface. In most cases, isotherms for adsorption of BSA attained the state of adsorption saturation. In the presence of barium sulphate, carbon and alumina, two steps in the isotherms were observed. Adsorption of BSA was affected by change in pH, ionic strength and temperature of the medium. In the presence of metallic chromium, adsorbed BSA molecules were either denatured or negatively adsorbed at the metallic interface. Due to the presence of pores in ion-exchange resins, adsorption of BSA was followed by preferential hydration on resin surfaces in some cases. Sometimes two steps of isotherms were also observed during adsorption of BSA on the solid resins in the

chloride form. Adsorption of BSA, beta-lactoglobulin, gelatin, myosin and lysozyme was negative on sephadex surface due to the excess adsorption of water by sephadex. The negative adsorption was significantly affected in the presence of $CaCl_2$, KSCN, LiCl, $Na_2SO_4$, NaI, KCl and urea. The values of absolute amounts of water and protein, simultaneously adsorbed on the surface of different solids, were evaluated in some cases on critical thermodynamic analysis. The standard free energies ($\Delta G^0$) of excess positive and negative adsorption of the protein per square meter at the state of monolayer saturation were calculated using proposed universal scale of thermodynamics. The magnitude of standard free energy of transfer ($\Delta G^o$)B of one mole of protein or a protein mixture at any type of physicochemical condition and at any type of surface was observed to be 38.5 kJ/mole.

Wassell and Embery (1996) studied the adsorption of bovine serum albumin (BSA) onto titanium powder as a function of protein concentration and pH, and in the presence of calcium and phosphate ions. Isotherm data revealed that the adsorption process did not follow the Langmuir model (inflection points). The time dependence, isotherm, and desorption data provided indirect evidence of possible conformational changes in the BSA molecule.

Hughes *et al* (1997) studied the adsorption of chandroitin-4-sulphate ($C_4s$) and heparin onto hydroxyapatite (HAP) in the absence and presence of bovine serum albumin (BSA). Isotherm data at pH 6.8 revealed that BSA in solution had no effect on C4S adsorption, whereas affinity and adsorption decreased. These data suggest that C4S and BSA bind to

different calcium sites on the HA surface. Heparin and BSA may compete for the same calcium sites or alternatively form heparin-BSA complexes leading to less binding due to steric effects. Evidence of an interaction between heparin and BSA in solution was shown. There was negligible interaction because C4S BSA adsorption from solution onto HAP decreased with increasing C4S/heparin solution concentration, which may be due to glycosaminoglycan-induced conformational change of BSA from a compact to an extended structure. For the HAP precoated with BSA, both C4S and heparin adsorption decrease above a certain solution concentration. An explanation to this was that precoated BSA masked binding sites for the C45/heparin. The percentage of BSA desorbed on the precoated HAP in the presence of C45 and heparin was < 10% and < 30% respectively, indicating that BSA was strongly bound to the HAP surface.

Gorbunoff (1984) studied the criteria for elution of protein from hydroxyapatite columns as a function of (1) protein isoelectric point (22 proteins with isoelectric points between 3.5 and 11.0); (2) ionic nature of elute (Na Salts of $PO_4^{3-}$ $F^-$ $Cl^-$ $SCN^-$, $ClO_4^-$ and $CaCl_2$); and (3) structural differences between related proteins. It was found that proteins can be classified into three groups: (1) basic proteins, which elute at similar moderate molarities of $PO_4^{3-}$, $F^-$, $Cl^-$, $SCN^-$ and $ClO_4^-$ and low (less than 0.003M) $Ca^{2+}$; (2) acidic proteins which elute at about equal moderate moralities of $PO_4^{3-}$ and F; but do not elute with $Ca_4^{2+}$ and usually not with $Cl^-$; (3) neutral proteins, which elute with $PO_4^{3-}$, $F^-$ and $Cl^-$, but showed a strong anion specificity, and do not elute with $Ca^{2+}$ or $SCN^-$. Furthermore, individual specific polar groups were not in general crucial to binding or desorption, and variations in structure, other than major loosening, did not influence strongly the pattern

of protein-hydroxyapatite interaction. Binding occured both by nonspecific attraction between protein positive charges and HAP and by specific complexing of protein carboxyls with calcium lock on the mineral. Elution can take place either as a result of the nonspecific ion screening of charges or by the specific displacement of protein groups form sites on the column with which they had complexed (Gorbunoff and Timasheff, 1984).

If a biomaterial contacts body liquids during implantation, the initial host response is characterised by fast interactions with water molecules and ions, followed by macro-molecular interactions. Thus, the complex initial reactions result in a conditioned surface which is faced by bacteria and tissues cells when they arrive at the biomaterial's surface. Among the surface parameter influencing the initial host response are: surface composition, surface charge, hydrophilicity and surface free energy as well as surface roughness and topography. (Gorbunoff and Timasheff, 1984).

It is important to realize that every surface interaction will change the above-mentioned parameters. Studies confirm that roughness influences surface wettability, whereby roughness and wettability both interfere with micromolecular interactions during formation of the conditioning film. Thus, there is a dynamic change of the surface and interfacial state during the initial phase of host contact. Dynamic contact angle analysis (DCA) was used on the basis of multiloop Wilhelmy-technique to study the interfering effects between protein adsorption/desorption and changes in wettability parameter such as advancing and receding contact angles and, wetting tension hysteresis. Different

experimental dental plaque reducing biomaterial surface modifications were investigated. Another investigation dealt with the effects of sand-blasting and acid-etching of titanium implant surfaces on their dynamic wettability behaviour (Rupp *et al.*, 2000).

Zhang and Sun (2001) studied the adsorption equilibrium of bovine serum albumin (B SA) γ-globulin, and lysozyme to three kinds of Cibacron blue 3GA (CB)-modified agarose gels, 6 % agarose gel-coated steel heads (6AS), Sepharose CL-6B, and a home-made 4 % agarose gel (4AB). Ionic strength had irregular effects on BSA adsorption to the CB-modified affinity gels by affecting the interactions between the negatively charged protein and CB as well as CB and support matrix. At low salt concentrations, the increase in ionic strength decreases the electrostatic repulsion between negatively charged BSA and the negatively charged gel surfaces, thus resulting in the increase of BSA adsorption. This tendency depended on the pore size of the solid matrix, CB coupling density, and the net negative charges of proteins (or aqueous-phase pH values). Sepharose gel has a larger average pore size, so the electrostatic repulsion-effected protein exclusion from the small gel pores was observed only for the affinity adsorbent with high CB coupling density (15.4 mM/L) at very low ionic strength (NaCl concentration below 0.05 M in 10 mM tris-HCl buffer, pH 7.5). However, because CB-6AS and CB-4AB have a smaller pore size, the electrostatic exclusion effect could be found at NaCl concentrations of up to 0.2 M. The electrostatic exclusion effect was even found for CB-6AS with a CB density as low as 2.38 mM/L. Moreover, the electrostatic exclusion effect decreased with decreasing aqueous phase pH due to the decrease of the net negative charges of the protein. For γ-globulin and lysozyme with higher isoelectric

points than BSA, the electrostatic exclusion effect was not observed. At higher ionic strength, protein adsorption to the CB-modified adsorbents decreased with increasing ionic strength. The hydrophobic interaction between CB molecules and the support matrix increased with increasing ionic strength leading to the decrease of ligand density accessible to proteins, and then the decrease of protein adsorption. Thus, due to the hybrid effect of electrostatic and hydrophobic interactions, in most cases studied, there exist a salt concentration to maximise BSA adsorption.

Rosengren *et al* (2003) studied the adsorption of protein from diluted human plasma on three ceramics: alumina, zirconia and hydroxyapatite, and two glass-ceramics, RKKP and AP40, by means of chromatography and two-dimensional poly acryl-amide gel electrosphoresis (2D-PAGE). It was found that less than 12 %, 13 % and 24 % of the surfaces of alumina, zirconia and hydroxyapatite, respectively, were covered by proteins, while RKKP and AP 40 were fully covered with proteins. In the case of RKKP and AP 40 even multilayers were found. Regarding preferential adsorption almost half of the studied proteins exhibited differences in absorptivity between the materials, whereas the rest were non-selective. Among the selective proteins were (albumin, apolipo protein J, fibronectin, prothrombin), inflammatory components ($I_gG$, Cls, C3), anti-inflammatory agents (ceruloplasmin), coagulation agent (fibrinogen, fibronectin prothrombin), acute-phase reactants (αlantichymotrypsin, cerulplasmin), bone formation inhibitors (albumin, α2 HS glycoprotein) and other various proteins (apoli poprotein D, α, β glycoprotein, SRBP).

The assembly of amelogenin protein into nanosphere was postulated to be a key factor in the stability of enamel entracellular matrix frameworks, which provides the scaffolding for the initial enamel apatite crystals to mediate and grow. Thus, adsorption isotherms were evaluated in order to investigate the nature of interactions of amelogenin nanospheres with hydroxyapatite crystals in solution, where their assembly status and particle size distribution are defined. The adsorption isotherm of a recombinant mouse amelogenin (rM179) on synthetic hydroxyapatite crystals can be described using a Langmuir model indicating that amelogenin nanospheres adsorb onto the surface of apatite crystal as binding units with defined adsorption sites. The adsorption affinity and the maximum adsorption sites were 19.7 x $10^5$ $dm^3/mol$ and 6.09 x $10^{-7}$ $mol/m^2$ respectively, with an $r^2$ value of 0.99. Knowing the composition and particle size distribution of amelogenin nanospheres under the condition of adsorption experiments, the number of nanospheres and the crystal surface area covered by each population of nanospheres was calculated at their maximum adsorption. It was found that total maximum binding covered 64 % of the area unit. This observation supported the speculation that amelogenin binding onto apatite surface is selective and occurs only at certain sites (Bouropulous and Moradian - Oldak, 2003).

Mura-galelli *et al* (1991) studied the adsorption of human albumin onto synthetic hydroxyapatite, using a radiotracer technique and a specific flow cell. Adsorption was studied under various conditions corresponding to different thermodynamic paths. It appeared that (i) as is the usual case, the isotherms obtained within a short time range (a few hours) do not correspond to a true equilibrium situation, (ii) when the adsorption

process is followed for longer times (which is necessary) at low bulk concentration, one always reaches the plateau surface adsorption, (iii) this plateau value is independent of the "history" of the adsorption process and corresponds well to the Jamming limit predicted by the random sequential adsorption model' (iv) surface denaturation, leading to enhanced surface binding.

Silvia *et al* (1995) studied the adsorption and desorption of bovine serum albumin and lysozyme on hydroxyapatite. The hydroxyapatite was synthesised through direct precipitation method, subjected to thermal treatments (dried at 100 $^{o}$C, and calcinated at 450 $^{o}$C and 900 $^{o}$C, and labeled as HAP-d, HAP 450 and HAP 900, respectively), and characterised through x-ray diffraction. The Ca/P ratio was determined by fluorescence (FRX), surface composition by XPS, surface area by $N_2$ adsorption at 77 K and size distribution by laser beam diffraction. Adsorption and desorption runs were carried out in stirred batch system. For both proteins, the larger amount adsorbed was close to the isoelectric pH of the protein. For BSA, the adsorption isotherm could be adjusted to the Langmuir's model even in adverse electrostatic conditions and the model parameter correlated well with the type of forces involved. For lysozyme, it was observed that a Langmuir Pattern only at favourable electrostatic condition. Desorption run was accomplished in the presence of indifferent and non-indifferent cations ($K^+$, $Ca^{2+}$ and $Mg^{2+}$). It was shown that lysozyme adsorbed by two types of interactions demonstrating that there are more than one adsorption site. The analysis of adsorption and desorption results revealed that the BSA adsorbed preferentially in the $Ca^{2+}$ sites of HAP even in

adverse electrostatic conditions and lysozyme adsorbed at $PO_4^{3-}$ sites at favourable electrostatic conditions. Both proteins desorbs completely in an appropriate medium.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Reagents

## 3.2 Adsorptive Characteristic of Mucin on Calcium Hydroxyapatite

All reagents used were of analytical grade except where otherwise stated. Distilled water was used in the preparation and dilution of solutions except where also otherwise stated.

### 3.2.1 Synthesis of Calcium Hydroxyapatite

Precipitation method was employed to prepare calcium hydroxyapatite powder. Disodium hydrogen phosphate solution (1100 $cm^3$ of 0.038M containing 5.3945g/1000 $cm^3$) was put in a conical flask and heated on a hot plate to 150 $^oC$, where it was maintained for 55 minutes. The precipitation of HAP was initiated by adding drop-wisely, 400 $cm^3$ of 0.016 M $(CH_3COO)_2Ca$ (containing 1.2654g/1000 $cm^3$) was added with stirring at about 250 r.p.m. using a magnetic stirrer (Ajibola, 1995). After precipitation, the content of the flask was refluxed for about one hour to enhance homogeneity and crystallinity of the apatite (Chiranjeevirao *et al.*, 1982).

The precipitate was filtered and washed repeatedly with distilled water to remove unwanted ions. The precipitate was left covered in distilled water for 14 days to allow "ageing". The precipitate was then filtered and dried in an oven at 105 $^oC$ for about four hours before using it. The resulting HAP cake was ground into a fine powder using an

agate mortar and pestle. X-ray diffraction studies were carried out to confirm the hydroxyapatite.

### 3.2.2 Adsorptive Characteristics of Mucin onto Hydroxyapatite and Calcium Hydrogen Phosphate Dihydrate.

The experimental absorbents used for this study were hydroxyapatite powder of two types. One, with a particle-size ranging from 10 to 20 μm, was prepared by precipitation method using the procedure described above. The other with particle size of 5 – 15 μm was obtained from Sigma-Aldric Co., USA. The control adsorbent was calcium hydrogen phosphate dihydrate powder ranging from 10 to 15 μm in particle size. Mucin obtained from Nacatai, Tesque Inc., Kyoto, Japan (Batch no. M1P960) was used for the adsorption studies.

### 3.2.3 Determination of Thermal Stability of Hydroxyapatite and Calcium Hydrogen Phosphate Dihydrate Procedure

Three sets of an adsorbent (300 mg each) were taken in preweighed crucibles and heated in a furnace (GLM 34PD, Carbonite, Inc., England) at 600 $^{o}$C. After 30 minutes, they were removed and allowed to cool in desiccators, and reweighed. The process of heating, cooling and weighing were repeated until the weight of each sample was constant. The average weight loss was calculated. The above procedure was repeated for all adsorbents used in this study.

Mucin solution (1 %, w/v) was prepared by dissolving 1.00 g of mucin powder in a 100 $cm^3$ volumetric flask and made up to the mark with distilled water.

Another set of mucin solution (5 %, w/v/) was prepared by dissolving 5.00 g of mucin powder in a 100 $cm^3$ volumetric flask and the solution made up to the mark with distilled water.

A Crisson 2000 pH meter coupled with a hand held Hanna ion specific HI 93710 pH meter was used to measure the pH of mucin solutions at 25 $^{o}C$ and at 37 $^{o}C$. The mucin solutions prepared were incubated (1H – 150, Gallenkamp Co., England) at 37 $^{o}C$ (being the physiological temperature).

### 3.3 Determination of Adsorption Capacity of Mucin to HAP and Calcium Hydrogen Phosphate Dihydrate (CHP).

Hydroxyapatite powder (50,100,150 and 300 mg) was respectively weighed into centrifuge tubes.

The *in-vitro* test for amount of mucin adsorbed on to hydroxyapatite was performed by adding 10 $cm^3$ of (1 % or 5 %) mucin solution to each set of the adsorbent. The mixtures were then shaken and incubated at the 37 $^{o}C$ for 30 minutes after which they were centrifuged (Baird and Tatlock Ltd., England) at 2000 r.p.m. for 20 minutes. The supernatant was decanted. Distilled water (10 $cm^3$) was added to the centrifuge tubes and shaken with the hydroxyapatite particles. The supernatant was also decanted. The

precipitate particles of hydroxyapatite with the adsorbed mucin were filtered using filter paper and then dried at 60 °C for 5 hours in an oven. When the coated hydroxyapatite samples were dry, they were weighed using an analytical balance (H15, E. Mettler Co., Switzerland, and accuracy $10^{-4}$ g). The samples were then heated to 600 °C for 30 minutes in a furnace in order to remove the mucin through burning, and the samples were weighed after cooling. The amount of adsorbed mucin was calculated by difference. All the HAP and control samples were treated similarly.

The other type of HAP produced by Sigma – Aldrich Co., USA was studied similarly following the above procedure. Calcium hydrogen phosphate dihydrate (the control material), was also studied using the same procedure above.

### 3.4 Adsorptive Strength to Mucin

An aliquot of 1 % (w/v) mucin solution (10 $cm^3$) was added to 500 mg of the hydroxyapatite sample as well as the calcium hydrogen phosphate dihydrate respectively in centrifuge tubes. The shaking, centrifugation, decanting, and rinsing were carried out under similar conditions as in section 2.3 above. Then 8 $cm^3$ of the supernatant solution of the hydroxyapatite was removed from the precipitates after centrifugation and 8$cm^3$ of distilled water at 37 °C was added to the centrifuge tube. This was also done for calcium hydrogen phosphate dihydrate. The centrifuge tubes were shaken for 1 minute to mix the solution and the hydroxyapatite particles, following which 8$cm^3$ of supernatant was again removed by the same method. The same rinsing procedure as described above was repeated 0, 1, 2, 4, 8 and 12 times in different experimental groups. The samples were

filtered through filter paper and heated to 60 °C for 5 hours. The weight of the samples in the different groups was determined with an analytical balance. The adsorptive strength was evaluated by comparing the weight difference between the non-rinsed and the multiple rinsed samples, and also by comparing the differences in the mass change between the two types of hydroxyapatite and calcium hydrogen phosphate dihydrate.

## 3.5 Mechanism for the Adsorption of Mucin to Hydroxyapatite

Hydroxyapatite (HAP) was obtained from industrial (approximately 25 % solids, suspended in 0.001M phosphate buffer and pH 6.8). A known quantity (4 $cm^3$) of the suspension yielded 1.1 g dry weight of the solid HAP. The calcium/phosphate ratio was 1.63 and a surface area of 25 $m^2/g$. The HAP used in these studies is termed "calcium deficient." Mucin powder was obtained from Nacatai Tesque Inc., Kyoto, Japan (Batch no. MIP960) (Lyohilized Powder, Analytical grade). It was used without further purification.

### 3.5.1 Pretreatment of Hydroxyapatite (HAP) Powder

A known amount (20 mg) of HAP was suspended in 2 $cm^3$ of 0.01M $CaCl_2$, 0.01 M NaCl, and 0.01M $Na_2HPO_4$ for 24 hrs at room temperature. HA powder suspended in double distilled $H_2O$ served as control. The powders were then washed three times with double distilled water and left to dry at room temperature for 24 hrs.

### 3.5.2 Monitoring Mucin Concentration (Protein assay)

Samples of 1.0 $cm^3$ mucin in the concentration range of 0.2-2.0 $mg/cm^3$ were placed in test tubes containing 5.0 $cm^3$ Bradford reagent (Biorad, Richmond CA) and the total volume was adjusted to 8.0 $cm^3$ with double – distilled water. The absorbance of each solution was measured at 595nm using a colorimeter (model 257 Sherwood Scientific Ltd., UK). A calibration curve of absorbance versus the concentration of mucin solution was plotted as shown in Appendix II.

### 3.5.3 Adsorption Isotherm

Another quantity of HAP (20 mg) was placed in a series of test tubes and suspended in 1.0 $cm^3$ of solution containing 0.2 – 1.8 $mg/cm^3$ mucin respectively. The suspensions were shaken for 2 hrs at 37 $^{o}C$ and then centrifuged for 5 minutes. The supernatant (0.2 $cm^3$) was transferred to a test tube containing 5.0 $cm^3$ Bradford reagent and the total volume adjusted to 8.0 $cm^3$ with double distilled water according to the Bradford assay. The absorbance of each solution was measured at 595 nm using a colorimeter. The amount of adsorbed mucin was calculated by subtracting the amount of unabsorbed (free) mucin remaining in the supernatant from the amount of mucin in the control. The results were plotted in the form of a Langmuir adsorption isotherm (see Fig 3.4). The maximum amount of adsorbed mucin and the mucin – HAP association constant were calculated according to the slope and the x-intercept respectively, of the linear curve in Fig 3.4.

$$F/B = 1/K\text{œ}N + 1/N \,.\, F \qquad (2.1)$$

where B = Bound mucin, F = free mucin

Kœ = association constant and N = maximum amount of mucin adsorbed.

The data were then replotted in the form of a scatchard plot (see Fig 3.5).

### 3.5.4 Effect of Cations and pH on Mucin Adsorption onto Hydroxyapatite

A known amount (20 mg) of HAP powder was taken in four replicates. They were respectively treated with $CaCl_2$, NaCl, and $Na_2HPO_4$. Each was suspended in 1.0 $cm^3$ of a solution containing 1.0 mg/$cm^3$ mucin. The suspensions were adjusted to pH 3.0 or 7.0 with citric acid – disodium phosphate buffer and shaken for 2 hrs at 37 $^oC$. They were then centrifuged for 5 minutes. The supernatant (0.2 $cm^3$) was transferred to a test tube containing 5.0 $cm^3$ Bradford reagent and the total volume adjusted to 8 $cm^3$ with double distilled water as before. The absorbance of each solution was measured as described above. The untreated HAP (the fourth replicate) was used as a control.

The y – axis is expressed as the amount of mucin adsorbed to untreated HA which served as control in this case subtracted from the amount of mucin adsorbed to the treated samples.

### 3.5.5 Effect of Incubation Time on the Adsorption of Mucin onto Hydroxyapatite

Untreated HAP (20 mg) and calcium chloride (treated, 20 mg) were respectively placed in test tubes and suspended in 1.0 $cm^3$ of mucin solution (1.0 mg/$cm^3$). The suspensions were shaken at 37 $^oC$ for different times ranging from 1 – 30 hrs. They were then centrifuged for 5 minutes and Bradford assay was performed on 0.2 $cm^3$ of supernatant. The amount of adsorbed mucin was calculated by subtracting the amount of unadsorbed (free) mucin remaining in the supernatant from the amount of mucin in the control

(mucin not suspended in HAP powder). The result was plotted (see Fig 3.6). All experiments were performed in triplicate.

## 3.6 Statistical Analysis

One-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 3.00 for Windows.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1. Results

Results of all the analyses described in chapter two are presented in Tables 3.1 to 3.5 and Figures 3.1 to 3.8. Results are expressed as the average of replicate experiments $\pm$ the standard error of the mean.

**Table 4.1:** Mass of HAP obtained from each set of precipitation of $Na_2HPO_4$ solution with $(CH_3COO)_2Ca$ solution.

| **No. of Replicates** | **Mass of HAP obtained per preparation (g)** |
|---|---|
| 1. | 3.723 |
| 2. | 3.697 |
| 3. | 3.703 |
| 4. | 3.714 |
| 5. | 3.706 |

Mean mass obtained = $3.710 \pm 0.905$

Table 4.2: Adsorption of 5% Mucin Solution to HAP and CHP

| Serial No. | Adsorbent | Weight of adsorbent (mg) a | Weight after adsorption ($60^0$C/5hrs) (mg) B | Weight of adsorbent loss (Correction factor (mg) c | Weight after adsorption ( ht $600^0$C/ 30 mins) (mg) d | c + d | Amount of adsorbed mucin (mg) b – (c + d) | Amount of mucin, % |
|---|---|---|---|---|---|---|---|---|
| 1. | HAP (Synthesised) | 50.0 | 41.30 | 2.46 | 36.40 | 38.86 | 2.44 | 4.88 |
| | | 100.0 | 92.30 | 4.93 | 79.10 | 84.03 | 8.27 | 8.27 |
| | | 150.0 | 136.00 | 7.40 | 112.30 | 119.69 | 16.31 | 10.87 |
| | | 300.0 | 281.10 | 14.79 | 236.70 | 251.49 | 29.61 | 9.87 |
| 2. | HAP (Industrial) | 50.0 | 59.30 | 1.90 | 41.00 | 42.9 | 16.40 | 32.8 |
| | | 100.0 | 121.10 | 3.80 | 93.60 | 97.4 | 23.70 | 23.70 |
| | | 150.0 | 179.20 | 5.70 | 142.30 | 148.0 | 31.20 | 20.8 |
| | | 300.0 | 341.70 | 11.40 | 286.10 | 297.5 | 44.20 | 14.73 |
| 3. | CHP | 50.0 | 40.20 | 3.62 | 34.40 | 38.02 | 2.18 | 4.73 |
| | | 100.0 | 101.20 | 7.24 | 90.50 | 97.74 | 3.46 | 3.46 |
| | | 150.0 | 151.70 | 0.86 | 133.50 | 144.36 | 7.34 | 4.89 |
| | | 300.0 | 310.50 | 21.72 | 271.80 | 293.52 | 16.98 | 5.66 |

Table 4.3: Adsorption of 1 % Mucin Solution to HAP and CHP

| Serial No. | Adsorbent | Weight of adsorbent (mg) a | Weight after adsorption ($60^0$C/5hrs) B | Weight of adsorbent loss (Correction factor) (mg) c | Weight after adsorption (ht $600^0$C for 30 mins) (mg) d | c + d | Amount of adsorbed mucin (mg) b – (c+d) | Amount of mucin % |
|---|---|---|---|---|---|---|---|---|
| 1. | HAP (Synthesised) | 50.0 | 50.70 | 2.46 | 45.2 | 47.66 | 3.04 | 6.08 |
| | | 100.0 | 104.10 | 4.93 | 92.9 | 97.83 | 6.27 | 6.27 |
| | | 150.0 | 152.60 | 7.40 | 132.4 | 139.80 | 12.80 | 8.50 |
| | | 300.0 | 313.70 | 14.79 | 282.1 | 296.89 | 16.81 | 5.60 |
| 2. | HAP (Industrial) | 50.0 | 58.50 | 1.90 | 49.3 | 51.2 | 7.30 | 14.60 |
| | | 100.0 | 110.90 | 3.80 | 92.3 | 96.1 | 14.80 | 14.80 |
| | | 150.0 | 163.30 | 5.70 | 142.4 | 148.1 | 15.20 | 10.10 |
| | | 300.0 | 315.60 | 11.40 | 284.9 | 296.3 | 19.30 | 6.40 |
| 3. | CHP | 50.0 | 47.00 | 3.62 | 41.8 | 45.42 | 1.58 | 3.16 |
| | | 100.0 | 92.80 | 7.24 | 83.9 | 91.14 | 1.66 | 1.66 |
| | | 150.0 | 146.20 | 10.86 | 133.2 | 144.06 | 2.14 | 1.43 |
| | | 300.0 | 301.50 | 21.72 | 276.2 | 297.92 | 3.58 | 1.19 |

Table 4.4: Adsorptive strength to mucin by HAP and CHP – 500mg of adsorbent used

| Serial | Adsorbent | No. of rinses | Weight of adsorbent loss (Correction factor) (mg) a | Weight of adsorbent and mucin (ht $60^0$C/5hrs (mg) b | a+b | Adsorptive strength to mucin (mg) 500 (a + b) | mucin adsorbed (%) |
|---|---|---|---|---|---|---|---|
| 1. | HAP (Synthesised) | 0 | 0.60 | 515.7 | 516.3 | 16.3 | 16.3 |
| | | 1 | 0.60 | 512.4 | 513.0 | 13.0 | 13.0 |
| | | 2 | 0.60 | 510.8 | 511.4 | 11.4 | 11.4 |
| | | 4 | 0.60 | 507.9 | 508.5 | 8.5 | 8.5 |
| | | 8 | 0.60 | 503.8 | 504.4 | 4.4 | 4.3 |
| | | 10 | 0.60 | 503.8 | 504.3 | 4.3 | 4.3 |
| | | 12 | 0.60 | 503.8 | 504.3 | 4.3 | 4.3 |
| 2. | HAP (Industrial) | 0 | 0.20 | 529.4 | 529.6 | 29.6 | 29.6 |
| | | 1 | 0.20 | 528.0 | 528.2 | 28.2 | 28.2 |
| | | 2 | 0.20 | 526.5 | 526.7 | 26.7 | 26.7 |
| | | 4 | 0.20 | 521.7 | 521.9 | 21.9 | 21.9 |
| | | 8 | 0.20 | 516.0 | 516.2 | 16.2 | 16.2 |
| | | 10 | 0.20 | 516.0 | 516.2 | 16.2 | 16.2 |
| | | 12 | 0.20 | 516.0 | 516.2 | 16.2 | 16.2 |
| 3. | CHP | 0 | 1.15 | 511.5 | 512.7 | 12.7 | 12.7 |
| | | 1 | 1.15 | 508.0 | 509.2 | 9.2 | 9.2 |
| | | 2 | 1.15 | 505.8 | 507.0 | 7.0 | 7.0 |
| | | 4 | 1.15 | 503.9 | 505.0 | 5.0 | 5.0 |
| | | 8 | 1.15 | 502.3 | 503.4 | 3.4 | 3.4 |
| | | 10 | 1.15 | 502.1 | 503.2 | 3.2 | 3.2 |
| | | 12 | 1.15 | 501.9 | 503.0 | 3.0 | 3.0 |

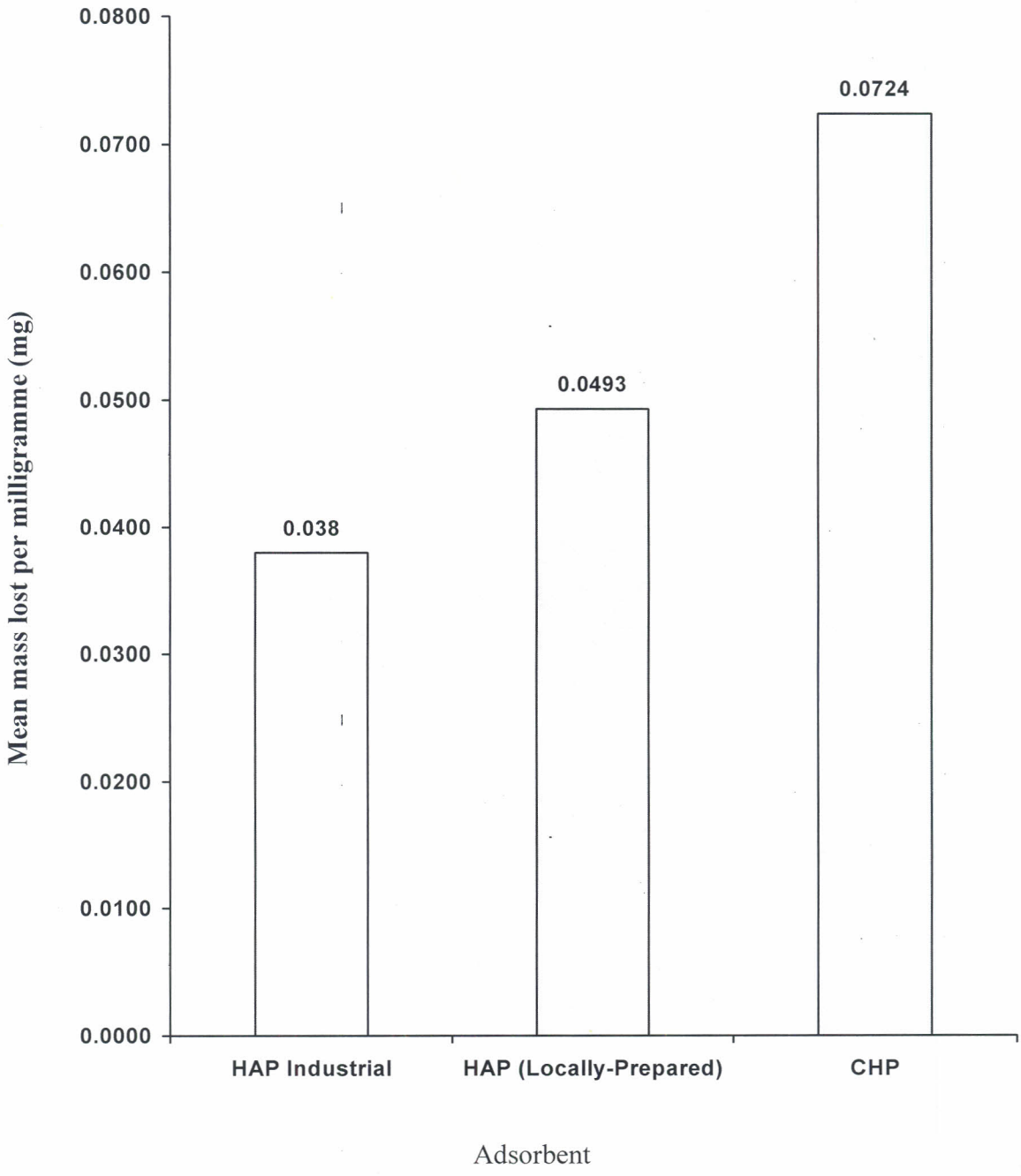


Figure 4.1: Mean mass loss of Adsorbents at 600 $^{o}$C Heating for 30 Minutes.

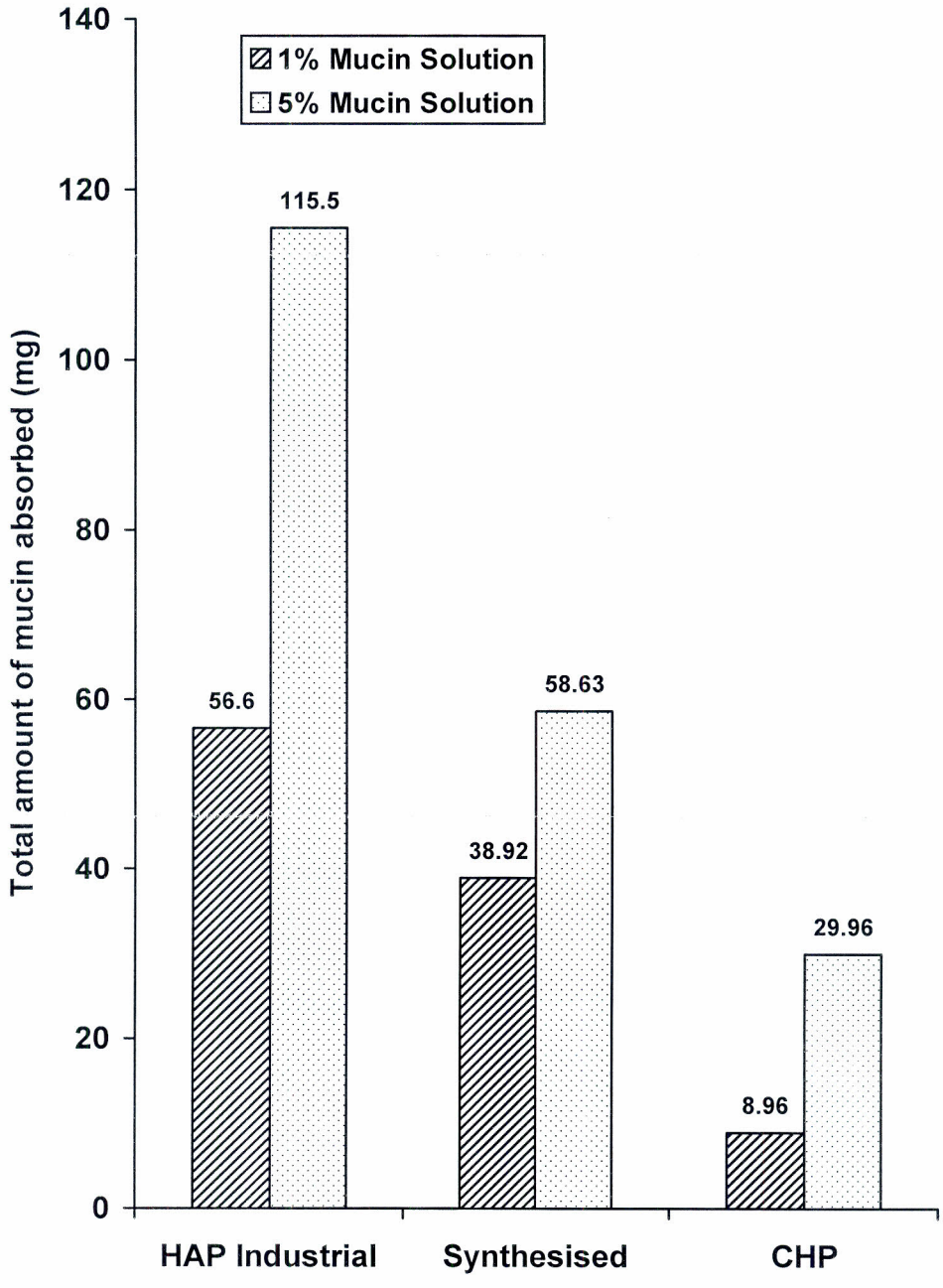


Figure 4.2: Total adsorption to mucin by HAP and CHP concentration dependence.

**Table 4.5: Free and bound mucin concentrations on hydroxyapatite**

| Concentration of mucin ($mg/cm^3$) | Free mucin (F) ($mg/cm^3$) | Bound mucin B (mg) | F/B | B/F |
|---|---|---|---|---|
| 0.20 | 0.12 | 0.08 | 1.50 | 0.667 |
| 0.40 | 0.31 | 0.09 | 3.40 | 0.290 |
| 0.80 | 0.70 | 0.10 | 7.00 | 0.143 |
| 1.00 | 0.90 | 1.10 | 9.00 | 0.125 |
| 1.40 | 1.30 | 1.10 | 13.00 | 0.077 |
| 1.80 | 1.70 | 0.10 | 17.00 | 0.056 |

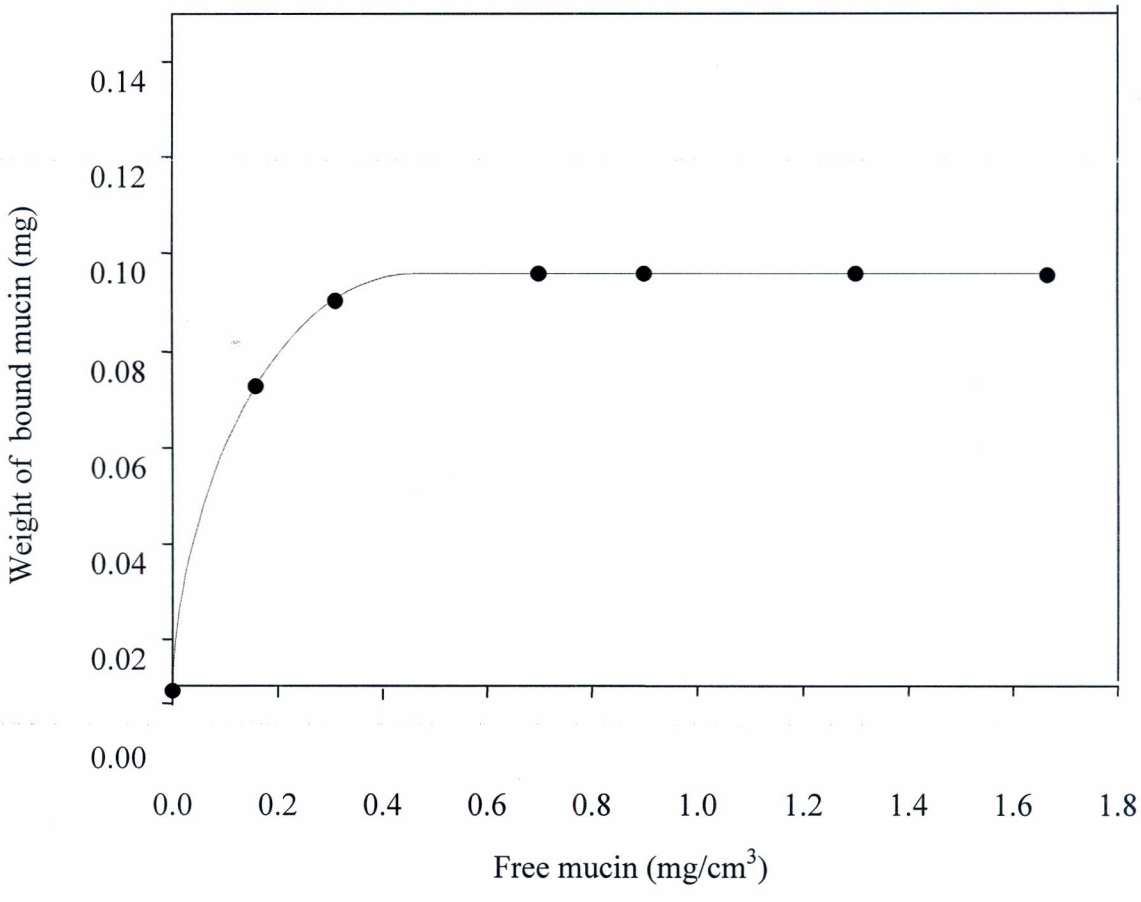


Figure 4.3: Adsorption isotherm of mucin onto hydroxyapatite

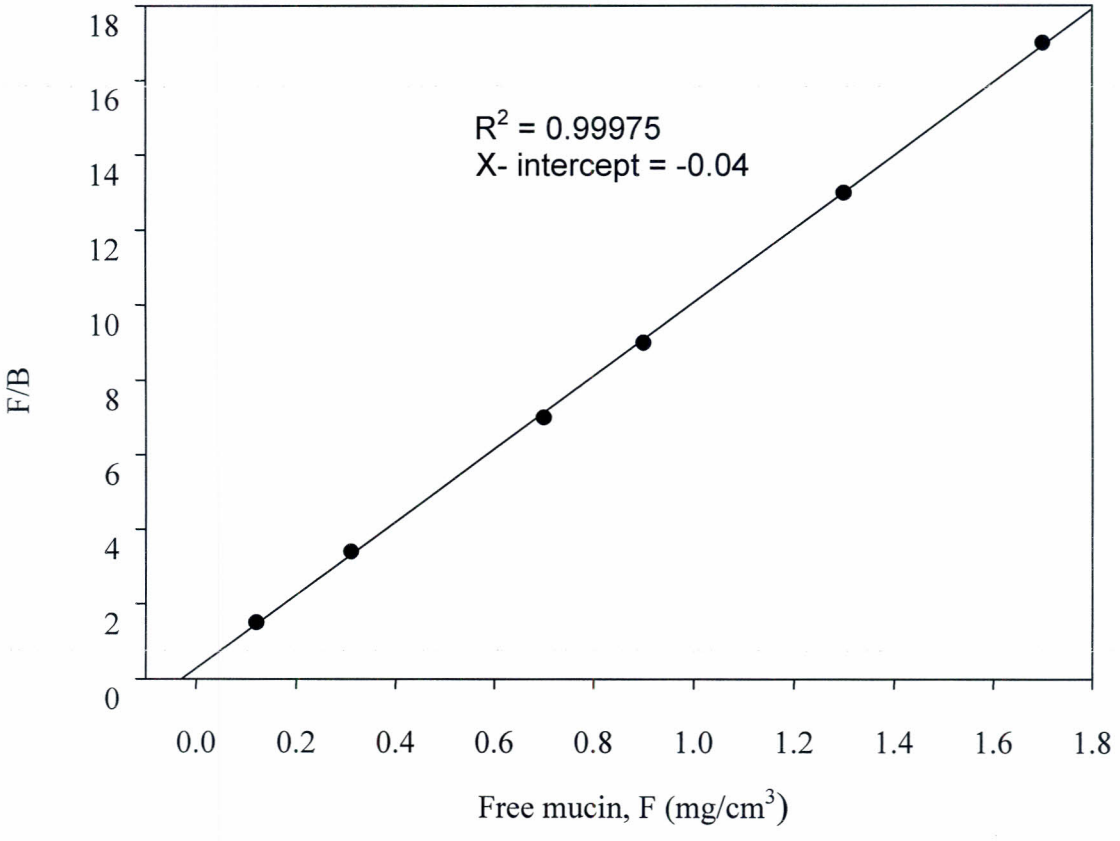


Figure 4.4: Langmuir adsorption isotherm of mucin adsorbed onto hydroxyapatite

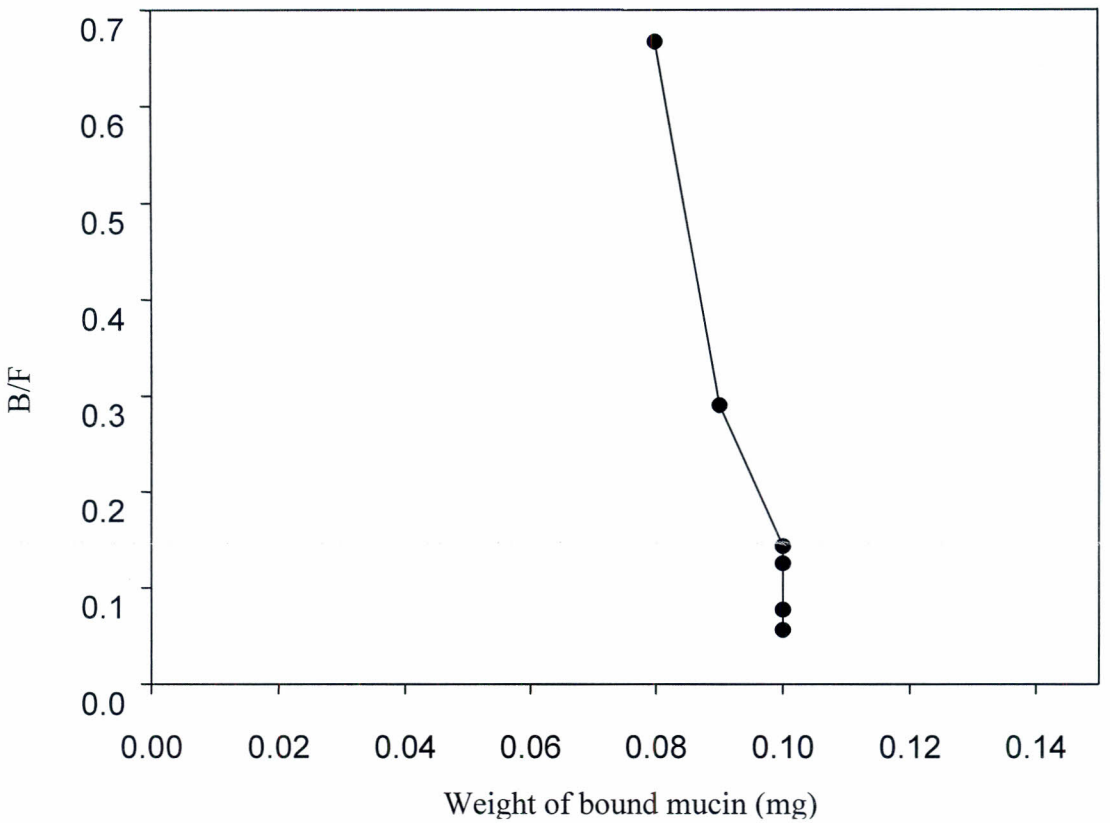


Figure 4.5: Scatchard plot demonstrating lack of linearity in the adsorption of mucin on hydroxyapatite powder

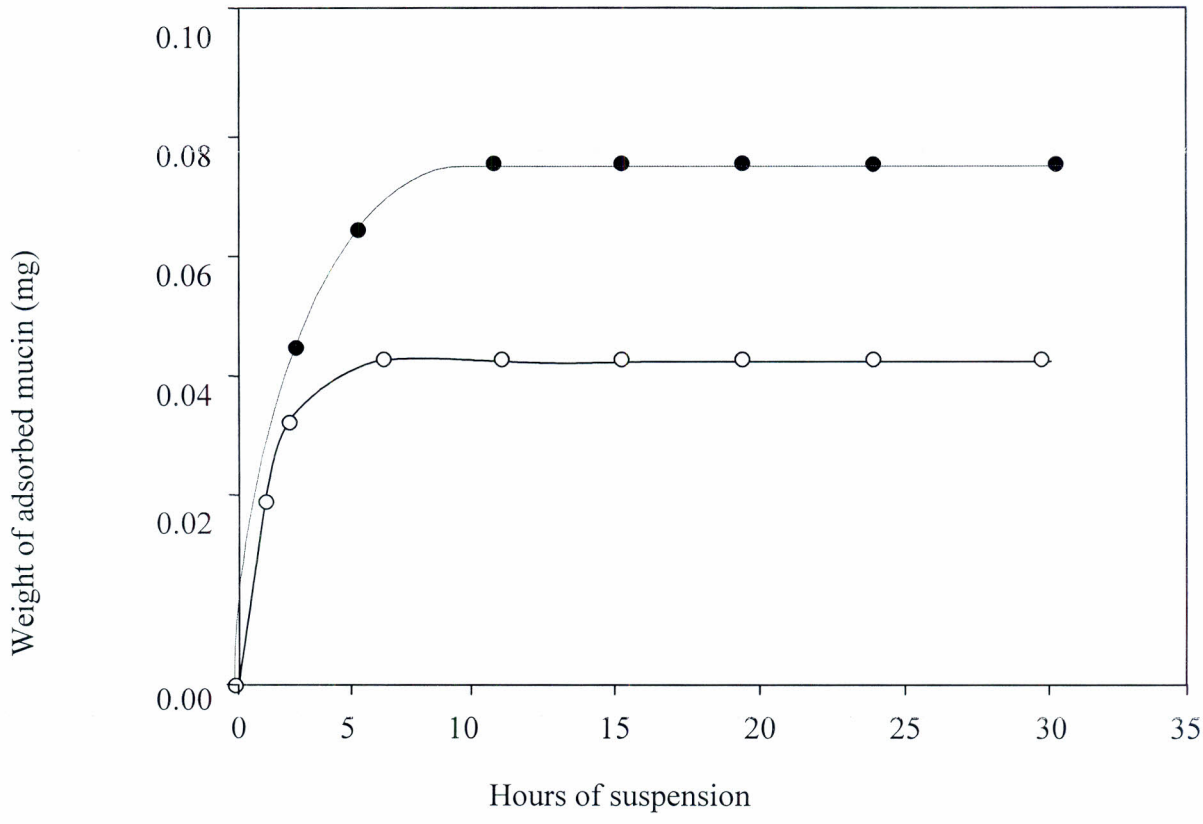


Figure 4.6: Effect of incubation time on the adsorption of mucin in the presence (●—) and absence (○—) of calcium ions

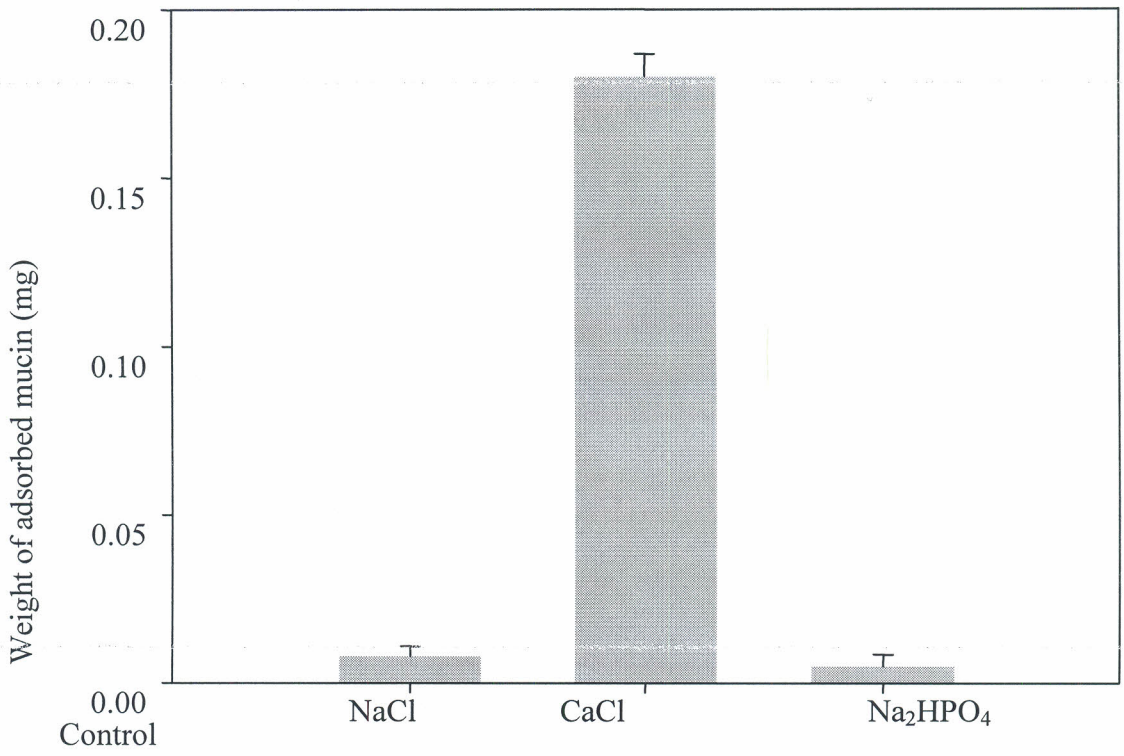


Figure 4.7: The adsorption of 1.0 mg/cm$^3$ mucin to hydroxyapatite pretreated with $CaC1_2$, NaCl or $Na_2HPO_4$

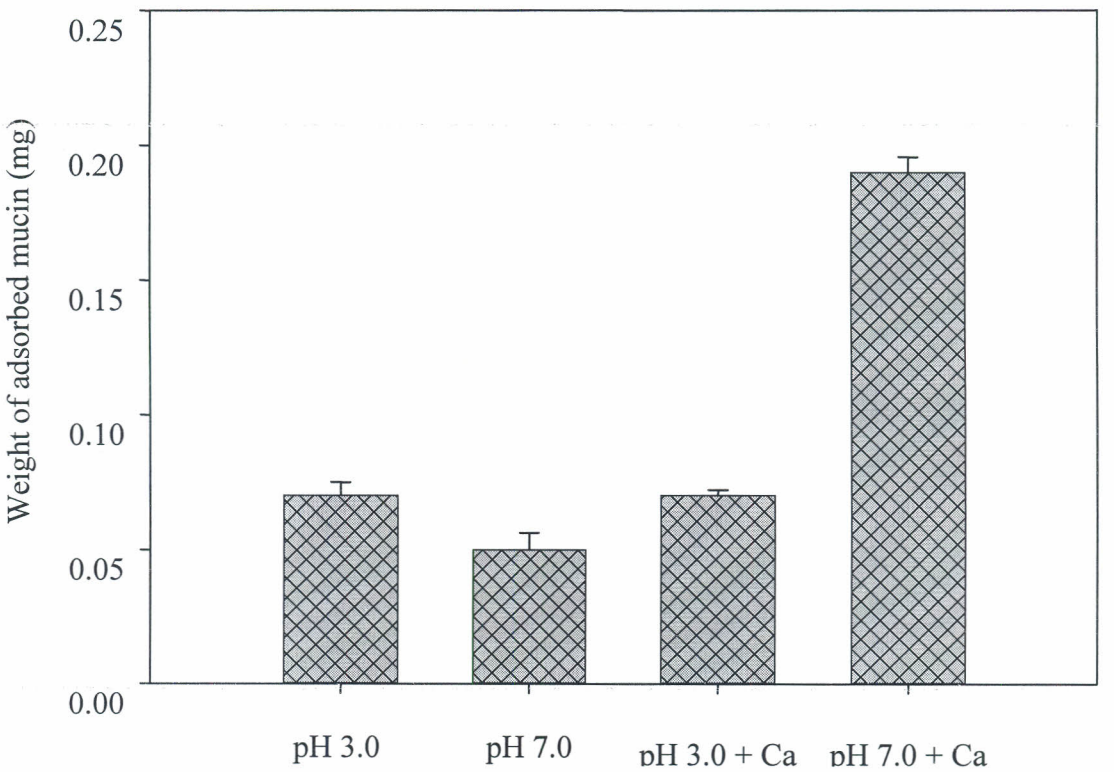


Figure 4.8: The adsorption of 1.0 mg/cm$^3$ mucin to untreated and calcium - treated hydroxyapatite at pH 3.0 or 7.0

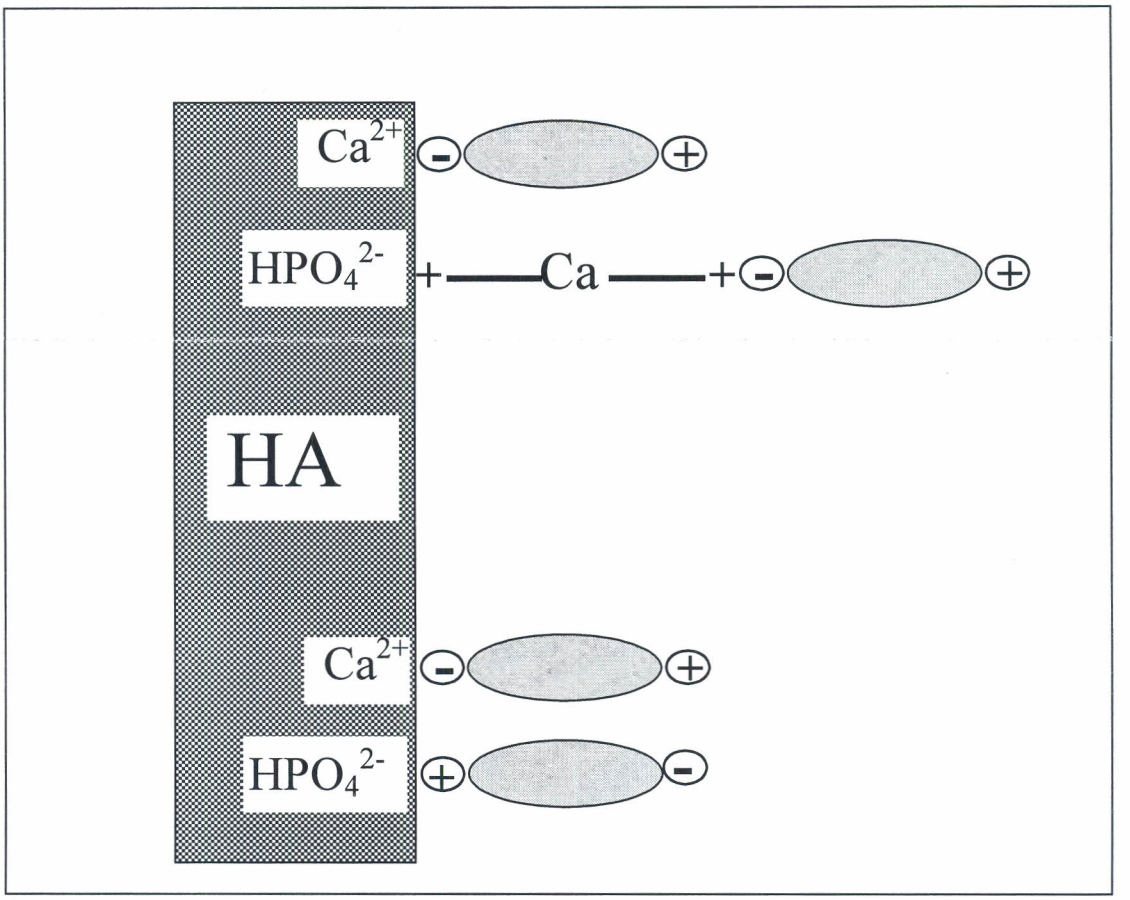


Figure 4.9: The possible mechanism for the initial adsorption of mucin to hydroxyapatite

## 4.2 Discussion

### 4.2.1 Thermal Stability

The following equation represents the decomposition of calcium hydrogen phosphate dihydrate.

$$2[CaHPO_4 2H_2O](s) \xrightarrow{\Delta} Ca_4 P_2 0_7 (s) + 5H_2O(g) \ldots 1$$

The weight of hydroxyapatite was decreased by 1.2% to 98.8% of its original weight after the water was removed by heating at 600 $^0$C for 30 minutes. Calcium hydrogen phosphate dihydrate changed to calcium pyrophosphate when heated to 600 $^o$C for 30 minutes. Theoretically, the weight loss from conversion of calcium hydrogen phosphate dihydrate to calcium pyrophosphate is 26.2% (Motoo *et al.*, 1999).

$$2[Ca_5(PO_4)_3OH]_{(s)} \xrightarrow{\Delta} 3Ca_3(PO_4)_{2(s)} + CaO_{(s)} + H_2O_{(g)} \ldots 2$$

The 95 % confidence limits of the mean mass loss for heating 300 mg were 14.8 $\pm$ 1.95, 11.4 $\pm$ 0.78 and 21.73 $\pm$ 0.13 for HAP synthesised HAP and $CaHPO_4.2H_2O$ respectively at 600 $^0$C for 30 minutes (Fig. 4.1).

### 4.2.2 Amounts of Mucin Adsorbed to HAP (mg)

The weight of mucin adsorbed (mg) to HAP (prepared), industrial HAP and calcium hydrogen phosphate dihydrate were 6.1 %, 14.6 % and 3.16 % in the 50 mg group; 6.27 %, 14.80 % and 1.66 % in the 100 mg group; 8.5 %, 10.13 % and 1.43 % in the 150 mg group; and 5.60 %, 6.43 % and 1.19 % in the 300 mg group, for 1 % mucin solution. (Table 4.3 and Appendix III).

The amounts of mucin adsorbed (mg) to HAP (locally – prepared), Industrial HAP and $CaHPO_4.2H_2O$ for 5 % mucin solution were 4.88 %, 32.8 % and 4.36 % in the 50 mg group; 8.27 %, 23.70 % and 3.46 % in the 100 mg group; 10.87 %, 20.8 % and 4.89 % in the 150 mg group, and 9.87 %, 14.73 % and 5.66 % in the 300 mg group (Table 4.2 and Appendix IV). The adsorption of mucin to hydroxyapatite and calcium hydrogen phosphate dihydrate increased with increase in adsorbent quantity. The amount adsorbed to industrial hydroxyapatite was the highest followed by that of synthesised hydroxyapatite in all experimental groups (see Fig. 4.2). The maximum adsorption ($Ad_{max}$) was recorded as 0.15 mg mucin/1.0 mg for Industrial HAP at 100mg mass of adsorbent using 1 % mucin solution.

The results indicate that the mass of mucin adsorbed to the hydroxyapatite produced by Sigma Co. USA were $1.8 \pm 0.13$ and $4.4 \pm 0.30$ times higher than that onto the synthesized hydroxyapatite and calcium hydrogen phosphate dihydrate respectively. Analysis of Variance (ANOVA) when $P < 0.05$ gives a significance in the adsorption of both types of HAP compared to $CaHPO_4.2H_2O$.

This result conforms with the claim of Motoo *et al* (1999) in the adsorptive characteristic of dextran and albumin onto hydroxyapatite that the percentages of albumin adsorbed to hydroxyapaptite and calcium hydrogen phosphate dihydrate were 17.4 % and 12.5 % in the 50 mg group, and 21.7 % and 17.8 % in the 100 mg group, 19.0 % and 13.3 % in the 150 mg group, 14.7 % and 10.2 % in the 300 mg group. This revealed more adsorption of albumin to hydroxyapatite than to calcium hydrogen phosphate dihydrate in all

experimental groups. The percentages of dextran absorbed to hydroxyapatite and calcium hydrogen phosphate dihydrate were 14.6 % and 7.38 % in the 50 mg group, 13.1 % and 10.3 % in the 100 mg, 12.7 % and 8.57 % in the 150 mg group, and 11.6 % and 7.09 % in the 300 mg group. The adsorptive capacity of mucin is therefore greater than that of dextran onto hydroxyapatite but less than that of albumin.

The experimental data from this work demonstrates that the amount of albumin and dextran adsorbed by hydroxyapatite is about 1.4 $\pm$ 0.13 and 1.6 $\pm$ 0.30 times higher than that of calcium hydrogen phosphate dihydrate. The data also reveal that the adsorptive strength of hydroxyapatite for albumin and dextran is stronger than that of calcium hydrogen phosphate dihydrate.The mechanism of adsorbing to organic substances by hydroxyapatite is considered to be related to hydrogen bonding and zeta potential change between adsorbent and substances which are adsorbed (Motoo *et al.,* 1999).

### 4.2.3 Adsorption Isotherm

As concentration of mucin was increased, there was an initial rapid adsorption followed by a slower approach to a limiting value of 0.95 mg/cm$^3$.

The maximum amount of mucin adsorbed and the affinity between the mucin molecule and the HAP surface were calculated according to the Langmuir adsorption isotherm in (Fig. 4.4). A maximum of 0.104 mg mucin was adsorbed per gramme of HAP (N = 1/slope) and the mucin - HAP association constant was 0.04cm$^3$/ng (Kά = - X intercept). However, the scatachard plot of the same data revealed a lack of linearity as in (Fig 4.5).

This implies a positive cooperativeness and the probable existence of an additional binding site to the one mediated by $Ca^{2+}$ ions.

### 4.2.4 Effect of Ions

Although a positive correlation was observed between the presence of calcium ions and degree of mucin adsorption, binding was evident in calcium – free medium as well (Fig. 4.6). HAP has its own native calcium sites for adsorption and this was increased by the calcium ion treatment which has put the calcium ions more on the surface of HAP. The effect of incubation time on the adsorption of mucin in the absence and presence of calcium ions is shown in Fig. 4.6. The results obtained imply that more than 80% of the adsorbed mucin was adsorbed within 1hr in either the absence or presence of calcium ions. Equilibrium adsorption was attained within 4 hrs.

The results of the use of HAP treated with NaCl, $CaCl_2$ and $Na_2HPO_4$ for binding studies with mucin are shown in Fig. 4.7. The Y – axis is expressed as the amount of mucin adsorbed to untreated HAP which served as the control in this case subtracted from the amount of mucin adsorbed to treated samples. Generally, the amount of unabsorbed mucin in the supernatant decreased following the suspension of mucin in calcium – treated HAP. There was very little adsorption as a result of HAP treatment with NaCl and $Na_2 HPO_4$.

Mucin is an acidic protein and therefore has a net negative charge at 7.0 or higher. This means that it will bind to the calcium groups of HAP through its COOH groups. The

binding of these sites may expose the $NH_4^+$ groups that will bind to the phosphate groups of HAP forming weak hydrogen bonds. $Na^+$ may compete with $NH_3^+$ groups for the phosphate sites on HAP. There was no significant difference ($P<0.05$) in the amount of mucin adsorbed to HAP in the presence or absence of NaCl. It has been reported that $Na^+$ ion has a weak affinity for BSA and HAP (Shimbayashi *et al.,* 1991). Therefore the bridging effect observed with divalent calcium may be absent or negligible. Phosphate addition makes HAP more negatively charged due to surface adsorption (Wang and Somasandaran, 1984).

The phosphate groups are reported to have a higher affinity for HAP surface than the COOH groups of proteins (Shimbayashi *et al.*, 1991). The parameters for the adsorption of various amino acids on HAP (Hay *et al.*, 1984) indicate that the strength of the phosphate bond is more than 20 times greater than that of the carboxyl bond.

The more hydrophilic nature of the phosphates removes water from HAP surface upon adsorption. The level of mucin adsorbed on HAP pretreated with $Na_2HPO_4$ is therefore probably due to the successful competition of phosphate for calcium groups of HAP, thereby lowering the number of binding sites ordinarily available to mucin on an untreated HAP (Fig. 4.7).

### 4.2.5 Effect of pH on Adsorption of Mucin onto Hydroxyapaptite

The net electric charge of a protein depends on its isoelectric pH (PI) and the pH of the environment. The pH of mucin solution in distilled water lies between 4.6 and 4.9 (Wang

and Somasandaran, 1984). Mucin undergoes a neutral – acid transition and becomes negatively charged at higher pH values while at lower pH values mucin undergoes a neutral – basic transition and becomes positively charged. The isoelectric point of HAP has been found to be between pH 6.4 and 8.5 depending on the experimental method used (Doss, 1976; Arends, 1979; Wang and Somasandaran, 1984). Since a high solid/liquid ratio is expected for the *in vivo* situation, in this work, a solid/liquid ratio of 20g/L of HAP was used. This means that at pH 7.0 the zeta – potential for HAP system will be positive (Wang and Somasandaran, 1984; Arends, 1979). Since mucin is negative at pH 7.0 (that this work was conducted principally), electrostatic attraction played a very prominent role in the interaction of mucin with HAP surface. This is substantiated by the result shown in Fig. 4.8. At a lower pH 0/3.0, the mucin molecules are positively charged. This makes the presence of $Ca^{2+}$ ions irrelevant to the adsorption of mucin to HAP surface. This can also be explained by the shift in the zeta – potential of HAP at this pH. As seen from Fig. 4.8 at pH – 7.0, the negatively charged mucin molecule and the HAP surface make the bridging action of the divalent calcium ions very relevant and increased adsorption resulted as shown in the Fig. 4.8 and illustrated in Fig 4.9.

In Fig. 4.9 mucin is mainly adsorbed by electrostatic attraction between the COOH group of mucin and the calcium ion on the surface of HAP. It has been reported that when labeled calcium and phosphate ions are added to HAP, only phosphate ions are released when acidic proteins are adsorbed. Acidic proteins therefore exchange with phosphate and are adsorbed to calcium ions (Rolla *et al.,* 1982). The neutral and positive parts of mucin bind weakly to HAP.

# CHAPTER FIVE

# CONCLUSION AND RECOMMENDATIONS

## 5.1 Conclusion

It can be seen that when a biomaterials is implanted into the body, blood proteins start to absorb on the surface and this causes an immune response to the biomaterial. The study has revealed that the amount of mucin adsorbed onto industrial hydroxyapatite was higher than that adsorbed onto synthesised hydroxyapatite and calcium hydrogen phosphate dihydrate. This indicates that hydroxyapatite is a better adsorbent than calcium hydrogen phosphate dihydrate.

The results suggest that electrostatic attraction is the main mechanism involved in the adsorption of mucin onto hydroxyapatite (HAP) and the adsorbtion process is described by the Langmuir mode. Treatment of HAP with phosphate and sodium ions did not increase the amount of mucin adsorbed.

The calcium ions serve as binder between the negatively charged mucin molecule and the HAP surface. In light of the abundance of calcium in saliva and the variations in pH occurring during and between meals, this study has revealed the mechanism of the binding of salivary components (mainly mucin) to HAP structure *in vivo.*

## 5.2 Recommendations

Based on the findings from this work, the following recommendations are made.

- The relationship between the stability of hydroxyapatite and ageing should be studied.
- The mechanism for the adsorption of mucin to hydroxyapatite was studied in *vitro*. There is need to study the mechanism in *vivo*.
- More than one protein should be studied at the same time, because many proteins will come in contact with the biomaterials in the body system simultaneously.

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## APPENDIX I

## EFFECT OF HEAT ON ABSORBENTS AT 600 °C

Table 1: The effect of heat on HAP ( prepared) at 600 °C for 30 mins.

| Serial No | Weight after heating (mg) | Weight loss (mg) |
|---|---|---|
| 1. | 284.3 | 15.7 |
| 2. | 285.5 | 14.5 |
| 3. | 285.8 | 14.2 |
| 4. | 285.5 | 14.5 |
| 5. | 284.3 | 15.7 |
| 6. | 285.8 | 14.2 |

Mean mass lost = 14.8 ± 0.458

Mass lost / mg = 0.0493 ± 0.0015

**Table 2: The effect of heat on Industrial HAP at 600 °C for 30 mins.**

| Serial | Weight after heating (mg) | Weight loss (mg) |
|---|---|---|
| 1. | 288.9 | 11.1 |
| 2. | 288.6 | 11.4 |
| 3. | 288.3 | 11.7 |
| 4. | 288.9 | 11.1 |
| 5. | 288.3 | 11.7 |
| 6. | 288.6 | 11.4 |

Mean mass lost = 11.4 ± 0.173

Mass lost / mg = 0.0380 ± 0.0006

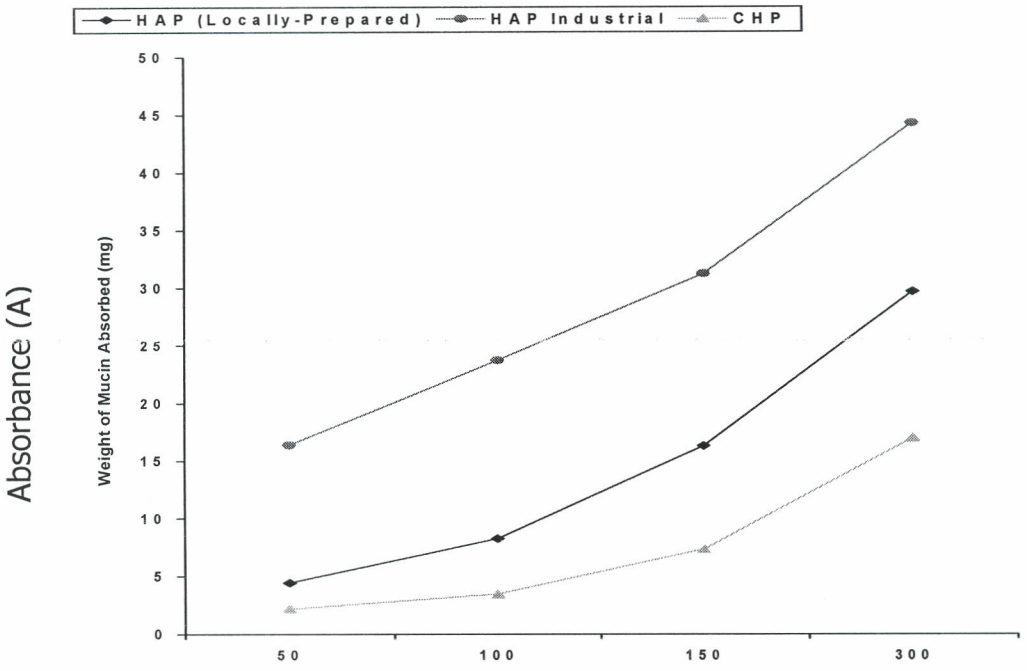
**Table 3: The effect of heat on $CaHPO_4.2H_2O$ at 600 °C for 30 mins.**

| Serial | Weight after heating (mg) | Weight loss (mg) |
|---|---|---|
| 1. | 278.2 | 21.8 |
| 2. | 278.3 | 21.7 |
| 3. | 278.3 | 21.7 |
| 4. | 278.2 | 21.8 |
| 5. | 278.3 | 21.7 |
| 6. | 278.3 | 21.7 |

Mean mass lost = 21.73 ± 0.0330

Mass lost / mg = 0.0724 ± 0.0001

# APPENDIX II




Concentration (mg/ $cm^3$)

Figure 1: Absorbance against concentration (mg/$cm^3$) of mucin

## APPENDIX III

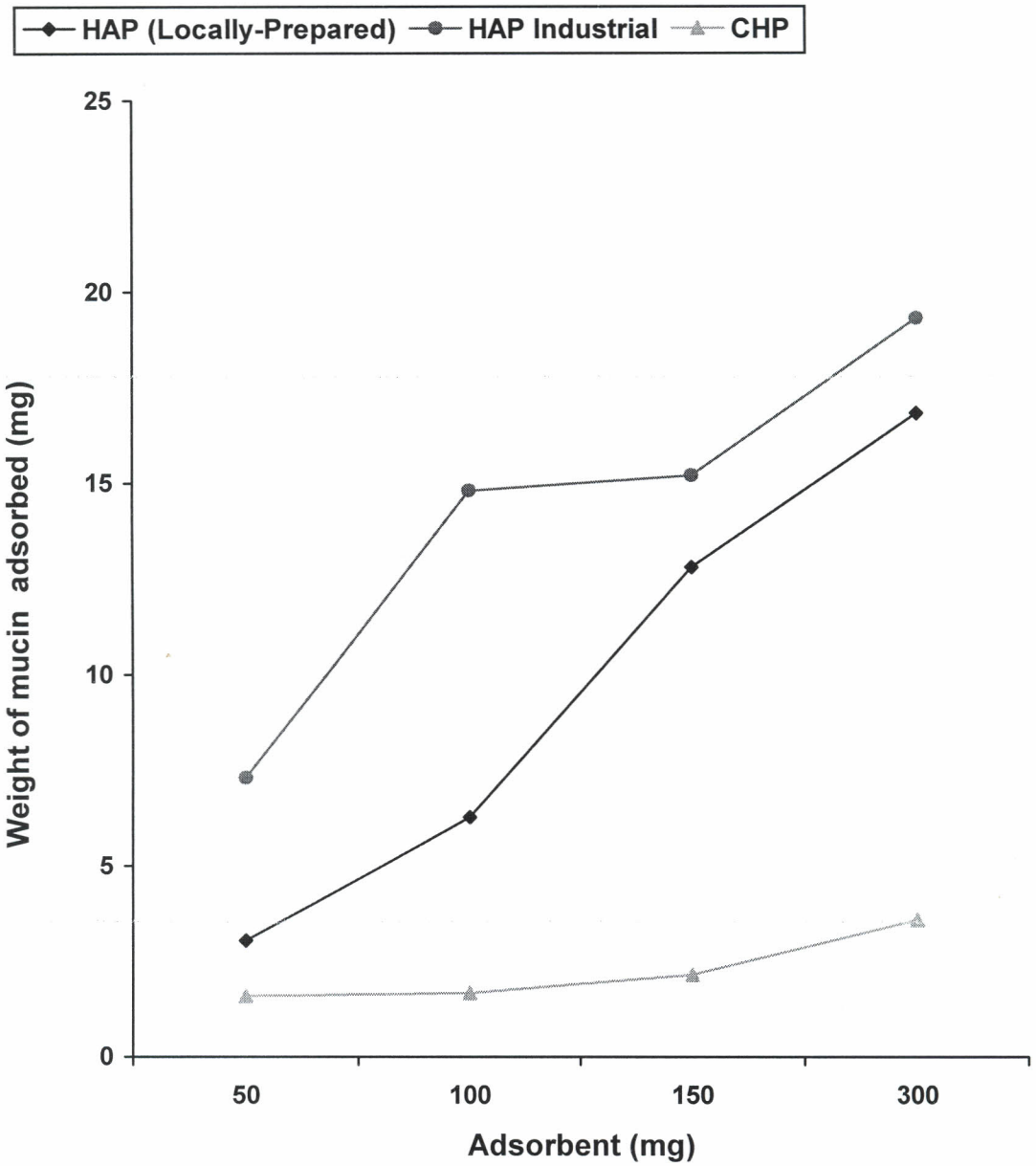



Figure 2: Adsorption to 1% mucin solution by HAP and CHP.

## APPENDIX IV

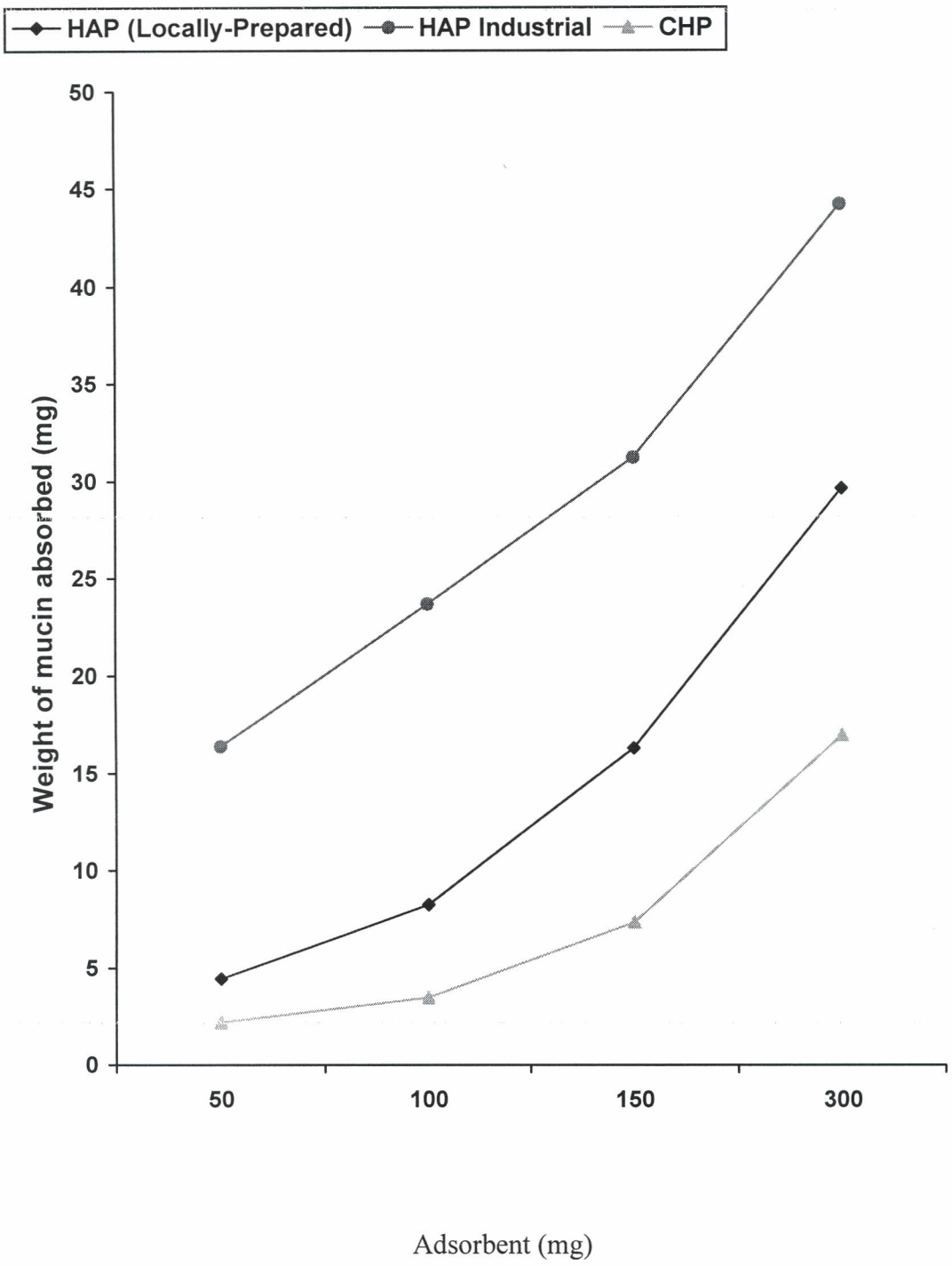



Figure 3: Adsorption to 5% mucin solution by HAP and CHP

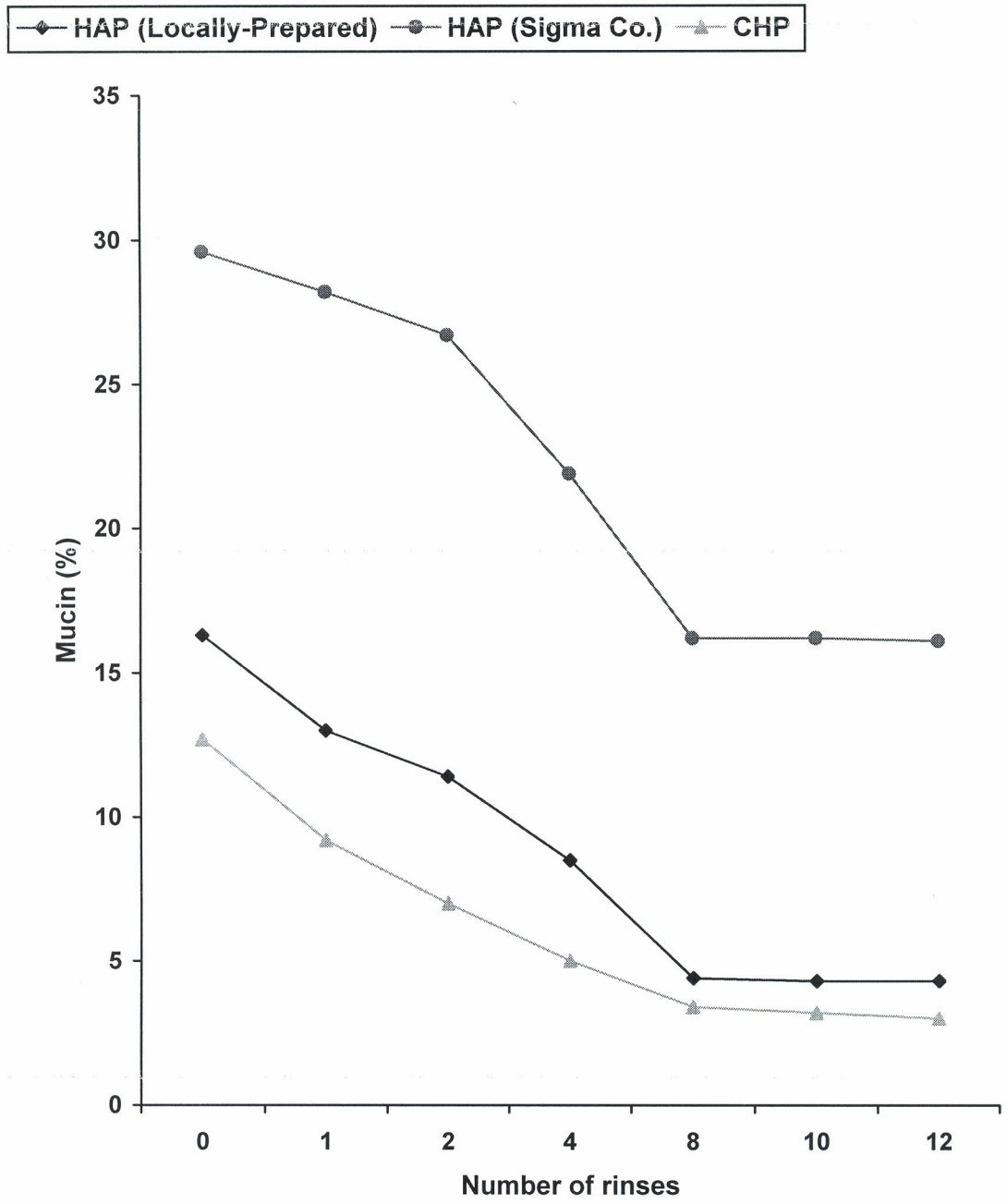
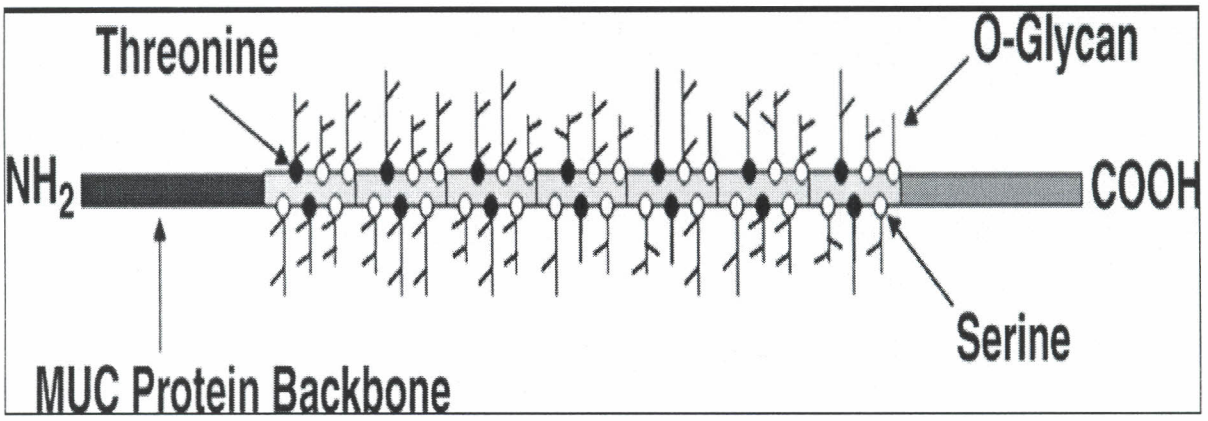



Figure 4: Percentage of mucin adsorbed after rinsing.

## APPENDIX VI




**TYPICAL STRUCTURE OF MUCIN**

# APPENDIX VII

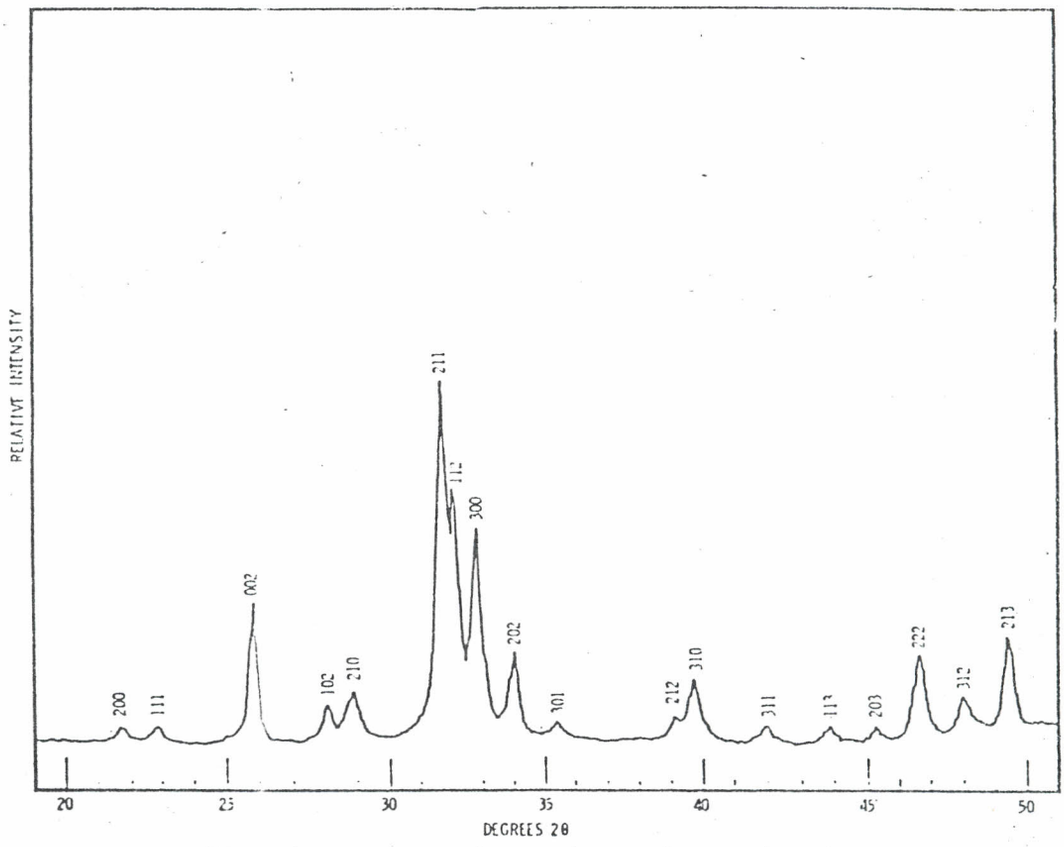



Figure 6: X-Ray diffraction patterns of synthetic hydroxyapatite. The Miller Indices are shown (Posner, 1969).