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Evidence for carbon monoxide binding to sickle cell polymers during melting

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Abstract

The melting of sickle cell hemoglobin (HbS) polymers was induced by rapid dilution using a stopped-flow apparatus. The kinetics of polymer melting were monitored using light scattering. Polymer melting in the absence of any hemoglobin ligand was compared to melting when the diluting buffer was saturated with carbon monoxide (CO). In this way the role of CO in polymer melting could be assessed. The data were analyzed using models that assumed that melting occurs only at the ends of polymers. It was further assumed that CO could only bind to HbS in the solution phase. However, our data could not be fitted to this model, where CO cannot bind directly to the polymer. Thus, CO probably binds directly to the polymers during our melting experiments. This result is discussed in terms of oxygen induced polymer melting and polymerization processes in sickle cell disease

Keywords: Sickle cell hemoglobin; Polymer; Melting; Kinetics; Ligand binding

1. Introduction

Sickle cell anemia is an inherited condition in which the replacement of hydrophilic Glutamate by hydrophobic valine at the β6 position of hemoglobin induces HbS to aggregate into polymers under hypoxic conditions [1]. It is believed, that only T-state HbS molecules polymerize [2]. Reoxygenation at the lungs drives polymer melting — the dissociation of HbS molecules from the polymer. The polymerization of HbS reduces red cell deformability. These sickled red cells contribute to occlusions of the microvasculature leading to tissue damage.

The double nucleation mechanism of HbS polymer formation, proposed by Ferrone et al., involves both homogeneous and heterogeneous nucleation [3]. The initial event in HbS polymerization is homogeneous nucleation. Local tempera-

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ture or concentration fluctuations may stimulate the formation of an aggregate from a few HbS molecules. The critical nucleus is defined by the number of hemoglobin molecules in the aggregate above which the favored process is growth and below which the favored process is dissociation. The critical nucleus gives rise to the HbS fiber and the fiber provides a large number of sites for heterogeneous nucleation. This mechanism of HbS polymerization, involving the critical nucleus, explains the observation of a long and stochastic delay time followed by autocatalytic growth. If complete melting of HbS polymers leaving the lungs takes longer than the transit time to tissue with low oxygen pressure, the remaining polymer can resume relatively rapid growth with no delay time. Some early studies indicate that polymer melting is slow on a time scale compared to the transit time of an erythrocyte during circulation [4,5].

Despite the importance of the subject, there is no established theory of sickle hemoglobin melting kinetics at present. Based on observations using differential interference contrast (DIC) microscopy, Briehl [6] proposed that polymer melting is the reversal of growth, with hemoglobin molecules coming off the polymer ends. Thus, polymer melting would begin with the shortening of the polymer and the number of polymers would be constant during the beginning of melting. Several investigators have used CO to induce sickle cell polymer melting instead of oxygen since one can avoid autooxidation and CO makes a good model for oxygen [6–8]. In earlier studies of HbS polymer melting we have seen evidence supporting direct binding of CO to the polymers [7,8]. In a recent, preliminary report, Briehl et al. have observed that at high concentrations of CO, melting occurs at the sides of polymers as well as at the ends and proceeds in a cooperative manner [9]. This preliminary result may also imply that CO binds directly to the polymer.

The purpose of this paper is to further assess the role of CO in HbS polymer melting. Melting of the HbS polymer has been induced in two ways. The first method, which we call dilution-only mediated melting, involves rapid mixing of a deoxygenated HbS polymer solution with an excess of deoxygenated buffer. The solubility, the concentration of the deoxy, solution phase HbS molecules in equilibrium, depends on the salt concentration of the diluting buffer and the temperature [10–12]. In equilibrium, the concentration of hemoglobin molecules in excess of the solubility constitute the polymer phase. Dilution of the solution decreases the concentration of the molecules in the solution phase, so the system is no longer in equilibrium. The polymers melt, increasing the concentration of the solution phase component, in order to bring the system into equilibrium (Le Chatelier's principle). The dissociation of molecules from the polymer continues until the concentration of the molecules in the solution phase is equal to the solubility, or until all the molecules go into solution.

The second method of inducing melting that we have used involves rapid mixing of a deoxygenated HbS polymer sample with CO-saturated buffer. We study the role CO binding plays in the melting kinetics. Consider a model where CO can only bind to solution phase HbS molecules. Fully ligated solution phase sickle cell hemoglobin molecules do not polymerize [2] and do not contribute to the exchange of molecules between polymer and solution phase HbS. A decrease in the concentration of deoxy, solution phase HbS induces melting. Thus, one may expect to see an increase in the melting rate when CO-saturated buffer, rather than a deoxy one, is used for a dilution. In other words, since CO bound hemoglobin molecules have a higher solubility than deoxy molecules, CO can increase the rate of polymer melting by binding to solution phase hemoglobin molecules. It is also possible that CO can increase the rate of melting by binding directly to the polymer phase HbS molecules. However, in the model denoted here as the simple model, we assume that CO can only bind to solution phase HbS tetramers. Although the dilution mediated melting is a part of the whole melting kinetics, we refer to this method as CO mediated melting. Here we show that the simple model cannot account for CO mediated melting kinetics, thus inferring direct binding of CO to HbS in the polymer phase.

We have developed a model that assumes that

the polymers can melt only from the ends. This is consistent with early observations reported by Briehl [6]. Furthermore, earlier experiments performed in our laboratory show that as the number of polymer ends in a given sample decreases the rate of melting also decreases [8]. If melting only occurs at the polymer ends, then, initially, the number of ends is approximately constant. Using this model we have determined the melting rate for a given HbS sample via the dilution-only mediated melting method. The model was modified further and applied to CO mediated melting for the same HbS sample preparation. We refer to the model that assumes (1) melting only occurs at the ends and (2) CO only binds to the solution phase molecules as the simple model. We found that, in order to account for the rapid CO mediated melting, CO must associate directly to HbS polymers.

2. Experimental

The HbS was obtained from blood donated by patients homozygous in HbS, with less than 6% fetal hemoglobin, following federal regulations and guidelines outlined by the National Institute of Health. Normal adult hemoglobin (HbA) was obtained from volunteer donors. The hemolysate was prepared as described previously [13]. Samples were pelleted in liquid nitrogen and stored at −80°C. The HbA was prepared similarly. In order to achieve polymerization with low concentrations of HbS [11,12] so that stopped-flow experiments could be performed, the experiments were carried out in high concentration phosphate buffer. After overnight dialysis in 1.8 M potassium phosphate buffer (pH 7.1) hemoglobin was diluted with 1.8 M potassium phosphate buffer to produce a final hemoglobin concentration of HbS $= 2.0 \pm 0.4$ mM. A solution of sodium dithionite (Sigma Chemical Company, St. Louis, MO) was made by adding it to deoxy buffer at a concentration of 150 mg/ml. A few drops of the dithionite solution were added to the samples to scavenge residual oxygen. The same procedure was performed on the HbA sample. The HbS sample was slowly polymerized by purging with argon prior to addition of sodium dithionite.

The time-resolved extinction measurements were performed on an OLIS RSM-1000 Spectrophotometer (Bogart, GA) with a stopped-flow apparatus. The dead time of the instrument was determined by the method of Gibson [14], using measurements of the binding rate of CO to myoglobin for molar ratios of 3:2 and 4:1 at 25°C and 35°C. The dead time was found to be 0.02 s. The melting experiments were performed on both HbA and HbS samples. Measurements on HbA were performed as a control. In order to avoid changes in the transmitted light intensity due to sinking of the hemoglobin or other sedimentary effects, the sample in 1.8 M phosphate buffer was mixed with 1.5 M buffer so that the final concentration of phosphate after mixing (1:10) was 1.53 M. We determined that the hemoglobin solution neither sinks nor floats at this phosphate concentration. For dilution-only mediated melting the hemoglobin sample was diluted with deoxygenated, 1.5 M, potassium phosphate buffer (pH 7.1) in ratio 1:10 (hemoglobin to diluting buffer). This dilution factor, $s = 1/11$, was chosen to ensure a decrease of HbS sample concentration below the solubility. No sedimentary effects were noticed for these mixing conditions. For CO mediated melting, the hemoglobin sample was diluted with CO-saturated buffer of the same salt concentration and in the same ratio. All measurements were performed at room temperature.

The solubility of HbS in 1.8 M phosphate buffer (used for polymerization) was calculated based on previous measurements of the solubility at 30°C, measurements of the delay time at various temperatures [11] and using the relationship $1/t_d = \lambda(C_{tot}/C_s)^n$ [4]. Here t_d is the delay time, C_{tot} is the HbS total concentration, C_s is the solubility concentration, λ is a constant of proportionality and $n = 2.8$ for 1.8 M phosphate buffer [12]. The solubility of HbS in 1.53 M phosphate buffer (used for dilution) was determined by centrifuging deoxy HbS at 7000 g for 20 min and measuring the supernatant concentration. At room temperature, the solubilities are $C_s^{1.53} = 0.4$ mM and $C_s^{1.8} = 0.04$ mM for buffer concentrations 1.53 M and 1.8 M, respectively.

The melting of the HbS sample was monitored by changes in the turbidity of the time-resolved extinction (absorption + scattering) spectra. The total concentration of the Hb was determined by examination of spectra taken when polymer melting was complete. The absorbance (at 555 for deoxy and 540 nm for carboxy hemoglobin) was divided by the product of the path length (0.35 cm) and the extinction coefficient [12.5 1/(mM cm) for deoxy and 13.5 1/(mM·cm) for carboxy hemoglobin] to give the concentration. Since the difference in absorption between deoxy and carboxy hemoglobin is small compared to contributions from scattering at 650 nm, the extinction at this wavelength was used to calculate the concentration of polymers, as described below. The initial extinction of the sample was calculated by taking the extinction measured at 20 ms (corrected using the dead-time) and projecting it to zero ms using an exponential function. The lifetime, τ_e, of the exponential function was obtained by a global fit to the spectral, kinetic data using Spectfit (Spectrum Software Associates, Boston, MA).

Conversion of the turbidity, measured at 650 nm to a concentration of polymers cannot be done with absolute accuracy. The reason for this is that light scattering, responsible for the turbidity, is not linearly proportional to the concentration of hemoglobin in the polymer phase. A given concentration of molecules in the polymer phase may scatter more if there are a few large aggregates compared to many small ones. Thus, one could imagine scenarios where there is a lot of melting with little change in the turbidity. For example, one may have initial melting from diffuse polymer ends that contribute little to the scattering. Nevertheless, many authors have used light scattering to quantify the amount of an aggregated phase in the past. In most cases, turbidity or light scattering was assumed to be linearly proportional to the concentration in the aggregated phase. This does not constitute an accurate account of the size and shape dependence of light scattering. The relationship between the size and shape of the polymer domains and the intensity of scattered light can be quite complicated. Our use of turbidity to quantify the polymer phase, in this study, is justified since, if the simple model is correct, then the relationship between the turbidity and the size and shape distribution of the polymer domains will be the same for both dilution-only and CO mediated melting. For both types of melting we use the same sample preparation, so it starts out with the same size distribution of polymer aggregates. If the simple model is correct, wherein CO only binds to solution phase molecules, then the molecules melting off the polymers do not sense whether CO is present or not. If the simple model is correct, the progression of size and shape distributions following dilution-only and CO mediated melting should be the same. Thus, according to the simple model, the relation between the amount of Hb in the polymer phase and the turbidity will be the same.

We used three different functions to convert the measured turbidity at 650 nm to a concentration of polymers: a square root; a linear and a quadratic conversion function. The conversion functions were: concentration of hemoglobin in the polymer phase, $C_p' = A \times \text{extinction}^Q + B$, where $Q = 0.5, 1, 2$ for the square root, linear and quadratic conversion (a, b and c, if not defined otherwise), respectively. The effect of the quadratic function would be similar to that of having initial melting of diffuse ends whereas the square root function describes a situation where small initial changes in the polymer mass would have large effects on scattering. The extinction was converted to a concentration of hemoglobin in the polymer phase, $C_p'(t)$, by assigning $C_p'(0) = s(C_{tot} - C_s^{1.8})$, $C_p(\infty) = 0$ and using either the square root, linear or quadratic conversion. Here C_{tot} stands for the total concentration of HbS molecules and $C_s^{1.8}$ stands for the solubility concentration of HbS molecule in the phosphate buffer of concentration equal 1.8 M. The concentration of HbS in the polymer phase was fit to the models developed below [Eqs. (1) and (3)] using IDL (Interactive Data Language, Boulder, CO).

Our argument is of the reductio ad absurdum type. We make a couple of assumptions and show that these are inconsistent with our measurements, thereby discrediting the assumptions. Our assumptions are those constituting the simple

model: (1) melting occurs from the polymer ends; and (2) CO can only bind to the solution phase Hb molecules. These assumptions lead to the following conclusions: (1) the number of ends will be constant for some time during melting (before the shortest polymer melts); (2) the dependence of turbidity on the amount of Hb in the polymer phase is the same when our samples are melted either by the dilution-only or by the CO mediated melting; and (3) the theoretical models developed below [particularly Eqs. (1) and (3)] should agree with our measured data for as long as the concentration of polymers is constant. Since we find that the conclusion (3) is not correct, we refute the simple model.

3. Results

The kinetic equations (and their solutions) for both dilution-only mediated melting and CO mediated melting are derived in Appendix A. When HbS polymers are mixed with excess deoxygenated buffer, in dilution-only mediated melting, the concentration of HbS molecules in the polymer phase is given by:

$$C_{\mathrm{p}}'(t) = s(C_{\mathrm{tot}}) - C_{\mathrm{s}}^{1.53} + (C_{\mathrm{s}}^{1.53} - sC_{\mathrm{s}}^{1.8}) \cdot e^{-t/\tau} \tag{1}$$

where:

$$\tau = \frac{C_{\mathrm{s}}^{1.53}}{C_{\mathrm{p}}} \cdot \frac{1}{k^d} \tag{2}$$

is the characteristic polymer melting time. $C_{\mathrm{p}}'(t)$ is the molar concentration of the HbS monomers in the polymer phase, C_{p} is the molar concentration of HbS polymers,[2] k^d is the dissociation rate of deoxy HbS monomers from the polymer ends, C_{tot} is the total molar concentration of HbS before dilution, s, is the dilution factor (1/11), and C_{s} is the solubility concentration with $C_{\mathrm{s}}^{1.8}$ and $C_{\mathrm{s}}^{1.53}$, reflecting the buffer phosphate concentrations for mixing. The sample was polymerized in 1.8 M phosphate buffer to achieve a large percentage of molecules in the polymer phase and it was diluted into 1.5 M buffer to avoid sedimentary artifacts.

The larger the concentration of polymers (and, thus polymer ends), the sooner the concentration of the solution phase in the diluted sample will reach the solubility. However, the larger the solubility, the longer the relaxation to the equilibrium will last. According to Eq. (2), as the ends concentration increases, the rate of melting also increases. This is consistent with our earlier findings [8].

Our goal here is to assess the importance of CO binding to the solution and polymer phase molecules in the kinetics of HbS polymer melting. The ligand affinity of polymerized HbS is much lower than that of solution phase HbS [2]. While it is not clear if the ligation of the polymer phase HbS contributes to the melting kinetics, the ligation of the solution phase HbS, including those that come off the polymer, is involved in melting in the manner discussed above — the solubility of CO bound molecules is higher than that of deoxy molecules. The CO binding rate to the solution phase HbS is the same as that to HbA [15,16]. We found that, under our experimental conditions using 1.8 M phosphate buffer, the CO binding rate to HbA is $k_m = 0.07 \pm 0.002\ (\mathrm{s}^{-1}\mu\mathrm{M}^{-1})$. The derivation of the kinetic equations describing CO mediated melting of HbS polymers is given in Appendix A. We find that the molar concentration of polymerized HbS monomers is given by:

$$C_p'(t) = s(C_{tot} - C_s^{1.8}) - C_s^{1.53} \cdot \frac{\tau - \tau^*}{\tau^2} \cdot t + C_{\mathrm{s}}^{1.53} \cdot \left(\frac{\tau^*}{\tau}\right)^2 \cdot \left(1 - s\frac{\tau}{\tau^*} \cdot \frac{C_{\mathrm{s}}^{1.8}}{C_{\mathrm{s}}^{1.53}}\right) \times \left(1 - e^{-\frac{t}{\tau^*}}\right) \tag{3}$$

where

$$\tau^* = \frac{\tau}{1 + \tau \cdot k_m[\mathrm{CO}]} \tag{4}$$

[2] By polymer we mean the 21 nm fiber with 14 hemoglobin molecules in its cross section.

When deoxy buffer is used to drive HbS melting, [CO] = 0 so that τ^* equals τ and the solution Eq. (3) reduces to Eq. (1), the solution for dilution-only mediated melting. As the concentration of CO increases, τ^* decreases, indicating that CO increases the rate of polymer melting.

We used time-resolved extinction measurements to determine the concentration of the polymerized component in the HbS sample exposed to both melting drives mentioned above. The same reactions were performed with HbA and a typical outcome is illustrated in Fig. 1. Fig. 1a presents an example of time-resolved absorption spectra of the diluted HbA sample when deoxy buffer is used. The absence of any time dependence of the spectra demonstrates that no diffusion or sedimentary effects are present. Fig. 1b presents time-resolved spectra for an HbA sample diluted with CO saturated buffer. Two characteristic peaks appear at wavelengths 540 and 570 nm, reflecting the transition of hemoglobin from the deoxy state to the ligated state.

Fig. 2 shows HbS polymer melting. The turbidity is due to scattering by the HbS in the polymerized phase. Fig. 2a presents data collected after deoxy HbS is mixed with deoxy buffer. The melt-

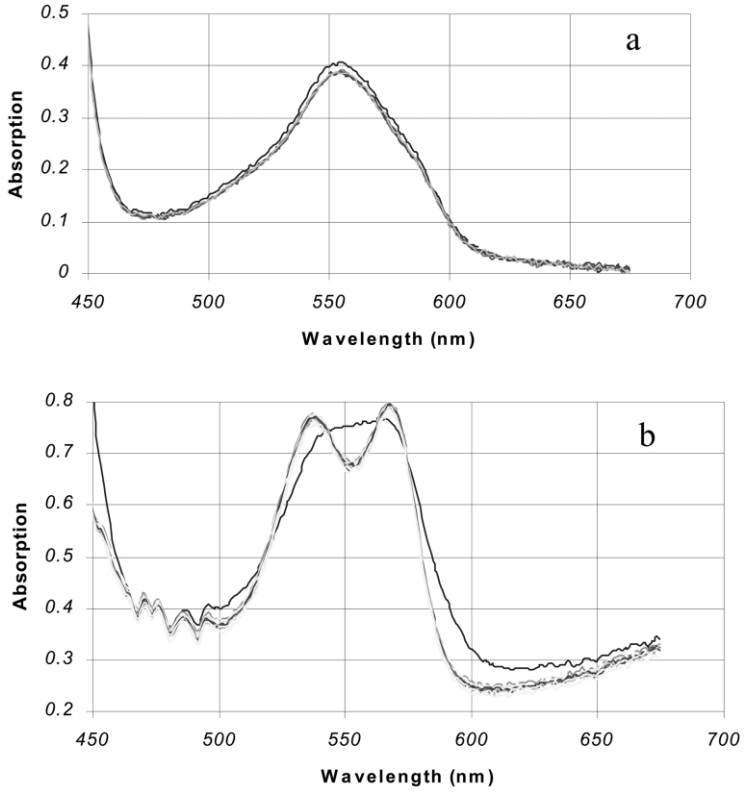

Fig. 1. Time-resolved absorption spectra of rapid mixing involving HbA. (a) Deoxygenated hemoglobin, HbA, in 1.8 M phosphate buffer mixed with deoxygenated 1.5 M phosphate buffer in a ratio of 1:10 by volume. Nine representative spectra are shown spaced 80 ms apart beginning with the one obtained at an effective time of 20 ms after mixing. The overlap of the spectra with each other indicates that there is no sedimentation or other artifacts that could account for the data shown in Fig. 2 for HbS. (b) Deoxygenated hemoglobin HbA in 1.8 M buffer was mixed with CO saturated 1.5 M buffer in a ratio of 1:10. As a result of transition of HbA from deoxy to the ligated state the absorption peak profile changes form peak at 555 nm to two peaks at 540 and 570 nm. Eleven representative spectra are shown spaced 80 ms apart beginning with the one obtained at an effective time of 20 ms after mixing. The CO binding is rapid. No turbidity is observed for HbA.

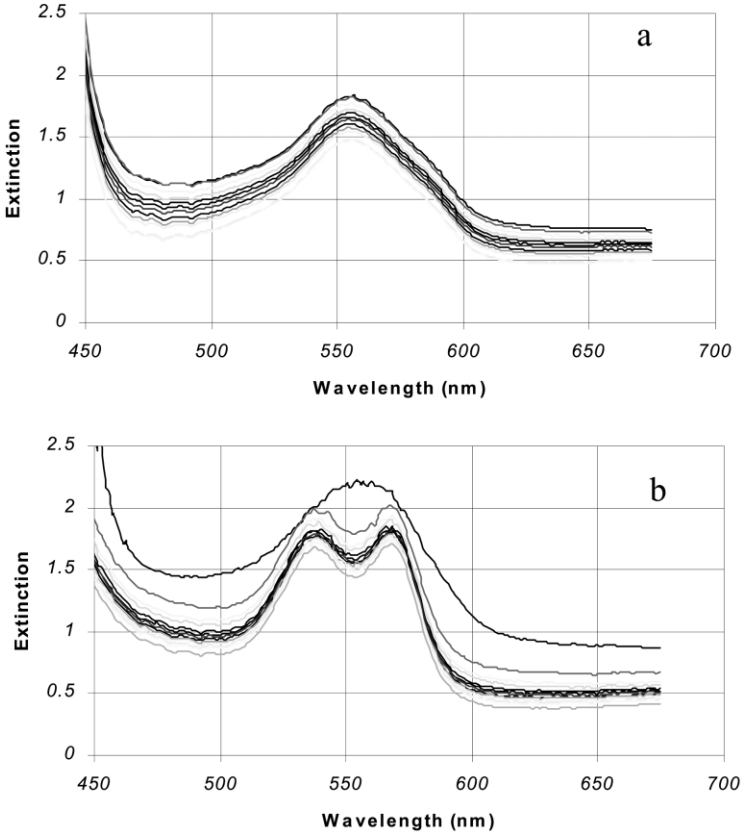

Fig. 2. Time-resolved extinction (absorption + scattering) spectra of deoxygenated hemoglobin HbS. (a) HbS in 1.8 M buffer mixed with 1.5 M deoxygenated buffer in a ratio of 1:10 by volume. The melting due to dilution does not affect the ligation state of hemoglobin and the single peaked, shape of the spectra remains. The turbidity due to scattering is apparent. The extinction of the spectra decreases as the polymers melt. Twelve representative spectra are spaced 80 ms apart beginning with the one obtained at an effective time of 20 ms after mixing. (b) Time-resolved extinction spectra of the same sample preparation of deoxygenated hemoglobin HbS mixed with 1.5 M CO saturated buffer in a ratio of 1:10. Twelve representative spectra are shown spaced 5 ms apart. CO binding is evidenced by the change in shape of the spectra while decreasing turbidity is indicative of polymer melting. The faster melting compared to the dilution mediated melting (Fig. 2a) is illustrated by the rapid decrease in turbidity.

ing of HbS polymers due to dilution does not affect their absorption spectra since there is no change in ligation state of the HbS molecules and HbS in the polymer phase has a similar absorption to that of monomer phase. Fig. 2b presents an example of time-resolved absorption spectra of the HbS + CO reaction. It displays two features: the change from a single peak to two peak spectra; and the decrease of the extinction due to decreased turbidity.

As discussed above, in the Section 2, we have used the extinction at 650 nm to calculate the concentration of hemoglobin molecules in the polymer phase. The relationship between this polymer mass and the turbidity may be quite complicated but if the simple model is correct it should be the same for both types of dilution. This implies that the conversion factors should be the same for both types of dilution. If the simple model is not correct, then the turbidity would be different for a given polymer concentration due to differences in the size distribution of aggregates during melting. Table 1 shows the conversion factors used to convert turbidity to polymer

Table 1
Coefficient A from equation $C_p' = A \times \text{extinction}^Q + B$, used to calculate concentration C_p' from extinction data of both dilution and CO mediated melting reaction

Deoxy HbS polymer and deoxy buffer	Deoxy HbS polymer and CO saturated buffer	Conversion mode
0.32 ± 0.13	0.34 ± 0.04	a
0.61 ± 0.05	0.62 ± 0.16	b
2.21 ± 0.33	2.29 ± 1.10	c

Three conversion modes — square root (a), linear — (b), quadratic — (c), having exponent $Q = 0.5$, 1, 2, respectively, as described in Section 2, were applied to the melting data. The average and standard deviations are shown for 15 mixtures from a typical sample preparation.

mass concentration for a typical sample preparation. It is seen that the conversion factors are similar for both types of induced melting (dilution-only vs. CO mediated) within each type of conversion function.

Fig. 3 compares data for the same HbS sample preparation, which has undergone melting by the dilution-only and CO mediated methods. Both the original data (a) and those (b, c, d) converted to $C_p'(t)$ using the three different conversion functions are shown. The faster melting when the CO binding is involved is apparent both in the original data and when converted to $C_p'(t)$ using the various modes.

The calculated values of $C_p'(t)$ were fit to the dilution-only and CO mediated melting models. The dilution-only mediated model [Eq. (1)] could not always fit the measured values of $C_p'(t)$ for the entire time span of the experiment. This is due to the fact that the number of ends is not constant towards the end of the melting progress curves. Fitting Eq. (1) to the data within shorter time spans yielded consistent results for τ. When the Eq. (1) was applied to longer time spans, the value of τ obtained got larger and the goodness of the fit (as measured by least-square) began to get worse. This is what is expected to happen according to the simple model when the time span gets into the regime where the number of polymer ends is no longer constant. The values of τ obtained from the fits and the values of least-

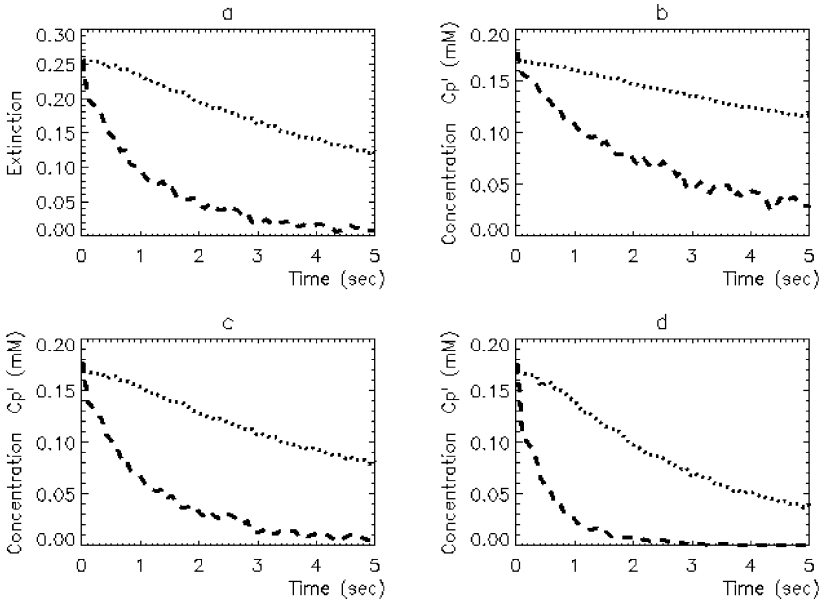

Fig. 3. The measured time course of polymer melting. Dilution mediated melting (dotted line) and CO mediated melting (dashed line) of HbS polymers are represented by the extinction at 650 nm (a). The same data are shown converted to concentration of HbS in the polymer phase using three conversion modes: b — square root; c —linear; and d — quadratic, as described in Section 2. Faster melting when CO binding is involved is apparent for all modes.

Table 2
The τ (Eq. (2)) obtained by fitting the ends model (Eq. (1)) to the dilution mediated melting data

(a)			(b)			(c)		
time s	$\tau \pm \Delta\tau$ s	least square	time s	$\tau \pm \Delta\tau$ s	least square	time s	$\tau \pm \Delta\tau$ s	least square
2.5	29.6 ± 2.9	3.50E-06	2.2	16.0 ± 1.9	1.40E-05	1.8	8.7 ± 1.2	5.30E-05
3	28.8 ± 2.4	3.60E-06	2.6	15.7 ± 1.3	1.20E-05	2.2	8.9 ± 0.9	5.30E-05
3.6	28.8 ± 2.1	3.40E-06	3.1	15.6 ± 1.1	1.20E-05	2.6	8.9 ± 0.6	5.20E-05
4.2	28.9 ± 2.0	3.00E-06	3.6	15.8 ± 1.0	1.20E-05	3	9.1 ± 0.5	5.40E-05
4.7	28.9 ± 1.9	3.00E-06	4.1	16.1 ± 1.0	1.20E-05	3.4	9.4 ± 0.5	6.80E-05
5.3	28.9 ± 1.8	3.20E-06	4.6	16.3 ± 0.9	1.30E-05	3.8	9.7 ± 0.4	8.30E-05
5.8	29.1 ± 1.6	3.90E-06	5	16.5 ± 0.8	1.60E-05	4.2	10.1 ± 0.4	1.00E-04
6.4	29.4 ± 1.5	5.20E-06	5.5	16.8 ± 0.8	2.00E-05	4.6	10.4 ± 0.3	1.30E-04
7.6	29.6 ± 1.4	6.30E-06	6	17.1 ± 0.7	2.70E-05	5	10.8 ± 0.3	1.70E-04
8.8	30.1 ± 1.4	7.90E-06	6.5	17.5 ± 0.7	3.70E-05	5.4	11.2 ± 0.3	2.10E-04
11.2	31.6 ± 1.2	1.70E-05	8.1	18.6 ± 0.6	6.60E-05	6.2	12.1 ± 0.3	3.30E-04
13.6	33.2 ± 1.2	3.60E-05	9.7	20.1 ± 0.6	1.30E-04	7.4	13.4 ± 0.4	5.20E-04
16	34.8 ± 1.6	6.30E-05	11.3	21.8 ± 0.6	2.30E-04	8.5	15.0 ± 0.5	7.90E-04

Three conversion modes-square root (a), linear — (b), quadratic — (c), as described in Section 2, were applied to the melting data. The first column is the time span over which the fit was performed. The second column presents the melting time, τ, averaged over the measurements on the same sample. The third column is the least square of the fit.

square for data taken from a representative sample is shown in Table 2.

Fig. 4 presents typical examples of fits to dilution-only mediated melting curves converted to $C_p'(t)$ using the various modes.[3] The model [Eq. (1)] fails to fit the data beyond the times indicated in the caption. The lack of fit at these later times is probably due to the decrease in the number of polymer ends, once some shorter polymers melt completely. The HbS polymer concentration corresponding to this time defines the limits of the validity of the assumption that the number of ends is constant when it is used for further analysis of CO mediated melting. Only within the range of these concentrations can the mechanism of CO involvement in melting be validated. The fit to the same HbS samples mixed with CO-saturated buffer typically yields results shown in Fig. 5. The values of τ used in Eqs. (3) and (4) are those determined by fitting Eq. (1) to dilution-only mediated melting data from the same HbS sample (that is one that has the same initial concentration of ends). On the same figure we also display the prediction generated by the dilution model [Eq. (1)]. One can see that the model for CO mediated melting [Eq. (3)] shows faster kinetics than the dilution-only mediated melting but it is still much slower than the observed data. In particular, the slope of the experimental data at early times is much steeper than that of model.

We summarize results from measurements taken on four sample preparations. The slopes at the initial moment and the half times of the melting for the observed data and the model are presented in Table 3. Based on the initial slopes, the kinetics of the data is approximately 100 times faster than those predicted by the model. The model of CO mediated melting has an initial melting rate equal to $[s(C_s^{1.8}) - C_s^{1.53}]/\tau$ [see Eq. (3)], which is the same for the model describing dilution mediated melting [Eq. (1)]. The half times for measured data are over fivefold shorter than those for the model.

4. Discussion

This work provides evidence for the involvement of CO binding directly to HbS polymers

[3] An inconsistant fit was defined, when τ was more than one standard deviation from the average value calculated for shorter time spans.

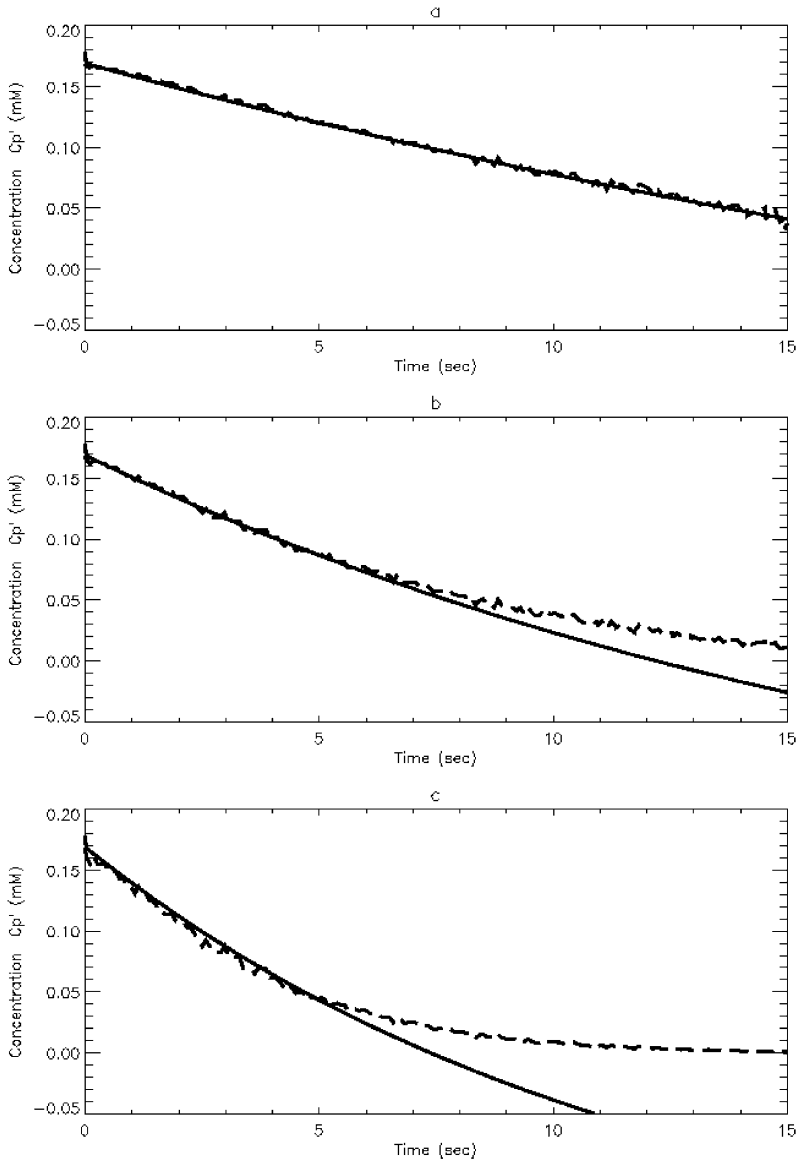

Fig. 4. Comparison of data and model for dilution mediated melting. The data are represented by dots and the solid line corresponds to the fit using Eq. (1). The extinction data have been converted to concentration of polymerized monomers, C_p' (dotted line), by its relation to the extinction at 650 nm as described in Section 2. Each of the three conversion modes are shown (a — square root, b — linear, c — quadratic). The time span used in the fit and the τ obtained and exported for fitting the CO mediated melting data are 4.5 s and 15.2 s. For all conversion modes (a, b, c) the ends model [solid line, Eq. (1)] fits the data through a significant fraction (approx. 35% of the time) of the complete melting, until C_p' is between 0.04 mM and 0.1 mM. Thus, we expect that the number of ends is constant through the first 41–76% of melting. This value of C_p', thus defines the time range for which we expect the model for CO mediated melting [Eq. (3)] to apply.

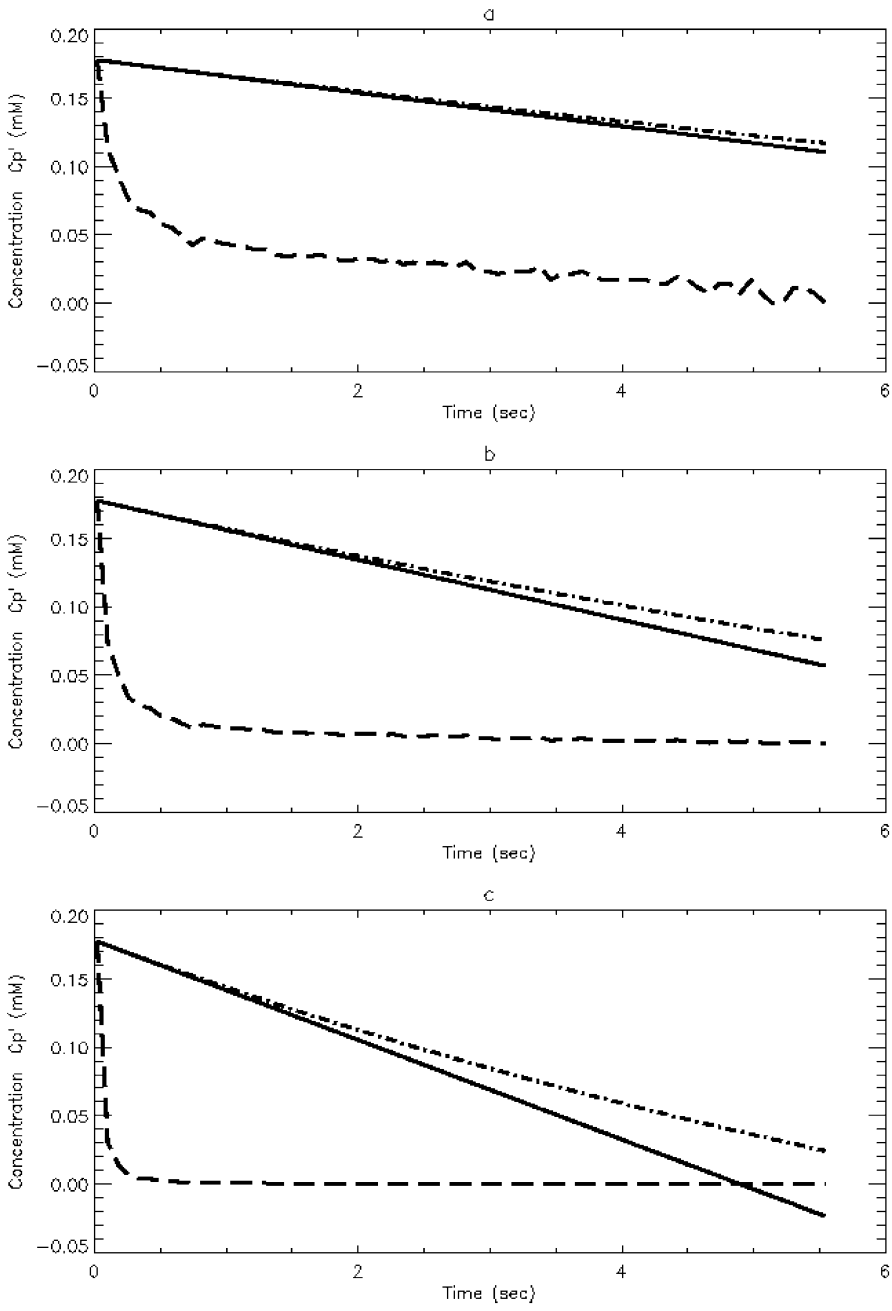

Fig. 5. Comparison of data and model for CO mediated melting for three conversion modes (a — square root, b — linear, c — quadratic). The experimentally measured concentration of HbS in the polymer phase is represented by the dashed lines. Incorporation of the reaction of CO binding to the solution phase HbS [Eqs. (A6) and (A7)] results in slightly faster melting kinetics [solid line, Eq. (3)] compared to the model considering only the dilution drive [dotted line, Eq. (1)]. The predicted decay time τ^* [Eq. (4)] used in the model [Eq. (3)] uses the value of τ obtained from fitting Eq. (1) to dilution experiments on the same HbS sample ($\tau_a = 30.1$ s; $\tau_b = 16.8$ s; $\tau_c = 10.1$ s); the value for k_m was measured to be 0.07 ± 0.002 1/(s mM) and the value for [CO] = 1000 mM $\times (1 - s)$, where $s = 1/11$ is the dilution factor. The time corresponding to the melting of e^{-1} fraction of the HbS aggregate is approximately 1.54 s. For all conversion modes (a, b, c) the model is clearly inconsistent with the data.

Table 3
Half-times (s) and slopes of the melting progress curves found for CO mediated melting data

Slope (model)	Slope (data)	1/2 time (model)	1/2 time (data)
(a)			
-0.015 ± -0.005	-1.16 ± -0.94	1.5 ± 1.0	0.33 ± 0.22
(b)			
-0.027 ± -0.009	-2.02 ± -1.60	1.5 ± 1.0	0.15 ± 0.14
(c)			
-0.047 ± -0.014	-3.36 ± -2.60	1.5 ± 1.0	0.09 ± 0.08

The average values are shown $\pm$ one standard deviation for a total of 55 mixtures of samples prepared on four different days. The initial slope obtained from the model [Eq. (3)] is $[s(C_s^{1.8}) - C_s^{1.53}]/\tau$. The part of the progress curves, corresponding to the time span of order ~ 1 s was used to find the slope of the experimental data. The time when the concentration of HbS polymerized monomers has fallen to one-half of the initial concentration $C_p'(0) = s(C_{tot} - C_s^{1.8})$ is defined as the 1/2 time. Three conversion modes — square root (a); linear — (b); and quadratic — (c), as described in Section 2, were applied.

during melting. Using a model based on the idea that the aggregate melts from the ends, we have shown analytically that the process speeds up with CO binding to the solution phase HbS. Nevertheless, our simple model involving CO binding to solution phase HbS could not fit our data, as illustrated in Fig. 5 and Table 3. Thus, CO binding to the polymer phase is implicated.

A key assumption of our model is that the concentration of polymers or polymer ends in the HbS sample remains constant during melting. If there are short polymers present, which are likely to be present in small aggregates, they might melt quickly so that the concentration of polymer ends is not constant. Thus, the presence of short polymers may be inconsistent with our analysis. However, in extinction measurements, the small polymers will not contribute to scattering. In previous measurements we have shown that smaller polymer aggregates do not scatter very much and would not contribute significantly to the measured changes in turbidity here [8]. Moreover, we found that the model for dilution-only melting [where the number of ends are assumed to be constant, Eq. (1)] can fit our data well for a significant fraction of the time of complete melting. The value for the fraction of the HbS remaining in the polymerized phase derived from this time determines when the kinetics of CO binding mediated melting must be valid. In Fig. 4 the dilution-only model diverges from the data when C_p' has fallen to approximately 40% (50 for linear and 70% for quadratic conversion modes) of its initial value. Based on this result, we would expect that the model involving CO binding to solution phase molecules [Eq. (3)] should be valid up until the time when C_p' has reached the limiting fraction of its initial value. Thus, examining Fig. 5, the simple model should fit the measured CO mediated polymer melting kinetics for the first 80–120 ms. However, the model and data diverge tremendously well before this time.

The dilution-only model assumes that melting only occurs at the polymer ends and that the number of ends is initially constant. One might propose that the reason that our models fail is due to the false assumption that melting only occurs at the ends, even when CO is not present. However, observations using DIC microscopy showing melting to proceed from the polymer ends proves otherwise [6]. Recently, Agarwal et al. have communicated preliminary results claiming that, whereas at low CO concentration polymer melting only occurs at the ends, at higher concentrations, melting occurs at the sides as well [9]. Those results were also based on observations using DIC microscopy. The most probable way for CO to influence whether an HbS molecule on the side of a polymer comes off is by direct binding of CO to the polymerized HbS. Melting from the sides of the polymer in the presence of high concentrations of CO also implicates direct binding of CO to the HbS polymer.

We think that the direct binding of CO to the

hemoglobin molecules in the polymer phase contributes to the dissociation of this monomer from the polymer. We expect this mechanism to be much faster than the dilution mechanism. Breakage of polymers becomes more likely if the binding sites are not restricted to the polymer ends. Hence the implications of CO binding to the molecules located on the polymer surface can be twofold — with and without fractionating of the polymer. The dissociation rate may be different for CO bound and CO free HbS molecules k^{d}, k^{co} respectively. Also, the number of polymer ends may increase dramatically during CO mediated melting with a rate relating to the rate of melting of CO bound HbS from the polymer surface. In general, this rate may differ from the rate of melting of CO bound HbS molecules from the polymer ends.

Incomplete melting of HbS polymers during circulation could contribute to microvascular occlusion. Persistent polymers could become seeds for relatively rapid polymerization with no delay time in hypoxic tissues. To the degree that polymer persistence contributes to occlusion events or other rheological abnormalities associated with sickle cell disease, new therapies can be developed to help reduce such effects. Such therapies must be based on knowledge of the mechanism of polymer melting. Our results provide strong evidence that the simple model, melting at the ends and CO binding to solution phase molecules only, is not adequate.

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Appendix A: Kinetic equations of melting of HbS polymers and their solutions

The model developed below for the melting kinetics of the HbS fiber is based on the observation that HbS fiber melting is the reversal of the growth [3,6]. According to Ferrone et al. [3], both melting and growth of HbS fibers, in the absence of a ligand such as CO or oxygen, are described by:

$$-\frac{dC_{m}(t)}{dt} = (k_{+}C_{m} - k_{-})\cdot C_{p} \tag{A1}$$

where k_{+} and k_{-} are rate constants for polymerization and melting of deoxy HbS, $C_{p}(t)$ is the molar concentration of the HbS polymers and $C_{m}(t)$ is the molar concentration of the solution phase HbS. In the above equation, we assume that the hemoglobin solution is dilute (as in high concentration phosphate buffer), so that we can ignore volume effects that were included by Ferrone et al. Using the equilibrium relation $k_{+} = \frac{k_{-}}{C_{s}}$, where C_{s} is the solubility concentration, one can rewrite Eq. (A1) in terms of the melting rate constant k_{-}. In order to distinguish the melting rate constant of deoxy and CO bound HbS molecules we will use k^{d} and k^{co}, respectively. Making these substitutions, we get:

$$\frac{dC_{m}(t)}{dt} = k^{d}\left(1 - \frac{C_{m}(t)}{C_{s}}\right)C_{p}(t) \tag{A2}$$

If the initial value $C_{m}(0)$ exceeds the solubility, C_{s}, then the equation above should describe the rate of polymerization. When the solubility is larger than the initial value $C_{m}(0)$, and $C_{p}(0) \neq 0$, the concentration of the HbS polymers decreases, and Eq. (A2) describes the melting.

Observations using DIC microscopy reveal that growth is an elongation and melting is a shortening of the HbS fiber from the ends [6]. Thus, only monomers at the ends of the polymer fibers contribute to melting. On a short time scale, the concentration of polymers remains constant. The concentration of polymer ends is twice the concentration of polymers.

The sum of the molar concentrations of hemoglobin molecules in the polymer phase, C_{p}' (prime), and that in the monomer phase, C_{m}, is constant, equal to the total concentration of hemoglobin molecules, C_{tot}. Taking the derivative

of the relationship $C_{tot} = C_m + C_p'$ and substituting in Eq. (A2) gives:

$$\frac{dC_p'(t)}{dt} = k^d \cdot \left(\frac{C_m(t)}{C_s} - 1 \right) \cdot (C_p) \tag{A3}$$

The initial conditions in our experiments are $C_p'(0) = s(C_{tot-}C_s^{1.8})$ and $C_m(0) = s(C_s^{1.8})$, where s is the dilution factor 1/11. The two solubility concentrations, $C_s^{1.8}$, $C_s^{1.53}$, reflect the buffer concentrations for the mixing condition mentioned above. The sample was polymerized in 1.8 M phosphate buffer to achieve a large percentage of molecules in the polymer phase and it was diluted into 1.5 M buffer to avoid sedimentary artifacts. The solution of Eqs. (A2) and (A3) is straightforward and the concentration of HbS in the polymer phase is:

$$C_p'(t) = s(C_{tot}) - C_s^{1.53} + (C_s^{1.53} - sC_s^{1.8}) \cdot e^{(-t/\tau)} \tag{A4}$$

where

$$\tau = \frac{C_s^{1.53}}{C_p} \cdot \frac{1}{k^d} \tag{A5}$$

is the characteristic polymer melting time.

In the simple model, we assume that CO can only bind to solution phase hemoglobin molecules. The CO binding rate to the solution phase HbS is the same as that to HbA [15,16]. We define the concentration of solution phase molecules that are CO bound as $C_m^{CO}(t)$, the concentration of deoxy HbS molecules in the solution phase as $C_m^d(t)$ and the concentration of deoxy HbS molecules in the polymer phase as $C_p'(t)$. The melting Eqs. (A2) and (A3) are modified to account for the CO ligated solution phase HbS.

$$\frac{dC_m^d(t)}{dt} = -k^d \cdot \left(\frac{C_m^d(t)}{C_s^{1.53}} - 1 \right) \cdot (C_p) - k_m[CO] \cdot C_m^d(t) \tag{A6}$$

$$\frac{dC_p'(t)}{dt} = +k^d \cdot \left(\frac{C_m^d(t)}{C_s^{1.53}} - 1 \right) \cdot (C_p) \tag{A7}$$

$$\frac{dC_m^{co}}{dt} = +k_m[CO] \cdot C_m^d(t) \tag{A8}$$

The initial conditions are $C_p'(0) = s(C_{tot} - C_s^{1.8})$, $C_m^d(0) = s(C_s^{1.8})$ and $C_m^{co}(0) = 0$. Eq. (A7) shows that the rate of polymer melting depends on $C_m^d(t)$, which is affected by melting and CO binding to solution phase molecules [Eqs. (A6), (A7) and (A8)]. The solution of Eqs. (A6), (A7) and (A8) is straightforward and the composition of the sample changes in time as follows:

$$C_m^d(t) = C_s^{1.53} \cdot \frac{\tau^*}{\tau} \cdot \left(1 - \left(1 - s \cdot \frac{C_s^{1.8}}{C_s^{1.53}} \frac{\tau}{\tau^*} \right) \cdot e^{-\frac{t}{\tau^*}} \right) \tag{A9}$$

$$C_m^{co}(t) = C_s^{1.53} \cdot \frac{\tau - \tau^*}{\tau^2} \cdot t - C_s^{1.53} \frac{\tau - \tau^*}{\tau^2} \cdot \tau^* \cdot \left(1 - s \frac{\tau}{\tau^*} \cdot \frac{C_s^{1.8}}{C_s^{1.53}} \right) \times \left(1 - e^{-\frac{t}{\tau^*}} \right) \tag{A10}$$

$$C_p'(t) = s(C_{tot} - C_s^{1.8}) - C_s^{1.53} \cdot \frac{\tau - \tau^*}{\tau^2} \cdot \tau + C_s^{1.53} \cdot \left(\frac{\tau^*}{\tau} \right)^2 \cdot \left(1 - s \frac{\tau}{\tau^*} \cdot \frac{C_s^{1.8}}{C_s^{1.53}} \right) \times \left(1 - e^{-\frac{t}{\tau^*}} \right) \tag{A11}$$

where

$$\frac{1}{\tau^*} = \frac{1}{\tau} + k_m[CO] \tag{A12}$$

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