Published in final edited form as:

J Biomol Struct Dyn. 2019 March ; 37(5): 1270–1281. doi:10.1080/07391102.2018.1456362.

Molecular Insights into the Irreversible Mechanical Behavior of Sick Hemoglobin

Sumith Yesudasan^a, Simone A. Douglas^b, Manu O. Platt^b, Xianqiao Wang^c, Rodney D. Averett^{a,*}

^aSchool of Chemical, Materials, and Biomedical Engineering, University of Georgia, Athens, GA 30602, USA

^bWallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA

^cSchool of Environmental, Civil, Agricultural and Mechanical Engineering, University of Georgia, Athens, GA 30602, USA

Abstract

Sickle cell disease is caused by the amino acid substitution of glutamic acid to valine, which leads to the polymerization of deoxygenated sickle hemoglobin (HbS) into long strands. These strands are responsible for the sickling of red blood cells (RBCs), making blood hyper-coagulable leading to an increased chance of vaso-occlusive crisis. The conformational changes in sickled RBCs traveling through narrow blood vessels in a highly viscous fluid are critical in understanding; however, there are few studies that investigate the origins of the molecular mechanical behavior of sickled RBCs. In this work, we investigate the molecular mechanical properties of HbS molecules. A mechanical model was used to estimate the directional stiffness of an HbS molecule and the results were compared to adult human hemoglobin (HbA). The comparison shows a significant difference in strength between HbS and HbA, as well as anisotropic behavior of the hemoglobin molecules. The results also indicated that the HbS molecule experienced more irreversible mechanical behavior than HbA under compression. Further, we have characterized the elastic and compressive properties of a double stranded sickle fiber using six HbS molecules, and it shows that the HbS molecules are bound to each other through strong inter-molecular forces.

Keywords

Sickle Cell; Molecular Dynamics; Erythrocytes; Molecular Mechanics; Thrombosis

*Corresponding author. raverett@uga.edu.

Supplementary information:

Accompanies this paper by two videos of HbS fiber compression.

Conflict of interest

There are no conflicts to declare.

Introduction

Sickle cell disease (SCD) is a group of inherited hemoglobinopathies with mutations that cause polymerization of hemoglobin molecules that deform RBCs into a sickle shape. Several health problems are associated with SCD, such as painful *sickle-cell crises*, anemia, swelling in the hands and feet, bacterial infections, and stroke (Konotey-Ahulu, 1974). Often, there is a direct connection between various types of blood related diseases and mechanical properties of blood cells (Mohandas & Evans, 1994). For example, hereditary elliptocytosis is related to decreased mechanical stability in the RBC membrane due to the mutated α and β spectrin (Lane et al., 1987). The role of the mechanical strength of RBCs in SCD and the possible implications in blood clot formation is poorly understood. Though many computational models (Lei & Karniadakis, 2012; H. Li, Ha, & Lykotrafitis, 2012; L. Lu, X. Li, P. G. Vekilov, & G. E. Karniadakis, 2016) exist which utilize coarse grain (CG) models to simulate HbS polymerization behavior, the mechanical properties in such models are empirically fitted from the experiments and are thus phenomenological in nature. In contrast, CG models developed from atomistic information can reproduce the accurate molecular mechanical behavior in compression and extension, and also the spontaneous polymerization of sickle fibers, which are crucial in understanding the bulk behavior of sickled RBCs in various physiological conditions. As a first step in developing such a molecular based CG model, in this work we investigate the mechanical properties of HbS (hemoglobin produced when homozygous for the sickle cell mutation), from a molecular mechanics perspective. We anticipate that our findings can be advantageous in creating a CG model of sickled RBCs to predict bulk mechanical properties of blood clots observed in SCD patients, which can give insights about hypercoagulation, and lead to new treatments for coronary related complications of SCD.

The molecular root cause of SCD is due to a single point mutation of the glutamic acid codon to a valine codon, in the sixth position of the amino acid sequence of the β -globin subunit of the hemoglobin. The difference in amino acid sequence was first reported for the HbS and HbA molecules by using paper chromatography (V. M. Ingram, 1956), followed by chemical characterization of HbS (V. Ingram, 1959), and precipitation rate studies related to unstable β sub unit (Asakura, Adachi, Sono, Friedman, & Schwartz, 1974). De-oxygenation promotes conformational changes that expose the valine codon to aqueous environment, inducing polymerization of HbS molecules into long strands called sickle hemoglobin fibers that deform the RBC membrane. HbS polymerization nucleus formation occurs because of the attraction of portions of HbS molecules containing Val- β 6 to the hydrophobic pockets Leu- β 88 and Phe- β 85 (Adachi, Reddy, Reddy, & Surrey, 1995). The molecular crystal structure of HbS (Wishner, Ward, Lattman, & Love, 1975), and the growth rate of HbS fiber polymerization (A. Aprelev, Liu, & Ferrone, 2011) have been experimentally investigated, showing that the sickle fiber consists of 14 strands (G. W. Dykes, R. H. Crepeau, & S. J. Edelstein, 1979), hexagonally packed with an elliptical cross section. Later, researchers showed that the 14 strands are actually organized into 7 double stranded pairs (D. W. Rodgers, R. H. Crepeau, & S. J. Edelstein, 1987). The half staggered longitudinal arrangement, positions of HbS molecules, and their polarities have been previously identified, including a hypothetical arrangement of HbS molecules to form a sickle fiber.

Additionally, researchers have concluded that decreasing intermolecular connections led to the limited radial growth of the fiber (Watowich, Gross, & Josephs, 1989). This was done using different strand-strand interactions by the Dykes-Rodgers model (Gene W Dykes, Richard H Crepeau, & Stuart J Edelstein, 1979; David W Rodgers, Richard H Crepeau, & Stuart J Edelstein, 1987) and Carragher model (Carragher, Bluemke, Gabriel, Potel, & Josephs, 1988), and a two-step mechanism of homogeneous fiber nucleus formation (Galkin et al., 2007). However, compared with other models, the Dykes-Rodgers model was better in elucidating the double strand packing (Creteigny & Edelstein, 1993) in HbS fibers.

Estimation of the mechanical properties of HbS fibers and sickled RBCs has been previously investigated. Mechanical properties of sickled RBCs such as membrane bending rigidity, and membrane extensional rigidity have been reported (Evans, Mohandas, & Leung, 1984), stating that the mechanical properties of sickle erythrocytes are strongly influenced by the state of cell hydration (density of hemoglobin). The shear elastic modulus and surface viscosity of irreversibly sickled RBCs (Nash, Johnson, & Meiselman, 1984) indicate a shear modulus twice that of RBCs that are still reversibly sickled. Using improved reconstruction methods, HbS fibers have been characterized with a higher degree of accuracy, including individual HbS molecule spacing, sequence of pairing, pitch length of the fiber, and polarity of the strands (Carragher et al., 1988), which were consistent with earlier findings of the fiber model (Gene W Dykes et al., 1979). Mechanical properties (bending and torsional rigidities) of HbS fibers were estimated using electron microscopy through thermal fluctuations and an anisotropic behavior was observed (Turner, Briehl, Wang, Ferrone, & Josephs, 2006). In other studies, the material mechanical properties of blood from a person with SCD were shown to play key roles in increased vascular rigidity (Belizna et al., 2012), and vaso-occlusions due to the polymerization of the HbS molecule (Alexey Aprelev, Stephenson, Noh, Meier, & Ferrone, 2013).

Researchers in this field have also developed mathematical models to investigate correlations between the sickled RBC and fiber formation. These studies can be categorized as: 1) coarse-grain molecular dynamics (CGMD) models to study mechanical properties, and 2) CGMD models to study the molecular basis of sickle fiber self-assembly. The first set of CGMD models were developed by adjusting the parameters of interaction to reproduce the bending and torsional rigidity (H. Li & Lykotrafitis, 2011) and other mechanical properties (H. Li et al., 2012) observed from experiments. In the second set of CG modeling simulations, the objective was to predict sickle fiber self-assembly and its effect on RBC distortion (X. Li, Caswell, & Karniadakis, 2012). These models were used to study morphological changes in RBCs under different polymer growth rates (Lei & Karniadakis, 2012), demonstrating that the growth rate is directly proportional to the HbS molecule concentration. The CG model (Lu Lu, Xuejin Li, Peter G Vekilov, & George Em Karniadakis, 2016) also confirms that the Dykes-Rodgers (Gene W Dykes et al., 1979; David W Rodgers et al., 1987) model reproduces the complex structure of the HbS polymer fiber (Lu Lu et al., 2016). However, this model assumes the nucleus of the sickle fiber is already formed and the fiber grows on it. Despite these advancements, little is known about the role of SCD in terms of molecular mechanical behavior of HbS, the spontaneous assembly of the sickle fiber nucleus, and the behavior of sickle cells and fibers during blood clotting. Some of the limitations of these models are the inability to simulate spontaneous

fiber formation, molecular basis for mechanical strength and polymerization, entirely different length scales between RBCs and computational models, and lack of explanation of fiber branching from the computational models.

In order to understand the molecular basis of the strength of RBCs and to develop CG models, calculations of the mechanical properties of the components such as the membrane and hemoglobin molecule are necessary. In this research, we performed a thorough study on the molecular mechanical properties of HbS and HbA to unveil compressive strength, internal chain elongation strength, and relaxation time after compression. We also investigated the elastic and compressive strength of a sickle fiber consisting of six HbS molecules using molecular dynamics (MD) simulations. The de-oxygenated state of HbS and HbA was used for these studies. Using the compression algorithm that has been previously developed (Yesudasan, Wang, & Averett, 2017), the solvated and non-solvated molecules were compressed and the resulting force versus deflection graph was used to estimate the stiffness of the molecules.

Methods

Molecular model

The molecular models for hemoglobin (Hb) stiffness estimation were taken from the online RCSB PDB repository. HbS (2HBS (Harrington, Adachi, & Royer Jr, 1997)) and HbA (2DN2 (Park, Yokoyama, Shibayama, Shiro, & Tame, 2006)) were oriented arbitrarily from the PDB repository (Fig. 1a and 1c), which were aligned to obtain a consistent geometric representation (Fig. 1b and 1d respectively). The alignment was performed by defining an imaginary tetrahedron with the iron atoms of the HEME residues as the corners and aligning its sides and corners consistently (Yesudasan et al., 2017) with the Cartesian coordinate system as explained next.

Figure 2a shows the molecular representation of the hemoglobin molecule with α -chains and β -chains. Even though the Hb molecule visually appears as a globular protein, it is not symmetric in any orientation. Hence, we defined a tetrahedron with corners as iron atoms of HEME residues as $\alpha 1$, $\beta 2$, $\alpha 3$ and $\beta 4$, which correspond to the respective chains. The Hb molecule was aligned based on this tetrahedron by (1) shifting $\beta 2$ to origin, (2) aligning side $\alpha 3$ - $\beta 2$ with the z-axis, and (3) aligning the normal of the $\alpha 1$ - $\alpha 3$ - $\beta 2$ plane with the x-axis. The vector that is perpendicular to the side $\alpha 3\beta 2$, passing through $\alpha 1$, is parallel to the y-axis and is called as “ $\alpha 3\beta 2$ - $\alpha 1\beta 4$ compression axis”. The vector that connects $\alpha 3$ and $\beta 2$ parallel to the z-axis is called the “ $\alpha 3\beta 2$ compression axis”. The vector that is normal to the plane $\alpha 1$ - $\alpha 3$ - $\beta 2$ and passes through $\beta 4$ is parallel to the x-axis and is called the “ $\alpha 1\beta 4$ compression axis”. The graphical representation and orientation of the x-axis ($\alpha 1$ - $\beta 4$), y-axis ($\alpha 3\beta 2$ - $\alpha 1\beta 4$) and z-axis ($\alpha 3\beta 2$) compression axes are shown in Fig. 2b, 2c and 2d respectively.

Two states of molecular models were considered for this study: 1) solvated using pure water and 2) non-solvated. The solvated models were generated using Visual Molecular Dynamics (VMD) (Humphrey, Dalke, & Schulten, 1996), spherically solvated, with 1.5 nm water as a skin layer on the periphery, which represents the Hb molecule in plasma. The non-solvated

models were used to understand the sensitivity of solvation on the mechanical strength of hemoglobin. MD simulations in this work were performed using Nanoscale Molecular Dynamics (NAMD) (Kalé et al., 1999). The time step of numerical integration for all simulations was 1 fs, with energy minimization for 5,000 steps at the initiation of the simulation. The force field used for the MD simulations was CHARMM27 (MacKerell, Banavali, & Foloppe, 2000) and the water molecular model used for solvation was TIP3P (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983). For constant temperature simulations, the system was kept at a constant temperature of 300 K (NVT ensemble) using a Langevin thermostat (Allen & Tildesley, 2017). The potential cut-off radius of the simulations was 1 nm with no linear center of momentum removal. The simulation box dimensions were not defined in the script, thus periodic boundary conditions (PBC) were not employed for the simulations. However, this was different from non-periodic and wall boundary conditions. In the absence of PBC, the long range Coulombic force calculation was not applicable; therefore, the Particle Mesh Ewald (PME) (Darden, York, & Pedersen, 1993) modules were not employed during the MD simulations. However, the Coulombic forces were calculated based on the CHARMM force field using a simple cutoff potential.

Compression strategy

To estimate the directional stiffness of the Hb molecules, we used two imaginary rigid walls to compress the de-oxygenated HbA and de-oxygenated HbS molecule in the solvated and non-solvated states. A distance dependent force was applied on the peripheral atoms of the spherically solvated (molecule inside a water sphere) Hb molecule, which mimics mechanical compression using two rigid walls (Fig. 3a). The imaginary rigid walls were initially kept at a distance d_0 from the center of the Hb molecule, which is also the origin of the coordinate system. During the MD simulation, these plates were moved toward the center at a constant velocity, $v = 1 \text{ Å/ps}$. At any instantaneous time, t , from the beginning of the simulation, the instantaneous location of the wall was given as: $d_0 - vt$. From this location, a region of influence was defined at a distance of r_c toward the inside of the molecule. This is different from the potential cut-off radius ($r_{cut} = 1 \text{ nm}$) and is given by $r_c = 0.8 \text{ nm}$.

Mathematically, if an i^{th} atom with $\vec{r}_i$ as the position vector is in the region

$|\vec{r}_i \cdot \vec{n}| > d_0 - vt - r_c$, then a force $\vec{f}_i$ was applied to it. Here, $\vec{n}$ is the normal of the imaginary rigid quadratic wall. The applied forces on atoms were distance dependent and intrinsically quadratic, to ensure a rigid wall. The force takes the form of a quadratic curve starting from zero at the point $(d_0 - vt - r_c)$ of influence (Fig. 3b), gradually increasing toward the imaginary plate. Equations 1 and 2 were used to calculate and apply the mechanical forces. For our studies, v was taken as 1 Å/ps and d_0 as 35 Å . To create a rigid imaginary wall, we set f_0 to 0.2 kcal/mol/Å . A lower value of f_0 would result in a soft wall.

$$\vec{f}_i = -F\vec{n} \frac{(\vec{r}_i \cdot \vec{n})}{|\vec{r}_i \cdot \vec{n}|} \quad (1)$$

$$F = \begin{cases} f_0 [\vec{r}_i \cdot \vec{n} - (d_0 - vt - r_c)]^2, & \text{if } |\vec{r}_i \cdot \vec{n}| > d_0 - vt - r_c \\ 0, & \text{if } |\vec{r}_i \cdot \vec{n}| \leq d_0 - vt - r_c \end{cases} \quad (2)$$

The gradient in Fig. 3a shows the magnitude of the force applied to the molecule, which is small near $d_0 - vt - r_c$ and significantly large near $d_0 - vt$. The force was applied only on the qualifying atoms of the Hb molecule in the region of influence at every 10 fs. A TCL script in conjunction with NAMD was used to impose these force criteria on the system.

Results and Discussion

Stiffness estimation of hemoglobin

Using the compression strategy mentioned above, we applied forces on the Hb molecules and computed the force versus deflection behavior for both solvated and non-solvated cases. Fig. 4a and 4b shows the variation in force required to compress Hb molecules along x-axis ($\alpha 1\beta 4$), y-axis ($\alpha 3\beta 2$ - $\alpha 1\beta 4$) and z-axis ($\alpha 3\beta 2$), for both HbS and HbA de-oxygenated molecules.

The results from the Hb compression simulations show an initial elastic behavior with an ensuing rigid mechanical behavior in both the solvated and non-solvated cases. The compression simulation results show a varying compressibility along each direction and among different types of Hb molecules. Clearly, the non-solvated Hb molecules were more easily compressed (Fig. 4b), compared with the solvated ones (Fig. 4a). The compression study implies that the initial soft region shows elastic properties while the stiff core compression leads to strain stiffening behavior of the Hb molecules, which we will refer to as the *elastic soft shell region* and the *stiff core region*. The strain stiffening deformation analysis may not be relevant from a physiological perspective; thus, we limit our stiffness estimation only to the elastic regions by considering the slope of the force versus deflection curve until 1 nm compression (Fig. 5). To account for the statistical nature of the results from the MD simulations, we repeated the compression simulations 5 times for each case (60 total runs) for estimating the directional stiffness.

The HbS molecules possess an increased stiffness compared to HbA molecules in x-axis ($\alpha 1\beta 4$) and y-axis ($\alpha 3\beta 2$ - $\alpha 1\beta 4$) compression (Fig. 5a and Fig. 5b). However, the z-axis ($\alpha 3\beta 2$) compression results show an overlap in the stiffness, indicating that there is little stiffness dominance by any type of Hb molecule. The average values of the stiffness show that the solvated HbS molecule is 46% stiffer than HbA along the $\alpha 1\beta 4$ -direction, and 27% stiffer along the $\alpha 3\beta 2$ - $\alpha 1\beta 4$ -direction. For the non-solvated cases, the HbS molecules are 32% and 40% stiffer along the $\alpha 1\beta 4$ -axis and $\alpha 3\beta 2$ - $\alpha 1\beta 4$ -axis, respectively. In all cases, the solvation of the molecules increased the stiffness by 30 – 45 % for both HbA and HbS molecules.

From the Hertz model, for a deformable spherical body under compression, the relationship between normal force and strain takes the form $F_N \propto \epsilon^n$ (Mukhopadhyay & Peixinho, 2011). Here, $\epsilon = (d_0 - d)/d_0$, d_0 is the diameter of the sphere and d is the compression thickness. We estimated the parameter n for our compression cases. Though, our model is not exactly

spherical, the estimation shows that $n = 1.5, 2.25, 1.58$ for HbA and $n = 1.45, 1.71, 1.66$ for HbS, which is similar (1.25) to what was found in the literature (Mukhopadhyay & Peixinho, 2011) for hydrogel spheres.

Relaxation of hemoglobin

Next, we studied the elasto-plastic irreversible compression behavior of the Hb molecules, by performing relaxation studies. This was done by compressing the molecules for a specific time or to a specific thickness and then allowing relaxation. All four states of Hb, including the solvated de-oxygenated HbA, solvated de-oxygenated HbS, and non-solvated de-oxygenated HbA and HbS molecules were considered in this study. For brevity, we limit the compression studies discussion only along the $\alpha 1\beta 4$ -direction (x-axis). In the first set of simulations, we compressed the Hb molecules by 1 nm along its diameter. Since each molecule has different stiffness values, the time required for compressing to a specific thickness varied. This is due to the characteristics of the quadratic wall, which compresses softer materials faster than stiffer ones. Hence, the time required for 1 nm compression was determined by trial and error for every Hb molecule. After 1 nm compression, the force application on the Hb molecules was ceased and the MD models were equilibrated (relaxed) at constant temperature 300 K for another 200 ps. The resulting thickness (along the diameter) evolution of Hb molecules was calculated (Fig. 6a). In the second set of simulations, the molecules were compressed for 20 ps (approximately 2.5 nm to 3 nm compression to reach the stiff core region) and then allowed to relax (Fig. 6b).

The relaxation study results indicate that for 1 nm compression, the Hb molecules were elastic, but subsequent to 20 ps of mechanical compression, the molecules experienced irreversible deformation. The uncompressed thicknesses (thickness of Hb molecule prior to force application) along the x-direction ($\alpha 1\beta 4$), for both HbS and HbA are shown in Fig. 6a and 6b for reference. The uncompressed thicknesses were the same for both solvated and non-solvated cases. Relaxation results also show that the solvated Hb molecule possesses a faster relaxation rate compared with the non-solvated cases. This indicates that the Hb molecule possesses elastic properties for smaller compressive forces, while large forces engender irreversible mechanical behavior. Another interesting fact we observed was that the HbS rate of relaxation was slower than that of HbA in both the solvated and non-solvated cases. These relaxation study results also corroborate the notion of an elastic soft shell region and a strain stiffening, stiff core region.

Temperature sensitivity on stiffness

To investigate the influence of temperature on stiffness of the Hb molecules, the compression studies were extended by varying the system temperature. Four cases of temperatures (280 K, 300 K, 320 K, and 340 K) were studied for both de-oxygenated HbA and HbS. Five sets of MD simulations were performed for every case. Again, for brevity, we have performed this study only for $\alpha 1\beta 4$ (x-axis) compression (Fig. 7a and 7b).

Figure 7a shows the temperature dependent stiffness of HbA and HbS structures for solvated states and Figure 7b shows the same for non-solvated states. The variation of the average stiffness between 300 K (26.8 °C) and 320 K (46.8 °C) is not significant (10 % for solvated

HbA, and 1% for the remaining structures). This also means that the stiffness values of HbA and HbS molecules are less sensitive to the changes in normal human body temperature (33.2 ° to 38.1 °C (Sund-Levander, Forsberg, & Wahren, 2002)). HbS shows dominance over HbA in mechanical strength across all temperatures. For solvated and non-solvated states, there was a maximum variation in mechanical strength of 38% and minimum variation of 15%.

Internal tetrahedral structure

We also investigated the internal structural stability of HbS molecules for the non-solvated case, by which we can distinguish between the behaviors of the Hb molecules under compression (when RBCs deform) and extension scenarios, especially during fiber formation. The two α -chains and two β -chains were interconnected through non-covalent interactions (Fig. 8a). The objective was to pull the hemoglobin structure along different directions internally to understand the internal structural behavior of the molecule under tension. For this, we used the same tetrahedral arrangement of the iron atoms mentioned in the previous sections. The locations of iron atoms present in the Hb molecule are enhanced and marked along with heme residues in Fig. 8b. By interconnecting the iron atoms, we formed the tetrahedron, with its center of mass (COM) shown as an orange sphere (Fig. 8c).

This tetrahedral structural model represents the internal structural framework of the Hb molecule and we assume that the model is constructed of beads interconnected with springs. Each side of the tetrahedron (Fig. 8c) represents a spring. To estimate the spring stiffness, a force was applied to one of the corners of the tetrahedron, while the other three corners were restrained. The pulling direction was along the normal of the vector connecting the center of mass and the respective pulling corner. This simulation was performed using steered molecular dynamics (SMD) in NAMD via an external TCL script. The pulling procedure was repeated for all corners of the tetrahedron and the spring stiffness was averaged from the solution of all equations. For example, if the corner FE2 (Fig. 8c) was pulled, then the normal pulling vector was $\vec{n}_{pull} = (\vec{r}_{COM} - \vec{r}_{FE2}) / |\vec{r}_{COM} - \vec{r}_{FE2}|$, and the corresponding spring forces are given by $F_{23} = \vec{F} \cdot \vec{n}_{23}$, $F_{24} = \vec{F} \cdot \vec{n}_{24}$, and $F_{21} = \vec{F} \cdot \vec{n}_{21}$ respectively. Here, the subscripts 1, 2, 3 and 4 represent the corners FE1 (α 1), FE2 (β 2), FE3 (α 3) and FE4 (β 4) respectively, $\vec{F}$ is the total force acting on the pulling bead, and $\vec{n}$ is the normal to the individual sides of the tetrahedron.

A constant force of 800 pN was incrementally added to the pulling corner bead during every step of the simulation for 10 ps. The deflections of the springs were estimated by calculating the instantaneous tetrahedron extension. Figure 9a–d shows the force vs. deflection behavior of the tetrahedron for different pulling conditions. To obtain a linear spring stiffness, we considered the force-deflection curve data for the initial 0.5 nm extension, and calculated the slopes. These stiffness values are reported (Fig. 9e) for various springs K_{12} , K_{13} , K_{14} , K_{23} , K_{24} and K_{34} which are graphically marked in Fig. 9f.

The maximum internal tetrahedron stiffness was 2.6 kN/m, while the maximum directional stiffness from the compression simulations was 100 kN/m. This indicates that the compression strength of the HbS molecule is approximately 40 times higher than its internal

elastic strength. In essence, the HbS molecule can sustain highly compressible mechanical forces in comparison to the weaker internal extensional strength.

Sickle fiber compression

To understand the behavior of the HbS molecules while forming a strand, it was necessary to study the compressive and elastic properties of multiple molecules. To determine the compression behavior, we arranged the HbS molecules in a row (Fig. 10a), with the Val β 6 aligned with the hydrophobic pockets Phe β 85 and Leu β 88 (Fig. 10b shows these interactions). The system consists of 6 molecules, solvated with a 1 nm thick water-box surrounding it, and equilibrated for 40 ps. The equilibrated HbS strand was then compressed from both sides (Fig. 10a), using the compression strategy of rigid walls (Yesudasan et al., 2017). The time elapsed molecular representation of this compression procedure is visualized (Fig. 10d–g), at 0 ps, 20 ps, 30 ps and 40 ps respectively. The compression procedure was conducted for a total of 5 runs and the resulting force vs. deflection curve was calculated (Fig. 10c). The trend shows an initial strong resistance to compression and then a corresponding irreversible behavior (elasto-plastic mechanical behavior). During the initial phase, the stiffness is estimated to be 213 N/m and during the later phase the stiffness is 33 N/m.

Interestingly, the HbS molecules remained in tact and did not reveal any mechanism of slipping off the compression axis. This demonstrates the strong hydrophobic attractions of the HbS molecules. This study also shows that the HbS molecules are so closely packed that they are strong in compression due to their tandem arrangement and also due to the inter-molecular bonding strength. The average compressive strength of a single HbS molecule is 90,000 N/m, which is 422 times greater than the fiber compressive strength.

Sickle fiber extension

To estimate the elastic properties of the HbS fiber, we used the same molecular model (Fig. 10a) to perform the extension analysis. The left most molecule was restrained and the right most HbS molecule was pulled along the axis of the fiber (Fig. 11a) using SMD simulations in NAMD. Default pulling parameters were used for SMD with a spring stiffness of 7 kcal/mol/Å² and a pulling velocity of 0.005 Å/fs. To avoid any localized deformations, the pulling spring was connected to all the atoms of the HbS molecule on the right. The time evolution behavior of the HbS strand extension is shown in Fig. 11d–h, with details of the hydrophobic interactions marked in Fig. 11(i–m). The extension study indicates that the HbS molecules do not shear off easily and are restrained by reasonable attractive forces.

The force vs. extension behavior of the HbS fiber was calculated (Fig. 11b). The initial linear region shows an elastic stiffness of 4.23 N/m, and the effect of hydrogen bonds on the stiffness was checked by calculating the number of hydrogen bonds formed over time (Fig. 11c). One of the interesting facts that we observed from the extension study is that the hydrophobic interactions in the HbS fiber were not significant and those regions remained stable during extension (Fig. 11i). The average elastic strength of a single HbS molecule is 1,900 N/m, which is 449 times greater than the fiber elastic strength. The ratio of the compressive strength to elastic strength in both the single HbS molecule and fiber cases is

47.36 and 50.35, respectively. This indicates that the sickle fiber can be modeled using a linear springs network. However, such a network will have sophisticated interactions between inter-molecular and intra-molecular springs which makes it a challenging problem to solve. Hence, we will limit our assumptions based on a simple elastic network which constructs the fiber with 4 strong intra-molecular springs (pulling and fixed molecules are exempted) with constant as k_1 , 4 tandem inter-molecular springs (non hydrophobic) with a stiffness of k_2 , and 5 zigzag hydrophobic springs with a stiffness of k_3 . Under the assumption of simple springs in series, and assuming $k_2 = k_3$, then $1/k_{equiv} = 4/k_1 + 9/k_2$. We know that $k_{equiv} = 4.23 \text{ N/m}$ and $k_1 = 1,900 \text{ N/m}$. Using this information, the stiffness of the inter-molecular strength is 38.41 N/m .

Limitations and Scope for Future Work

In this section we discuss some of the limitations of this work and some suggested improvements to the mechanical models. In the present work, a constant compression rate of $v = 1 \text{ \AA/ps}$ was used. Higher compression rates necessitate usage of low time integration steps which leads to unstable simulations and lower compression rates lead to very long simulation times. Indeed the viscous behavior (if any) of the molecule can be characterized by running multiple simulations with varying compression rates. Another improvement that can be made is to estimate the bulk compression properties of hemoglobin by having many molecules in a system while compressing them. This would require high computational power, careful designing of the compression strategy, but may be more representative of the physiological condition of hemoglobin inside the RBCs. Again, the sickle hemoglobin stiffness estimated in this work can be used in the models of (H. Li et al., 2012) to develop an improved coarse grain model.

Conclusions

We performed all-atom molecular dynamics simulations for calculating the directional stiffness of both HbS and HbA molecules. The solvated and non-solvated Hb molecules were compressed along three different directions and the corresponding directional stiffnesses were computed. The results showed an anisotropic behavior of the HbS molecules, in particular with high stiffness along $\alpha 3\beta 2-\alpha 1\beta 4$ (y-axis) compared to $\alpha 1\beta 4$ (x-axis). The results also showed that the HbS structure is 27-46% stronger than HbA. By varying the hemoglobin system temperature, we did not notice any significant strength differences in the normal human body temperature range; however, an increased stiffness was observed at lower temperatures. Solvation of the molecules also played an important role in stiffness, increasing it by approximately 40%. The stiffness results indicate a soft initial resistance and a stiffer resistance over time. This is due to the *soft elastic* molecules at the periphery of the Hb molecules. The soft and stiff regions were shown to be elastic and irreversible based on results from the relaxation simulations. Our extended studies with the internal tetrahedral model based elastic analysis indicates a weak elastic strength of the Hb molecule. The stiffness computed from the internal perturbation was 40 times weaker than the bulk compressive strength. Further, the compression and extension studies with the HbS strand indicates that the hydrophobic and Coulomb interactions among the HbS molecules are not insignificant and play an important role in strengthening the fiber. The inter HbS

attractive spring constant was estimated as 38.41 N/m , which represents a significant force of attraction. Our studies also suggest that the compressive properties of sickle fibers are reasonably high that mere buckling or shear sliding will likely not occur due to the considerable inter HbS attractive forces. Using our elastic simulation results, a CG model development could elucidate the actual mechanical behavior of sickled RBCs. This work can also serve as the foundation toward the development of a realistic CG Hb model that can be used to simulate the spontaneous formation of sickle fibers through molecular self-assembly and conformational changes of a sickle cell in various physiological and pathological states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Research reported in this publication was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health under Award Number K01HL115486. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This study was also supported in part by resources and technical expertise from the Georgia Advanced Computing Resource Center, a partnership between the University of Georgia's Office of the Vice President for Research and Office of the Vice President for Information Technology.

References

- Adachi K, Reddy LR, Reddy KS, & Surrey S (1995). Mutational analysis of phenylalanine beta 85 in the valine beta 6 acceptor pocket during hemoglobin S polymerization. *Protein Sci*, 4(7), 1272–1278. doi: 10.1002/pro.5560040703 [PubMed: 7670370]
- Allen MP, & Tildesley DJ (2017). *Computer simulation of liquids*: Oxford university press.
- Aprelev A, Liu Z, & Ferrone FA (2011). The growth of sickle hemoglobin polymers. *Biophys J*, 101(4), 885–891. doi: 10.1016/j.bpj.2011.05.064 [PubMed: 21843479]
- Aprelev A, Stephenson W, Noh HM, Meier M, & Ferrone FA (2013). The Physical Foundation of Vaso-Occlusion in Sickle Cell Disease. *Biophysical Journal*, 104(2), 677a. [PubMed: 23442918]
- Asakura T, Adachi K, Sono M, Friedman S, & Schwartz E (1974). Mechanical stability of hemoglobin subunits: An abnormality in β S-subunits of sickle hemoglobin. *Biochem Biophys Res Commun*, 57(3), 780–786. [PubMed: 4827832]
- Belizna C, Loufrani L, Ghali A, Lahary A, Primard E, Louvel J-P, ... Ifrah N (2012). Arterial stiffness and stroke in sickle cell disease. *Stroke*, 43(4), 1129–1130. [PubMed: 22198986]
- Carragher B, Bluemke DA, Gabriel B, Potel MJ, & Josephs R (1988). Structural analysis of polymers of sickle cell hemoglobin: I. Sickle hemoglobin fibers. *J Mol Biol*, 199(2), 315–331. [PubMed: 3351926]
- Corey RB, & Pauling L (1953). Molecular models of amino acids, peptides, and proteins. *Review of Scientific Instruments*, 24(8), 621–627.
- Cretegnay I, & Edelstein SJ (1993). Double strand packing in hemoglobin S fibers: Elsevier.
- Darden T, York D, & Pedersen L (1993). Particle mesh Ewald: An $N \cdot \log(N)$ method for Ewald sums in large systems. *The Journal of chemical physics*, 98(12), 10089–10092.
- Dykes GW, Crepeau RH, & Edelstein SJ (1979). Three-dimensional reconstruction of the 14-filament fibers of hemoglobin S. *J Mol Biol*, 130(4), 451–472. [PubMed: 480359]
- Dykes GW, Crepeau RH, & Edelstein SJ (1979). Three-dimensional reconstruction of the 14-filament fibers of hemoglobin S. *J Mol Biol*, 130(4), 451–472. [PubMed: 480359]
- Evans E, Mohandas N, & Leung A (1984). Static and dynamic rigidities of normal and sickle erythrocytes. Major influence of cell hemoglobin concentration. *The Journal of clinical investigation*, 73(2), 477–488. [PubMed: 6699172]

- Galkin O, Pan W, Filobelo L, Hirsch RE, Nagel RL, & Vekilov PG (2007). Two-step mechanism of homogeneous nucleation of sickle cell hemoglobin polymers. *Biophysical Journal*, 93(3), 902–913. [PubMed: 17449671]
- Harrington DJ, Adachi K, & Royer WE Jr (1997). The high resolution crystal structure of deoxyhemoglobin S1. *J Mol Biol*, 272(3), 398–407. [PubMed: 9325099]
- Humphrey W, Dalke A, & Schulten K (1996). VMD: visual molecular dynamics. *Journal of molecular graphics*, 14(1), 33–38. [PubMed: 8744570]
- Ingram V (1959). Abnormal human haemoglobins. III the chemical difference between normal and sickle cell haemoglobins. *Biochim Biophys Acta*, 36(2), 402–411. [PubMed: 13852872]
- Ingram VM (1956). A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin. *Nature*, 178(4537), 792. [PubMed: 13369537]
- Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, & Klein ML (1983). Comparison of simple potential functions for simulating liquid water. *The Journal of chemical physics*, 79(2), 926–935.
- Kalé L, Skeel R, Bhandarkar M, Brunner R, Gursoy A, Krawetz N, ... Schulten K (1999). NAMD2: greater scalability for parallel molecular dynamics. *Journal of Computational Physics*, 151(1), 283–312.
- Konotey-Ahulu FI (1974). The sickle cell diseases. Clinical manifestations including the “sickle crisis”. *Arch Intern Med*, 133(4), 611–619. [PubMed: 4818434]
- Lane PA, Shew RL, Iarocci TA, Mohandas N, Hays T, & Mentzer WC (1987). Unique alpha-spectrin mutant in a kindred with common hereditary elliptocytosis. *J Clin Invest*, 79(3), 989–996. doi: 10.1172/JCI112911 [PubMed: 3818958]
- Lei H, & Karniadakis GE (2012). Predicting the morphology of sickle red blood cells using coarse-grained models of intracellular aligned hemoglobin polymers. *Soft Matter*, 8(16), 4507–4516.
- Li H, Ha V, & Lykotrafitis G (2012). Modeling sickle hemoglobin fibers as one chain of coarse-grained particles. *Journal of Biomechanics*, 45(11), 1947–1951. [PubMed: 22673758]
- Li H, & Lykotrafitis G (2011). A coarse-grain molecular dynamics model for sickle hemoglobin fibers. *Journal of the Mechanical Behavior of Biomedical Materials*, 4(2), 162–173. [PubMed: 21262494]
- Li X, Caswell B, & Karniadakis GE (2012). Effect of chain chirality on the self-assembly of sickle hemoglobin. *Biophysical Journal*, 103(6), 1130–1140. [PubMed: 22995485]
- Lu L, Li X, Vekilov PG, & Karniadakis GE (2016). Probing the Twisted Structure of Sickle Hemoglobin Fibers via Particle Simulations. *Biophys J*, 110(9), 2085–2093. doi: 10.1016/j.bpj.2016.04.002 [PubMed: 27166816]
- Lu L, Li X, Vekilov PG, & Karniadakis GE (2016). Probing the twisted structure of sickle hemoglobin fibers via particle simulations. *Biophysical Journal*, 110(9), 2085–2093. [PubMed: 27166816]
- MacKerell AD, Banavali N, & Foloppe N (2000). Development and current status of the CHARMM force field for nucleic acids. *Biopolymers*, 56(4), 257–265. [PubMed: 11754339]
- Mohandas N, & Evans E (1994). Mechanical properties of the red cell membrane in relation to molecular structure and genetic defects. *Annu Rev Biophys Biomol Struct*, 23, 787–818. doi: 10.1146/annurev.bb.23.060194.004035 [PubMed: 7919799]
- Mukhopadhyay S, & Peixinho J (2011). Packings of deformable spheres. *Physical Review E*, 84(1), 011302.
- Nash GB, Johnson CS, & Meiselman H (1984). Mechanical properties of oxygenated red blood cells in sickle cell (HbSS) disease. *Blood*, 63(1), 73–82. [PubMed: 6689955]
- Park S-Y, Yokoyama T, Shibayama N, Shiro Y, & Tame JR (2006). 1.25 Å resolution crystal structures of human haemoglobin in the oxy, deoxy and carbonmonoxy forms. *J Mol Biol*, 360(3), 690–701. [PubMed: 16765986]
- Rodgers DW, Crepeau RH, & Edelstein SJ (1987). Pairings and polarities of the 14 strands in sickle cell hemoglobin fibers. *Proceedings of the National Academy of Sciences*, 84(17), 6157–6161.
- Rodgers DW, Crepeau RH, & Edelstein SJ (1987). Pairings and polarities of the 14 strands in sickle cell hemoglobin fibers. *Proc Natl Acad Sci U S A*, 84(17), 6157–6161. [PubMed: 3476937]
- Sund-Levander M, Forsberg C, & Wahren LK (2002). Normal oral, rectal, tympanic and axillary body temperature in adult men and women: a systematic literature review. *Scandinavian Journal of Caring Sciences*, 16(2), 122–128. [PubMed: 12000664]

- Turner M, Briehl R, Wang J, Ferrone F, & Josep R (2006). Anisotropy in sickle hemoglobin fibers from variations in bending and twist. *J Mol Biol*, 357(5), 1422–1427. [PubMed: 16490203]
- Watowich SJ, Gross LJ, & Josep R (1989). Intermolecular contacts within sickle hemoglobin fibers. *J Mol Biol*, 209(4), 821–828. [PubMed: 2585512]
- Wishner BC, Ward K, Lattman E, & Love WE (1975). Crystal structure of sickle-cell deoxyhemoglobin at 5 Å resolution. *J Mol Biol*, 98(1), 179–194. [PubMed: 1195378]
- Yesudasan S, Wang X, & Averett RD (2017). Molecular dynamics simulations indicate that deoxyhemoglobin, oxyhemoglobin, carboxyhemoglobin, and glycated hemoglobin under compression and shear exhibit an anisotropic mechanical behavior. *Journal of Biomolecular Structure and Dynamics*, 1–13. [PubMed: 26727155]

Figure 1: Molecular model of de-oxygenated HbS (a) before rotation (b) after rotation. De-oxygenated HbA model (c) before rotation (d) after rotation. These models were rotated and aligned systematically to obtain a consistent configuration.

Figure 2:

Alignment and directional nomenclature of the Hb molecules. (a) The molecular model of Hb is shown with α -chain (red) and β -chain (blue). (b) The iron atoms of heme residues of every chain are labeled as $\alpha 1$, $\beta 2$, $\alpha 3$ and $\beta 4$ and represented as solid spheres, with transparent chains in the background. The y-axis is the direction of the vector connecting the edges $\alpha 3$ - $\beta 2$ and $\alpha 1$ - $\beta 4$, while the direction of $\alpha 1$ - $\beta 4$ represents the direction of the x-axis. (c) $\beta 2$ is the origin of the coordinate system and $\alpha 3$ - $\beta 2$ represents the z-axis direction. (d) Alignment of the molecule with x-axis and z-axis is shown from a different orientation. (Molecular details are semitransparent to enhance visualization of the tetrahedron arrangement in Fig. 2b-d)

Figure 3:

Compression strategy used for the hemoglobin molecule. (a) Two imaginary rigid walls travel at constant velocity toward the center of the Hb molecule and exert a distance dependent force on the atoms (Magnitude of the force applied is shown as the intensity of red) (b) The applied force magnitude as a function of distance, where the horizontal axis represents the distance from the origin (left). The force acting on atoms in the region of influence at a particular time t is shown in the shaded diagonal pattern.

Figure 4:

Compressive force vs. deflection of the Hb molecules for (a) solvated and (b) non-solvated cases. The x-axis shows the deflection of hemoglobin, corresponding to the compression axis and the y-axis shows the applied force (mN). The initial peak represents the elastic soft shell region and the steeper region is indicative of strain stiffening of the Hb molecule (stiff core region).

Figure 5: Stiffness variation along x-axis, y-axis, and z-axis during compression. Average stiffness values of sickle and normal de-oxygenated hemoglobin molecules are shown for (a) solvated and (b) non-solvated cases. (The error bars show the minimum and maximum stiffness values.)

Figure 6:

Relaxation behavior of hemoglobin for different compression states. (a) The hemoglobin molecules were compressed for 1 nm and then allowed to relax for 200 ps for both solvated and non-solvated cases. (b) Hemoglobin molecules compressed for 20 ps and then allowed to relax for 200 ps. The graph shows the thickness along the x-direction of the molecules during relaxation.

Figure 7: Temperature sensitivity on Hb stiffness. Stiffness results for (a) solvated HbA and solvated HbS molecule, (b) non-solvated HbA and HbS molecule at temperatures of 280 K, 300 K, 320 K and 340 K.

Figure 8:

Tetrahedral modeling of de-oxygenated HbS molecule. (a) Molecular model of HbS with α -chains and β -chains. The HEME residues are also visualized using CPK molecular representation (Corey & Pauling, 1953). (b) The iron atoms of HEME residues visually enlarged to show their location, and the possible imaginary tetrahedral arrangement. (c) A tetrahedron is visualized by connecting all iron atoms. The center of mass (COM) of these four atoms is shown (orange sphere in center).

Figure 9: Elastic stiffness of the internal tetrahedral arrangement. (a-d) Force vs. deflection of the sides of tetrahedron for different pulling directions. (e) Estimated linear stiffness for the sides of the tetrahedron. (f) Nomenclature for the springs of the tetrahedron.

Figure 10:

Six HbS molecules arranged in fiber formation with (a) Val-β6 associating into the hydrophobic pockets Leu-β88 and Phe-β85. This solvated fiber arrangement was compressed along the axial direction from both ends. (b) The hydrophobic interaction locations (circles). (c) Force vs. deflection mechanical behavior of the fiber strand (5 runs). Time elapsed behavior showing the compression of the HbS strand at (d) 0 ps, (e) 20 ps, (f) 30 ps and (g) 40 ps.

Figure 11:

Elastic strength testing of the HbS fiber. (a) SMD was used to restrain the left end molecule of the fiber and to pull the right most molecule outwards. (b) Force vs. deflection curve of the sickle fiber during extension (Upper x-axis shows the time during extension). (c) Number of hydrogen bonds formed between HbS molecules during extension. Time evolution of the HbS molecules under tension is shown from (d-h) (Water hidden for clarity). Time elapsed behavior of HbS strand (i-m) with Val-β6---Leu-β88---Phe-β85 interaction (circles).