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ARTICLE

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Shear-induced alignment of self-associated hemoglobin in human erythrocytes: small angle neutron scattering studies

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Abstract Small angle neutron scattering (SANS) was performed on suspensions of actively metabolising human erythrocytes in the constant shear field induced by a Couette cell. The SANS pattern recorded on a two-dimensional detector was a function of the shear rate; at zero shear, the SANS pattern had radial symmetry around the direction of the beam. The radial average of the SANS pattern consisted of a broad intensity maximum superimposed on a decay. The intensity maximum at $q = 0.1$ Å^{-1} was attributed to isotropically oriented self-associated complexes of the tetrameric oxygen transport protein hemoglobin inside the erythrocytes. A flow curve of the cell suspension was used to identify at what shear rate a suspension of uniaxially oriented ellipsoidal cells is produced. The radial symmetry of the SANS patterns persisted until the shear rate was sufficient to produce a suspension of uniaxially oriented ellipsoidal cells. Again, an intensity maximum was present in directions parallel and orthogonal to the shear axis, but this intensity maximum was superimposed upon quite different intensity decays in each direction from that of the primary neutron beam. The angular range of the SANS instrument was limited, however the results from shear-induced structural changes is consistent with a model that involves hemoglobin complexes that are aligned with respect to the plasma membranes of the elongated cells.

Keywords SANS · Shear · Erythrocytes · Hemoglobin · Hydrodynamic alignment

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Introduction

The highly penetrating and non-ionising nature of neutrons make them an ideal probe for investigating the physical chemistry of the cytoplasm of cells while allowing the maintenance of their integrity and active metabolism. Here, we report experiments on the two-component system of human erythrocyte suspensions in saline solution placed under shear. Erythrocytes are highly deformable and are known to form uniaxially aligned ellipsoids under shear (Schmid-Schönbein and Wells 1969; Bessis et al. 1980; Mazeron et al. 1997a, 1997b). Scattering methods are very sensitive to orientational effects in a sample (Wagner 1998; Penfold 1988), and in this work, we examined the neutron scattering patterns produced by suspensions of erythrocytes, in structural equilibrium, with increasing shear rates. The results illustrated the use of neutron scattering from biological materials in shear to study structures where the hydrodynamic interaction (Salem and Fuller 1985) produces a preferential alignment. These results also provided a unique insight into the distribution of hemoglobin within the erythrocyte. This insight has implications for the metabolic regulation of cell volume.

Human erythrocytes are biconcave disks that transport oxygen around the body. All such mammalian cells are highly differentiated and specialised, since they are not nucleated, have no organelles, and consist largely of the protein hemoglobin confined within the cytoplasm. For a blood concentration of hemoglobin of ~150 g/dm^{-3}, the concentration inside the erythrocyte is ~350 g/dm^{-3}. At this concentration, the volume fraction of protein inside the cell is ~30% (Minton 1980).

It has been suggested, on the basis of data from small angle X-ray scattering, that tetrameric hemoglobin (Hb_4) inside erythrocytes exists in an aggregated condensed state in dynamic equilibrium with a less condensed state (Bateman et al. 1953). The position of the equilibrium between aggregated and free Hb_4 may depend on the cell volume (Bateman et al. 1953). An

alternative model used to explain the small angle neutron scattering from whole erythrocytes was proposed by Krueger and Nossal (1988). This model is of a concentrated solution of interacting particles (protein molecules), where the scattered intensity has been accounted for by considering the intracellular protein solution and the surrounding lipid membrane. Thus, the reductionist model of the erythrocyte of these workers is a concentrated solution of hemoglobin surrounded by the lipid bilayer where other cell components, while certainly important in a biochemical sense, are not present in sufficient volume fraction to make a resolvable contribution to the scattering pattern. In a later work which modelled the SANS of concentrated Hb_4 solutions, limited self-aggregation of interacting Hb_4 units was included (Krueger et al. 1990).

Certainly, the cell interior is a very concentrated environment and the ability of hemoglobin to obstruct its own free diffusive motion and hence crowd the cytoplasmic environment is evident from pulsed-field gradient NMR measurements of diffusion of hemoglobin in whole erythrocytes (Kuchel and Chapman 1991). The molecular crowding of Hb_4 in the cell has been proposed as a mechanism by which the erythrocyte "senses" its volume (Zimmerman and Minton 1993). In this proposed mechanism, it is the concentration of hemoglobin at the cell membrane that is important since the pumps that actively regulate cell volume are membrane bound and crowding could obstruct the diffusion of the substrate, ATP, to the pumps. In this context, the observation of the enhanced surface accumulation of Hb_4 at an air/water interface close to the isoelectric point of the protein (Maruyama et al. 2000) is relevant since this is close to the intracellular pH (Stewart et al. 1986). It would seem that if hemoglobin is relatively surface active and the cell membrane of the erythrocyte is oriented in shear, then there would be orientation of the hemoglobin aggregates.

Materials and methods

Erythrocytes

Fresh whole blood was collected from a healthy volunteer (C.J.G) by cubital fossa venipuncture. The blood was first washed in isotonic saline (154 mM NaCl) and glucose (10 mM) in H_2O by centrifugation (2,000 *g*, 5 min). The supernatant, a yellow clear solution, and the interfacial region, "buffy" coat (platelets and white cells) were removed by aspiration. The resulting cell suspension was then washed twice more in the same manner. After resuspending in additional medium, the cells were then bubbled with CO to convert the hemoglobin iron into the low-spin diamagnetic state and centrifuged again. This treatment improves the stability of Hb_4 and avoids the changing SANS pattern associated with oxygenation of the protein (Krueger and Nossal 1988). The pellet of intact erythrocytes was resuspended in isotonic saline made with D_2O and washed three times in this solution. The volume of erythrocytes in the solution was then determined with a capillary centrifuge (Clements, NSW Australia) to be 80(±3)%. For rheology experiments the steps in which the cells were washed in D_2O saline were omitted.

SANS

SANS patterns were collected on the instrument at Lucas Heights (ANSTO, NSW, Australia) from a quartz Couette-geometry shear cell. The details of this geometry and the relationship of the neutron beam to the shear cell are shown in Fig. 1. The operational details of the SANS instrument and the shear cell are given on the webpage: http://www.ansto.gov.au/ansto/bragg/hifar/aus.html

The representation of small angle scattering data is usually based on the assumption that scattering is an elastic process, i.e., momentum is conserved thoughout interactions (Guinier and Fournet 1953). In this way the magnitude of the momentum transfer vector, **q**, can be related to the angle of scattering, θ, and the wavelength of the neutrons, λ:

$$q = \frac{4\pi}{\lambda}\sin\left(\frac{\theta}{2}\right) \tag{1}$$

The q range of a SANS instrument is determined primarily by the length of the instrument, the collima-

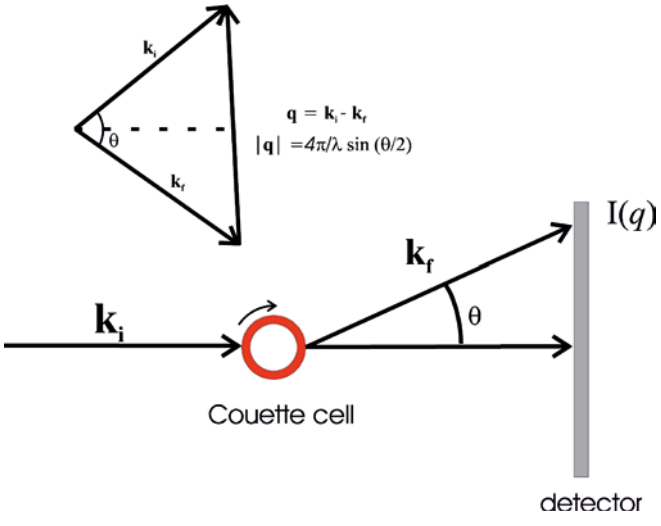

Fig. 1 Schematic diagram of the small angle scattering experiment using a Couette-geometry shear cell. The Couette cell consists of two cylinders, the external one being fixed with respect to the interior which rotates at a specified rate (units, s^{-1}). The representation of the small angle scattering experiment is in terms of the variation of the scattering intensity with the scattering vector, ***q***. As the process is assumed to be elastic, ***q*** which is the sum of the incident vector, $\boldsymbol{k}_i$, and a final vector $\boldsymbol{k}_f$ is related to the angle between the direction of the incident radiation and the scattered radiation, θ (see Eq. 1)

tion of the beam, the neutron wavelength, and the area of the detector. For the ANSTO SANS instrument, the q range is $0.01 < q < 0.14$ Å^{-1}. When an intensity maximum arises from constructive interference due to a repeat structure within the sample, the Bragg equation gives a simple relationship between the repeat distance, d, λ and θ (Guinier and Fournet 1953):

$$n\lambda = 2.d \sin\left(\frac{\theta}{2}\right) \quad (2)$$

Thus from Eqs. (1) and (2), the simple relationship between the position of a maximum, q_{max}, and the repeat distance is:

$$d = \frac{2\pi}{q_{max}} \quad (3)$$

This equation also indicates the length-scales of the structures that are interrogated by the SANS instrument (500 to 40 Å).

Rheology

Rheological characterisations of the erythrocyte suspensions were performed at 309 K, using a HAAKE CS-150 controlled stress rheometer (Haake Mass-Technik 2000, Karlsruhe, Germany). The cell suspension described above at a hematocrit of 80(±3)% was transferred to a thermostated double-cone closed Sensor System DC60/2. The sensor is designed for low viscosity fluids and for fluids that tend to react with air. The steady-state flow curve was measured in the range 0–20 Pa. The flow curve was measured with increasing stress, "up-curve", and with decreasing stress, "down-curve", to investigate thixotropy and viscoelasticity of the sample.

Results and discussion

SANS with shear

The evolution of the SANS patterns as the shear rate was increased from 0 to 3,000 s^{-1}, is shown in Fig. 2. At the shear rate of 1,000 s^{-1} there was a slight change in the SANS pattern. This change became more pronounced as the shear rate was increased to 3,000 s^{-1}. A sample of the cell suspension was then removed in order to examine if shearing had disrupted the integrity of an appreciable number of the erythrocytes. This was done through determination of the hematocrit of the cell suspension. The supernatant was clear, and there was no significant red colour of hemoglobin. The hematocrit and zero shear SANS pattern after the maximum shear rate were identical. In other words, there was no significant hemolysis during the increments of shear, and the change in the SANS pattern was therefore due to a reversible process brought about by shearing the cells.

q-Space

The radially symmetrical pattern at zero shear was typical of that found, or assumed by other workers, in the small angle scattering region from intact normal human erythrocytes (Bateman et al. 1953; Guinier and Fournet 1955; Damaschun et al. 1975; Krueger and Nossal 1988). Radial averaging of the pattern at a zero shear rate and calculation of the value of q, according to Eq. (1) and geometry shown in Fig 1, produce the intensity profile shown in Fig. 3. This profile is expressed in the form of absolute intensity, I(q) (units, cm^{-1}), as a function of q (Å^{-1}). In common with other results (Bateman et al. 1953; Guinier and Fournet 1955;

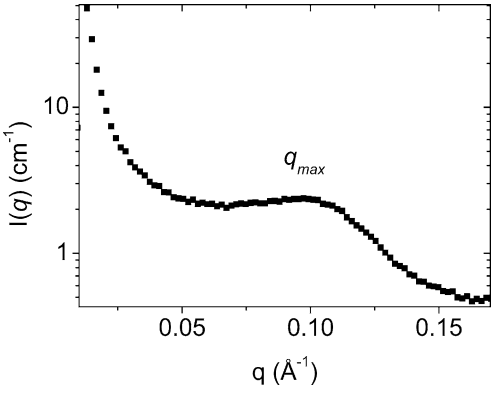

Fig. 3 Radial average of a SANS pattern from the erythrocytes in zero shear in Fig. 2A with the position of the intensity maximum, q_{max}, labelled; it has the value 0.1 Å^{-1}

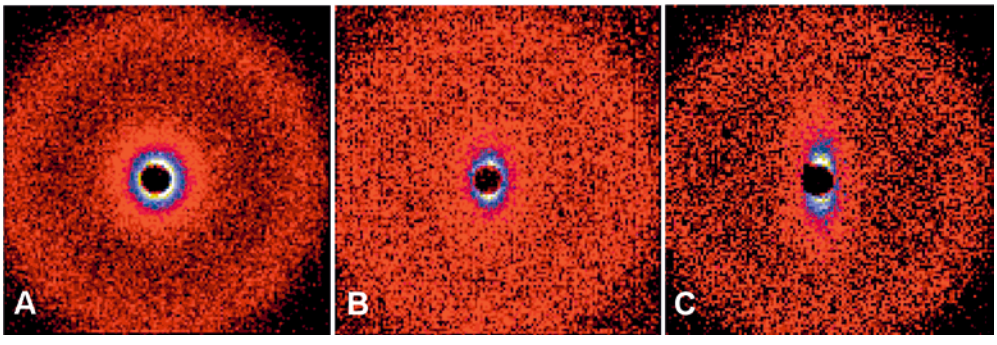

Fig. 2 SANS patterns from a suspension of erythrocytes at 80% haematocrit in 154 mM NaCl/D_2O at shear rates of: **A** 0 s^{-1}, **B** 1,000 s^{-1}, and **C** 3,000 s^{-1}. The axis of rotation of the Couette cell is parallel to the long side of the page

Damaschun et al. 1975; Krueger and Nossal 1988) for whole erythrocytes in isotonic saline, there was an intensity maximum at a q value of 0.1 $Å^{-1}$. Within the interpretation of Bateman et al. (1953), a repeat distance can be calculated by using Eq. (3); it corresponds to a distance of 63 Å. Krueger and Nossal (1988) call this feature in the SANS pattern an "interaction" peak, though as noted previously these workers later acknowledge the self-association of Hb_4 in concentrated solution (Krueger et al. 1990). Such maxima can be observed in solutions of monodisperse spheres of varying volume fraction, where they may be due to (1) form-factor scattering, a solution structure factor (Hayter and Penfold 1981); or (2) where there is equilibrium between a condensed and a diffuse phase of monodisperse structural units (Ise et al. 2001). In the q-range considered by the SANS instrument used here, only the latter possibility, self-associated Hb_4, would produce a scattering anisotropy in shear provided it adopted a preferential orientation with respect to the cell membrane.

Inferred hydrodynamic radius

The interparticle distance 63 Å is the hydrodynamic diameter of a protein core surrounded by a layer of immobile water, and it agrees with the hydrodynamic diameter of hemoglobin calculated from the diffusion co-efficient in dilute solution (Jones et al. 1975) and the limiting small angle neutron (Schelten et al. 1975) and X-ray (Guinier and Fournet 1955) scattering from dilute solutions. It has been suggested from previous small angle scattering studies (Bateman et al. 1953; Guinier and Fournet 1955) and pulsed-field gradient NMR spectroscopy (Garvey et al., unpublished results), of the thermal motion of Hb_4 inside the cell, that Hb_4 exists in an aggregated state in concentrated solutions such as exists within the erythrocyte. In this model, the radial symmetry of the SANS pattern in the stationary cells is indicative of a completely isotropic spatial orientation of Hb_4 and its aggregates, that is in effect a powder pattern (Warren 1992).

Rouleaux formation under shear

The response of a suspension of erythrocytes to an applied shear stress has been used to infer structural information on larger length scales that those considered by the SANS experiment, i.e., the dimensions of the erythrocyte (μm). When a suspension of erythrocytes is sheared incrementally, it will first, at low shear rates, form larger cellular aggregates with no preferred spatial orientation of the cells. This has been observed optically (De Roek and Mackely 1998) and inferred indirectly from the thixotropic behavior of cell suspensions (De Roek and Mackely 1998; Kang 2002). At higher shear rates, these aggregates are no longer present and the cells become aligned as prolate ellipsoids with the shear field. The distortion of erythrocytes as ellipsoids with their longest axis aligned with the direction of shear has been studied and well characterised by light scattering methods (Bessis et al. 1980; Mazeron et al. 1997a, 1997b). The shear rate which results in the formation of a suspension of prolate ellipsoids corresponds to the anisotropic SANS pattern shown in Fig. 2C.

Thixotropic response of red blood cell suspensions

A representative shear/flow curve is shown in Fig. 4. At the lower shear rates, up to 425 s^{-1}, the sample manifests the shear thinning effect of a pseudoplastic fluid as the erythrocytes form rouleaux aggregates (Kang 2002). In very low shear rates, often called the "zero shear viscosity", Brownian motion, and possibly the endogenous motion of the cytoskeleton, keeps all the rouleaux aggregates at random orientation in spite of the initial effects of shear orientation. With increasing shear rates, the rouleaux suspended in the isotonic saline will be turned lengthwise in the direction of flow (Kang 2002). In the range of shear rate from 425–1,700 s^{-1}, we have observed the first Newtonian range, where the viscosity remains constant with a value of 8.5 mPas, independent of the applied shear rate. This behaviour can be explained by the rouleaux aggregates being completely aligned in the direction of flow (Kang 2002). With further increase in the shear rate from 1,700 up to 2,000 s^{-1}, a second shear thinning region was revealed. This is related to reshaping the long aggregates/rouleaux with reduced diameters of the elastically deformable erythrocytes. Shear can also induce irregular break up of the aggregates to flow faster, becoming less viscous at a given shear rate.

Shear rates higher than 2,000 s^{-1} resulted in a second Newtonian region in which the viscosity was constant, and independent of further increases in shear rate. We

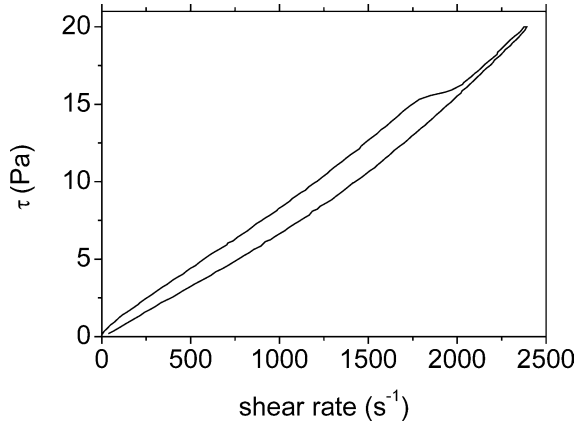

Fig. 4 Shear stress, τ, of an 80% haematocrit suspension of erythrocytes in 154 mM $NaCl/H_2O$ as a function of shear rate. The *top curve* was recorded when the shear rate was increased (up curve) and the *lower curve* was obtained as the shear rate was decreased (down curve)

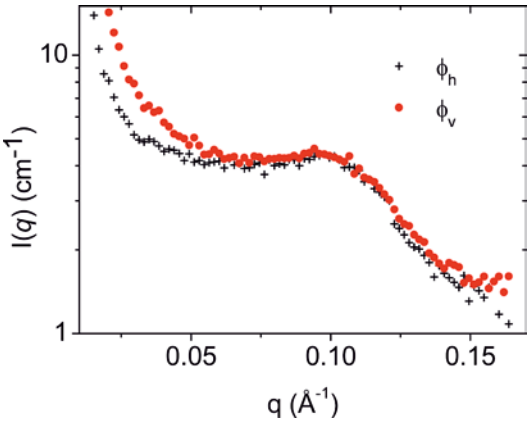

Fig. 5 The radially averaged SANS intensity from erythrocytes at 80% haematocrit in 154 mM NaCl/D_2O at a shear rate of 3,000 s^{-1} (Fig. 3**C**) using 90° sectors in the same direction as the axis of rotation, ϕ_v, and at right angles to the axis of rotation, ϕ_h

deduce that the alignment of the erythrocytes was achieved. These rheological investigations were consistent with the observation that the SANS patterns changed with the applied shear rates (Fig. 2).

The flow curve in Fig. 4 illustrates the rheological phenomenon of thixotropy (De Roek and Mackely 1998; Kang 2002). It is typical of many dispersions that not only show potential for orientation but additionally for time-related particle/molecule-interaction. This leads to bonds creating a three-dimensional network structure. When the network is disrupted, the viscosity drops with the duration of shear until it asymptotically reaches the lowest possible value. A thixotropic fluid is defined by its potential to reform the pre-stress structure. The hysteresis between the "up-curve" and "down-curve" surrounds an area that defines the magnitude of the thixotropy of the sample, (27,443 Pas^{-1} in the present case).

Evidence of anisotropy

In the SANS experiments, as the shear rate was increased, the radially symmetrical pattern assumed an anisotropic structure at low q. Radial averages were taken from 90° sectors parallel and orthogonal to the axis of rotation of the shear cell (Fig. 2C). The I(q) behaviour is shown in Fig. 5. Evident in the direction parallel and orthogonal to the rotation axis is an intensity maximum in the same position as the zero shear radial average i.e., $q = 0.1$ $Å^{-1}$. It appears from these plots that the small angle diffraction peak was superimposed upon quite different decays for the directions parallel and orthogonal to the shear cell's rotation axis. This result is typical of anisotropically shaped objects oriented in shear (Penfold 1988). It is not possible to draw any quantitative conclusions from these data sets because of the limited q-range of the SANS instrument (http://www.ansto.gov.au/ansto/bragg/hifar/aus.html). It is possible, however, to consider the length scales of the various structures within the erythrocyte that are aligned with the shear field.

Membrane-protein interactions

Figure 5 suggests that the structures that are contributing greatest to the anisotropic scattering pattern are structures on length scales $q < 0.05$ $Å^{-1}$, or of the order 100 Å and that there is no preferential alignment of structures of the order tens of Å. SANS is a technique sensitive to the volume fraction of scattering particles. Contrast variation experiments were carried out on cell suspensions by Krueger and Nossal (1988), and they concluded that the major contributors to scattering are the protein of the hemoglobin and the lipid of the cell membranes. Within the context of the q-range of the SANS instrument used here, it is the width of the cell membrane (~50 Å, Deuticke 2003) and the hemoglobin molecule which should contribute greatest to the scattering in the available q-range. The cell membrane structure will be able to cause scattering anisotropy if there is sufficient scattered intensity, whereas the protein structure is globular and will not exhibit any anisotropy without forming larger structures from the unitary Hb_4. A radially symmetric pattern, and thus no scattering anisotropy, was observed on the length-scale of tens of Å. Contrary to the results of Krueger and Nossal (1988), this suggests that contributions to the scattered intensity by the lipid membrane are minimal since, although the cell membranes would be oriented in the shear field, no scattering anisotropy was observed over this length scale. In the length scale corresponding to the lateral dimension of the cell membrane, there was no resolvable change in the scattering in the direction of the rotation or at right angles to the rotation. We suggest therefore that the oriented structures are of dimensions intermediate to the length of the oriented cell and the diameter of a Hb_4 molecule, or the lateral dimension of the cell membrane.

Conclusions

Hb_4-membrane interaction

We conclude that there is a preferred orientation of a structure with respect to the axis of prolate ellipsoids that are formed by human erythrocytes under shear. We suggest that this structure is self-associated Hb_4 molecules interacting with the cell membrane since there is no scattering anisotropy in the length scales which correspond to the other oriented structure, the cell membrane. It is known that hemoglobin will have an enhanced interfacial concentration (surface active) close to its isoelectric point and the charge on the protein has a strong influence on this surface activity (Maruyama

et al. 2000). Although the actual chemical activity of H^+ is experimentally difficult to define inside the erythrocyte, the value of pH measured by ^{31}P NMR methods gives an effective pH value of 7.2 (Stewart et al. 1986). This value lies close to the isoelectric point of hemoglobin, 6.9 (Edsall and Wyman 1958), thus the charge on Hb_4 and repulsive electrostatic interactions between Hb_4 units are likely to be small. Thus alignment is likely to be the result of the surface activity of hemoglobin on the interior of the cell membrane and the average alignment of the erythrocytes in the shear field. That there is probably a pronounced enhancement of the concentration of hemoglobin at the internal surface of the cell membrane would have a number of consequences with respect to the gas-exchange functions of the cell.

Mechanistic postulate

The erythrocyte membrane contains a variety of metabolically active ion pumps and channels that regulate the cell volume (Mongin and Orlov 2001; Maher and Kuchel 2003). It has been speculated that crowding effects of hemoglobin may be an important mechanism by which the metabolic activity of these pumps and channels is regulated (Zimmerman and Minton 1993). The experimental observations reported here suggest that crowding effects close to the interior of the cell membrane may be more pronounced than would be expected from the excluded volume due to the bulk concentration of hemoglobin inside the cell (Han and Herzfield 1993).

Summary

In summary, enhancement of the concentration of hemoglobin at the cell membrane implies important consequences for the primary function of the cell, oxygen transport. When oxygen is transported, the hemoglobin closest to an important exchange surface would act co-operatively and enhance the rate of the exchange process and the average distance that free oxygen would need to diffuse to the cytoplasmic solution would be reduced.

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