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EVALUATION OF THE CHARACTERISTICS OF THE ADSORPTION OF FIBRINOGEN ONTO HYDROXYAPATITE

***Jalija, D.O.[1] and Lori, J.A.[2]**
[1]Ahmadu Bello University, School of Basic and Remedial Studies, Funtua, Nigeria
[2]Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria
*Correspondence author: danieljalija@yahoo.com

ABSTRACT
Three chemical substances namely Hydroxyapatite, HAP (Sigma), HAP (locally prepared) and calcium hydrogen phosphate dehydrate (CHP), were investigated for the adsorption of fibrinogen onto them. Of the three, the Sigma sample of HAP adsorbed the highest amount of fibrinogen. Increase in the concentration of fibrinogen up to 0.8mg/cm^3 resulted in increased adsorption onto HAP. The adsorption followed the Langmuir model (which is typically monolayer adsorption). For the Sigma sample of HAP, there was increase in the amount of fibrinogen adsorbed with time but it remained constant after 120 minutes. Desorption after this time is attributable to conformational changes in the molecule. Treatment of HAP with calcium and magnesium ions increased the amount of fibrinogen adsorbed onto it as against treatment with potassium ion (a monovalent ion). Electrostatic attraction on the surface of the treated HAP and hydrogen are responsible for the adsorption. The results are useful in fabricating bone and teeth implants that are compatible with body fluid and blood.

Keywords: Hydroxyapatite, Fibrinogen, Biocompatibilty, Implants

INTRODUCTION

There has been a huge development in biomaterials that are specially designed to repair and reconstruct damaged or diseased parts of the human body (Hench, 1991). The characterization of these materials is of utmost necessity for the optimization of their biological and chemical properties (Akiyosi *et al*., 1994). These materials often are detected as foreign bodies by the patient's immune system and sometimes interact with the body in an undesirable manner.

Hydroxyapatite is the most commonly used, specially designed biomaterial for repair and reconstruction of damaged or diseased parts of the human bone due to its widely accepted biocompatibility. Hydroxyapatite is especially suitable for implantation since it is chemically close to the calcium phosphate that is the mineral phase of bone. This similarity encourages the bone to accept the material as its own with minimal adverse reactions (Constatino *et al*., 1992;). Hydroxyapatite is widely utilised as a component in oral hygiene agents. It has been discovered that hydroxyapatite adsorbs, amino acids, lipids and polysaccharides. Tooth plaque contains water, proteins and also dextrin – a polysaccharide produced by bacteria in the mouth. Hydroxyapatite particles could be added to toothpaste as an important component for removing plaque adhered to tooth surfaces and/or to eliminate mouth odour caused by oral disease (Aoki *et al*., 1982; Tamura *et al*., 1995).

The adsorption of proteins on the surface of biomaterials has been considered to be the most important issue in evaluating their biocompatibility (Shi, 2000). The purpose of this study is to characterize the adsorption of fibrinogen, a plasma protein on the biological calcium hydroxyapatite of bones and teeth. It has been undertaken as part of an on-going attempt to fabricate biomaterials for bone and teeth implants with biocompatibility with body fluid and blood.

The adsorptive characteristics of protein on biomedical materials especially, hydroxyapatite has not been reported extensively. The project is also aimed at providing scientific data to help in understanding the adsorptive capacity and characteristics of fibrinogen on hydroxyapatite. A comparative study has also been carried out using calcium hydrogen phosphate dihydrate, which is also an important component in a number of oral hygiene products.

MATERIALS AND METHODS

Preparation of Calcium Hydroxyapatite

The precipitation method of Ajibola (1995) was used to prepare the HAP powder. In this preparation, 700cm^3 of 0.30M orthophosphoric acid was placed in a conical flask and heated on a hot plate at 150^{o}C. The precipitation of HAP was initiated by adding dropwisely, 500cm^3 of 0.5M $Ca(OH)_2$ to the boiling acid, with stiring at about 250 r. p.m. using a magnetic stirrer. After precipitation, the content of the flask was refluxed for one hour to enhance homogeneity and crystallinity of the apatite (Chiranjeevira *et al*., 1985). The precipitate was filtered and washed repeatedly with distilled water to remove unwanted ions. The HAP precipitate was aged in distilled water for 14 days. It was then filtered and dried in an oven (Gallenkamp Co., England) at 105^{o}C for four hours before use. The resulting HAP cake was ground into a fine powder using a mortar and pestle.

Protein Assay

The Biuret method (Holme and Peck, 1998) was used to determine fibrinogen concentration throughout this study.

Samples of 0.1cm^3 fibrinogen solution were placed in test tubes and the volumes were made up to 1.0 cm^3 with distilled water. Biuret reagent (4.0 cm^3) were added to each test tube thus bringing the total volume of each sample to 5.0 cm^3. The absorbance of the solutions were measured at 540nm using CECIL 1020 UV – visible spectrophotometer. The concentration of fibrinogen was calculated with reference to a calibration curve of standard solutions of bovine serum albumin (Sigma).

Evaluation of the Characteristics of the Adsorption of Fibrinogen onto Hydroxyapatite (HAP) and Calcium Hydrogen Phosphate Dihydrate (CHP)

The adsorbents used for this study were two samples of HAP powder. One of these with a particle size ranging from 5-15 µm mesh size was purchased from Sigma – Aldrich Co., USA. The other with a particle size ranging from 10-20 µm was prepared by the precipitation method described above. The CHP powder ranging from 10-15 µm mesh size served as control.

Adsorptive Amount of Fibrinogen to HAP and CHP

Varying amounts of the HAP (Sigma) powder (50,100,150, 200 and 300 mg) were respectively weighed into five centrifuge tubes. The fibrinogen solution prepared earlier was placed in an incubator (1H-150, Gallenkamp Co., England) at the physiological temperature of 37^{o}C. The test for the adsorptive amount of fibrinogen onto HAP was performed by adding 1.0 cm^3 of 1.0 mg/ cm^3 (or 0.1% w/v) fibrinogen solution to each set of the adsorbent. The mixtures were then shaken while incubating at the physiological temperature (37^{o}C) for 1 hour. They were then centrifuged using bench auto-centrifuge (Baird and Tatlock Ltd., England) at 2000 r.p.m. for 5 minutes. The supernatants were removed and the protein assay described above was performed on 0.1cm^3 samples of the supernatants to determine the amount of unabsorbed protein present and hence by difference determine the amount of fibrinogen absorbed in each set of adsorbent. The prepared HAP and the CHP were also studied using this same procedure. The results are presented in Figure 1.

Langmuir Adsorption Isotherm of Fibrinogen Adsorbed onto HAP

One hundred milligram of HAP powder (Sigma Co.,USA) was placed in each of six test tubes and suspended in 1.0cm^3 of solutions containing 0.2-1.8mg/ cm^3 fibrinogen. This was followed constant shaking for 2 hours at 37^{o}C. The suspensions were allowed to settle and the supernatants were collected . The amount of protein adsorbed was calculated by subtracting the amount of unabsorbed (free) protein These results are similar to the findings of Motoo *et al*, (1999) on the adsorptive characteristics of dextran remaining in the supernatant from the amount of protein in the control (fibrinogen not suspended in HAP powder sample) and plotted in the from of a Langmuir adsorption isotherm. The maximum amount of adsorbed fibrinogen and the fibrinogen-HAP association constant were calculated from the slope and the x-intercept respectively of the linear curve

$F/B=1/K_{\infty}N + 1/N.F$

where B = bound fibrinogen, F = Free fibrinogen;

K_{∞} = association constant and N= maximum amount of fibrinogen adsorbed.

The results are shown in Figure 2.

Effect of Incubation Time on the Adsorption of Fibrinogen onto HAP

One hundred milligram of HAP powder samples were placed in five centrifuge tubes. Each sample was then suspended in 1.0cm^3 of a 1.0mg/ cm^3 fibrinogen solution for different periods of time (30, 60, 90,120 and 180 minutes respectively) in an incubator at 37^{o}C. Each of the sample tubes was removed and the contents were centrifuged for 5 minutes and protein assay was performed on the supernatants collected from each sample. The same procedure was followed for the CHP powder. The results are presented in Figure 3.

Effect of Cations on the Adsorption of Fibrinogen onto HAP

Pretreatment of HAP powder was carried out according to the procedure described by Lori and Nok (2004) . One hundred milligrammes of Sigma HAP powder was suspended for 24 hours at room temperature in 1.0 cm^3 of 0.1M $CaCl_2$, $MgCl_2$ and 0.1M KCl. HAP powder suspended in double distilled water served as control. The powders were then washed three times with double-distilled water and left to dry at room temperature for 24 hours.

Effect of Pretreatment of HAP on Adsorptive Amount

One hundred milligrammes each of untreated HAP, calcium treated HAP, magnesium treated HAP and potassium treated HAP powders (prepared as described above) were suspended in 1.0cm^3 of a solution containing 1.0mg/ cm^3 of fibrinogen. The suspensions were shaken for 2 hours at 37^{o}C and the adsorbed fibrinogen was measured as described earlier. The results are presented in Figure 4.

RESULTS AND DISCUSSION

Amounts of 0.1% (w/v) Fibrnogen Adsorbed onto HAP and CHP

As Figure 1 bellow shows the adsorption of fibrinogen onto HAP and CHP increased with increase in adsorbent quantity. The adsorbed amounts of fibrinogen to HAP (Sigma) showed a sharp increase from the use of 100 mg of the adsorbent while in the case of HAP (prepared), the sharpest increase was observed with the use of 150 mg of the adsorbent. The amount of fibrinogen adsorbed onto HAP (Sigma) was the highest followed by the HAP (prepared). It is evident that CHP is not a good adsorbent compared to HAP (150 mg mass of the adsorbent).

and albumin onto HAP in their attempt to eliminate mouth odour and tooth plaque using these

substances. Their results indicate that more albumin and dextran were adsorbed onto HAP than CHP in all experimental groups.

Langmuir Adsorption Isotherm

The adsorption isotherm for the adsorption of fibrinogen onto HAP is shown in Figure 2. It shows that increasing the concentration of fibrinogen from 0.2 to 0.8mg/cm^3 resulted in increase in adsorption of fibrinogen onto HAP. Higher concentrations of fibrinogen did not result in an increase in fibrinogen adsorption to HAP.

The Langmuir adsorption isotherm (Figure 2) reveals a linearity in the adsorption process with a maximum fibrinogen adsorption at 0.109mg/100mg HAP powder (N= 1/slope). The fibrinogen–HAP association constant was 0.088 cm^3/mg (K∞ = −x intercept). This shows that the adsorption process of fibrinogen to HAP follows the Langmuir model which depicts a monolayer adsorption.

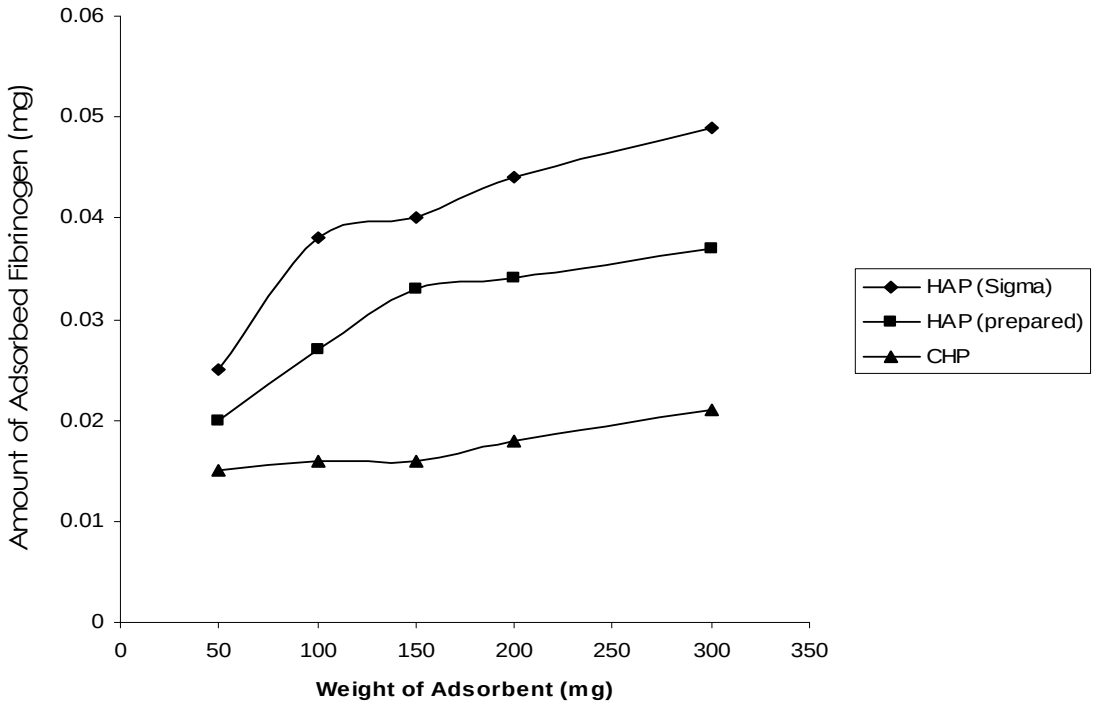

Figure 1: Adsorption of 0.1% fibrinogen solution onto HAP and CHP

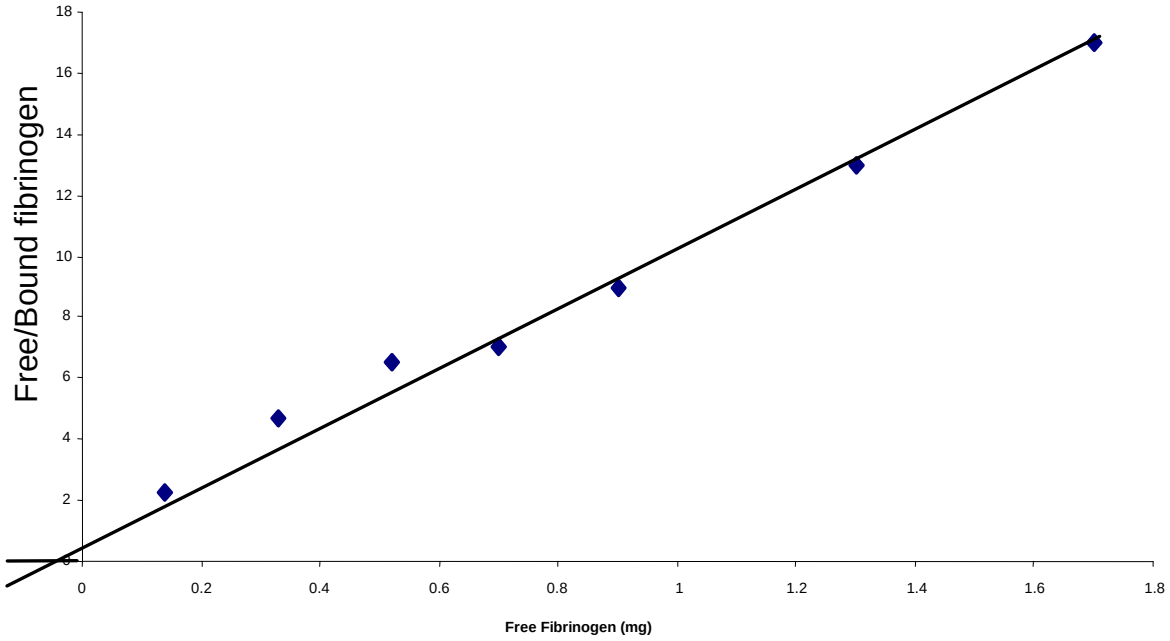

Fig.2 Langmuir adsorption isotherm of fibrinogen Adsorbed onto HAP

Effect of Incubation Time on Adsorption of Fibrinogen onto HAP and CHP

As Figure 3 shows, for the HAP (Sigma), optimum adsorption occurred at or before 60 minutes. There was a decrease in the amount of fibrinogen adsorbed after that time and the amount adsorbed became constant after 120 minutes. For the prepared HAP, optimum adsorption was recorded at 90 minutes, with the values becoming fairly constant after that time. For the CHP, there was little change in the amount of adsorbed fibrinogen as a function of time. The results are similar to those obtained by Soderquist and Walton (1980) and Van and Norde (1983). These authors observed a maximum adsorption of Bovine Serum Albumin onto titanium at 10 and 15 minutes for 0.1 and 0.5 gl^{-1} BSA respectively after which there was a decrease in adsorption to a steady state. Previous studies of albumin adsorption on to other surfaces also recorded this kinetic 'overshoot' (Soderquist and Walton, 1980). These studies therefore indicate that desorption after sometime may be the result of rearrangements in the structure of already adsorbed molecules. Conformational changes in the molecules lead to some unfolding which result in an increased number of protein sites contacting the surface. Some protein molecules may become detached in favour of 'spreading' of the other adsorbed molecules.

Effect of Cations on Adsorption of Fibrinogen onto HAP

From Figures 4, it is evident that the amounts of fibrinogen adsorbed by HAP in the presence of KCI, $CaCI_2$ and $MgCI_2$ respectively were 0.039 mg, 0.066mg and 0.068mg. The amount of fibrinogen adsorbed in the control (i.e. untreated HAP) was 0.037mg. It can be seen here that the divalent cations (Ca^{2+} and Mg^{2+}) increased the amount of fibrinogen adsorbed onto HAP over and above the amount adsorbed by untreated HAP while the monovalent cation (K^+) did not affect the adsorption in the same way. Lori and Nok (2004) reported similar results in respect of their work on the mechanism of the adsorption of mucin onto titanium. The results here are also similar to that reported by Hughes-Wasssel *et al*, (1995) in their work on the adsorption of BSA onto HAP. They reported that the addition of calcium ion leads to an increase in A_T (maximum amount of protein adsorbed) and K (affinity constant) for the adsorption of the protein onto HAP. Calcium is known to bind to many blood proteins (Shimabayashi *et al.*, 1991) and the increase in A_T and K values is therefore possiblly due to calcium ion bridging between fibrinogen and HAP.

The calcium ion possesses two positive charges. With one it binds to the OH^- on the surface of the HAP and with the other it binds with the negatively charged fibrinogen molecule. This leads to the enhanced adsorption of the protein by the HAP.

It has been shown by Rolla *et al*, (1982) 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 ions and are adsorbed to the calcium ions. The treatment with calcium ion addition makes the HAP positively charged due to surface adsorption. The interaction of protein with HAP is likely to occur by participation of charged domains with specific surface sites and by hydrogen bonding of neighboring parts of the molecule with polar surfaces.

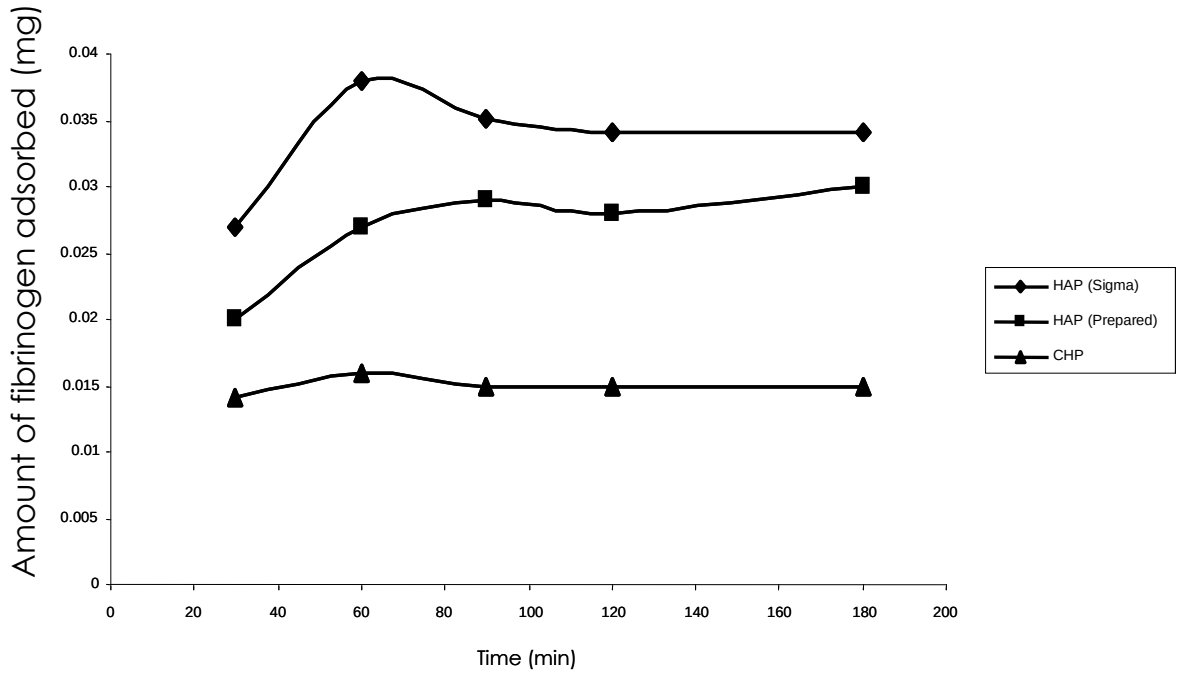

Fig. 3: Effects of incubation time of adsorption of fibrinogen onto HAP and CHP

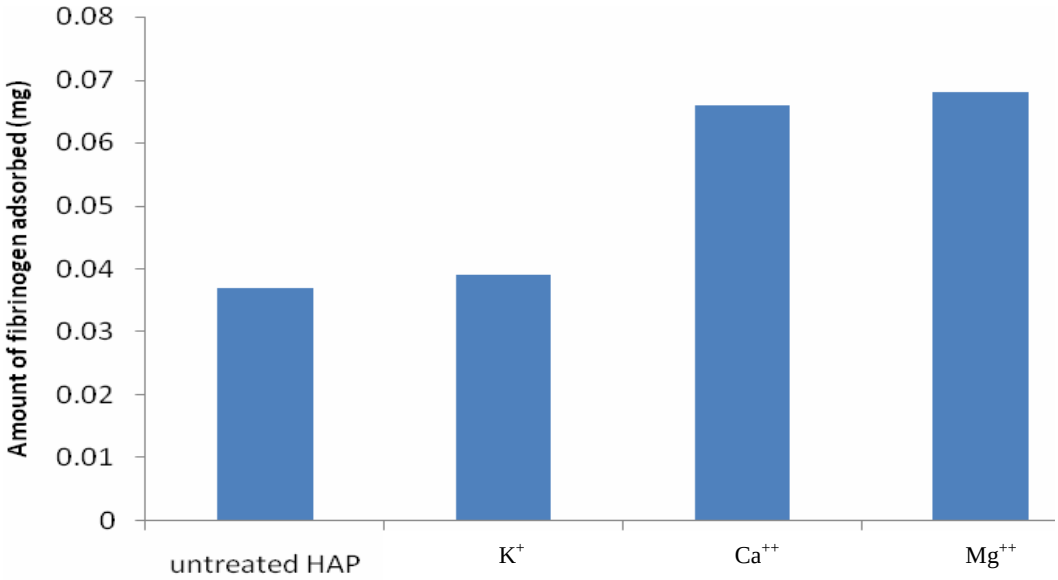

Fig. 4 Effect of cations on the adsorption of fibrinogen onto HAP

CONCLUSION

It can be concluded from this work that the adsorption of fibrinogen onto HAP follows the Langmuir model and that treatment of the HAP with calcium and magnesium ions increased the adsorption of fibrinogen. The treatment with calcium ion makes the HAP positively charged due to surface adsorption. The interaction of protein with HAP is likely to occur by participation of charged domains with specific surface sites and by hydrogen bonding of neighbouring parts of the molecule with polar surfaces. This knowledge is very essential in the design and production of biocompatible materials that the body will not reject. From the adsorptive characteristics of HAP described in this study it is evident that HAP is a material with good biocompatibility and is suggested for use as a biomaterial.

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