

Appendix 4: Materials and Methods

Hemoglobin Preparation. Bovine blood was collected in a heparinized (10 units/ml) tube. The plasma and buffy coat were removed after centrifugation at $800 \times g$ for 10 min. The cells were resuspended and washed three times in a buffer containing 40 mM Hepes, 120 mM NaCl, 5 mM glucose at pH 7.4, 282 milliosmolar (mOsm). After each wash, the cells were centrifuged at $800 \times g$ for 10 min. RBCs were purified by filtration through a mixture of α -cellulose and microcrystalline cellulose. Aliquot was frozen at -80°C overnight, then centrifuged at $22,000 \times g$, 4°C , for 12 min to get cell lysate. Oxygenated hemoglobin (oxyHb) was isolated by passing the cell lysate through a Sephadex G-25 fine column. To prepare methemoglobin (MetHb), $\text{K}_3\text{Fe}(\text{CN})_6$ (0.2M) was added to oxyHb isolated above at 2-fold excess. MetHb was purified by passing the solution through a Sephadex G-25 fine column. To prepare cyano-methemoglobin (CyanoHb), 0.2M $\text{K}_3\text{Fe}(\text{CN})_6$ and 0.1M KCN were added to purified oxyHb at 2-fold excess. The extra chemical components were removed by passing the solution through a Sephadex G-25 fine solution. Mixtures of Hbs at different concentrations were generated by mixing pure solutions, and buffer was added to obtain the desired final concentration.

Spectrophotometric Measurements. The absorbance spectra of various Hb solutions were measured by using a UV/visible spectrophotometer (Beckman DU640) at wavelength from 380 to 700 nm. Spectra data were collected for a wavelength increment of 1 nm.