

## Hydration State of Single *cytochrome c* Monolayers on Soft Interfaces via Neutron Reflectivity

**W**ater is critical, not only for the correct folding of proteins but also for the maintenance of this folded structure. The internal molecular motions in proteins, which are necessary for biological activity, are very dependent on the degree of flexibility which is determined by the level of hydration. The number of water molecules hydrating a functioning protein is thus an issue of great interest. Given an appropriate model system, neutron scattering, particularly sensitive to hydrogen, provides a way to measure this water content.

Previous optical spectroscopy studies have shown that yeast *cytochrome c* (YCC) covalently bound to a soft interface (Fig. 1) and partially hydrated by a moist helium atmosphere can be fully functional with respect to the oxidation-reduction chemistry of its iron porphyrin prosthetic group [1, 2, 5, 6]. This system thus provides an opportunity to examine the water distribution required to maintain the structure and function of the protein YCC.

Unlike x-rays, neutrons scatter very differently from hydrogen and deuterium. This fact allows the water distribution in the monolayer profile structure of YCC to be obtained by comparison. The neutron scattering length density profile derived from neutron reflectivity for a YCC monolayer hydrated by D<sub>2</sub>O is compared to the profile for identical hydration with H<sub>2</sub>O.

The silicon surface layer of an iron-silicon (Fe/Si) or iron-gold-silicon (Fe/Au/Si) multilayer solid substrate can bind a self-assembled monolayer (SAM) to form the soft

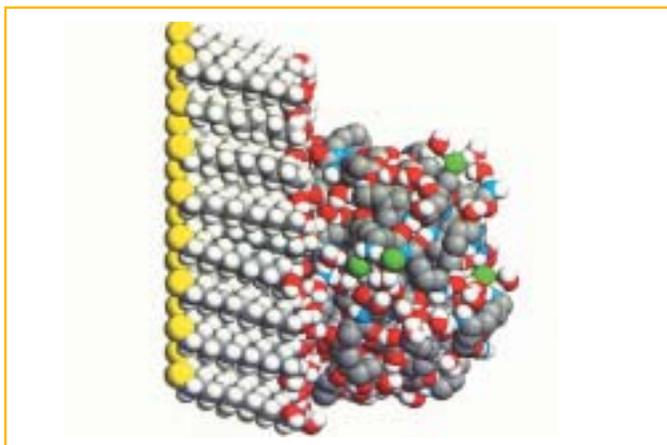


Fig. 1. Molecular dynamics “snapshot” of YCC tethered to the soft surface of an uncharged-polar self-assembled monolayer (SAM).

surface used to tether the YCC monolayer. (See diagrams at the top of Fig. 3.) Such a multilayer substrate has two key advantages in a neutron (or x-ray) reflectivity experiment: a multilayer substrate dramatically enhances this scattering for momentum transfer normal to the substrate surface, and a multilayer substrate also provides an important reference profile structure for the unique interferometric phasing of the reflectivity data.

Neutron reflectivities (Fig. 2) were collected on the NG-1 reflectometer for both H<sub>2</sub>O and D<sub>2</sub>O hydration cases for two such SAM/YCC samples. One SAM formed a nonpolar surface (-CH<sub>3</sub>/-SH = 6:1 mixed endgroups), and

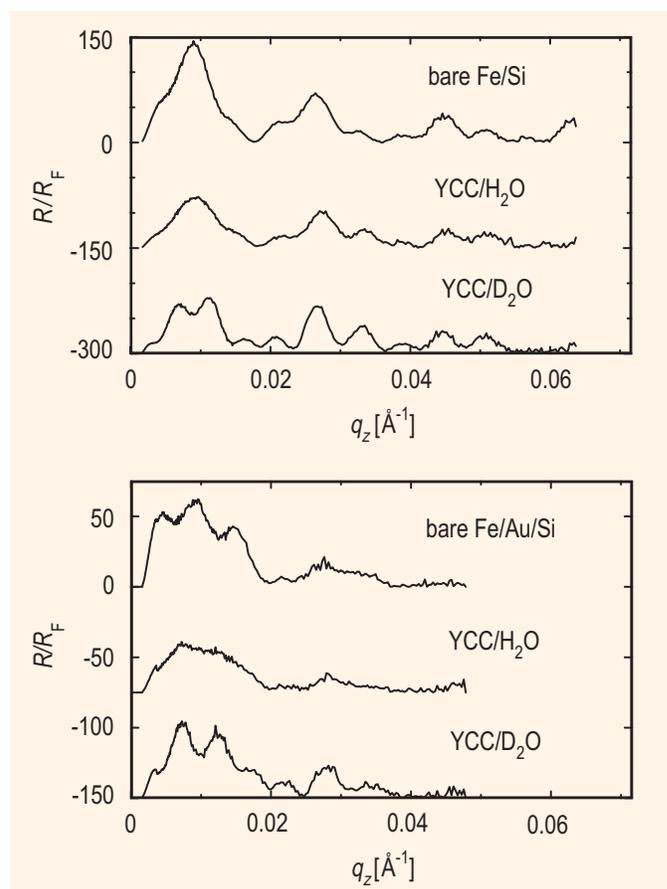
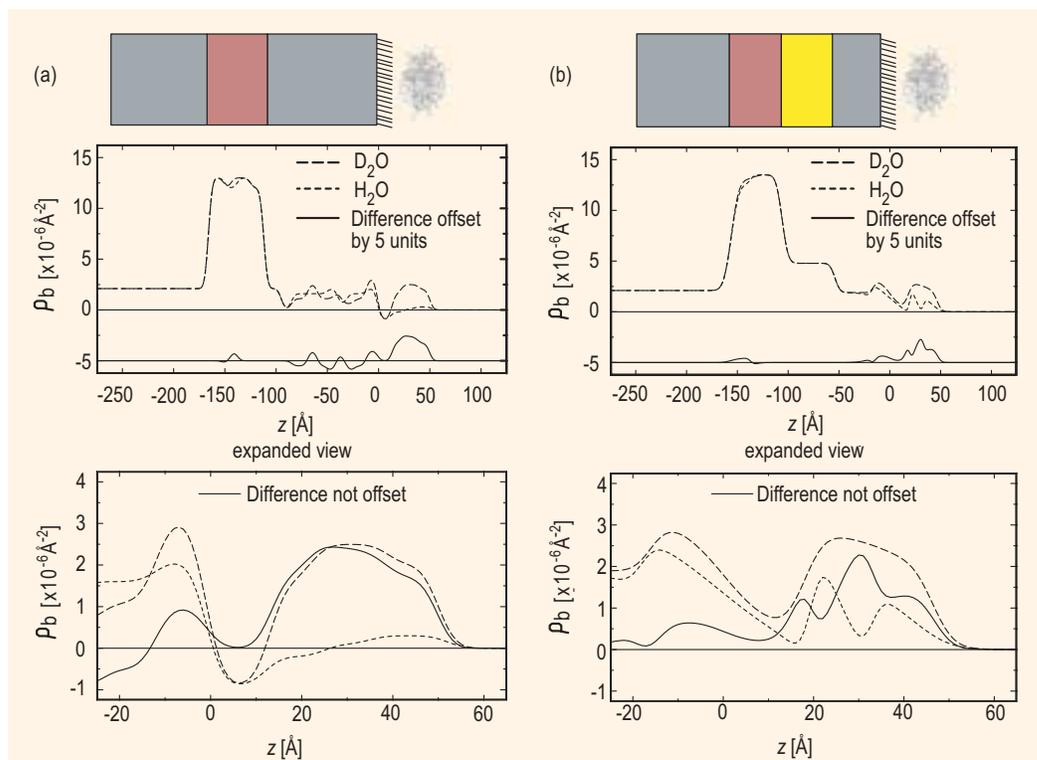


Fig. 2. Top: normalized reflectivity data with incident neutron spins parallel to the iron magnetization for the bare multilayer substrate, substrate plus nonpolar SAM plus YCC/D<sub>2</sub>O, and substrate plus nonpolar SAM plus YCC/H<sub>2</sub>O. Bottom: data for similar arrangements with the uncharged polar SAM on a Fe/Au/Si substrate. In the top plot the H<sub>2</sub>O data are offset by 150 units and the D<sub>2</sub>O data by 300 units on the ordinate, and in the bottom plot the H<sub>2</sub>O data are offset by 75 units and the D<sub>2</sub>O data by 150 units on the ordinate. (Note:  $q_z = 2s \sin \theta / \lambda$ .)

the other SAM formed an uncharged polar surface (-OH/-SH = 6:1 mixed endgroups). These data were analyzed using a new interferometric phasing method that makes use of two features: the neutron scattering contrast between the Si and Fe layers in a single reference multilayer structure, and a constrained refinement approach using the finite extent of the gradient of the profile structures for the systems [3]. The water distribution profiles for the two SAM/YCC monolayers provided by this analysis are shown in Figs. 3a and 3b.

For hydration with  $D_2O$ , these profiles show that the protein monolayer is 3 Å to 4 Å closer to the substrate surface for the uncharged-polar SAM compared to the nonpolar SAM. This finding is in excellent agreement with simulations of these systems [3, 4], and arises because residues on the protein's surface interact strongly with the polar SAM's hydroxyl endgroups via hydrogen-bonding, thus drawing the YCC closer to the SAM surface.

Given these water distribution profiles, the number of water molecules hydrating the YCC monolayer at each SAM can be calculated. Allowing for proton exchange in the *cytochrome c* molecule itself ( $\approx 17$  polypeptide backbone hydrogens and  $\approx 104$  side chain hydrogens), we obtained values of  $\approx 167$  water molecules/YCC at the uncharged polar SAM (exposed to He at 81 % relative humidity) and  $\approx 297$  water molecules/YCC at the nonpolar SAM (exposed to He at 88 % relative humidity) with relative errors of order 20 % to 25 %. These findings allow quantitative comparison to molecular models, opening an important window to understanding the role of water in protein functioning.



**Fig. 3.** The absolute neutron scattering length density profiles for partial hydration with  $D_2O$  and  $H_2O$  and their difference profile for both the nonpolar SAM (a) and the uncharged polar SAM (b) cases. The boundaries for the *cytochrome c* protein region of the profiles used for calculation of the amount of water hydrating the protein are  $z = 10$  Å and  $z = 60$  Å. Schematics of the composite structures are shown above their respective scattering length density profiles approximately to scale.

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