# Supporting Information

Change of hemoglobin isoelectric point at the air/water interface probed by the orientational flip-flop of water molecules

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- A. Im  $\chi^{(2)}$  spectra of phosphate buffer solutions
- B. Charged groups, methyl groups and aromatic rings in HbA
- C. Control experiment of protein damage under laser irradiation
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### A. Im $\chi^{(2)}$ spectra of phosphate buffer solutions



**Fig. S1.** Im  $\chi^{(2)}$  spectra of 50 mM phosphate buffer at bulk pH 5, 6, 7.4 and 9. Im  $\chi^{(2)}$  spectra of air/neat H<sub>2</sub>O and D<sub>2</sub>O interfaces are also shown for comparison.

	α-chain	β-chain	tetramer
Positive charges	15	24	78
Negative charges	13	28	82
CH <sub>3</sub>	94	96	380
Aromatic rings	11	7	36
Number of residues	141	146	574

#### B. Charged groups, methyl groups and aromatic rings in HbA

**Table S1.** Number of positive and negative charges, CH<sub>3</sub> groups, aromatic rings and total number of residues in HbA, in the case of full deprotonation and protonation of acid and basic residues



C. Control experiment of protein damage under laser irradiation

**Fig. S2.** Im  $\chi^{(2)}$  spectra of 10  $\mu$ M HbA solution in 50 mM phosphate buffer at pH 7.4 as a function of exposure time (a) in the CH stretch region and (b) in the OH stretch region. Solution was continuously irradiated at the same focus point. Recording of one spectrum takes 2 minutes.

D. Concentration dependence of Im  $\chi^{(2)}$  spectra of HbA at the air/water interface and analysis of the varied Im  $\chi^{(2)}$  spectra at the concentration of 0.1  $\mu M$ 



**Fig. S3.** (a) Im  $\chi^{(2)}$  spectra in the CH and OH stretch regions of HbA at a concentration of 0.1, 1, 10 and 40  $\mu$ M in 50 mM phosphate buffer at pH 7.4 compared to neat water at the air/water interface. (b) Fitting of three representative Im $\chi^{(2)}$  raw spectra of 0.1  $\mu$ M HbA (solid orange lines) and their fits (dotted blue line) that are calculated by linear combinations of the Im $\chi^{(2)}$  spectra of neat water (black solid line) and 10  $\mu$ M HbA solution (red solid line) in the OH region.

Three spectra representative of the variability observed at [HbA] = 0.1  $\mu$ M are shown in Fig. S3. The Im $\chi^{(2)}$  spectra of 0.1  $\mu$ M HbA were fitted by linear combinations of experimental Im $\chi^{(2)}$  spectra of neat water  $R_w(\bar{v})$  and 10  $\mu$ M HbA solution  $R_{Hb}(\bar{v})$  between 3000 and 3800 cm<sup>-1</sup> in the form:

$$R(\bar{v}) = a_w \cdot R_w(\bar{v}) + a_{Hb} \cdot R_{Hb}(\bar{v})$$

The 10  $\mu$ M HbA solution corresponds to a saturating condition with a full surface coverage by the protein. Fitting was performed by the relative mean squared error method. The weight factors  $a_w$  and  $a_{Hb}$  for neat water and 10  $\mu$ M HbA solution, respectively, are given in Table S2.

Fitting	Imχ <sup>(2)</sup> HbA 0.1μM	Imχ <sup>(2)</sup> HbA 0.1μM	lmχ <sup>(2)</sup> HbA 0.1μM
parameters	spectrum 1	spectrum 2	spectrum 3
$a_w$	0.90	0.44	0.15
$a_{Hb}$	0.10	0.56	0.85

Table S2. Fitting parameters of  $\text{Im}\chi^{(2)}$  spectra of 0.1  $\mu\text{M}$  HbA at pH 7.4

The area per molecule reported in the literature for a film of hemoglobin at the air/water interface<sup>1</sup> is ~1.2 nm<sup>2</sup>. This full surface coverage requires a concentration of 0.4  $\mu$ M (without taking into account possible spreading at lower concentration) if we assume that all molecules are adsorbed at the interface. This value is consistent with the observed saturated spectrum at [HbA] = 1  $\mu$ M as well as the unsaturated surface coverage at 0.1  $\mu$ M because the available surface per molecule would be 4.8 nm<sup>2</sup>.

### E. Charged residues in Mb structure



**Fig. S4.** Two-side views on localization of positively charged and negatively charged residues in Mb (structure PDB 1YMB). The heme group embedded in a hydrophobic pocket is represented in black.

## F. Im $\chi^{(2)}$ spectrum of HbA in pure water



**Fig. S5.** Im  $\chi^{(2)}$  spectra of 10  $\mu$ M HbA in pure water at the air/water interface. Im  $\chi^{(2)}$  spectra of the air/water interface is also shown for comparison.

G. Calculation of the charge densities of acid and basic residues



**Fig. S6.** Calculated atomic charge of the protonated side chains of Lys and Arg (top) and deprotonated side chains of Asp and Glu (bottom). The total charges of the side chains are indicated in frames. Calculations were performed with Gaussian09 program package. The charge densities were calculated with Natural Bond Orbital (NBO) analysis for isolated amino acids. Their structures were optimized at the B3LYP/6-31++G(d,p) level. The calculated charges of the side chains reported by Price *et al.*<sup>2</sup> are also indicated in parentheses for comparison.

### H. Hemoglobin purification

Human adult hemoglobin was purified according to standard procedures with some modifications. Briefly, erythrocytes were extracted from the whole blood and washed several times in isotonic saline solution. Hemolysis was done by mixing equal amounts of packed red cell with deionized water with 10% v/v toluene. The upper organic layer containing cell membranes was separated by centrifugation at 5,000 g and then discarded by aspiration. Cellular debris and unbroken cells were discarded as precipitate at 10,000 g. The resulting crude hemoglobin solution was extensively dialyzed against water at 4 °C, passed through Sephadex G-25 fine (GE Healthcare) column in 10 mM Tris, pH 7.4, followed by AG 501-X8 (D) resin (Bio-Rad) in water.

#### References

- 1 K. S. Birdi and A. Nikolov, J. Phys. Chem., 1979, 83, 365–367.
- 2 S. L. Price, C. H. Faerman and C. W. Murray, J. Comput. Chem., 1991, **12**, 1187–1197.