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Metastable gels: A novel application of Ogston theory to sickle hemoglobin polymers

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ABSTRACT

Sickle hemoglobin differs from normal adult hemoglobin by its ability to polymerize, which occurs at relatively high concentrations since the solubility for polymerization is typically above 160 mg/ml. We have recently found that the gel formed by polymers is metastable if the gel is not centrifuged or aged for long times in that polymerization ceases before the monomer concentration has decreased from its original value to the solubility. We have proposed that this effect is due to the obstruction of ends by other polymers in the crowded gel. Here we use Ogston's theory describing spaces amid arrays of random rods to provide a framework for describing the failure of the polymers to propagate. We find good agreement between fiber diameter and minimum void spaces. This novel application of a well-established theoretical framework for crowding may apply to other dense gels as well.

Introduction

Sickle hemoglobin polymerization is the fundamental phenomenon that underlies sickle cell disease, which, by virtue of its being one of the oldest as well as structurally the simplest of aggregation diseases, has acted as a paradigm for their study. Sickle hemoglobin is a point mutation of normal hemoglobin, and behaves like a hard sphere in many ways, which simplifies the analysis of non-ideal effects on polymerization. The polymers that form are thick, 14 stranded structures of 22 nm diameter [1, 2], and are generated by a combination of homogeneous nucleation (in the bulk solution) and heterogeneous nucleation (of new polymers onto others) [3]. Assembly does not occur when hemoglobin is liganded since the presence of ligands causes a small shift in the position of the 4 hemoglobin subunits and precludes the registry of the contacts that are necessary to stabilize the polymer [4]. Polymers can form once ligands are removed and solubility is exceeded, which in physiological buffers means concentrations above 160 mg/ml (~12% volume occupancy). Since the hemoglobin concentration in red cells is typically around 320 mg/ml, and may be significantly higher, it is evident that the assembly process invariably occurs under highly crowding conditions. The effects of crowding on the thermodynamics of polymer formation have been well studied. The polymer formation process, being described by nucleation, is intimately related to thermodynamics. Theoretical relationships, based mainly on scaled particle theory, have been developed and are found to be remarkably accurate in describing the effect of crowding on kinetics. These aspects of crowding on the reaction are the subject of a recent review [5].

In addition, we have quite recently found another unexpected manifestation of crowding in this reaction. Although a solubility characterizes the reaction, and possesses the usual thermodynamic properties of path independence, and independence of initial conditions, the reaction appears to cease before the monomer concentration falls to the solubility unless the solutions are centrifuged or without a very long waiting time (on the order of days) [6, 7]. Without centrifugation [8, 9] or waiting for very long times [10] the final concentration *does* depend on the path, and the initial conditions, and is evidently a metastable state of the system. This finding was only made recently despite the many years of study devoted to this assembly system because of the lack of non-invasive methods to measure the concentration in gelled samples. A newly developed micromethod revealed the existing metastable behavior [6], which was confirmed by a second method, based on modulated photolysis [7]. The proposed metastability was further shown to resolve a long-standing discrepancy between van't Hoff and calorimetric enthalpy [7].

The difference in concentration between solubility and the measured terminal concentration is significant, and we have found that these results can be summarized by the empirical rule that only about 2/3 of the expected mass of polymers is formed [7] (Fig. 1). We have hypothesized that the metastability arises because the ends of polymers, where monomer attachment must take place, encounter other polymers and become obstructed, preventing their growth, as illustrated in Fig. 2. Nor is it possible for new polymers to form. Heterogeneous nucleation, being highly concentration dependent [11, 12], becomes strongly suppressed after a substantial amount of polymer has formed and consumed free monomers, and thus there is no further pathway for adding monomers to polymers, even though addition of monomers is still thermodynamically favorable. Homogeneous nucleation is even more strongly suppressed than heterogeneous nucleation.

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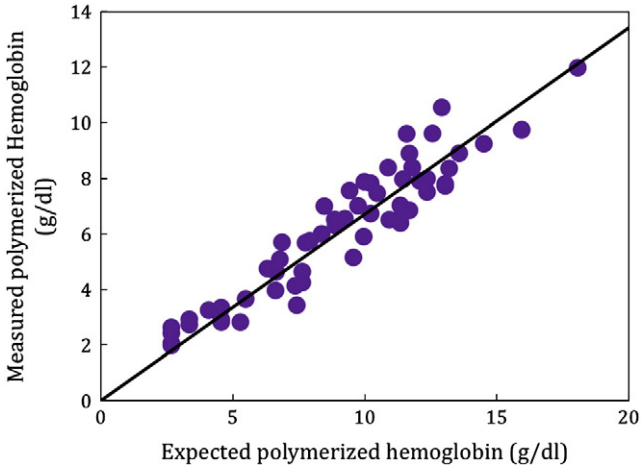

Fig. 1. Comparison of expected and measured concentration in gels. This data is taken from Weng et al. [7] The expected concentration of polymerized hemoglobin is computed as the difference in initial concentration and solubility, while the measured concentration of polymerized hemoglobin is the difference in initial concentration and the terminal concentration. The line is the best fit, constrained to pass through zero, and is 0.67. This illustrates the metastability of gels that are neither aged nor centrifuged.

The basis of this metastability has heretofore had no theoretical framework to unify these concepts. In this paper we modify the standard equations of polymer growth by using a theory developed by Ogston [13] to describe the spaces in collections of infinite length rigid rods. We find that the theory can indeed unify the diverse findings, and provide useful insights.

Theory

Ogston described the volume of a gel available to a spherical solute of radius r. We shall designate this available volume fraction Φ. The fibers that make up the gel are assumed to have a radius a and be long enough that their length is irrelevant. The volume fraction occupied by the hemoglobin fibers is given by ϕ_p. In the theory, the volume fraction taken up by monomers is not included because the intent is to discover the space that can be available. Monomers, being mobile, do not have the equivalent occlusive effect. Then the available volume fraction is given by

$$\Phi = \exp\left[(1 + r/a)^2 \phi_p\right]. \tag{1}$$

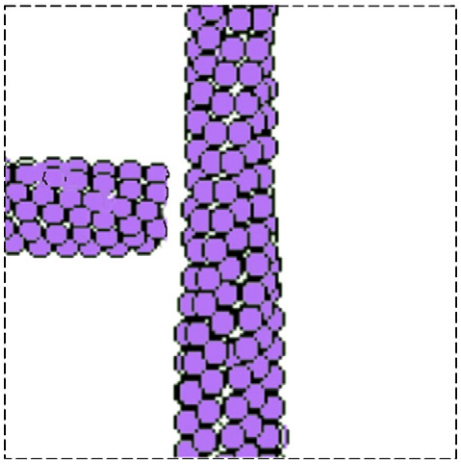

Fig. 2. Representation of a mechanism for metastability. Because polymers grow from their ends, if an end is occluded by the presence of another polymer, as shown in the illustration, its growth is frustrated. If polymers are broken by external force, or disassembled partially by spontaneous length fluctuations, the frustration can be relieved. External force can be provided by centrifugation, while length fluctuations involve long times.

Polymer growth involves a monomer addition rate constant, denoted k_+ and a dissociation rate constant denoted k_-. If the concentration of monomers is given by c, and the monomer activity coefficient is γ where $\gamma = \gamma(c)$, then the growth rate J is given by

$$J = k_+ \gamma c - k_-. \tag{2}$$

Growth is identically zero when the monomer concentration reaches solubility, so $k_- = k_+ \gamma_s c_s$ where c_s is the solubility and $\gamma_s = \gamma(c_s)$. Monomer activity coefficients are conveniently expressed in terms of the monomer volume fraction, ϕ. If the specific volume of monomers is v, then $\phi = vc$. By using the leading term in the Carnahan–Starling expression, it is easily shown that

$$\ln\gamma = 8\phi / (1-\phi)^2 \tag{3}$$

which is virtually indistinguishable from a full power series over the range of data for which almost all HbS polymerization is studied [5].

If we incorporate Ogston theory into the model described by Eq. 2, we modify the elongation rate term in J to indicate that a fiber can grow only where space exists. Thus, we propose

$$J = \Phi\ k_+ \gamma c - k_- = k_+(\Phi\ \gamma c - \gamma_s c_s). \tag{4}$$

The metastable state occurs when J in Eq. 4 is zero, giving an expression

$$\Phi \gamma' c' = \gamma_s c_s \tag{5}$$

where c' is the metastable concentration. The volume fraction of polymer, ϕ_p, is related to the final concentration of monomers c' as well as the initial monomer concentration c_o. If the specific volume of the polymer is v_p which is approximately $1/(69\ \text{g/dl})$, then

$$\phi_p = v_p(c_o - c'). \tag{6}$$

We can combine Eqs. 1, 5, and 6 and solve numerically for r/a, the size of the pores relative to the fiber radius, for given values of c_o and c'. Note that while the mathematical description does not formally require it, the physical basis of this description only makes sense if $r/a > 1$, since penetration of a fiber through the voids in a gel would require a hole radius r at least the size of the polymer radius a.

Results

The above analysis has been carried out for the data of Weng et al. [7]. In this analysis we used $2/3(c_o - c_s)$ rather than $c_o - c'$ as the polymerized Hb concentration for comparison with the theory (as shown in Fig. 1). This has the effect of averaging the data. When the Ogston model is applied, we find that at 15 °C $r = 1.5a$ while at 25 °C and 35 °C, $r = 1.4a$. These values give an excellent representation of the data at various initial concentrations and temperatures and accompanying metastable concentrations c' as shown in the correlation plot in Fig. 3. Blue diamonds designate data for 15 °C, green circles are for 25 °C and red triangles are for 35 °C. The Φ values deduced here span the range from 0.4 to 0.8.

In this application of the Ogston model, the radius of the holes for the polymer growth is 40–50% larger in diameter than the fiber diameter itself. The propagation of a fiber through a gel entails a slightly different geometry than specified by the Ogston model, which is designed to consider spheres amid rods. For example, the smallest

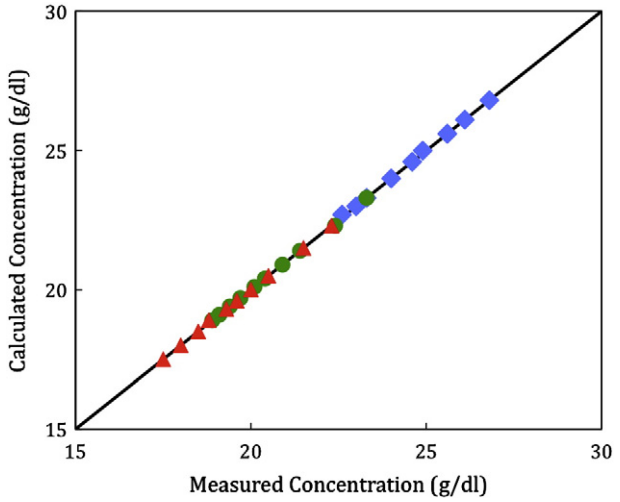

Fig. 3. Correlation of measured and calculated terminal concentration. Measured concentrations were determined using the relation shown in Fig. 1, i.e. 0.67 times the difference in initial concentration and solubility. Calculated concentrations were determined by using Ogston theory adapted to the growth model presented here, and fitting the radius of the pore size required. A single pore size r is used for each temperature: blue diamonds designate data for 15 °C, green circles are for 25 °C and red triangles are for 35 °C. If a is the radius of a single Hb fiber, for 15 °C, $r = 1.5a$, while for 25 °C and 35 °C, $r = 1.4a$. The line is that of perfect correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cylinder that would enclose a sphere of radius a will have a volume that is 1.5 times the volume of the sphere. Moreover, the fibers have an elliptical cross section that varies from 20 to 25 nm going from minor to major axis. Thus, the maximum radius (25 nm) is 10% greater than the geometric mean (22 nm). Therefore, the fact that the required diameter is greater than that of the fiber is not unexpected, although a precise calculation is beyond the scope of the simple model of Ogston used here.

Discussion

The formulation developed provides an excellent quantitative description of the effect of fiber crowding in generating a metastable gel, which is especially remarkably given that the Ogston model does not explicitly include the long range correlations that permit polymer growth. The description we have developed also provides a simple explanation for the observed path-dependence, since ϕ_p and thus Φ depends on the conditions under which the gel was formed. Thus, if conditions (e.g. temperature) change once a gel already arrives at metastability, then the appropriate Φ is not that of the new temperature but rather that at which the gel was formed. Eq. 5 shows clearly that a different terminal concentration is obtained, depending on conditions of gel formation. If alignment were to occur, the fiber diameters (a) would increase, and the terminal concentration would decrease towards the solubility since Φ approaches unity. In such an alignment process, the assumption that the gel is a collection of random rods must eventually break down so it is unclear how far such an analysis may be extended.

Φ is effectively an activity coefficient, and the product $\Phi\gamma$ can be considered as the net activity coefficient accounting for monomers as well as the fiber network in the association process. It is interesting to note that $\Phi < 1$, while excluded volume activity coefficients are always >1. Expressing the total activity coefficient as a product is the equivalent of viewing the crowding energies for monomers and for fibers as additive terms. It is possible that a further reason the $r > a$ in the above analysis has to do with this assumption of additivity.

Conclusions

The approach adopted here provides an excellent description of the effect of fiber crowding in generating a metastable gel and appears to be easily adapted to any system in which a dense mass of fibers forms and thus may thwart further growth. In the future it should be possible to adapt this theory to account for the flexibility of the fibers, since the ability of fibers to thread their way through a network will not only depend on the porosity of the network but also their own rigidity.

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