Small-angle X-ray scattering probe of intermolecular interaction in red blood cells *

LIU Guan-Fen(刘广峰) WANG We-Jia(王文佳) XU Jia-Hua(徐建华) DONG Yu-Hui(董宇辉)[1)]

Beijing Synchrotron Radiation Facility, Institute of High Energy Physics,
Chinese Academy of Sciences, Beijing 100049, China

Abstract: With high concentrations of hemoglobin (Hb) in red blood cells, self-interactions among these molecules could increase the propensities of their polymerization and aggregation. In the present work, high concentration Hb in solution and red blood cells were analyzed by small-angle X-ray scattering. Calculation of the effective structure factor indicates that the interaction of Hb molecules is the same when they are crowded together in both the cell and physiological saline. The Hb molecules stay individual without the formation of aggregates and clusters in cells.

Key words: small-angle X-ray scattering, protein-protein interaction, hemoglobin

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1 Introduction

Biological macromolecules evolve and function within intracellular environments that are crowded with other macromolecules. Ellis mentioned that it is crucial to study the protein-protein interaction at biologically relevant concentrations [1, 2].

Small-angle scattering of X-rays (SAXS) and neutrons (SANS) are effective methods to study the structure and interactions of macromolecules in solution [3]. In recent years, the SAXS of colloidal systems and protein solutions have been actively studied both theoretically and experimentally [4–13]. These works offer protein-protein interaction information under many conditions, such as in different salt concentration, pH and temperatures [14]. It is necessary to study the protein-protein interaction inside a real cell, e.g., under in vivo conditions. In the present work, we measured the hemoglobin (Hb) solution and red blood cell solution in different concentrations by SAXS in order to study intracellular interaction of the Hb.

2 Materials and methods

2.1 Sample preparation

The samples we used were extracted from venous blood of normal adults. The red blood cells were separated by centrifugation. A 3-fold volume of physiological saline (0.9% NaCl, pH 7.3) was added to the blood sample, then it was centrifuged at 3000 rpm for 5 min at 277 K and the supernatant was removed. This step was repeated three times. After centrifugation, the samples were divided into two parts. We stored one part as the cell sample in 277 K; the other part of the red blood cells was crushed using an ultrasonic cell disruptor, then the solution was centrifuged at 12000 rpm for 30 min at 277 K. The supernatant was collected and filtered through a 0.22 μm membrane filter. The Hb solution was concentrated to 120 mg/mL. Cell and Hb samples were diluted into different concentrations. The final protein concentrations used for experiments were determined by near-UV absorption spectroscopy at 280 nm of the tryptophan and tyrosine residues using a Nano-Drop (PUEX UV Spectrophotometer). Samples with different concentrations of Hb (1.5, 2.5, 5.0, 10.0, 25.0, 120.0 mg/mL) and red blood cells (30.0, 120.0, 240.0 mg/mL) were prepared. It should be noted that the concentration of proteins inside a cell is constant; therefore, the concentration of a cell can be expressed as the one of hb since the method we used can only provide the concentration of protein.

For the second experiment, we concentrated the Hb into 300 mg/mL, and the cell concentration was 200 mg/mL. The sample preparation was the same as described above.

2.2 Data collection

Just prior to SAXS data, 2 mM dithiothreitol was

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1) E-mail: dongyh@ihep.ac.cn

added into the sample collection to avoid radiation damage [15]. SAXS measurements were performed at the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY, Hamburg, Germany) on the X33 beamline of the European Molecular Biology Laboratory (EMBL) with a PILATUS detector [16] (Dectris, Baden, Switzerland). The scattering signal was recorded in the range of the scattering vector $0.02<q<0.55$ Å^{-1} ($q=(4\pi\sin\theta)/\lambda$, where 2θ is the scattering angle, and λ=1.5 Å is the X-ray wavelength). The exposure time of one frame is 15 seconds. Eight successive frames were collected for one sample in order to monitor the possible radiation damage. During the experiments, no radiation damage was observed.

This second measurement was performed at station 1W2A in the Beijing Synchrotron Radiation Facility (BSRF) using a Mar CCD detector [17]. The scattering was recorded in the range of the scattering vector $0.02<q<0.247$ Å^{-1}. All measurements were carried out in a cuvette with mica sheet windows. The exposure time of one frame was 100 s. No radiation damage and sample aggregation were observed during the measurements.

2.3 SAXS data processing

The SAXS data were processed using the ATSAS program package [18]. The data were normalized by the intensity of the transmitted beam, averaged and the solvent scattering was subtracted using PRIMUS [19]. The corrected curves were then extrapolated to infinitive dilution using standard procedures.

The scattering intensity from an interacting suspension of globular particles measured as a function of scattering vector q,

$$I(q)=N\Delta\rho V^2\langle P(q)\rangle S_{\rm M}(q). \quad (1)$$

Here, N is the number density of particles; $\Delta\rho$ and V are their average scattering contrast and volume, respectively. The form factor $\langle P(q)\rangle$, which is the averaged particle scattering over the ensemble of sizes and orientations, is related to the profile of the particle. The effective structure factor, $S_{\rm M}(q)$, provides information about the inter-particle interactions in the solution. For a very dilute solution of non-interacting particles $S_{\rm M}(q)\approx1$.

For globular proteins, the effective structure factor can be obtained from the ratio of the experimental intensity $I_{\rm exp}$ at a concentration c to that obtained by extrapolation to infinite dilution or measured at a sufficiently low concentration c_0 where all correlations between particles have vanished:

$$S_{\rm M}(q,c)=c_0I_{\rm exp}(q,c)/cI(q,c_0). \quad (2)$$

$S_{\rm M}(q)$ has a peak at small angles, and the position of the peak $q_{\rm p}$ is related to the average distance (d) between the neighboring particles: $d\approx2\pi/q_{\rm p}$ [4].

The Hayter-Penfold method in SANS Analysis [20], which calculated the structure factor for a system of charged, spheroidal objects in a dielectric medium [21, 22], was used to fit the effective structure factor.

3 Results

3.1 The scattering curve of red blood cells

The cells and purified Hb were measured under physiological conditions (pH=7.3), which had the same pH value and ion concentration as in red blood cells. SAXS data for Hb at concentrations up to 120 mg/mL are shown in Fig. 1(a). The concentrations for the samples were 2.5, 5.0, 10.0, 25.0, 120.0 mg/mL. The values of low q parts of the scattering profiles decrease with increasing concentration, showing that the interference effect is more severe with the increasing concentration.

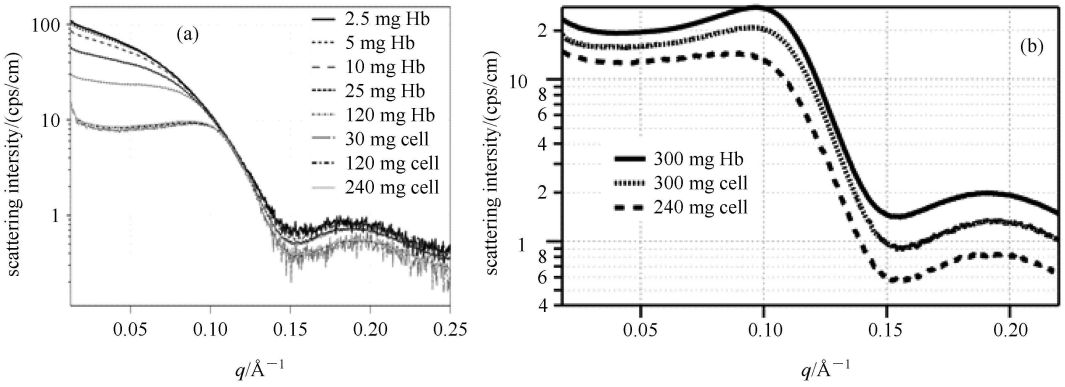

Fig. 1. Results of SAXS experiments of Hb and cells in physiological saline. (a) Scattering curves for different concentrations of Hb and different concentrations of cells collected on the X33 beamline of the EMBL at DORISIII, DESY; (b) Comparison of the scattering curves of high concentration Hb, different concentrations of cells. The curve labeled "240 mg cell" is the scattering of cells from the X33 beamline as in panel A, the curve labeled "300 mg cell" and the one labeled "300 mg Hb" were measured in 1W2A beamline of BSRF.

The differences between the scattering curves of red blood cells of different concentrations can be seen in Fig. 1(a). The concentrations of red blood cells vary from 240 mg/mL to 30 mg/mL, but the curves of three samples overlap exactly. That means the curve reflects the scattering behavior of red blood cells regardless of the concentration of samples.

As shown in Fig. 1(b), the scattering curve of the high concentration Hb (≈300 mg/mL) is very similar to the scattering curves of red blood cells. The concentration of Hb in red blood cells is also about 300 mg/mL. This result means that the scattering behavior of red blood cells is the same as the equivalent concentration of the Hb solution.

3.2 The effective structure factor

We calculated the effective structure factors using Eq (2). From Fig. 2, increasing the Hb concentration in solutions leads to a shift of the first peaks of the effective structure factor, leading to a decrease of the average distance between neighboring Hb molecules. The peak for 120 mg/mL Hb is at 1.07 nm^{-1}, corresponding to a mean nearest-neighbor distance of 5.87 nm. For the cell, the peak is at 1.13 nm^{-1}, and the mean nearest-neighbor distance is 5.56 nm. According to the high resolution structure of Hb (PDB Reference: 2DN1) [23], Hb is an ellipsoid with a radii of r_a=3.2 nm, r_b=2.7 nm and r_c=2.5 nm. In terms of the ellipsoid parameters, the effective hard-sphere diameter $R=2(r_a r_b r_c)^{1/3}$=5.57 nm for mean spherical approximation of Hb. That means Hb inside the cell shows dense packing.

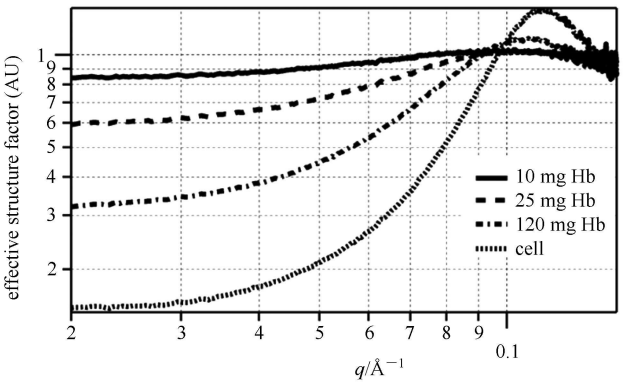

Fig. 2. The effective structure factors of different concentrations of Hb(10 mg/mL, 25 mg/mL and 120 mg/mL) and the cell.

3.3 The intracellular interaction

At a low protein concentration with the screening effect of adding salt, the solutions approach ideal behavior. The scattering intensity of 1.5 mg/mL Hb solution was successfully fitted using an oblate ellipsoid with radii of 3.2 nm, 2.7 nm, and 2.5 nm.

Maintaining the low ionic strength but with a high protein concentration, the screened Coulomb repulsive interaction dominates the overall interaction between protein molecules. In the Hayter-Penfold method, a screened Coulomb potential [20] was used to fit the experiment curves. The diameter of the particle (Hb molecule), salt concentration, surface charge, and volume fraction are fitting parameters, while other parameters in the screened Coulomb potential model, such as temperature and dielectric constant of solvent keep constants. The fit parameters for cells (Fig. 3(a)) and 120 mg/mL Hb (Fig. 3(b)) are summarized in Table 1. Except the volume fraction, which ranges from 0.15 for 120 mg/mL Hb to 0.27 for the cell sample, other parameters are the same. This means that the interaction potentials for Hb in solution and cell are the same.

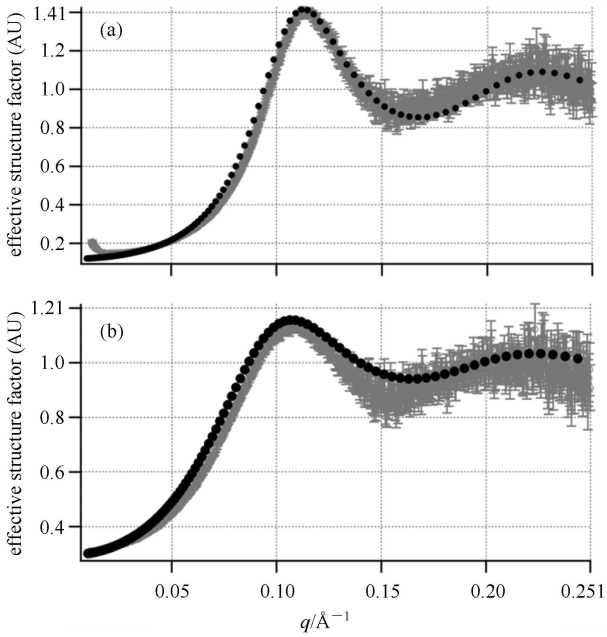

Fig. 3. Fit of the effective structure factors using the Hayter-Penfold MSA model in SANS Analysis: (a) the cells, (b) 120 mg/mL Hb.

Table 1. Fitting parameters of the Hayter-Penfold mean spherical approximation model for Hb in solution and cell, calculated by SANS Analysis [20].

parameters	120 mg/ml Hb	cell
diameter/Å	55.4	55.4
surface charge z	5	5
volume fraction	0.15	0.27
temperature/K	298	298
monovalent salt conc. (M)	0.15	0.15
dielectric constant of solvent	78	78

4 Discussion and conclusion

Human red blood cells are nucleoid-free cells in their mature form. They also lose all other cellular organelles such as mitochondria, Golgi apparatus and endoplasmic reticulum to carry Hb. Hb molecules account for more than 95 percent of cell's dry weight [24]. Our results have proved that the scattering signal of the cells we measured is the signal of high concentrations of Hb.

In the work of Ross and Minton [25], the structure of Hb in solutions can be exlained as the quasi-spherical model, according to the concentration dependence of osmotic pressure and the sedimentation equilibrium of concentrated Hb solutions. Our work shows that, at high protein concentration, the screened Coulomb repulsive interaction dominates the overall interaction among protein molecules. The interference effect between Hb molecules cannot be ignored when protein concentration is high, as well as in the cells.

In conclusion, the intracellular interactions of human Hb were analysed using small-angle X-ray scattering. Calculation of the effective structure factor indicates that the interaction of Hb is the same when they are crowded together in both the cell and physiological saline. The Hb molecules stay individual without the formation of aggregates and clusters in cells.

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