

**ORIGINAL ARTICLE**

## **Ligands Induced Formation of Heme Radicals at pH's 5.0 and 7.2: Implication in Malaria Resistance**

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### **ABSTRACT**

*Crude hemoglobins (Hbs) were extracted from blood samples of identified normal (AA), sickle trait carrier (AS), and sickle (SS) individuals by employing centrifugation techniques. The crude hemoglobins were dialysed at 4°C for 12 hr against 50mM Tris - HCl buffer (pH 8.5). The dialysis was repeated for another 12 hr against 50mM Tris - HCl buffer (pH 7.2). The effects of sodium dodecyl sulphate, hydrogen peroxide and linoleic acid on the hemoglobins were studied at pH 5.0 and 7.2 with UV – VIS Titration spectrophotometry. The study showed that sodium dodecyl sulphate (SDS) at pH 5.0, unfolds the hemoglobins. These can be likened to destabilization of hemoglobin structure by proteases such as plasmepsins and falcipains in the acidic environment of malaria parasite food vacuole due to malaria parasite infection. SDS, linoleic acid and hydrogen peroxide at pH 5.0 and 7.2 decreased the concentration of oxyhemoglobin and increased the concentrations of methemoglobin and deoxyhemoglobin of the studied proteins. The results also show that hemoglobins are deoxygenated due to interaction with sodium dodecyl sulphate, hydrogen peroxide or linoleic acid. Formation of methemoglobin is associated with lipid oxidation. Deoxygenation of hemoglobin as a result of their interaction with SDS, hydrogen peroxide or linoleic acid can be likened to pathological condition where by malaria parasites infection reduce the oxygen tension of erythrocytes of their host which can lead to production of membrane-associated hemin similar to repeated formation of sickle hemoglobin polymers in sickle cell and sickle cell trait individuals but not in individual of genotype AA. Due to protease activities and acidic environment of the food vacuole of the malaria parasites, It is possible that membrane lipid oxidation of the parasite as a result of deoxygenation of hemoglobin and formation of methemoglobin (which can be likened to as membrane-associated hemin in this in vitro study) may be responsible for resistance to malaria parasitism by some individuals with hemoglobin variants such as HbAS.*

**Key words:** Hemoglobin, Malaria, Hemin, Titration, Erythrocytes, Ligands

### **INTRODUCTION**

Hemoglobin (Hb) is an iron- containing oxygen transport metalloprotein in the red blood cells of vertebrates[1], and tissues of some invertebrates. Normal hemoglobin is a globular protein made up of four polypeptide chains, 2 $\alpha$  and 2 $\beta$ . A mutation which results in the replacement of glutamate with valine at position six of the two  $\beta$ -chains of HbA gives rise to mutant form of hemoglobin called sickle hemoglobin (HbS)[2]. It is well known that the main physiological function of hemoglobin is transport of oxygen. Other functions of hemoglobin are enzymatic such as lipoxygenase, peroxidase, oxygenase as well as catalase- like activities[3]. Hb catalyzes at low concentrations a quasi- lipoxygenase reaction with remarkably high substrate specificity[4]. The activity of the hemoglobin-catalyzed oxygenation of linoleate is comparable with those of true lipoxygenase[4]. Hemoglobin can oxidize phenols, amines, and aromatic hydrocarbons in the presence of hydrogen peroxide, via a high valent Fe (IV) oxo intermediate, in a manner similar to horseradish peroxidase[5]. Peroxidase activity of hemoglobin has been observed in vivo and this may have significant implications in tissue damage subsequent to injury, stroke, or heart attacks. The reaction of hydrogen peroxide with oxy-hemoglobin (oxy-Hb) forms ferry species as an intermediate but the final product is met-hemoglobin[6]. Heme radical formation may have physiological/medical importance in malaria-host relationship.

Malaria is an infectious disease that has killed many people across the globe. It is believed that it exploits the physiology of its host to its advantage in causing this problem to the society. The plasmodium parasite that causes malaria is transmitted from infected female anopheles mosquito to humans. The parasites spend part of their life cycle in the mosquito and part of it in the human host. It is believed that some individuals with hemoglobin variants such as hemoglobin AS (HbAS) are more resistant to malaria parasitism than individuals with hemoglobin A (HbA) and that heme radical formation may have physiological/medical importance in malaria-host relationship. Malaria

parasite like all organisms must acquire nutrients from the environment and convert these nutrients to other molecules as well as energy which are used to maintain its homeostasis, growth and reproduction. They can synthesize protein using amino acids from three sources: Denovo synthesis, host plasma proteins and degradation or digestion of host hemoglobin. It appears that the parasite prefers degradation of hemoglobin of its host in the quest for its survival. The precise mechanism by which sickle cell trait imparts resistance to malaria is unknown. Hence, investigation was carried out on ligands induced formation of heme radical with a view of deducing contributory factors responsible for resistance to malaria parasitism by some HbAS individuals.

## **MATERIALS AND METHODS**

### **Materials**

Sodium dodecyl sulphate (SDS), Linoleic acid and other chemicals used in this work obtained from BDH, England and Sigma, Germany and are of analytical grade. All reagents were freshly prepared unless otherwise stated.

### **Methods**

#### **Collection of Blood Samples**

Four milliliters (4ml) of blood samples were collected from each of the two identified individuals of genotype AA and AS after informed consent. Two milliliters (2ml) of blood sample was collected from an identified individual of genotype SS after informed consent and when the individual was not in crises. In each case, the blood sample was collected with an ethylene diamine tetracetic acid (EDTA) bottle.

#### **Isolation and Purification of Hemoglobin**

Four milliliters (4ml) each of the collected blood samples were combined with 6ml of cold normal saline in 50mM Tris-HCl pH 8.5 (wash buffer) and kept in the fridge for 10 min. In the case of the 2ml blood sample, 3ml of cold normal saline in 50mM Tris – HCl pH 8.5 was added and was also kept in the fridge for 10 min. The resulting solutions were centrifuged for 10 min at 4000 rpm[7]. Thereafter, the supernatants were removed by aspiration. The same amount of wash buffers were appropriately introduced into the pellet and kept in the fridge for 20 min. The above steps were repeated for 2-4 times until a clear supernatant is gotten in each case. The clear supernatants were removed and the resulting pellets in the case of 4ml blood samples were made up to 5ml while that of 2ml blood sample was made up to 2.5ml using 50mM Tris- HCl buffer, pH 8.5. The samples containing 50mM Tris- HCl were kept in the freezer in order to lyse the red cells. After lysing, 5% NaCl was added to the resulting volume and centrifuged for 10 min at 4000 rpm to remove inorganic phosphates and other ions from the sample. After the centrifugation the resulting supernatants (crude hemoglobin) were collected into separate vials and labeled appropriately. Each of the crude hemoglobin (i.e HbA, HbAS and HbS) was dialysed at 4°C for 12hr against 50mM Tris-HCl buffer, pH 8.5. The dialysis was carried out again at 4°C for another 12hr using 50mM Tris- HCl buffer, pH7.2. The dialyzed hemoglobin samples were collected and stored at -20°C for further experiments.

#### **UV – Visible Titration**

One hundred microliters (100 $\mu$ l) of 0.01mM of each of the hemoglobin samples calculated on heme basis by using  $\epsilon_{415} = 1.25 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$  [8], or  $\epsilon_{523} = 7.12 \text{mM}^{-1} \text{cm}^{-1}$  [9], were scanned from 250nm to 650nm using JENWAY 6405 UV- VIS Spectrophotometer in the absence and presence of different concentrations of ligands(sodium dodecylsulphate (SDS), hydrogen peroxide and linoleic acid) in 50mM buffers of pH 5.0 and 7.2 after appropriate buffer baselines. The titrations were done by fixing 0.1ml of the hemoglobins in 4ml cuvette containing a fixed volume of the buffer (2.1ml each for SDS and H<sub>2</sub>O<sub>2</sub> and 2.3ml for linoleic acid) then various volumes (0 to 0.6ml) corresponding to different concentrations of the ligands (0 to 1.043mM for SDS and H<sub>2</sub>O<sub>2</sub> and 0 to 0.400mM for linoleic acid) were added in stepwise manner from stock concentration of the ligand (5mM for SDS and H<sub>2</sub>O<sub>2</sub> and 2mM for linoleic acid), mixed and scanned from 250nm to 650nm. Spectrum readings were recorded at each titration point (after each addition of the ligand solutions). The results were analysed by monitoring absorbance changes at different wavelengths of the hemoglobin spectra (275nm, 340nm, 415nm, 542nm, 560nm, 576nm, and 630nm) and concentrations of Oxy-, Deoxy-

and Met-hemoglobin were calculated according to equation 1-3 as reported by Reza et al[10]. In the equations below,  $A_{576}$  means absorbance at 576nm,  $A_{630}$  means absorbance at 630nm etc.

$$[\text{Oxy}] = (1.0154 A_{576} - 0.2772A_{630} - 0.742A_{560}) \times 10^{-4} \text{ mol} \dots\dots\dots(1)$$

$$[\text{Deoxy}] = (1.335A_{560} - 0.7356A_{576} - 0.6254A_{630}) \times 10^{-4} \text{ mol} \dots\dots\dots(2)$$

$$[\text{Met}] = (2.6828A_{630} + 0.174A_{576} - 0.3414A_{560}) \times 10^{-4} \text{ mol} \dots\dots\dots(3)$$

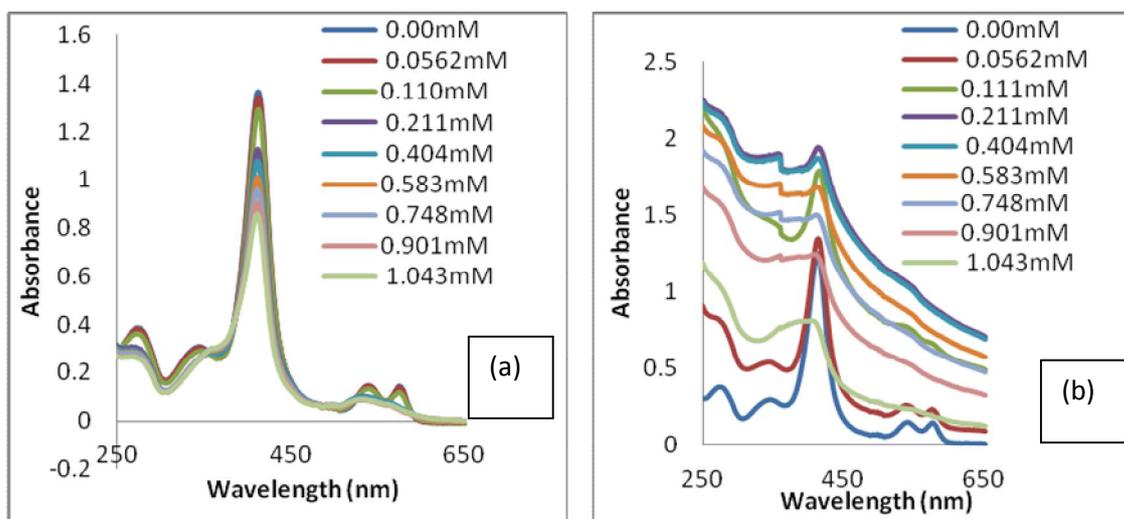
## RESULTS AND DISCUSSION

### Spectra of hemoglobin

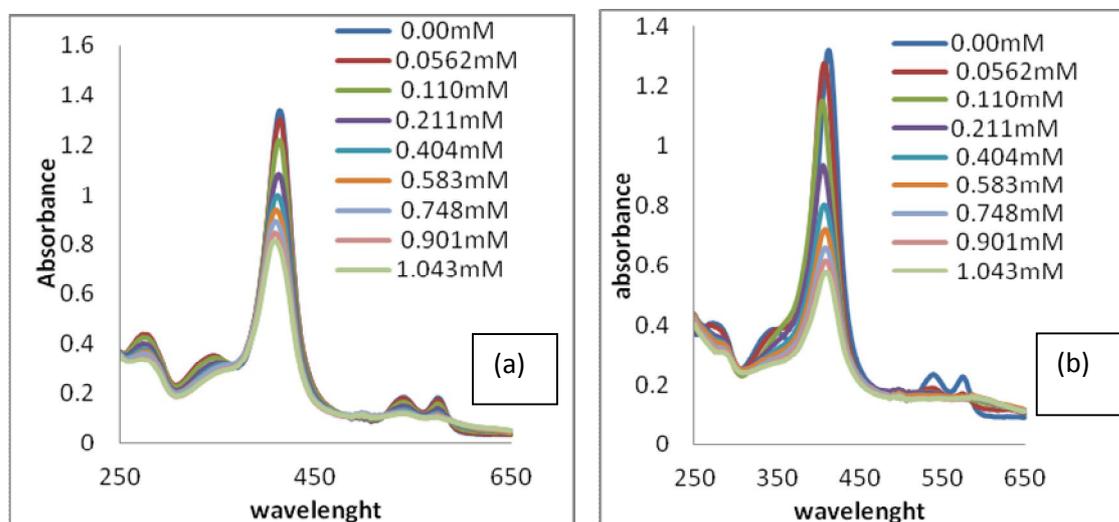
Interaction of SDS, hydrogen peroxide or linoleic acid with hemoglobin causes change on the spectrum of hemoglobin. These changes are increase or decrease in the peak absorbance of the soret band (415nm), oxyhemoglobin bands (542nm and 576nm), aromatic bands (275nm) and 630nm, disappearance of the delta band ( $\delta$ -band) (345-360nm) and spectral shift at increasing concentration of the ligands. See figures (1-3). SDS caused spectral shift of the soret band from 415nm to between 417 to 420nm with disappearance of the  $\delta$ -band. The spectral shift is an indication of formation of low-spin derivative of hemoglobin[11]. According to Matsui et al[12], spectral shift or decrease in the peak absorbance of the soret band is an indication of structural transformation of the hemoproteins while increase in the aromatic band is indicative of a protein that is unfolding[13]. This unfolding suggests destabilization of hemoglobin structure by SDS just like cleavage of the hinge region of hemoglobin by protease as a result of malaria parasite infection. Decrease in absorbance at 340nm which was observed when  $\text{H}_2\text{O}_2$  was used as ligand refers to the stretching or weakness of the non-covalent bond between histidine of globin and heme iron[6], of the studied hemoglobins (HbA, HbAS, and HbS). Therefore increase in absorbance observed at 340nm when both SDS and linoleic acid were used as ligands can be referred to as hardness of the non-covalent bond between histidine of globin and heme iron of HbA, HbAS and HbS while the decrease in absorbance observed in presence of SDS from concentration of 0.404 - 1.043mM can be due to breakage of the non-covalent bond which led to the weakness or stretching. Usually, hemoglobin has three conformational forms namely: Oxy-, deoxy- and met-hemoglobin conformations. Interaction of hemoglobin with ligand can lead to conversion of hemoglobin from one conformational form to another which can be monitored from the spectra of hemoglobin. For instance, the degree of conversion of oxyhemoglobin to met hemoglobin depends on the degree of unfolding of the protein which leads to the existence of a hybrid of low spin and high spin states as it appears from shift toward shorter wavelength of the soret band[6].

### Effect of SDS and linoleic acid on Aromatic and Soret bands of Hemoglobin

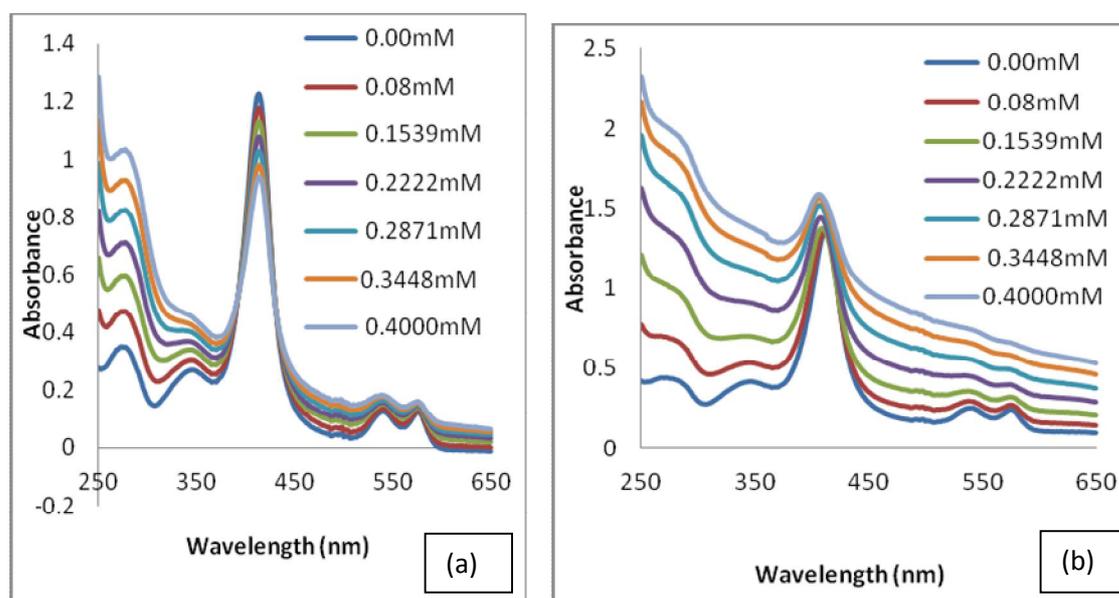
SDS increased the absorbance of soret and aromatic bands of HbA, HbAS and HbS at pH 5.0 and decreased the absorbance of these bands at pH 7.2. (see figures 4 and 5). The increase in absorbance at pH 5.0 on these bands reached maximum at 0.211mM SDS and started decreasing from 0.404mM SDS. linoleic acid at pH 5.0 and 7.2, decreased the absorbance of the soret band and increased that of aromatic band of the HbA, HbAS and HbS. However, the absorbance of the soret band of the proteins started increasing noticeably from 0.22mM linoleic acid pH 5.0. Observable difference in the extent of increase or decrease in absorbance at these bands were not seen in the three hemoglobins (Figure 6). The increase in the absorbance of the aromatic band refers to dynamic motion of the molecule and its deviation from normal structure and function[6,14], or unfolding of the of the haemoglobins[13,12]. These can be likened to destabilization of haemoglobin structure by proteases such as plasmepsins and falcipains in the acidic environment of malaria parasite food vacuole as a result of malaria parasite infection. This unfolding exposes the heme moiety and buried aromatic amino acids of the proteins which explains the increase in absorbance observed at the soret and aromatic bands of the haemoglobins by SDS. The ferrous iron ( $\text{Fe}^{2+}$ ) of the exposed heme moiety can oxidize to ferric iron ( $\text{Fe}^{3+}$ ) forming met-hemoglobin[15]. The decrease in absorbance on these bands by SDS at pH 7.2 suggests that the proteins are folding. The decrease in the absorbance of the soret band of the hemoglobins in the presence of linoleic acid at pH 5.0 and 7.2 suggests that linoleic acid reacts with the heme moiety of the proteins, while the increase in absorbance at the soret band at pH 5.0 from 0.22mM linoleic acid (Figure 6) suggests exposure of the heme moiety of the haemoglobins.



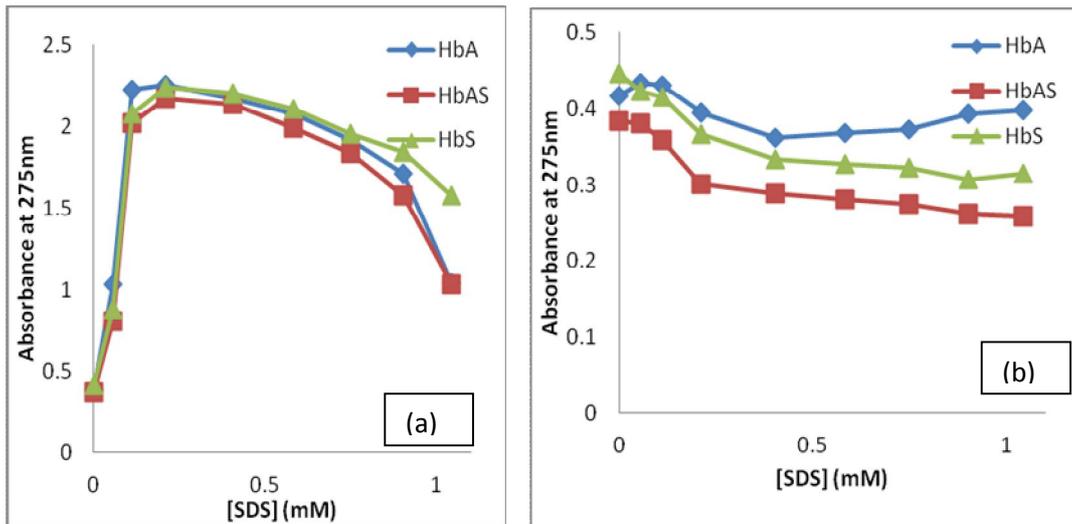
**Figure 1:** Absorption spectra of HbAS in the presence of varying concentrations (0 - 1.043mM) of SDS at (a) pH 7.2 and (b) pH 5.0.



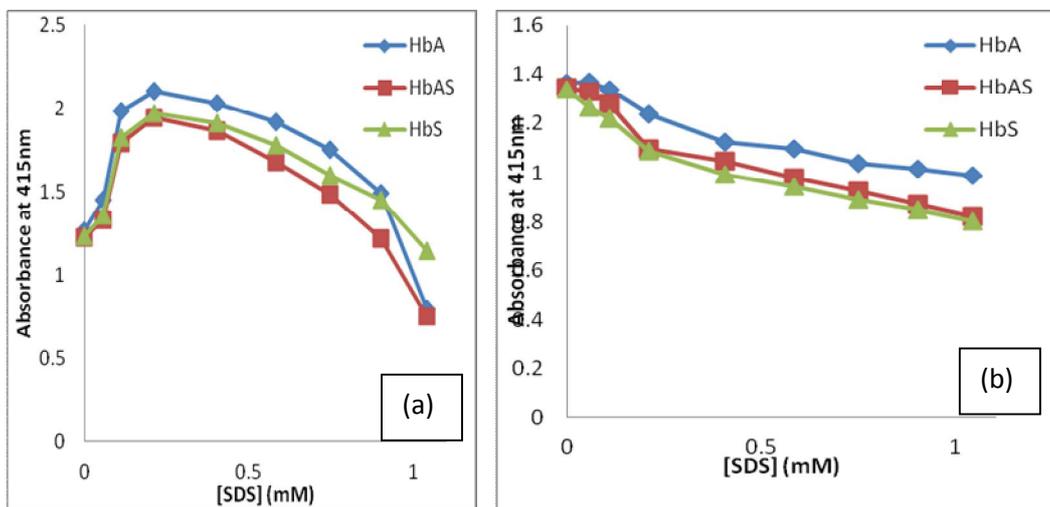
**Figure 2:** Absorption spectra of HbAS in the presence of varying concentrations (0 - 1.043mM) of hydrogen peroxide at (a) pH 7.2 and (b) pH 5.0.



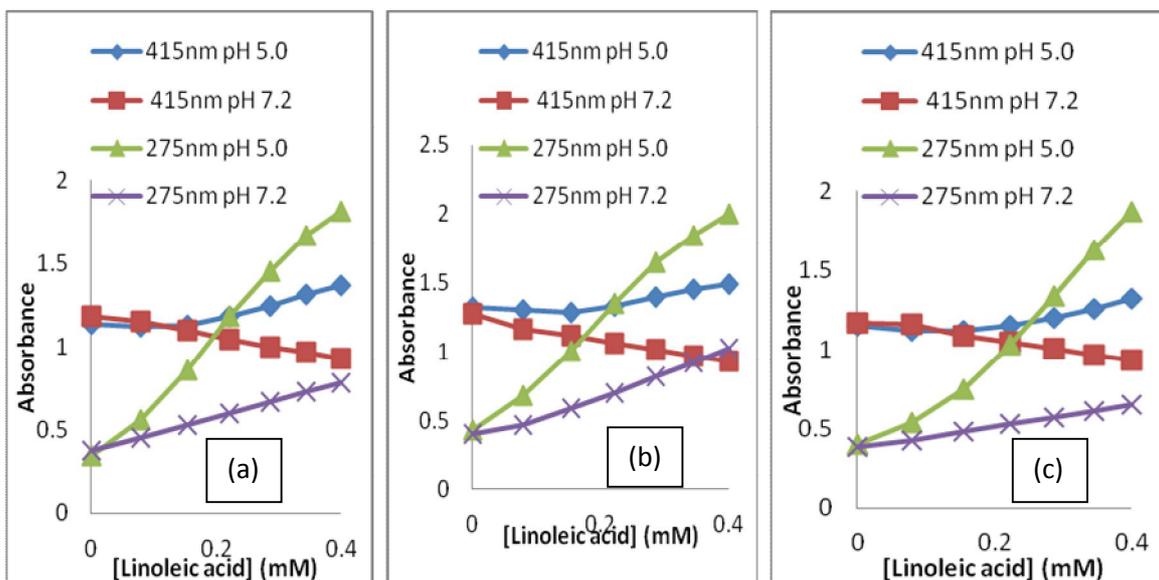
**Figure 3:** Absorption spectra of HbAS in the presence of varying concentrations (0 - 0.4000mM) of linoleic acid at (a) pH 7.2 and (b) pH 5.0.



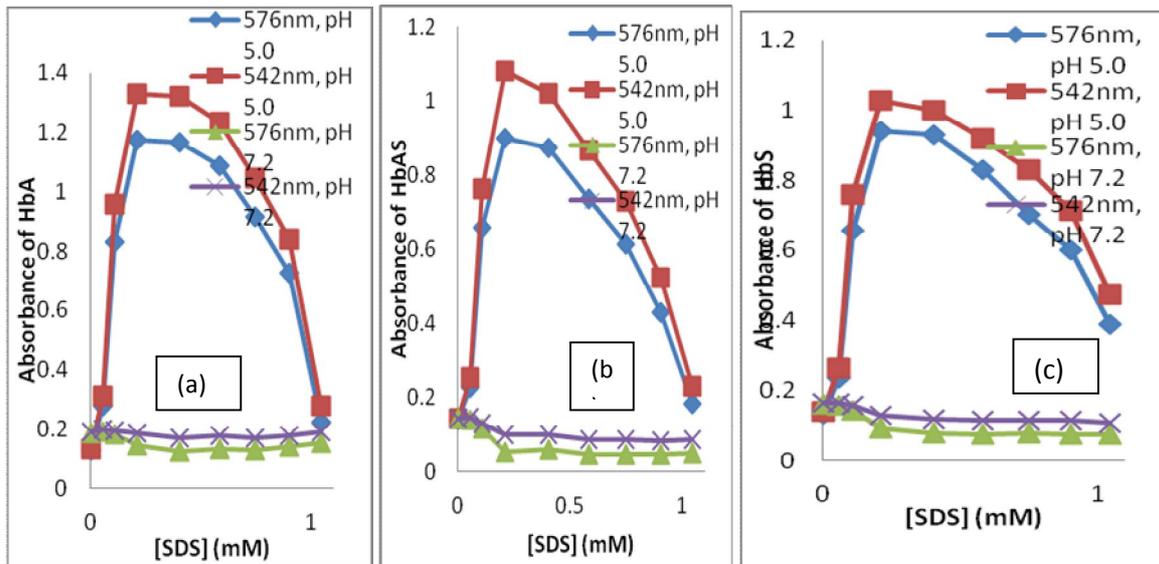
**Figure 4:** Changes in peak absorbance of aromatic band of hemoglobin at 275nm in the presence of varying concentrations (0.0 - 0.2087mM) of SDS at (a) pH 5.0 and (b) pH 7.2.



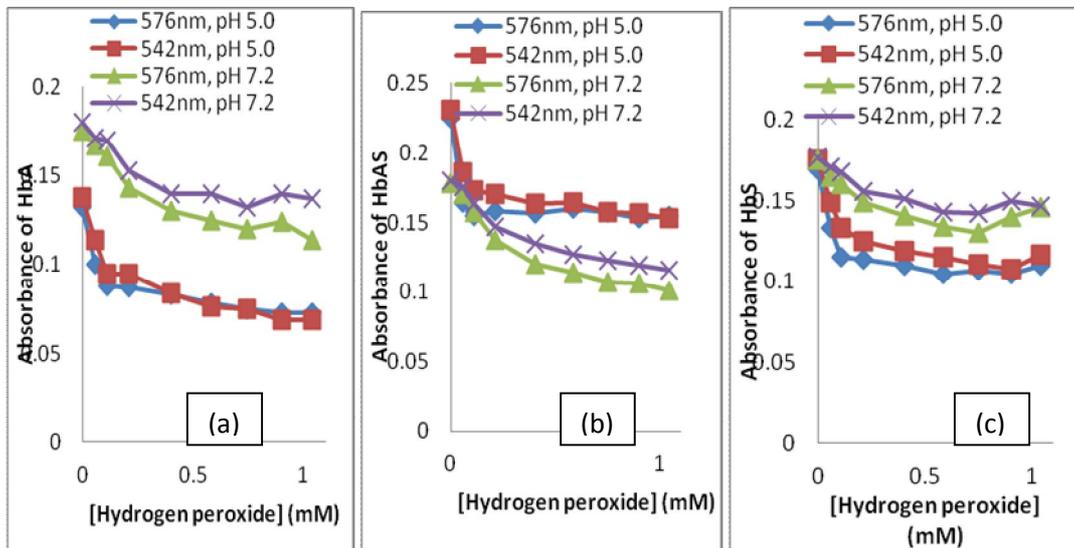
**Figure 5:** Changes in peak absorbance of soret band of hemoglobin at 415nm in the presence of varying concentrations (0.0 - 0.2087mM) of SDS at (a) pH 5.0 and (b) pH 7.2.



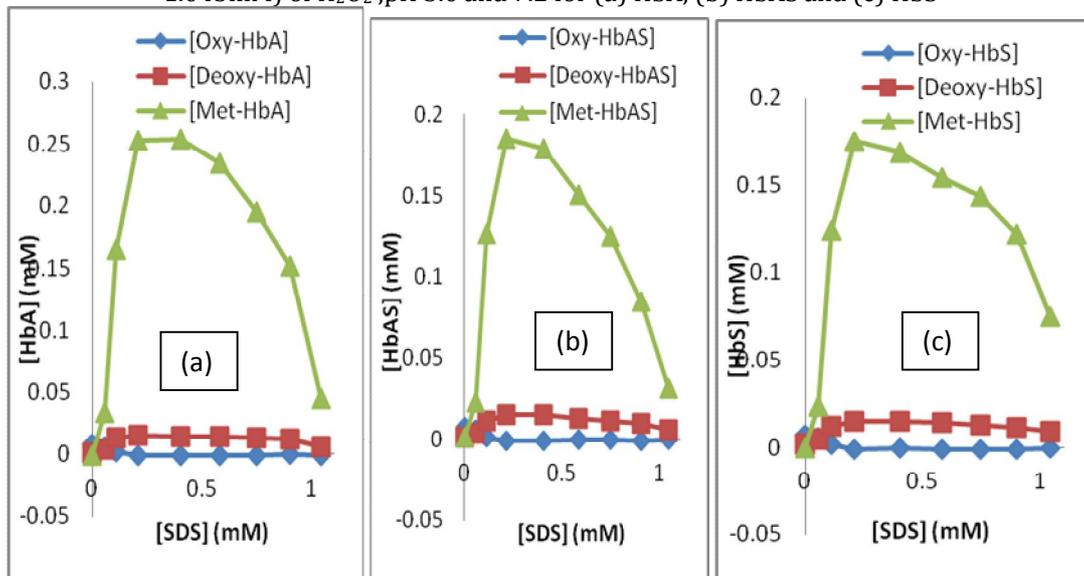
**Figure 6:** Changes in absorbance of hemoglobin at 415nm and 275nm in the presence of varying concentrations (0.0 - 0.400mM) of linoleic acid for (a) HbA, (b) HbAS and (c) HbS



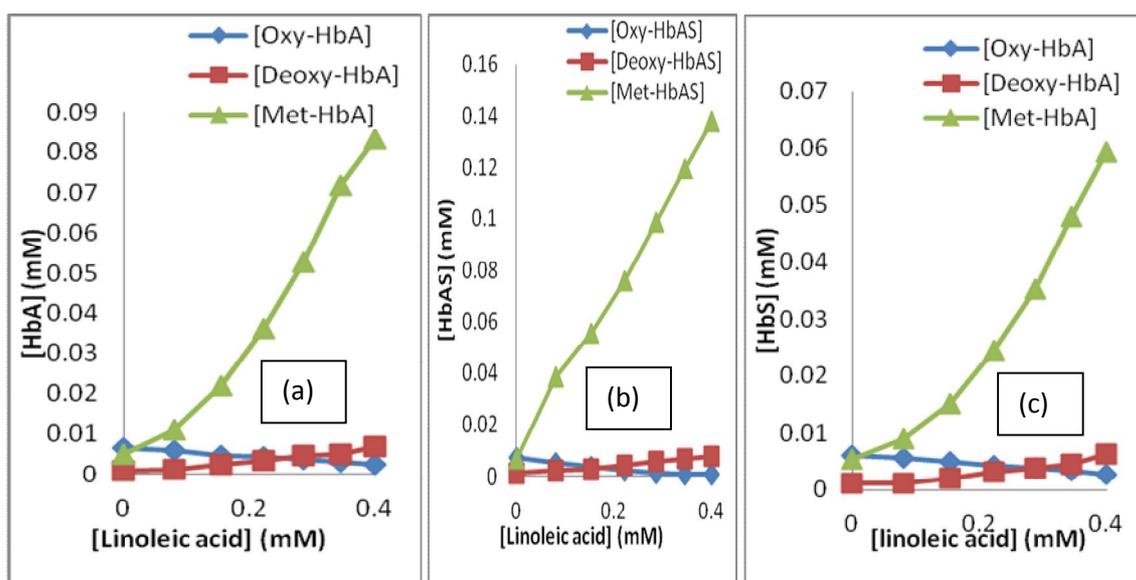
**Figure 7:** Changes in absorbance at 576nm and 542nm in the presence of varying concentrations (0.0 – 1.043mM) of SDS ,pH 5.0 and 7.2 for (a) HbA, (b) HbAS and (c) HbS



**Figure 8:** Changes in absorbance at 576nm and 542nm in the presence of varying concentrations (0.0 – 1.043mM) of H<sub>2</sub>O<sub>2</sub> ,pH 5.0 and 7.2 for (a) HbA, (b) HbAS and (c) HbS



**Figure 9:** Concentrations of Oxy- , Deoxy-, and Met-conformations of hemoglobin in varying concentrations (0.00 -1.043mM) of SDS at pH 5.0 for (a) HbA, (b) HbAS and (c) HbS



**Figure 10:** Concentrations of Oxy-, Deoxy-, and Met-conformations of hemoglobin in high concentrations (0.00 -0.400mM) of linoleic acid at pH 5.0 for (a) HbA, (b) HbAS and (c) HbS.

### Effect of SDS, H<sub>2</sub>O<sub>2</sub> and Linoleic acid on Oxy-hemoglobin bands

SDS increases the absorbance of the oxyhemoglobin bands in all the hemoglobin studied. Of the three hemoglobins, HbA has the highest absorbance at the oxy-hemoglobin bands followed by HbAS while HbS had the least. The absorbance started decreasing from 0.404mM SDS. At pH 7.2, SDS had no observable change on the oxy-hemoglobin bands in all the studied hemoproteins (Figure 7). This may suggest that malaria parasites do not utilize their host hemoglobin at physiologic pH. H<sub>2</sub>O<sub>2</sub> at pH 5.0 and 7.2 decreased the absorbance of oxy-hemoglobin bands of the haemoglobins (Figure 8). This may suggest structural transformation of the hemoglobins and its conversion from oxy-hemoglobin to other forms of hemoglobin. The decrease were more at pH 5.0 than at pH 7.2. and the decreases in absorbance of HbA, HbAS and HbS were not different. Linoleic acid has a concentration dependent increase in absorbance on the oxy-hemoglobin bands at pH 5.0. The increases were more at higher concentration of linoleic acid. At pH 7.2 increase in absorbance were not observed in the three hemoglobins as in the case of SDS at pH 7.2.

Increase in the oxy-hemoglobin bands in the presence of SDS and linoleic acid at pH 5.0 (Figure 7) and concomitant decrease in the solet band and increase in the absorbance at 630nm (Figures 1 and 3) suggest that oxy-hemoglobin conformation of the proteins are being converted to met-hemoglobin (Figures 9 and 10). At pH 7.2, SDS has no effect on the oxy-hemoglobin bands of the studied proteins (Figure 7).

### Effect of SDS, H<sub>2</sub>O<sub>2</sub> and Linoleic acid on Hb Conformation (Oxy-, Deoxy- and Met-conformations)

Figures (9 - 10) show that interaction of SDS, H<sub>2</sub>O<sub>2</sub> or linoleic acid with Hb decreased the concentration of oxy-hemoglobin in a concentration dependent manner. The figures also show that interaction of these ligands with hemoglobin increased the concentrations of deoxy-hemoglobin and met-hemoglobin which started decreasing in a concentration dependent manner from SDS concentration of 0.404mM (pH 5.0). HbA had the highest concentration of met-hemoglobin followed by HbAS while HbS had the least concentration of met-hemoglobin in the presence of SDS at pH 5.0. HbAS had the highest concentration of met-hemoglobin in the presence of linoleic acid followed by HbA while HbS had the least at pH 5.0. Reasonable changes in met-hemoglobin concentration were not observed in the presence of H<sub>2</sub>O<sub>2</sub> pH 7.2. Decrease in oxy-hemoglobin concentration and increase in deoxy and met hemoglobin concentrations suggests that the oxy-hemoglobin are being converted to deoxy-hemoglobins (i.e decrease in the concentration of oxy-hemoglobin and increase in the concentration of deoxyhemoglobin means that oxyhemoglobins are being deoxygenated) which its Fe<sup>2+</sup> can oxidize to Fe<sup>3+</sup> to form met-hemoglobin (Figures 7 and 8). Formation of met hemoglobin is correlated to lipid oxidation[16]. Deoxygenation of hemoglobin as a result of interaction with these ligands may be likened to pathological conditons where metabolism by malaria parasites reduce the oxygen tension of erythrocytes of their host which can

lead to production of membrane-associated hemin secondary to repeated formation of sickle hemoglobin polymers in sickle cell and sickle cell trait individuals but not in individuals of genotype AA. Membrane-associated hemin can oxidize membrane lipids and proteins[17]. The decrease in the concentration of met-hemoglobin and deoxyhemoglobin from SDS concentration of 0.4.04mM and above is an indication that the oxy-hemoglobin in the reaction system is diminishing. Due to protease activities and acidic environment of the food vacuole of the malaria parasites, It is possible that membrane lipid oxidation of the parasite as a result of deoxygenation of hemoglobin and formation of methemoglobin (which can be likened to as membrane-associated hemin in this in vitro study) may be responsible for resistance to malaria parasitism by some individuals with hemoglobin variants such as HbAS.

## CONCLUSION

Hemoglobins are being deoxygenated in the presence of SDS, H<sub>2</sub>O<sub>2</sub> or linoleic acid. The ferrous iron (Fe<sup>2+</sup>) of the deoxygenated hemoglobin can oxidize to ferric iron (Fe<sup>3+</sup>) to form met-hemoglobin and formation of met-hemoglobin is correlated to lipid oxidation[16]. Deoxygenation of hemoglobin as a result of interaction of SDS, H<sub>2</sub>O<sub>2</sub> or linoleic acid can be likened to pathological condition where by malaria parasites infection reduce the oxygen tension of erythrocytes of their host which can lead to production of membrane-associated hemin secondary to repeated formation of sickle hemoglobin polymers in sickle cell and sickle cell trait individuals but not in individuals of genotype AA. This membrane-associated hemin can oxidize membrane lipids and proteins[17] or may play role in damaging malaria parasites. Due to protease activities and acidic environment of the food vacuole of the malaria parasites, It is possible that membrane lipid oxidation of the parasite as a result of deoxygenation of hemoglobin and formation of methemoglobin (which can be likened to membrane-associated hemin in this in vitro study) may be responsible for resistance to malaria parasitism by some individuals with hemoglobin variants such as HbAS.

## ACKNOWLEDGEMENTS

The authors are grateful to Mrs Eze Fransisca O. of deans office, faculty of biological sciences for typing the manuscript.

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