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Supporting Material

Free heme and the polymerization of sickle cell hemoglobin

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Supplementary Information for

Free Heme and the Polymerization of Sickle Cell Hemoglobin

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Methods

Protein purification and solution preparation and dialysis. Hemoglobins S and A were isolated from the blood of sickle cell patients or healthy donors and purified as discussed in Refs. (2-3). The sickle cell blood was kindly provided by Drs. G. Airewele and B. Dinu from Texas Children's Hospital and Baylor College of Medicine in Houston. As last purification steps, the HbS solutions were run through a Q-Sepharose anion exchange column (XK 50, Amersham Biosciences) and dialyzed for 24 hours using Slide-a-Lyser cassettes (Pierce Scientific) with a cutoff of 2000 g mol^{-1} in 1 l of 0.15 M phosphate buffer, pH = 7.35, at 3°C. After concentration by ultrafiltration, the stock in oxy-form was stored in liquid nitrogen at –195.8 ºC. Prior to experiments, a solution sample was thawed, converted to CO- or deoxy- form, and ~ 0.05 M $Na_2S_2O_4$ (sodium dithionite) was added to capture residual oxygen species in the solution (3). The solution was then diluted to a desired concentration, which was verified using the Drabkin method (2-3).

Hemin (Fluka) was initially dissolved in 0.1 M NaOH. It was added to the HbS solution, as needed, as stock with concentration 1.2 mM and pH = 7.35 in the same 0.15 M phosphate buffer as used in the HbS solutions.

In several series of experiments, oxy-HbS stock solution was aged at room temperature for up to three consecutive days and CO-HbS was prepared from the aged stock. Polymerization experiments were performed immediately after preparation of CO-HbS.

Characterization of the polymerization kinetics. To monitor the evolution of HbS polymerization and to determine the nucleation rate and delay time and the growth rate of HbS polymer fibers, CO-HbS solution samples of chosen concentration were prepared and loaded in slides of uniform 5 μm thickness (2); polymerization was induced by laser photolysis of CO-HbS to deoxy-HbS and CO(aq.) at wavelength 532 nm of a ~90 μm wide area (3). Only polymers formed within a 50 μm wide central part were considered (3), so that the test solution volume was ~10^{-8} cm^3 = 10 picolitres. The time elapsed after initiation of polymerization was recorded with 0.1 s accuracy (2-3). The slides were observed with differential interference contrast (DIC) microscopy, which allows detection and monitoring of individual polymers (2-5). DIC images of the slides containing supersaturated deoxy-HbS solution were recorded about once per second. Because of their fast growth rates, individual polymers were detected ~ 0.2 s after their nucleation (3). The time of nucleation of the individual polymers, their orientation in the slide and the increase of their length were determined from the images. For experiments statistics, these determinations were repeated under identical conditions from 81 to 200 times. From all data sets, the nucleation rate of the polymers J, i.e., the number of polymers nucleating in unit volume per unit time, the nucleation delay time θ, i.e., the time within which the probability of having even one nucleus is zero, and the polymer growth rate R were determined in a straightforward manner (2-3). The reproducibility of the determinations of these three variables in independent experiments starting from HbS purification was within 10 % (3, 6). For further

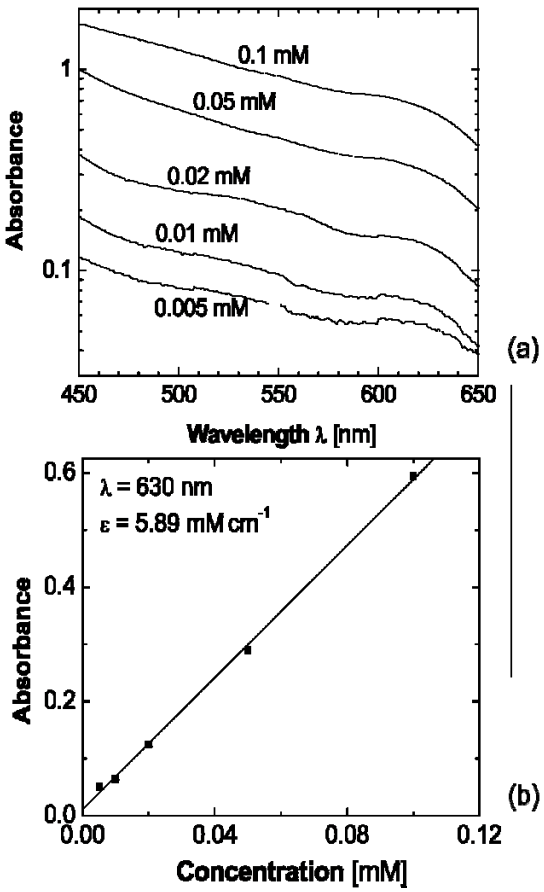
details of the procedures employed for the determination of the *J*, θ and *R* and their tests, see Refs. (2-3, 6)

Static and dynamic light scattering. The formation of dense liquid clusters in deoxy-HbS solutions, held in sealed cuvettes, was characterized by dynamic light scattering (7); interactions between deoxy-HbS molecules in solution and their modifications by free heme were characterized by static light scattering (8-9). Both methods employed a ALV-5000/EPP static and dynamic light scattering device (ALV-Gmbh, Langen, Germany) with a 35 mW He-Ne laser operating at wavelength λ = 632.8 nm (Uniphase), for further experimental details, see Refs. (7-8).

Hemoglobin characterization. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out using Voyager-DE STR spectrometer (Applied Biosystems). Sinapinic acid (molecular weight 224.2 g mol^{-1}), which gave low levels of noise, was used as matrix. Fast protein liquid chromatography was performed using a Pharmacia unit with a 280 nm wavelength detector and respective anion exchange or size exclusion columns (10). Native polyacrilamide gel electrophoresis was performed with a Pharmacia Phast system (10).

Determination of free heme concentration in polymerizing HbS solutions.

The spectra of heme in 0.15 M phosphate buffer at pH = 7.35 saturated with CO and in the presence of 50 mM sodium dithionite are shown in Supplementary Figure 1a. These spectra are similar to those of met-Hb and indicate that the heme iron is in Fe^{3+} form and heme is in the form of hemin, despite the presence of dithionite. A comparison of the heme spectra in Supplementary Figure 1a to those of oxy- and CO- HbS reveals that in the wavelength range λ > 600 nm, heme absorbance is ~10 to 100 × stronger than that of these two HbS liganded species. Thus, we choose λ = 630 nm for determination of heme in the presence of hemoglobin. From the data in Supplementary Figure 1b, the extinction coefficient of heme at λ = 630 nm is ε = 5.89 mM^{-1} cm^{-1}; in solutions saturated with O_2 (data not shown) this coefficient is 6.07 mM^{-1} cm^{-1}.

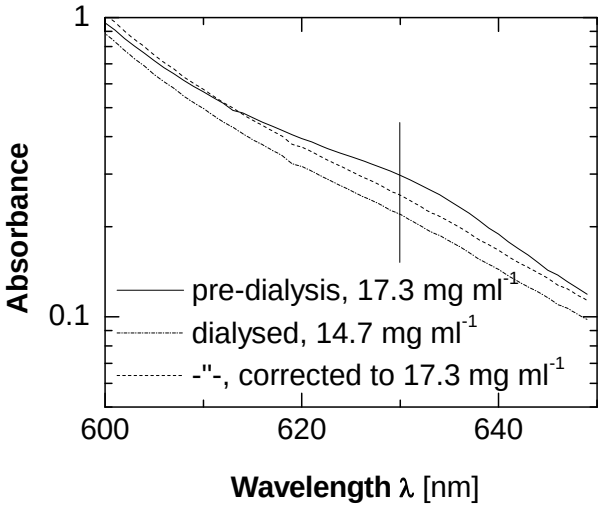


Supplementary Figure 1. (a) Spectra of heme at five concentrations in solution saturated with CO in 0.15 M phosphate buffer with pH = 7.35 at 25°C, in the presence of 50 mM sodium dithionite. (b) Determination of the extinction coefficient of heme from the dependence of solution absorbance at λ = 630 nm, from data in (a), on heme concentration

Since certain amounts of met-HbS, which absorbs significantly in the wavelength range 600 – 650 nm remain in the solution, direct spectroscopic determination of the heme concentration would be inaccurate. To detect free heme and quantify its concentration in the presence of met-HbS, we employed three methods: spectroscopy after dialysis, binding to albumin, and conversion of met-HbS to cyanmet-HbS

Spectroscopy after dialyses. We prepared HbS solutions identical to those used in the kinetics determination, but with concentration from 10 to 25 mg ml^{-1}, at which concentrations the absorbance of light of wavelength $\lambda > 600$ nm is in the 0.1 – 1 range. The solutions were dialyzed for 15 hours through a membrane (Pierce Scientific) with molecular mass cutoff of 2000 g mol^{-1} against 0.15 mM phosphate buffer. To minimize continued release of heme during dialysis and the formation of met-HbS, dialysis was carried out at 3 ^{o}C.

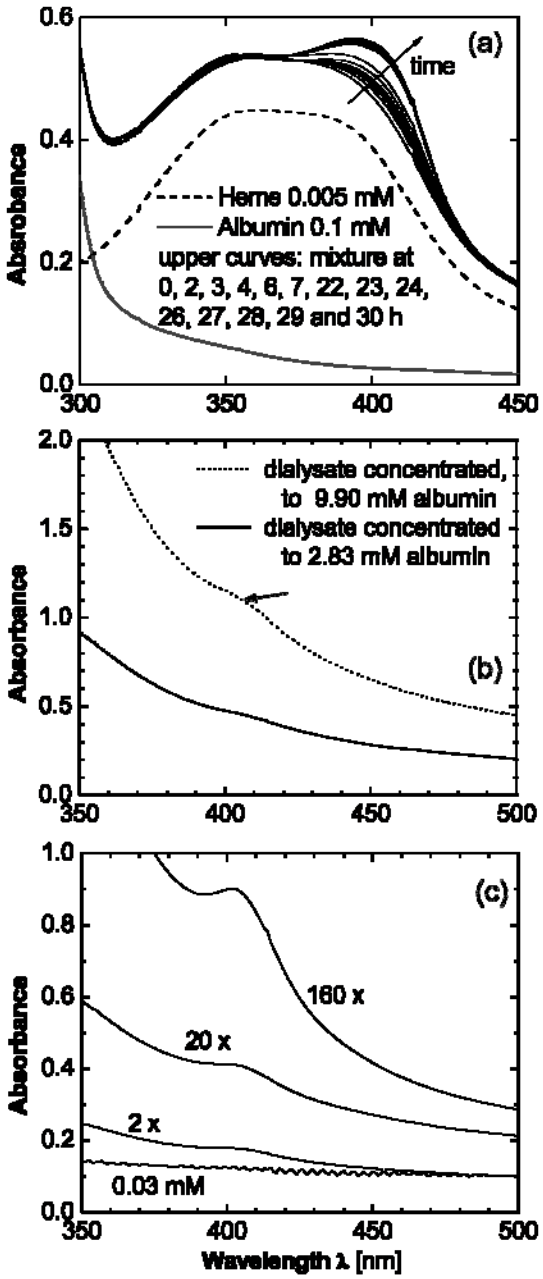
Heme concentration was determined from the difference of absorbance at 630 nm before and after dialysis, Supplementary Figure 2. Prior to the spectrophotometry determinations HbS was converted to its CO-form: the solution samples were treated with 50 mM sodium dithionite and saturated with CO. With the above ε, Supplementary Figure 2 yields a concentration of 7 μM of free heme in the analyzed solution, equivalent to 0.026 mole heme/mole HbS. Other determinations yielded heme-to-HbS molar ratios within 20 % of this value. Importantly, extended dialysis for up to 24 hours did not lower the solution absorption at $\lambda = 630$ nm further, indicating that free heme and apo-globin are not produced by dialysis. Hence the heme determined from the data as in Supplementary Figure 2 was present in the tested solutions, including during the characterization of polymerization kinetics.



Supplementary Figure 2. Spectra of HbS solution used in experiments shown in Figs. 1c and 2 of main test before and after dialysis through a membrane with a 2000 g mol^{-1} molecular weight cut-off for 15 h. The difference between the two absorbances is used to determine the free heme concentration.

Detection of heme in the dialysate by binding to albumin. Heme forms a complex with albumin, which has a distinctive absorbance peak in the wavelength range 370 – 420 nm (11-13). Supplementary Figure 3a shows that over about 30 hours bovine serum albumin (BSA), which we use, forms a heme complex with this characteristic spectroscopic signature. To utilize this complex for the detection of heme in the dialysate, we added between 0.03 and 0.06 mM BSA to the buffer, against which HbS—and HbA controls—were dialyzed. Upon completion of dialysis, the dialysate was concentrated by centrifugation and spectra were taken.

Tests revealed that BSA is soluble up to 4 mM in low ionic strength aqueous solvents with near physiological pH. However, upon concentrating the dialysate, BSA precipitated and/or formed a dense liquid phase coexisting with the “normal” dialysate. The threshold BSA



Supplementary Figure 3. Detection of heme in dialysate by binding to albumin. (a) Spectra of heme, albumin, and a mixture of heme and albumin with the same respective concentrations. Mixture spectra are shown immediately after mixing and at times indicated in the plot. Arrow through mixture spectra shows direction of time increase. Peak between 370 and 420 nm is characteristic of heme-albumin complex. (b) Spectra of dialysate containing 0.014 mM albumin after dialysis of 20 mg ml^{-1} HbS solution for 15 hours before and after concentrating the dialysate to indicated albumin concentrations. The peak at 404 nm, indicated by an arrow, is partially concealed by the solution turbidity. (c) Spectra of dialysate containing 0.03 mM albumin after dialysis of 20 mg ml^{-1} HbA solution for 15 hours, and after concentrating the dialysate by factors indicated in the plots. Because of lower turbidity of dialysate from HbA, the peak at 404 nm is apparent.

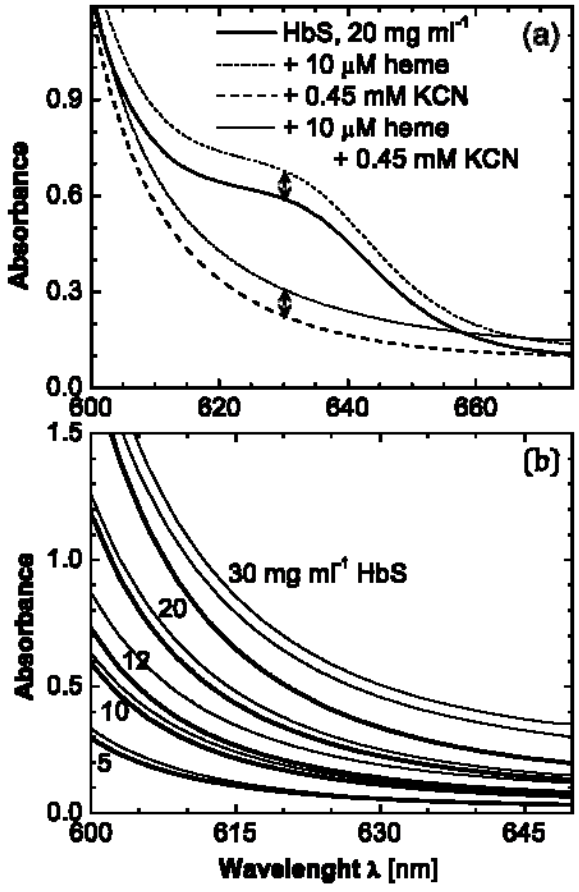
concentrations for precipitation or phase separation varied, but were significantly lower for HbS dialysate than for HbA dialysate.

The spectra of the BSA-containing HbS dialysate, at two concentration levels, and HbA dialysate, at four concentration levels, are shown in Supplementary Figures 3b and c, respectively. Both figures reveal significant solution turbidity likely due to BSA precipitation; the turbidity of the HbS dialysate is greater because of the stronger precipitation. The peak centered at 404 nm and characteristic of heme-BSA complex is well expressed in the spectra of the HbA dialysate and nearly masked by the higher turbidity in the HbS dialysate spectra. For both HbS and HbA, the height of this peak cannot be determined accurately because of the turbidity. Even if the peak height could be measured it could not be used to determine the

amount of the heme-BSA complex in the dialysate because of the precipitation and phase separation.

It is likely that the stronger precipitation during dialysis of HbS solutions (compared to HbA solutions) reflects the greater amounts of heme released by this variant, see below (14).

Conversion of met-HbS to cyanmet-HbS. The absorbance of cyanmet-HbS in the wavelength range 600 – 650 nm is about 4× weaker than that of met-HbS and is ~ 5 × stronger than of oxy-HbS; the CN^- ion does not bind to oxy-or deoxy-HbS (15, p. 33). Since CN^- ions do not absorb in the visible range, the spectra of HbS and heme in the presence of excess KCN (data not shown) are identical to those without KCN in, respectively, Fig.1 of the main text and Supplementary Figure 1a. Since < 10 % of HbS in the tested solutions is in met form (3), conversion of the met-HbS to cyanmet-HbS makes its contribution to the absorbance at 630 nm less significant. To determine the concentration of heme in the presence of oxy- and met-HbS, we add KCN in at least three-fold excess with respect to the met-HbS in the solution. As illustrated in Supplementary Figure 4a, heme concentration is determined from the difference between the absorbance at 630 nm of a tested HbS solution sample with added cyanide and that of a benchmark containing 93 % HbS and 7 % cyanmet-HbS. This latter concentration of cyanmet-HbS was chosen as the approximate average of the 5 – 10 % concentration range of met-HbS in the tested HbS solutions (3). Since the heme concentration is determined as the difference of two greater numbers, this determination has an inherent error of ~ 50 %, Supplementary Figure 4b.



Supplementary Figure 4. Determination of free heme by the addition of CN^-. (a) Spectra of freshly dialyzed HbS, i.e., in which no free heme is present, with and without the addition of heme and KCN at indicated concentrations. The shoulder between 600 and 660 nm in the spectra of solutions without added KCN is due to the absorbance by met-HbS: ε_{630}(met-Hb) = 16 l cm $mmol^{-1}$ Ref. (1) and free heme ε_{630}(heme) = 5.89 l cm $mmol^{-1}$ from Supplementary Figure 1b. Upon addition of KCN, met-HbS is converted to cyanmet-HbS, which has ~ 4 × lower absorbance (ε_{630}(cyanmet-Hb) = 3.88 l cm $mmol^{-1}$, determined by us after complete conversion of Hb to cyanmet-Hb by the Drabkin reagent). Absorbance by heme at 630 nm is marked by double sided arrows (b) Spectra of HbS solutions of indicated concentrations. Thin lines: after the addition of 0.45 mM KCN, thick lines: calculated spectra for of 93 % HbS and 7 % cyanmet-HbS. Determination at 30 mg ml^{-1} was duplicated. The difference between thin and thick lines is assigned to absorbance by free heme.

A summary of all determinations is shown in Fig. 2 of the main text in which the concentration of free heme is plotted as a function of the HbS concentration. This figure contains results of the dialysis and cyanide methods: binding of heme to albumin is accompanied by heme-induced albumin precipitation and cannot be used for quantification of the heme concentration. The scatter in the free heme concentrations in Fig. 2 of the main test reflects the inherent error of the cyanide method of about 50 %; the two points determined after dialysis have lower errors. With this caveat, the results of the two methods are consistent.

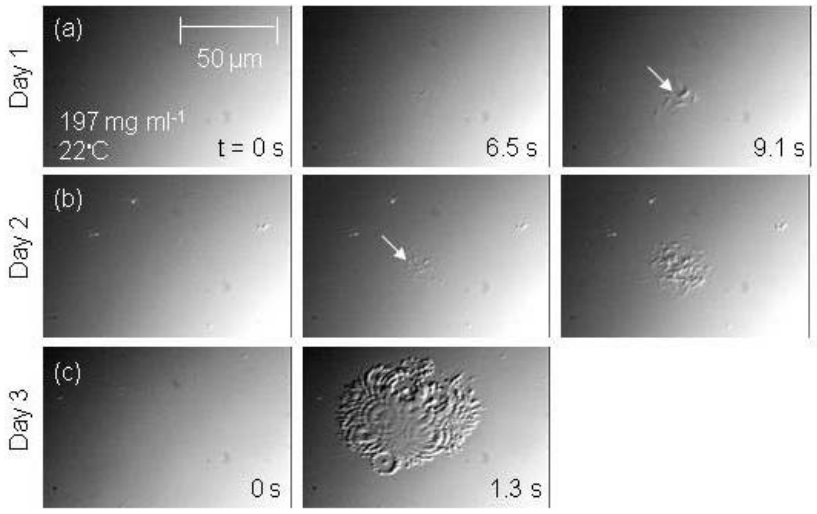
The consistency of the amount of free heme determined directly in the tested HbS solutions and the amount found in the dialysate, seen in Fig. 2 of the main text indicates that dialysis does not contribute to the release of heme by the HbS molecules and to the production of apo-HbS. This is concurrent with the observation, discussed above, that dialysis extended for additional eight hours (after the first 15 hours) does not lead to additional release of heme. These two observations lead to the conclusion that dialysis does not produce apo-globin and free heme.

Free heme content in HbA solutions. The three methods were applied to determine the concentration of free heme in solutions of HbA. The spectra of the HbA solutions before and after dialysis were nearly identical. i.e., the dialysis method did not detect any free heme in HbA solutions. This is likely due to the lower sensitivity of this method—heme was released in the dialysate and detected by its binding to albumin, as seen in Supplementary Figure 2c. Since the BSA precipitation in the HbA dialysate was relatively mild, it allowed a rough estimate of the concentration of free heme in HbA solutions and the ratio of free heme to HbA. This ratio was confirmed by several determinations employing the cyanide method as 0.002 mole heme/mole HbA. This is (10 – 20) × less than in HbS solutions.

Enhanced polymerization of HbS upon solution ageing.

Enhancement of the rate of HbS polymerization upon ageing of the HbS solution was observed in the classical study of the HbS polymerization mechanism (16), where it was attributed to formation of crystals upon storage of one day and longer. However, crystal formation lowers the HbS concentration in the solution and should lead to slower, rather than faster, polymerization rates. To distinguish the effects of ageing from those of crystal formation, we tested if polymerization is enhanced upon ageing of solutions of HbS concentrations < 280 mg ml^{-1}, where crystals do not form. We prepared three identical samples of oxy-HbS stock. After conversion to CO-HbS, the test solutions prepared from these stock samples had concentration 197 mg ml^{-1}. The first CO-HbS solution was prepared and tested immediately after thawing (the oxy- HbS stock solution is kept under liquid nitrogen) and it exhibited nucleation and growth of HbS polymers with moderate rates; a representative sequence of images illustrating the evolution of polymerization in such solutions in shown in Supplementary Figure 5a. The second stock sample was stored at room temperature for one day. After photolytic deoxygenation of the respective CO-HbS solution, more polymer fibers formed and they grew larger in a shorter time, i.e., the nucleation and growth rates were faster; this is illustrated in Supplementary Figure 5b. In solutions prepared from stock kept at room temperature for two days, Supplementary Figure 5c within 1 s after start of polymerization the supersaturated solution volume was fully occupied by polymerized HbS, indicating extremely fast nucleation and growth rates.

We also observed that polymerization did not occur in supersaturated solutions of freshly isolated and purified deoxy-HbS: the experiments in Supplementary Figure 5 were carried out



Supplementary Figure 5. Effects of aging on the polymerization of sickle cell hemoglobin. The panels in each row represent the evolution of polymerization under a set of conditions, as in Fig. 3 of the main text. Time after start of photolysis is indicated in each panel. White arrows point at some of the HbS polymer domains. Evolution of polymerization in solution prepared from stock stored under liquid nitrogen for one month: (a) solution prepared from freshly thawed stock; (b) solutions prepared from stock held at room temperature for one day. (c) solutions prepared from stock held at room temperature for two days.

with one-month old stock. Solutions prepared from oxy-HbS stock stored under liquid nitrogen at –198.8 ^{o}C for longer than a week exhibited nucleation and growth, illustrated in Fig. 1a of the main text, which were slower than those in solutions prepared from stocks older than a month, compare Supplementary Figure 5a to Fig. 3a of the main text. The rates obtained in solutions prepared from the older stock very close to those in Refs. (17-18). It is likely that the enhanced rates of nucleation and growth of HbS polymers in solutions stored under liquid nitrogen and tested over extended periods are due to the consequences of the repeated thawing and re-freezing, rather than to processes occurring at liquid nitrogen temperatures (19).

The observation of lack of polymerization in freshly purified HbS solutions contradicts results on HbS polymerization in literature, e.g., Ref. (16), in which polymerization started immediately after purification. Our experimental procedures differ from those in Ref. (16) by the type of dialysis membrane employed in the final step of purification: unidentified membrane in Ref. (16) and Slide-a-Lyser cassettes (Pierce Scientific) with a molecular weight cutoff = 2000 g mol^{-1} in our experiments. Heme, which at pH = 7.35 is mostly in form of dimers with molecular weight 1232 g mol^{-1}, Supplementary Figure 6b, may be retained by the former but would be removed from a solution dialyzed through the latter type of membrane. To test this hypothesis, we dialyzed freshly purified HbS solutions through membranes with molecular weight cutoff 500 and 1000 g mol^{-1}. In these solutions, HbS polymerization proceeded immediately after purification.

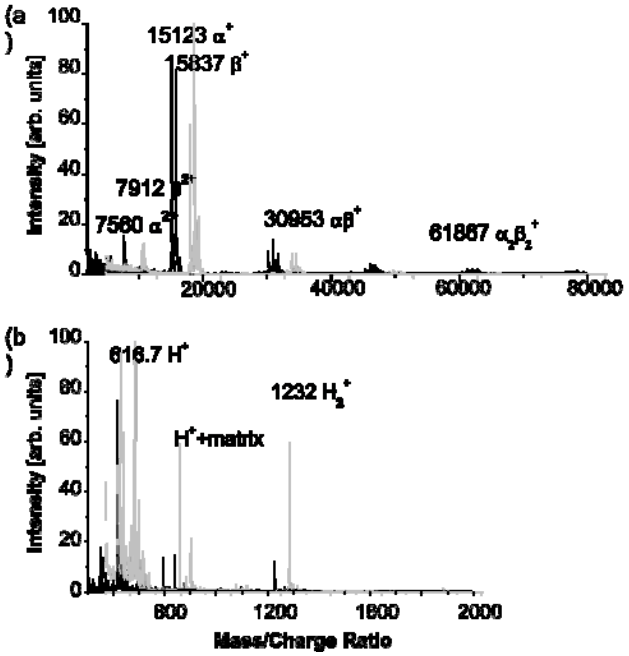
Heme release as source of ageing effects.

Mass spectroscopy characterization of the solutions used in the aging experiments in Supplementary Figure 5, see Supplementary Figure 6, reveals that no unexpected species, e.g., fragments of protein chains, appear in the solution upon ageing, and that heme is the only solution component, which appears to increase its concentration, Supplementary Figure 6b (mass spectroscopy cannot be used for concentration determination). Fe^{3+}, while not seen in the mass

spectra, has solubility in the attomolar range at the solution pH and, hence, is an unlikely source of enhancement.

Enhancement of autoxidation and heme release by laser illumination.

These tests aimed to find if the illumination by a laser with a wavelength 532 nm during the quantification of the polymerization kinetics may increase the free heme concentration. The concentration of met-HbS and free heme were determined using the cyanide method. In freshly purified and dialyzed oxy-HbS solution the spectra in Supplementary Figure 7 reveal the presence of ~ 6 % met-HbS. After the addition of cyanide, the resulting spectrum nearly coincides with the one from a respective mixture of oxy- and cyanmet-HbS. This correspondence indicates that the amount of free heme in this solution is < 1 μM. The solution with total HbS concentration ~ 200 mg ml^{-1} was divided into two. One part was placed in a cuvette with 2 mm thickness and 200 μl volume and irradiated for 3 hours with a laser beam expanded to 1 cm diameter and total power of 140 mW. Since the irradiation leads to solution heating to 28 ^{o}C (measured in the cuvette), the second part was placed in the dark in an incubator at the same temperature. The absorbance at 630 nm of these solutions in Supplementary Figure 7 reveals that irradiation increases the amount of met-HbS to ~9 %, while ageing in the dark—to ~ 7 %. The spectra taken after the addition of cyanide show that irradiation leads to the release of ~ 5 μM free heme. The amount of heme released by the control aged at 28 ^{o}C is, within the accuracy of the method, comparable to this.

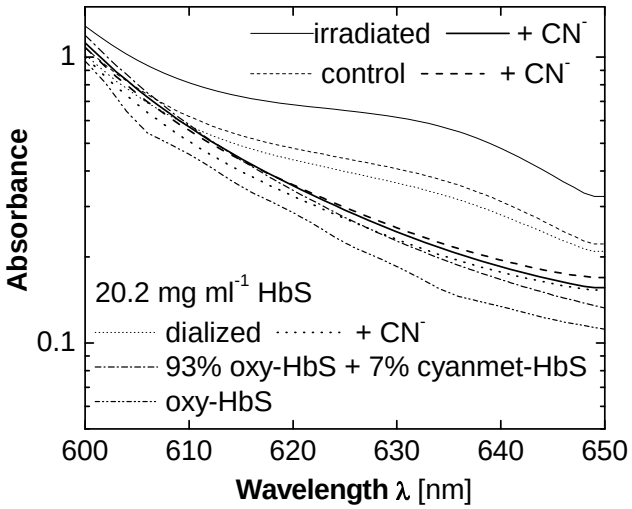


Supplementary Figure 6. Cation mass spectra characterization of species released upon ageing of oxy-HbS at 22 ^{o}C . (a) Mass-to-charge ratio region 2 000 – 80 000 g mol^{-1}. (b) Mass-to-charge ratio region 450 – 2 000 g mol^{-1}. The peaks corresponding to α and β-chains and their complexes and to heme and its dimer are labeled. Black and grey denote, respectively, solutions prepared immediately after thawing of the stock and solutions kept at 22 ^{o}C for one day. The second-day spectra are shifted to the right for clarity.

The total energy absorbed by the irradiated HbS for the 3 hour period (calculated from the extinction coefficient at this wavelength) was ~ 170 kJ $mmol^{-1}$. This is only slightly lower than the ~250 kJ $mmol^{-1}$ absorbed by CO-HbS and deoxy-HbS during a kinetic determination, in which a 10 mW beam is focused on a 90 μm spot for 10 s (the extinction coefficients at 532 nm of CO-, oxy- and deoxy- hemoglobins are close (1)). This absorption of energy leads to solution overheating and photolysis of CO-HbS to CO(aq.) and deoxy-HbS. Supplementary Figure 7 reveals that another consequence of the absorption of energy is the production of met-HbS and free heme in the oxy-HbS solution. Since autoxidation has several non-oxygen mechanisms (15, 20), irradiation induced autoxidation would be possible in CO- and deoxy-HbS.

Two factors limit the bias, due to the production of heme by irradiation, in the determinations of the kinetics of HbS polymerization in Fig. 4 of the main text and Refs. (2-3). First, the solutions tested in those experiments contain significant amounts of heme even prior to irradiation, and hence the newly released heme only mildly increases the total concentration of free heme. Second, the irradiated spots on the slide containing HbS, from which data are collected, are several millimeters apart. The interval between irradiation of two spots is about 20 s, for which time the characteristic diffusion distances of free heme and met-hemoglobin are, respectively, 100 μm and 30 μm. Thus, the methemoglobin and free heme produced at one spot cannot affect the rate of polymerization in other spots.

On the other hand, if the laser photolysis method is applied to study polymerization inside live sickle erythrocytes, in which the initial free heme concentration is low, or if significantly greater laser power is employed, the amounts of released heme and the related acceleration of polymerization may be significant.



Supplementary Figure 7. Generation of met-HbS and free heme by irradiation with wavelength 532 nm. Irradiated samples, controls kept in the dark at the temperature of during irradiation, and freshly dialyzed HbS solution, before and after the addition of KCN are compared to the spectra of 93 % oxy-HbS and 7 % cyanmet-HbS and with the spectrum of pure oxy-HbS.

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