Perspective

Chemical Engineers and the Fundamental Understanding of Human Disease

Peter G. Vekilov
Depts. of Chemical and Biomolecular Engineering and Chemistry, University of Houston, Houston, TX 77204

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The Challenges of Modern Life and Chemical Engineers

Numerous lists of the challenges and pressing problems facing humankind have been proposed. Those put forth by politicians necessarily contain terrorism and conflict, those assembled by economists focus on trade barriers and poverty. Scientists and engineers have come up with relatively few such compilations. The most prominent of these seems to be the one by Richard Smalley, the co-discoverer of the bucky balls, which, in one of its forms, is shown in Table 1. Several items are present among the top 10 in almost all such lists, regardless of whether they are composed by politicians, scientists, or economists: energy, the environment, terrorism, and human disease.

Chemical engineers have been actively working on solutions to the energy crisis facing us, in fact, the formulation and optimization of energy carriers is one of the classical subjects of chemical engineering. Alternative energy sources, such as solar, geothermal, and others are a popular research area in our community. The preeminent environmental problems, air and water pollution and global climate change, are other foci of research activities. Even the fight against terrorism, of which the socioeconomic and military aspects seem to be overwhelmingly more important that the scientific ones, has found volunteers among us, who mostly address detection and prevention issues.

In the fight against disease, chemical engineers have expanded several biomedical fields. Chemical engineers are active in medicinal nanotechnology. Nanoparticles of carbon (nanotubes and bucky balls) or transition metals are employed to identify and affect disease centers. Newly developed methods rely on nanoparticles functionalized in a way to selectively bind to tumor cells, and destroy them by local heating induced by irradiation with a radiofrequency, see [1] for example. The contribution of chemical engineers has been crucial in the development of several drug and gene delivery systems: embedded in polymer matrix for a slow sustained release,[2] and as inhalable preparations,[3] which allow direct access to the blood stream through the lungs. Chemical engineers have elucidated significant problems in the vast areas of stem cell therapy,[4] tissue engineering,[5] bacterial population growth,[6] and many others. This list is by far not conclusive, and only contains a few illustrative examples.

There is a class of diseases for which the contribution of chemical engineers and physical chemists goes beyond the development of means to affect known processes, to the search for fundamental knowledge about the disease pathophysiology. These are the condensation diseases, in which the formation of macroscopic solid and liquid protein phases is a main component of the pathophysiology. While the processes of formation of new phases are relatively complex and not a typical part of the biology and biochemistry curricula, they are within the domain studied in physical sciences. A classical example of condensation diseases is eye cataract, whose various forms are associated with the formation of crystals or disordered aggregates, or with liquid-liquid separation in the eye lens.[7] The amyloid diseases will be a major scourge in the coming years: Alzheimer's affects, in some form, about half of people above 85 years of age, and there will be more and more such patients as life expectancy increases. The processes of formation of amyloid fibrils were untangled with a decisive contribution by chemical engineers: it was established that the fibrils nucleate and grow by the addition of monomers, that a precursor cluster is crucial for nucleation, that several of the initial fibrils, sometimes referred to as proto-fibrils, can associate.[8,9]

Another disease, in which physicists, physical chemists, and chemical engineers have crucially contributed, is sickle-cell anemia.[10] The main clinical manifestation of the disease is the vasooclusion, i.e., the obstruction of blood flow, by red blood cells. 50% of the volume of human blood is occupied by the red blood cells, which carry out blood's main function: the supply of oxygen. With this high-volume fraction, blood flow is only possible because the red cells are soft and deformable. The vasculature is complex: the capillaries are a few micrometers wide so that the red cells, which, when free, are disks of about eight micrometers in diameter, would slowly squeeze through and efficiently deliver to the tissue the oxygen that they carry. The venules are up to several tens of micrometers wide and the flow through them has relatively high-shear val-

Correspondence concerning this article should be addressed to P. G. Vekilov's at peter.vekilov@mail.uh.edu.

Table 1. The Pressing Problems Facing Humankind, According to Richard Smiley

1	Energy
2	Water
3	Food
4	Poverty
5	Disease
6	The environment
7	Terrorism
8	Population growth
9	Economic development
10	The spread of democracy

ues. The veins and arteries are up to several millimeters wide, with arterial flow being pulsed, and venous flow-relatively smooth. Thus, blood flow is a process of inherent low-stability. This stability is lost if the red cell count is increased relatively mildly, in a condition called erythrocytosis or polycythemia.[11] Another means to lose stability of these blood flows is by increasing the rigidity of the red blood cells, and this is the mechanism of sickle-cell anemia. Below, we focus on the advances in the fundamental understanding of the pathophysiology of sickle-cell anemia achieved by employing physicochemical approaches, and on the challenges and opportunities that developing a treatment of sickle-cell anemia poses to chemical engineers.

Sickle-Cell Anemia Pathophysiology Poses Questions for Physical Chemists

Sickle-cell anemia was first diagnosed in 1910. In 1949 the group of Linus Pauling (the most famous American chemist had an undergraduate degree in chemical engineering) found that hemoglobin from sickle-cell patients had excess of positive charge,[12] and this difference was attributed to a mutation from glutamate, an acidic aminoacid, to hydrophobic valine at the sixth site of the two β-chains of hemoglobin.[13] The mutated protein (sickle cell hemoglobin or HbS) forms 14-member fibers[14] when in its T-conformation in deoxy-state, in the venous circulation. The presence of valine allows hydrophobic contacts between it and three other hydrophobic aminoacid residues from an adjacent HbS molecule. The fibers stretch and deform the red blood cells. The increased rigidity of the red blood cells increases the blood viscosity, which make blood flow more susceptible to obstruction. In turn, blood flow obstruction causes tissue and organ damage, pain, and often death.

This sequence polymerization—cell deformation—vasoocclusion provided a straightforward scenario for the evolution of sickle-cell crises. A "double-nucleation mechanism" was put forth for polymerization, according to which homogeneous nucleation of single fibers is followed by their growth, and branching by heterogeneous nucleation of new fibers on top of existing ones.[14] This mechanism explained many of the clinical features of the disease. A treatment avenue was suggested by the structures of the sickle-cell fiber: to find a hemoglobin ligand, which prevents the hydrophobic contact and the formation of the fiber. About 200 molecules were found which *in vitro* bind to hemoglobin and prevent polymerization. Unfortunately, all of them were inapplicable in patients, because of the high- concentration of hemoglobin inside the erythrocytes $\sim$ 5 mol l^{-1}, which requires unacceptable concentrations of a ligand targeting most HbS molecules.

There have been numerous observations which appear to contradict the polymerization scenario.[15,16] It was found that more than half of all red cells of sickle-cell patients undergo sickling on every passage through the venous circulation, yet sickle-cell crises are significantly rarer, that the propensity for sickling of different red cells is not correlated to their density,[16] and others. Other contradictions involved features of the disease which cannot be explained by solubility and activity, the only parameters in the double-nucleation mechanism.[15,16] The most significant is the realization, coming from several clinical studies, that the clinical manifestations of sickle-cell anemia are dramatically different in patients with identical concentrations of HbS in their red blood cells,[17,18] with no identified factors that could result in HbS solubility variations.

Thus, it was suggested that the HbS polymerization is just one of the events in the sickle cell anemia pathophysiology, and that other factors, such as adhesion of the outside of the red cell membranes, adhesion of the endothelial walls, flexibility and permeability of the red cell membrane, and others, may be equally or more important for the onset and frequency of sickle-cell crises.[19] The complexity of the physiological factors and clinical manifestations of the disease was characterized as "chaos".[16]

Besides transfusions with normal blood, two treatments for sickle-cell anemia are currently applied in the clinic: bone marrow transplantation and hydroxyurea. The former is a difficult and risky procedure, strongly dependent on the availability of a suitable donor. Unfortunately, less than half of all sickle-cell patients treated with hydroxyurea benefit from it.[18] Thus, fundamental studies of novel potential treatment pathways are still underway. These studies are focused on red cell adhesion, endothelial activation, platelet activation, membrane damage and many other factors.[16] In view of the failures of the polymerization scenario, discussed earlier, it is perhaps understandable why the current clinical treatment strategies do not include attempts to directly inhibit hemoglobin polymerization.

There is a potent recent finding, which suggests that polymerization may still be the primary pathogenic event in sickle-cell anemia. Transgenic mice expressing human HbS were employed.[20] Their HbS was genetically modified leaving valine at the β6 position intact, but removing the residues with which it forms hydrophobic contacts in the fiber. In this way, the sickle-cell gene was not touched, and its suspected pleiotropic consequences, i.e., those other than the hemoglobin modification, would not be affected. It was found that the additional mutations inhibit incorporation of the modified HbS into the polymer. The modification was found to delay polymerization, strongly reduce the fraction of sickled red blood cells, and reduce the severity of sickle crises. Significantly, two features of the disease, red cell dehydration and the count of irreversibly sickled cells, sometimes considered as independent factors for the disease, were also reduced.[20] These results provide a cure for sickle-cell anemia, at least in mice, other than bone marrow transplantation. The remarkable fact is that the cure works through delay of HbS polymerization.

While this delay was achieved through a genetic modification of hemoglobin, it is likely that such gene therapy in humans will be delayed by at least 20 years (the interactions between genes, which make humans, with ~25,000 genes, more complex organisms than the plant Arabidopsis, with about the same number of genes, are still largely a mystery); hence, other means to delay polymerization should be sought.

The finding that delaying HbS polymerization leads to sickle-cell anemia cure is in apparent contradiction with the slate of facts about the disease which defy the primary role of polymerization. Some of the contradictions can be resolved even within the framework of the current knowledge on HbS polymerization: the increased adhesion and other modifications of membrane properties of sickle red cells may be due to the repeated cycles of sickling and deformation that such cells undergo. Clearly, endothelial activation must be a factor independent of red cell sickling, which likely increases the severity of the disease. However, the disease variability in patients with identical expression of HbS, the venules as primary location of vasoocclusion, the uncorrelated cell density and sickling propensity cannot be rationalized in this way.

Many of these contradictions were resolved by recent results on the mechanism of HbS polymerization, which provide pathways of high-sensitivity of HbS polymerization to parameters other than HbS concentration and solubility. It was put forth that this high sensitivity, combined with variability of the identified parameters, may be the missing explanation. The primary focus was homogeneous nucleation of new fibers because its rate, as any other nucleation process, may be a strong function of the parameters in the red blood cells of sickle-cell patients: composition, shear rates, hemoglobin decay, and others. In what follows, we review these results and highlight their clinical implications as an example of the application of methods and approaches of chemical engineering and physical chemists the search for fundamental understanding of a disease.

New Insights into Nucleation of Sickle-Cell Hemoglobin Polymers

Nucleation kinetics

The quantification of the kinetics of nucleation of individual fibers from image sequences such as the one in Figure 1, in terms of nucleation rate, i.e., number of nuclei appearing per unit volume per unit time, and nucleation delay time, i.e., the

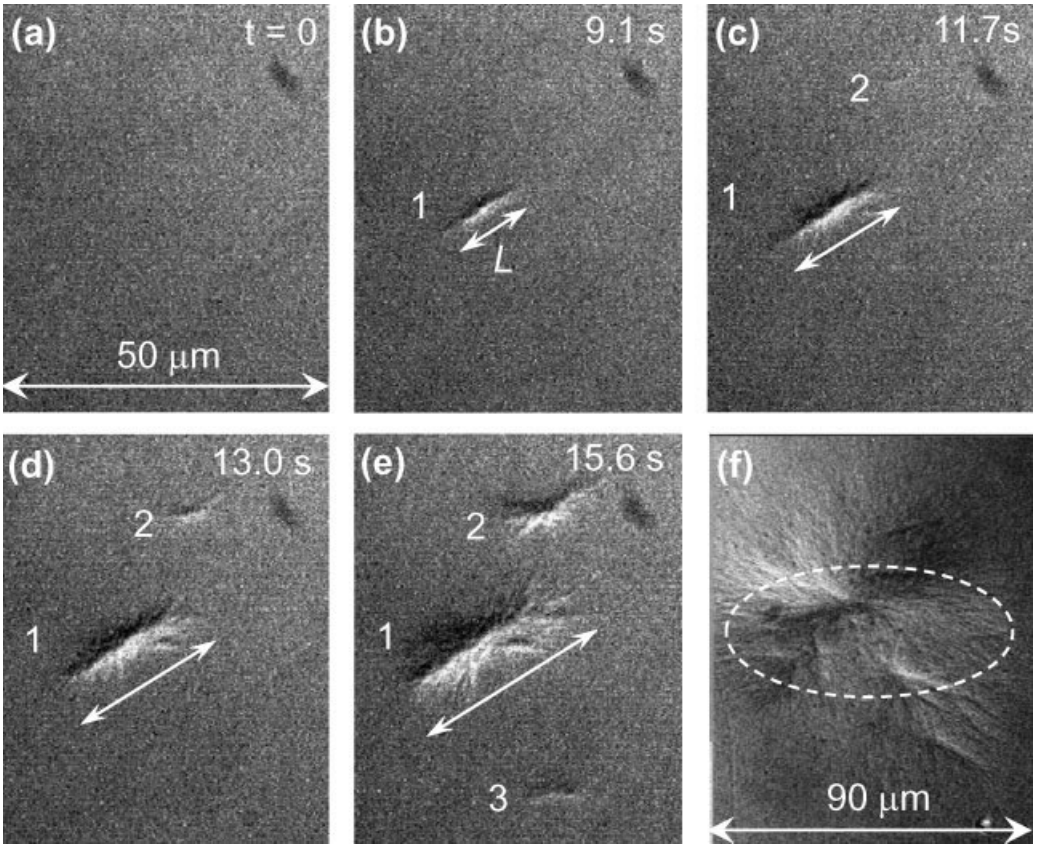

Figure 1. Evolution of HbS polymerization monitored by differential interference contrast microscopy in a 25 μm thick slide of supersaturated HbS solution.

Such observations allow counting of polymer spherulites, determination of the time of their detection, and of their length. Times for a–e indicated on panels, image in f corresponds to equilibrium between polymers and solution reached after ~ 1 min of polymerization. Elongated spherulites in b–e evolve into isometric spherulites in f. Width of panels a–e is shown in a. Determinations of fiber length L is illustrated in b. Individual spherulites traced through b–e are labeled with numbers.

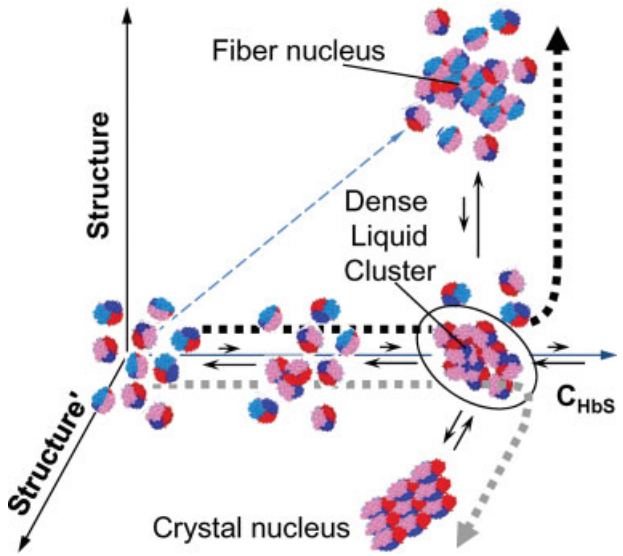

Figure 2. Schematic representation of the two-step nucleation mechanism of HbS polymers.

The nucleation pathway in the space of order parameters. Horizontal axis is HbS concentration, along which dilute solution and dense liquid can be distinguished. Axes of evolution of ordered structures are orthogonal to concentration axis, only two leading to polymer fibers and crystals, respectively, are shown. Other possible structure axes include other crystal polymorphs, disordered aggregates, and gels. Thin dashed arrow along diagonal indicates pathway of the one-step nucleation of the polymer fibers. Thick short-dashed lines indicate pathways of the two-step mechanism leading to polymers or crystals, respectively.

time during which the probability of having even one nucleus is zero, is discussed in.[21–23] The data on the nucleation rate and delay times at different HbS concentrations and temperatures provide insight on the mechanism of homogeneous nucleation of HbS fibers. In studies to-date, it has been assumed that this nucleation is a one-step process: the disordered HbS molecules from the solution assemble into an ordered nucleus, which has the same structure as long HbS fibers. A different outlook on nucleation of ordered structures has been suggested by recent results on another first-order phase transition with proteins: formation of crystals. Both experiment and theory revealed that, for protein crystallization, the formation of dense liquid droplets may precede and facilitate the formation of ordered nuclei.[24–26] The dense liquid may in some cases be stable with respect to the dilute solution, or, in other cases, it may be metastable.[27,28] The applicability of the two-step mechanism has been demonstrated for crystallization in a variety of systems: colloid materials,[29] molecular,[30] and ionic[31] small-molecule compounds, and even for the formation of another ordered solid phase of proteins, the amyloid fibrils.[32]

In several recent articles it was demonstrated that the two-step mechanism, illustrated in Figure 2, applies to the nucleation of sickle cell hemoglobin fibers.[22,23,33] It was shown that *stable* dense liquid does not exist in the HbS solutions in which the nucleation kinetics was studied.[34] However, dynamic light scattering monitoring of these solutions revealed *metastable* dense liquid clusters in these solutions (Figure 3).[33] The metastable clusters are similar to those seen with the proteins lumazine synthase,[35] and lysozyme. In deoxy-HbS solutions, the clusters are present immediately after solution preparation and overall their radius is relatively steady. They exist in broad temperature and Hb concentration ranges. The clusters have macroscopic lifetimes and occupy $\phi = 10^4$–10^2 of the solution volume.[33]

The precursor clusters

According to the two-step mechanism of homogeneous nucleation of the HbS polymers, illustrated in Figure 1, the polymer fibers nucleate inside metastable dense liquid clusters of sizes several hundred nanometers. As highlighted previously, a radically new understanding is emerging in the protein aggregation community that the concentrated liquid is an essential kinetic pathway to the formation of all *solid* protein phases, both ordered and disordered, including plaques, fibers (as in sickle-cell anemia), and crystals. The protein clusters are, thus, a main kinetic precursor to all major classes of solid protein aggregates, including plagues, fibers, such as in sickle-cell anemia and crystals. The understanding of the molecular origin and properties of these novel mesoscopic phases would be a major step toward controlling the formation of these solid aggregates.

According to a recent theory,[36] the clusters form via hydration forces between protein molecules. Since hydration forces almost always act between protein molecules in water, this explains the ubiquity of the clusters under a broad range of

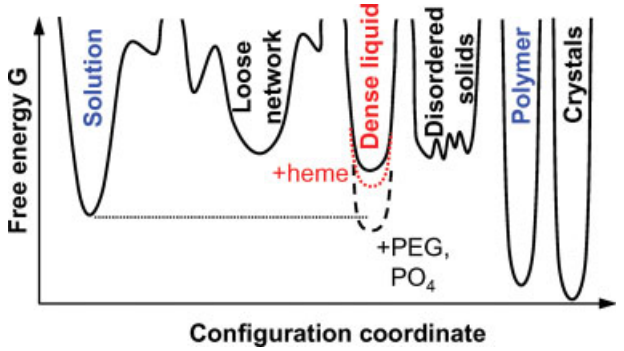

Figure 3. Free energy *G* landscape for different phases possible in HbS solutions.

The abscissa is a one-dimensional (1-D) projection of the of the full set of order parameters characterizing the phases in the haemoglobin + solvent system. This coordinate can be approximately thought of as HbS concentration + degree of ordering of the HbS molecules. Lower *G* corresponds to higher stability, thus, the dense liquid is metastable with respect to the solution in HbS solutions, and becomes stable upon the addition of poly(ethylene glycol) (PEG), or phosphate salts in concentrations greater than several millimolar. The addition of heme in micromolar concentrations lowers the free energy G of the dense liquid, but does not make it stable with respect to the solution.

conditions,[33,35,37,38] and their sensitivity to the chemical composition of the solution. The proposed theory provides a universal scaling law for cluster dynamics and predicts a crossover of cluster relaxation to critical-like density fluctuations at high-concentrations. It allows estimates and tests for the size and size-relaxation times of the clusters, the dependence of the cluster volume fraction on the bulk protein concentration, and their response to external parameters. This understanding of the cluster properties suggests a physicochemical pathway for the control of protein aggregation: via the structure of the water shell around the protein molecules in solution.

Such control may lead, among other things, to the development of treatment strategies for protein aggregation diseases that are based on radically novel, *physicochemical* principles, and do not involve using chemicals foreign/toxic to the cell. In contrast, the existing strategies are based on biochemistry or molecular biology and have had only limited success, often because of side effects. Furthermore, as protein crystallization is often the major bottleneck in structural biology, its enhancement has been a major challenge in the field.

Sensitivity of HbS polymerization to low-concentration solution components

The huge clinical variability of sickle-cell anemia has been attributed to processes occurring independently of the HbS polymerization.[15,16] On the other hand, the experiments with transgenic sickle-cell mice, discussed in the Introduction earlier, reconfirm that the primary pathogenic event of the disease is HbS polymerization.[20] If polymerization follows the accepted scenario of one-step homogeneous nucleation, followed by growth and branching, the rate of HbS polymerization is entirely determined by the HbS activity. The HbS activity is mostly determined by the HbS concentration since the HbS molecules only interact through their excluded volume.[14] Since most nonhemoglobin components of the red cell cytosol occupy low-volume fractions, HbS activity is unaffected by variations in their concentrations. Thus, the primary role of HbS polymerization has been hard to reconcile with the clinical variability among patients with identical expression of HbS in the erythrocytes.[39]

The clusters are metastable objects, whose stabilization, according to the theory highlighted previously, relies on the fine details of the intermolecular interaction potential. Thus, the sizes, properties, and volume fractions of the dense liquid precursors could be modified by components of the red cell cytosol at submillimolar concentrations, which modify the structuring of the water at the hemoglobin surface, or in other ways affect the integrations between the hemoglobin molecules.[34] In this way, the precursor clusters allow for a multitude of governing parameters of the fiber nucleation process, and reconcile the clinical variability of sickle-cell anemia with the primary role of HbS polymerization.

The effects of heme

One such molecule, whose effects on HbS polymerization were tested, is the heme. Heme is excessively released in sickle erythrocytes due to auto-oxidation of hemoglobin to met-hemoglobin, which has lower stability. It was recently shown that micromolar amount of free heme may increase the rates of nucleation and growth of sickle-cell hemoglobin polymer fibers by two-orders of magnitude. Removal of the free heme completely arrests polymerization. The fraction of the volume occupied by the dense liquid precursor to fiber nuclei increases, in the presence of heme, by orders of magnitude, which leads to a corresponding increase in the nucleation rate. The likely mechanism of heme action is through enhanced attraction between the hemoglobin molecules, which lowers the free energy of the precursor clusters, as illustrated in Figure 3.

The concentrations of heme, which significantly affect the rate of polymerization, are low and could be released by a minor fraction of the hemoglobin molecules in solution. The combination of the low heme amount and several heme removal processes, operating in the erythrocytes, could easily lead to significant variability of the concentration of free heme in the red cell cytosol. If efficient removal dominates over heme release, the concentration of free name in the red cell cytosol will be low, and sickle-cell hemoglobin will not sickle. On the contrary, if the removal is slow and heme release dominates, high heme concentration would lead to fast polymerization, even at low-concentrations of sickle-cell hemoglobin.

The effects of the free heme on polymerization could explain several of the current mysteries of the clinical picture of sickle-cell anemia. One of them is the occurrence of sickle-cell anemia crises in patients undergoing hydroxyurea therapy, or those with the Asian haplotype. Both groups express strongly fetal hemoglobin and have, as a result, lower concentration of sickle-cell hemoglobin in their red blood cells. While the frequency and severity of sickle crises is, on the whole, lower in such patients, even those with high fetal hemoglobin expression are not completely protected from sickle crises. This may be a consequence of high heme concentration is their erythrocytes. Another mystery is that many homozygous patients with the African haplotype, also common to patients in the US and the Caribbean, nearly all of whose hemoglobin is sickle, do not ever suffer sickle-cell crises. Evidence for such cases is accumulating in the last few years, and it is possible that the sickling in such patients is less severe due to efficient removal of heme in their red cells. Other inconsistencies in the clinical picture of sickle-cell anemia include the fact that the cells which predominantly sickle are not those with the highest HbS concentration, where HbS polymerization is most likely. From the viewpoint provided by the finding of the role of free heme, these could be the cells with highest concentration of free heme: the amount of free heme may vary between cells due to variations in the amount of oxidative species.

The finding of the effects of the heme provides another target in the search for treatment strategies. So far, all strategies have been based on lowering the concentration of sickle cell hemoglobin: by increasing the expression of fetal hemoglobin via hydroxyurea; by binding of so-called anti-sicklers to hemoglobin; or by surgically replacing the bone marrow, which produced sickle-cell hemoglobin. The most successful of these approaches, the use of hydroxyurea, is only efficient in less than half of all patients of all patients. It is likely that removal of free heme, e.g., by binding agents, or decreasing its release by introducing antioxidative drugs in the red cell cytosol, is an alternative pathway to treatment.

It was shown that the enhanced sickle-cell hemoglobin polymerization in the presence of heme is due to stronger intermolecular attraction. This effect was attributed to enhanced attraction in the presence of very low heme concentration, which is due to the known weak binding of heme to the outside of the hemoglobin molecule. This suggests a new paradigm in biophysics and biochemistry: the role of weak nonspecific binding in biological regulation. Furthermore, the current dogma in drug design is that only compounds with strong specific binding could be potential drugs. This outlook is useful for cases where the concentration of the target protein is low, however, if the target protein is at high-concentration, this paradigm often leads to ligands, which would be applied at concentrations above their toxicity limits.

In the broader context of evolution and the deletion of deleterious genes, the finding of the effects of the free heme provides a missing element between sickle-cell disease and malaria. It is accepted that the sickle cell trait protects against malaria. The suggested mechanism of this protection is that sickling is enhanced in parasitized sickle trait cells and the sickled cells, which in the early stages of infections are few, are removed by the spleen together with the contained parasites. The sickling enhancement is supposed to come from lowering of the oxygen concentration, i.e., hypoxia, in the parasitized red cells, however, laboratory tests have failed to confirm this element of the mechanism. The novel explanation is that during their metabolism of hemoglobin, the malaria plasmodia release heme, which helps sickling even in the sickle trait red blood cells, where the concentration of sickle-cell hemoglobin is lower.

Finally, this finding highlights the crucial role of small-molecule compounds in the regulation of physiological and pathophysiological processes. This may be the first example where it is shown at the molecular level that the presence or absence of such a compound may lead to variability between individuals with identical genome, containing the sickle cell gene, and identical expression of sickle-cell hemoglobin. The amounts and the corresponding effects of heme are not subject to tight genetic regulation in the erythrocytes, where most of the heme metabolic pathways are absent. Thus, the finding of the crucial role of the heme is a molecular-level argument against genetic determinism.

Open Questions for Chemical Engineers and Physical Chemists on the Fundamentals of HbS Polymerization

The first open question is how small molecules can affect the interactions between protein molecules in solution. Only several such mechanisms are typically discussed[40]: ions screen the Coulomb forces and in this way affect the long-range electrostatic interactions, molecules with size between that of the solvent and the protein enhance attraction by the so-called depletion mechanism, molecules that bind to certain sites on the protein surface may serve as specific bridges or blockers of attachment, ions may participate in the buildup of water structures, and in this way enhance repulsion between proteins. In all of these cases, the additives are active only at concentrations comparable or significantly higher than that of the protein. Hemoglobin is the component of the red cell cytosol with by far the highest concentration. Thus, these mechanisms cannot account for the variability of HbS polymerization, and they cannot provide the basis for the search of a sickle-cell treatment based on blocking of polymerization. Experimentalists and theorists in the area of biophysics should direct their efforts on putting forth and testing a mechanism, whereby solution components at concentrations lower than that of a protein could affect the interactions between the protein molecules. Clearly, such a mechanism would have implications far beyond the realm of sickle-cell anemia, but its applicability to the understanding of HbS polymerization may be immediate.

While the composition of the red cell cytosol is relatively well-known, and the identity and concentrations of about 100 components are available, the data comes from pooled blood of different donors. No data on the variability of the cytosol composition in healthy adults, or on the differences in composition between healthy individuals and patients with different hemoglobin disorders exist. In the absence of such data any conclusions about the role of the red cell cytosol components on the pathophysiology and severity of sickle-cell anemia cannot be made.

Two groups of open questions are related to the role of the free heme. The first one is about the variability of the free heme concentration among sickle-cell patients. They are related to the mechanisms of interaction of the heme with the red cell membrane, and with the components of the blood plasma, which may facilitate the passage through the membrane of erythrocyte. An important second group deals with the possibility of capturing or modifying the heme released by hemoglobin inside the red cells, so that its harmful effects on the sickle-cell hemoglobin polymerization could be prevented.

The occurrence of blood flow obstruction in the relatively wide venules suggests that shear flow may be involved in HbS polymerization in sickle-cell patients, and in the disease pathophysiology. While data on the flow rates in the different vessels of the venous circulation are available, from which the respective shear rates can be evaluated, the understanding of the effects of shear flow on HbS polymerization has two wide gaps: a theory of tank-treading motion adjusted for the rigidity, and other properties of the red cell membranes is needed to evaluate the shear rate inside the red cell. Theory of the effects of shear flow on the nucleation rate and mechanisms of HbS polymers should be developed and experimentally tested.

Acknowledgments

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