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Facile spectroscopy and atomic force microscopy for the discrimination of α and β thalassemia traits and diseases: A photodiagnosis approach

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ABSTRACT

Thalassemia (Thal) is an inherited blood disorder endemic to the Mediterranean and Middle East (*e.g.*, KSA and UAE). This disease is caused by defects in the synthesis of one or more hemoglobin chains in red blood cells (RBCs). Alpha (α) Thal is caused by a reduced or absent alpha globin segment. Similarly, beta (β) Thal is caused by a defect in the beta globin segment. We divided the diseases into four groups: α Thal trait, α Thal disease, β Thal trait, and β Thal disease. The α or β Thal traits are milder variants of these diseases and do not require treatment; but β Thal disease (and to a lesser extent, α Thal) causes hemolytic anemia, splenomegaly, and bone deformities and requires repeated lifelong blood transfusions. This paper presents results regarding the identification of Thal variants using fluorescence spectroscopy of blood biomolecules and atomic force microscopy analysis of the morphologic features of red blood cells. The combined results provide new insights into the characteristics of these diseases. Furthermore, this study shows why β Thal disease subjects are often transfusion-dependent, and α Thal disease subjects are only occasionally transfusion dependent.

1. Introduction

Hemoglobin is critical to the normal function of red blood cells, the fundamental roles of which are to transport oxygen from the lungs to tissues and CO_2 from tissues to the lungs. A normal hemoglobin molecule comprises two α- like globin polypeptide chains and two β-like globin chains; each globin molecule is associated with a heme group, which comprises a porphyrin ring with ferrous iron (Fe^{+2}) at the center. Each heme binds with one oxygen molecule; thus, the four hemes of a hemoglobin molecule carries four oxygen molecules [1].

The α chains are encoded on chromosome 16, and the β chains are encoded on chromosome [11,2]. The most abundant adult hemoglobin is HbA (α2β2) followed by a much smaller contribution from HbA2 (α2δ2; typically 1.5–3.5% of adult hemoglobin). Fetal hemoglobin HbF (α2γ2) has a higher oxygen affinity than adult hemoglobin and facilitates more efficient oxygen transfer between maternal and fetal circulations. Typically, the synthesis of α-like and β-like chains is balanced. An imbalance between the production of α and β chains is the pathophysiological basis of Thal [3].

Two genetic loci exist for α globin; thus, there are four alleles in diploid cells. Two alleles are maternal and two alleles are paternal. The severity of α- Thal is correlated with the number of affected α-globin loci: the more alleles that are affected, the greater the severity of the disease. When noting the genotype, "α" indicates a functional α- chain, and '-' indicates a pathological α- chain. Thus, α-Thal silent is (- α/α α). Three normal α-globin genes are sufficient to permit normal hemoglobin production without any clinical symptoms. When two alleles are affected, the condition is α Thal trait. Two α genes permit a nearly normal production of red blood cells, but a mild microcytic hypochromic anemia is observed. α - Thal trait can exist in two forms: α -thal-1 (- -/α α) involves a cis deletion of both α genes on the same chromosome; α -thal-2 (- α/- α) involves a trans deletion of α genes (this occurs on different chromosomes). When three alleles are affected (- -/- α) the condition is called hemoglobin H disease. Two unstable

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hemoglobin molecules are present in the blood: hemoglobin Bart's (with tetrameric γ chains) and hemoglobin H (tetrameric β chains). Both molecules are unstable and have higher affinity for oxygen than normal hemoglobin, when all four alleles are affected (- -/- -) the condition is called α major. The hemoglobin molecule comprises all tetrameric γ chains. Infants with α - Thal major are stillborn with hydrops fetalis.

Similarly, there are various grades of β Thal disorder. In β thal minor, only one of the β globin alleles bears a mutation (heterozygous form, β+/β βo/β). Individuals suffer from microcytic anemia. Detection usually involves lower than normal mean corpuscular volume value (< 80 fL). A more severe condition is thalassemia intermedia (β+/β+ βo/β+). Affected individuals can often manage a normal life but may need occasional transfusions, *e.g.*, at times of illness or pregnancy, depending on the severity of their anemia[4]. The severest form is Thal major (Cooley anemia βo/βo). This grade is a homozygous form and occurs when both alleles have Thal mutations. This is a serious microcytic, hypochromic anemia. If a subject is left untreated, Thal major causes splenomegaly and severe bone deformities and progresses to death before age 20. Treatment consists of periodic blood transfusion, splenectomy for splenomegaly, and chelation of transfusion-related iron overload [4–11].

While there are broad ranges and gradations in the severity of these diseases, Thal can be broadly classified by α or β Thal traits and Thal diseases for the purpose of this discussion. When a male with Thal trait and a female with Thal trait reproduce, out of four children, one will live a normal life, two will have chances of becoming a carrier, and one will be a Thal disease patient. Because of this KSA, the gulf countries of Turkey and Cyprus have required premarital screening [12]. However, when a female with Thal disease and a male with Thal disease reproduce (which is rare), most of their children will likely have Thal disease and require repeated blood transfusions. This present study is limited to a set of samples clinically classified as (a) α thal trait, (b) β thal trait, (c) HbH, and (d) β thal major.

The World Health Organization estimates that 1.5% of the world's population is a carrier of Thal [5]. This prevalence rate is region dependent and is quite high among Arab populations. The incidence and prevalence rates depend upon geographic and cultural characterization. For example, Thal is more common in border islands (*e.g.*, Maldives) or peninsular regions (*e.g.*, KSA). In these areas, falciparum malaria had been rampart and has been considered to influence Thal rates. Similarly, in Arab communities, consanguineous marriages and intrafamilial unions are more common than in north European or North American countries [6,7]. Arab nations, with an overall population of approximately 350 million, have carrier rates of 1–11% for β- Thal, 1–58% for α- Thal, and 0.3–30% for sickle cell trait [8–12]. Furthermore, the prevalence rate for α vs. β traits is also region dependent. For example, the β and α traits are 3% and 24% in Bahrain however in Iraq 4.4% and 1% respectively [11].

It is under this circumstance that the present investigation is important. Spectral and atomic force microscopy (AFM) assays provide insight into the composition and structural (morphological) differences among the four variants of Thal. Laser spectrometery can be developed as a portable instrument for premarital screening in remote areas.

Optical biopsy is an emerging novel technique and is based on the interaction of light with biomolecules. When photons of a particular wavelength interact with biomolecules, most photons are scattered as radiation with or without shifts in their wavelengths (*i.e.*, Raman or Raleigh scattering, respectively). A few photons are absorbed by the biomolecules which results in excited states. The molecules stay at the excited states for a few nanoseconds and subsequently release energy as fluorescence radiation. Raman shifts provide unique measures of vibrational energy levels and electronic energy levels of fluorescing molecules. Based on the presence and intensities of these signals, the relative proportions of these biomolecules could be determined. Such assays provide significant information about disorders or diseases associated with the tissues or fluids of a particular subject. A number of related studies have been published for the diagnosis of cancers [13–18] and blood disorders [19–21], based on the spectral features of biomolecular components of blood and urine [22,23]. Fluorescence spectroscopy can be considered for molecular diagnosis.

Atomic force microscopy is another novel technique utilizing light on a microlever to provide structural and morphological profiles of samples. The micro-lever tracks the surface of samples. Light reflected off the lever is received by a photodetector; these light signals are analyzed with the result of displaying a three-dimensional topography. Atomic force microscopy can complement or supplement scanning electron microscopy [24,25].

2. Materials and methods

2.1. Spectrofluorometric analysis

A conventional spectrofluorometer, such as Perkin Elmer (LS 50 or 55), capable of collecting excitation, emission and synchronous spectra in the range of 200–800 nm. The current spectroscopic experiment was used to acquire FES of acetone extracts of RBCs from 425 to 700 nm upon excitation at 400 nm with a spectral width of 10 nm (Xe lamp source). The same instrument was used to obtain synchronous fluorescence excitation spectra (SXS) of different biomolecules of blood plasma (*e.g.*, tyrosine tryptophan, NADH, and FAD) from 200 to 700 nm [21]. This was performed using an offset of 70 nm between excitation and emission gratings [13–19].

Patients were informed about the investigation, and proper consents were obtained. The authorization for this investigation was obtained from the Institutional Review Board [E – 17–2267 dated 27 March 17] King Khalid University Hospital of King Saud University of Riyadh, KSA. The spectral and AFM study were performed in Research Chair in Laser Diagnosis of Cancer under the guidelines of Ministry of Higher Education, King Saud University in Saudi Arabia. The distribution of subjects and their hematological parameters are given in Table 1.

Among the subjects, the normal controls were friends and relatives of the KKUH staff. The α or β thal trait subjects were identified in routine screening for marriage registration certificates. The β thal major and HbH subjects were regular patients under once or twice per month transfusions and supervision with or without chelation therapy.

Briefly, 5 ml blood were collected i.v. from each subject in an EDTA (ethylene diamine tetraacetic acid) vial containing standard anticoagulant. The collected blood vials were gently rocked five times and centrifuged for 15 min at 3000 rpm to separate plasma from cellular components. The top supernatant yielded greenish yellow liquid, plasma which was collected and subjected to SXS. The bottom thick,

Table 1
Hematological parameters of five types of subjects under study.

Samples Type	Quantity (Nos)	Age	Hb g/dL	MCV (fl)	MCH (pg)	HbA%	HbA2%	p- value
Normal	35 (20 male / 15 female)	14-43	12.56 ± 0.29	87.2 ± 1.81	30.5 ± 0.08	97.2 ± 0.12	2.3 ± 0.14	p < 0.10
β TT	28 (18 male/10 female	20-33	11.08 ± 0.78	76.3 ± 1.32	27.4 ± 0.91	94.3 ± 0.78	3.6 ± 0.45	p < 0.10
β TD	25(15 male/10 female)	10-23	7.8 ± 0.91	68.9 ± 1.30	23.3 ± 0.46	78.2 ± 0.13	5.4 ± 0.32	p < 0.10
α TT	20(15 male/10 female)	20-34	12.01 ± 0.89	81.6 ± 0.91	28.4 ± 0.17	95.6 ± 0.27	2.9 ± 0.39	p < 0.15
α TD(HbH)	12(8 male/4 female	10-20	9.87 ± 0.75	76.0 ± 1.12	25.6 ± 0.58	93.2 ± 0.10	3.2 ± 0.85	p < 0.15

semisolid paste of blood containing mostly RBC was lysed with acetone (1:2 v:v) and centrifuged to obtain a clear, transparent liquid. This contained the biomolecules for FES fluorescence study [13–19].

2.2. Atomic force microscopy classification

Droplets of erythrocytes of each subject were spread as monolayers on clean 12-mm diameter round glass cover slips. The smear was allowed to air dry prior to AFM. All AFM images and measurements were obtained by an AFM (Multimode, Bruker, USA) operating in tapping mode. A silicon probe with aluminum reflective coating on its back side (TEPSA, Bruker, USA) was employed in the AFM imaging. The probe had a spring constant of 20–80 N/m, a tip curvature radius of 8 nm, and a resonant frequency of 342–394 KHz.

To direct the AFM probe to the desired cells, we used a top-view optical microscope. To analyze cell membrane alterations, 5–7 cells from different areas of each smear were randomly selected and scanned. The experiments were repeated several times to exclude artifacts. The AFM images were processed using Nano-Scope Analysis 1.3 (Bruker, USA).

2.3. Statistical analysis

All data were analyzed using Student's *t*-test (available in SSP software). Mean, variance, standard deviation, and p value were calculated in each data set. Significant results were determined among all data sets.

3. Results

Fig. 1 shows the fluorescence emission spectra (FES) of normal blood and two inherited blood disorders: (a) normal vs. β Thal trait (βTT) and (b) normal vs. α Thal trait (αTT). Three major spectral bands were observed with peaks at 470, 585, and 630 nm. The spectra were normalized to the intensity at 470 nm (*i.e.*, the background Raman spectrum of acetone). The intensity ratio between the peaks at 630 nm and 585 nm (*i.e.*, the neutral and basic forms of porphyrin) is a measure of oxygen carrying capacity. This measurement was different for all variants of Thal. To highlight the differences, a ratio parameter, defined as $R_1 = I_{630}/I_{585}$, is given in Table 2.

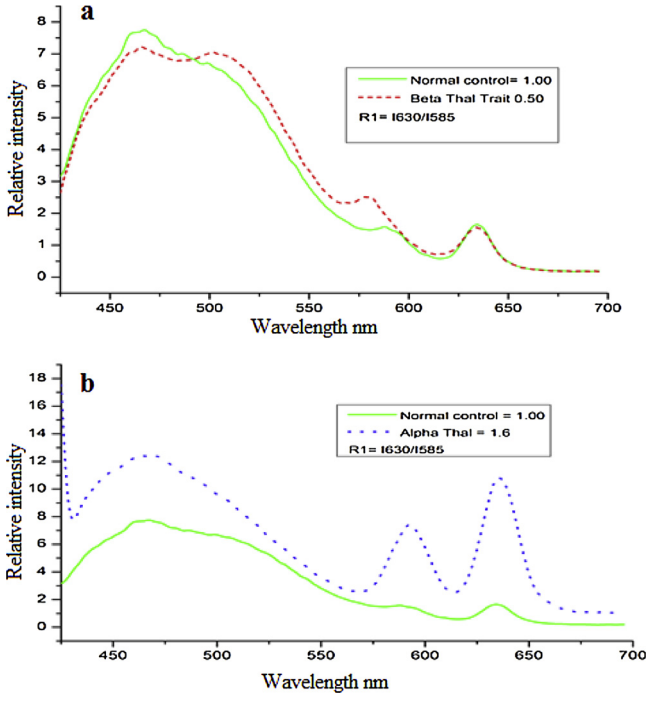

Fig. 1. Fluorescence emission spectra (SES) of normal and other types of inherited blood disorders (a) normal control vs beta thal trait (βTT) (b) normal vs alpha thal trait (αTT).

The presented ratio parameter was the average of number samples of each variant. The standard deviation was ± 8% ($p < 0.01$); thus, each variant was unambiguously classified.

Fig. 2 shows the SXS of the plasma of two subjects: (a) normal and (b) β Thal trait. In the spectra, normalization was not performed because the SXS spectra varied dramatically between variants (see below table). To highlight the characteristics features, two ratio parameters were defined: $R_2 = I_{270}/I_{290}$ (*i.e.*, a ratio of the concentrations of tyrosine to tryptophan) and $R_3 = I_{450}/I_{370}$ (*i.e.*, a ratio of the concentrations of FAD enzyme and NADH coenzyme). I_{270} represents the spectral intensity at 270 nm; the other variables follow similar definitions. The ratio parameters for the different disorders based on the plasma components spectra are shown in Table 3.

Each ratio parameter varied by ± 10% ($p < 0.012$). Thus, each disorder was distinguishable. An important non-spectroscopy parameter for variant classification is the ratio between plasma volumes to cellular components volume, as shown in Table 4. Representative AFM images of normal and Thal RBCs are shown in Fig. 3(a–e). As seen in Fig. 3(a), healthy RBCs have a biconcave shape with an average diameter of 7.30 ± 0.4 μm, whereas the overall RBC shapes of Thal samples displayed some degrees of deformities and reduced sizes (5–6 μm; Fig. 3(b–e)). A significant percentage of RBCs (approximately 27%) from αTT patients displayed various abnormal shapes and sizes, whereas the remainder of RBCs exhibited normal morphology. The abnormal cells tended to be spherical, as shown in Fig. 3(b). RBCs taken from βTT patients exhibited some degree of abnormalities in Fig. 3(c). However, the overall shapes were only marginally different from those of healthy RBCs. To highlight the contrast, the morphological appearance of abnormal RBCs from α-thalassemia intermedia (HbH disease) patients is shown in Fig. 3d. HbH disease manifested as significant alterations in shapes and sizes. For the RBCs of patients of βTD, more than 70% of scanned RBCs were severely distorted and agglomerated, which increased the probability of vascular occlusions. Though various abnormalities were observed, elongated RBCs was the most common (Fig. 3e).

Fig. 4(a–c) shows the topographical ultrastructure of RBC membranes from normal, βTT and βTD subjects. The ultrastructure of the normal RBC membrane was smooth and almost uniform, whereas for RBCs taken from βTT patients, surface erosion and unevenness were evident. For βTD, the ultrastructure of RBC membranes was distorted with many large holes (Fig. 4c).

4. Discussion

The most common method of Thal diagnosis is CBC coupled with hemoglobin quantification by electrophoresis or HPLC. Most persons with the Thal trait are incidentally identified when their complete blood count shows mild microcytic anemia. The spectroscopic and AFM investigations of this paper are not intended to replace these conventional methods. Instead, this study adds significant insights to the characteristics of these inherited disorders.

The ratio parameter $R_1 = I_{630}/I_{585}$ is a measure of the oxygen carrying capacity of RBCs [15–18]. Hemoglobin (Hb) is an oxygen carrier. Each Hb has four heme groups. Each heme contains an iron complex of porphyrins, and each iron atom can bind one oxygen molecule. This is because a heme group is nonplanar with a central iron atom extending out by 0.04 nm. By absorbing an oxygen molecule, iron radius shrinks and reaches a planar alignment with the porphyrin ring. The oxygen carrier in blood is the iron of porphyrin rings of the RBC heme groups [26,27].

Endogenous porphyrins and ALA-labeled and metabolized exogenous porphyrins exhibit at least two or three peaks one at 585 nm,

Table 2
Ratio parameter for different disorders from the RBC fluorescence.

Ratio parameter	Normal control	αTT(Alpha Thal Trait)	αTD(Alpha Thal Disease)	βTT(Beta Thal Trait)	βTD(Beta Thal Disease)
$R_1 = I_{630}/I_{585}$	1	1.6	2.5	0.7	0.5

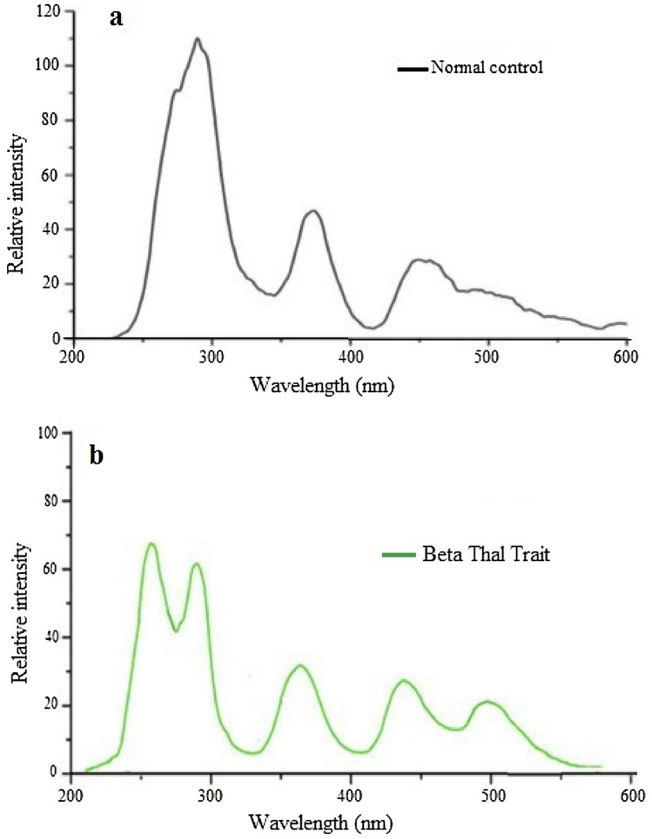

Fig. 2. Synchronous excitation spectra (SXS) of plasma of two of all five subjects. (a) normal control and (b) beta thal trait (βTT).

Table 3
Ratio parameter for different disorders from the excitation spectra of plasma components.

Parameter	Normal	βTT	βTD	αTT	αTD
I peak	100	60	5	80	20
$R_2 = I_{270}/I_{290}$	0.6	1.1	1.8	0.9	1.3
$R_3 = I_{450}/I_{370}$	0.6	0.9	20	1.0	10

Table 4
Ratio parameter ($R4 = V_{plasma}/V_{cellular\ component}$) for different disorders.

Parameter	Normal	βTT	βTD	αTT	αTD
R_4	1.22	1.5	1.7	0.9	0.7

630 nm, and 670 nm. Such peaks have been observed in a variety of malignant tissues, blood plasma, and malignant cell lines [16,17]. However, there is considerable disagreement in the identification of species responsible for each peak. For example, Masilamani et al. [22] examined porphyrin II in different pH environments and identified peaks at 585 nm, 630 nm, and 670 nm as the basic, neutral, and acidic forms of porphyrins. Others have associated these peaks with metalloporphyrin [27,28], in particular, zinc porphyrin. Such disagreements are understandable because porphyrins are complex molecules observed in equally complex matrices (*e.g.*, malignant tissues and plasma). In any case, in acetone extracts of RBCs of controls, cancer patients, and Thal subjects, two peaks (585 nm and 630 nm) are unmistakably present at different proportions. The relative intensity ratios of these peaks have been considered indicators of particular diseases.

For the sake of comparison, we normalized this quantity as 100% for normal, age-adjusted subjects. The relative value is 70% for βTT and 40% for βTD due to anemia. However, the ratio showed two different values for αTT. For 40% of αTT subjects, the ratio varied from 0.6 to 0.8 (mean 0.7), and for another set (60%), the ratio varied from 1.2 to 1.8 (mean 1.5 ± 0.2). This was in clear contrast to βTT, for which R_1 varied only from 0.6 to 0.8. Of the hundreds studied in a βTT screening, none exhibited ratios greater than 0.8 [27].

These results may be due to two possible variants to αTT (-α/ -α) and (–/α α). In the former case (cis type), one α is absent in each chromosome. In the latter case (trans type), two genes are absent in the same chromosome. There was no way of determining whether the cis or trans types manifests for each sample $R_1 < 1$. For HbH, all samples were β chains ($R_1 < 1.5$) in most cases (with only 15% less than 1). This is because HbH is considered abnormal due to a strong affinity for oxygen and poor delivery to the tissue cells [29]. RBCs in HbH essentially "hoarded" oxygen. This indicated that HbH patients will have unexpected health issues associated with iron overloading. After transfusion, βTD/HbH patients would experience "self-iron overloading". Because of this, transfusions are sparingly recommended for HbH patients.

The next most important blood component is plasma, which carries essential amino acids, enzymes, and RBC metabolites. The SXS of plasma provides data. The intensity of the peak at 290 nm is a measure of the wellness of the subject because it is the characteristic fluorescence excitation peak of essential amino acid tryptophan. Healthy RBCs are normalized to 100, whereas this value is 75 for αTT, 60 for βTT, 30 for HbH, and 5 for βTD. The poor health of βTD patients is evident in this gradation [30]. Similarly, $R_2 = I_{450}/I_{370}$ is 0.6 for normal cells but 1.2 for βTT cells. R_2 is only 1 for αTT, but it is 10 for HbH and 20 for βTD. I_{450} is a measurement of FAD, and I_{370} is that of NADH. The ratio R_2 is a measure of RBC degeneration [31,32].

Increasing values of R_2 indicates that αTT and βTT subjects will have marginal health issues. αTT subjects are relatively healthier than βTT subjects. However, βTD subjects will have serious hemolysis requiring repeated blood transfusion. Their RBCs have short life spans of approximately 40–50 days. HbH patients will have significant hemolysis but will not require transfusions. While βTD and HbH patients will have chronic diseases, the latter will not suffer from dramatic symptoms as much as the former.

The ratio parameter $R_3 = I_{270}/I_{290}$ is a ratio between tyrosine and closely related amino acid tryptophan. This is roughly proportional to the concentrations of immature to mature RBCs [33,34]. αTT subjects have 50% more immature cells (microcytosis); βTT have approximately 75% and HbH have almost 100%, but βTD have 300% (*i.e.*, RBC inefficiency increases in this order).

The ratio parameter R_4 is the ratio of plasma volume to cell volume. R_4 is 1.22 for normal cells, 1.7 for βTT, and 3 for βTD. The concentration or number of RBCs is lower for β Thal. However, RBC concentrations are higher than normal for αTT and HbH. The RBC concentration (R_4) is almost twice what it is in another inherited blood disorder, G6PD. HbH and G6PD exhibit similar clinical manifestations of anemia and increased hemolysis during viral infection or medical allergic reactions [35].

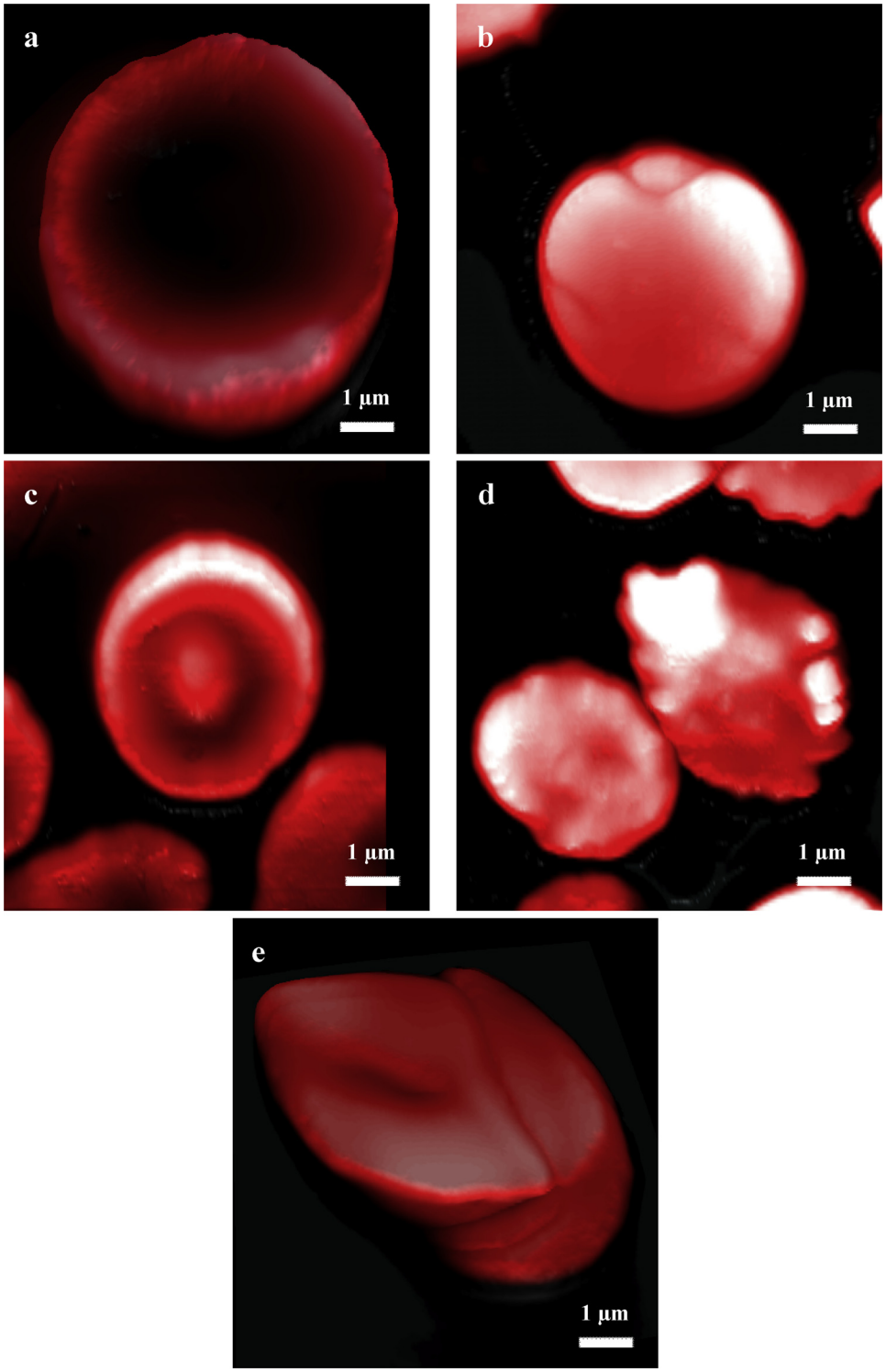

Fig. 3. Representative AFM images of the morphological appearance of red blood cells (RBCs) of subjects; (a) normal RBC, (b) RBC of alpha thal trait (αTT), (c) RBC of beta thal trait (βTT), (d)) RBC of alpha thal intermedia (HbH disease), (e) RBC of beta thal disease (βTD). The scale bar is 1 µm.

In this study, microscopy investigations using AFM showed obvious morphological differences between normal and Thal RBCs. The measured sizes of all types of Thal RBCs were smaller than those of healthy RBCs. A small proportion of αTT or βTT RBCs showed structural abnormalities. Decreases in the number of abnormal RBCs may explain why β (or α) Thal trait is generally asymptomatic. However, there remain risks of anemia, iron overload, and blood flow restriction in small blood vessels. These risks increase with increasing numbers of abnormal cells and increasing distortion.

Thal RBCs are characterized by their abnormal structures, reduced deformability, and increased aggregation [36–38]. This study showed that the aggregation of RBCs is strongly influenced by the degree of distortion. This observation was confirmed by the formation of larger clusters in βTD when compared with other types of Thal and by the high incidence of aggregates in RBCs of βTD with elongated shapes compared with other shapes, leading to increased thrombotic

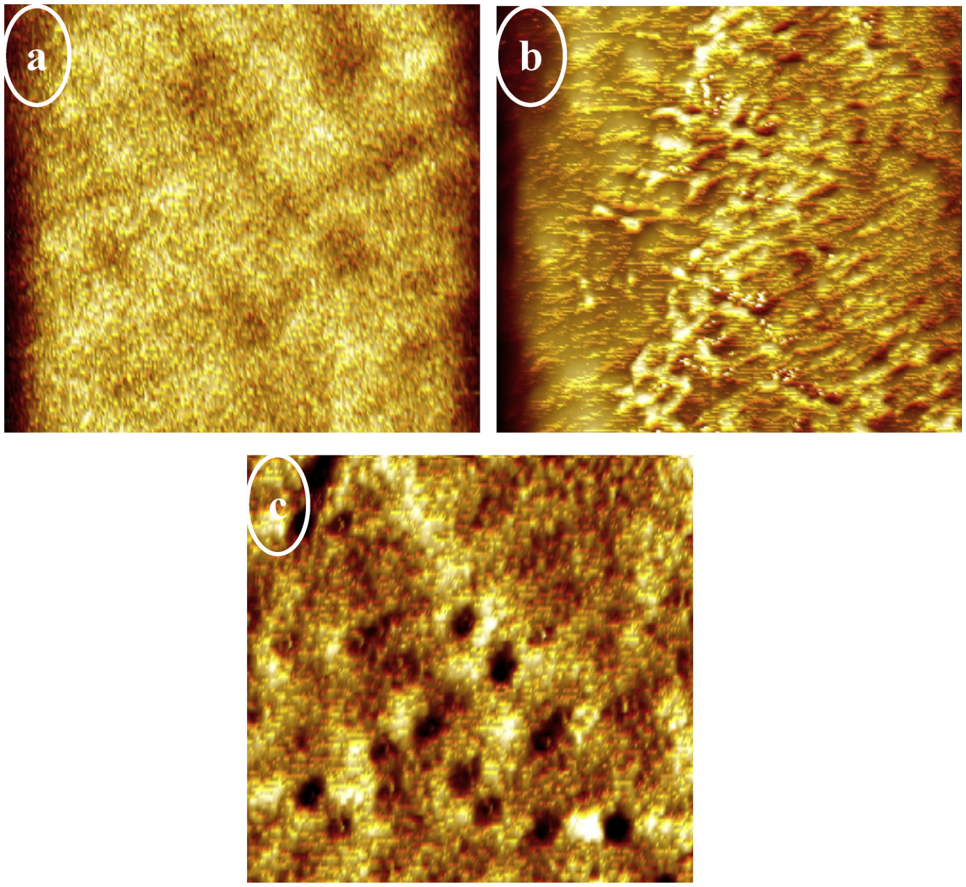

Fig. 4. The surface ultra structure of RBC (a) normal (b) beta thal trait (β TT) and (c) beta thal disease (βTD). Scan size 500 nm × 500 nm.

tendencies. The increased aggregation between cells may be attributed to the appearance of phosphatidylserine (PS) at cell surfaces. The cell membrane maintains phospholipid asymmetry by ATP activity [39]. Outer membrane leaflets are dominated by cholinephospholipids, such as phosphatidylcholine, whereas PS is confined to inner leaflets [40]. We expected such asymmetry may be missing in Thal RBCs. Correlations between ATP concentrations, the presence of PS, and RBC shape were identified.

5. Conclusion

Spectroscopy and AFM investigations on blood components of four types of Thal (αTT, αTD, βTT, βTD) has provided reasonable explanations for their clinical manifestation based on intrinsic molecular and morphological characteristics. The severity of these diseases increases in the order of αTT, βTT, HbH, to βTD. There were one-to-one correspondences between their clinical problems, structures, and spectroscopic features. This report provides a plausible explanation for clinical differences between HbH and βTD based on the abnormal presence of β components of globins.

Conflicts of interest

The Authors declare no conflicts of interest.

Contributions

SD, KA, VM, and MSA made substantial contributions in terms of spectroscopic and microscopic analysis, data collection and drafting of the original manuscript. FA and KF provided the blood samples. DC has interpreted the hematological data. All authors have read and approved the final manuscript.

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