# Development of a diagnostic test based on nanoscale distance measurements between the fatty acid binding sites in human serum albumin

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## Introduction

Human serum albumin (HSA) is the most abundant protein in human blood with a concentration (on average) of 0.5-0.8 mM. [1]. It has been widely studied in the past, also by magnetic resonance methods and it has extraordinary capabilities to bind a manifold of different ligands [1-3]. These ligand binding properties make it an important protein in the blood plasma for two reasons: *i*) it can act as a transport protein, e.g. transporting different types of especially hydrophobic drugs, and *ii*) many diseases may express e.g. small protein segments into the blood that can bind to HSA. Especially the latter point has raised interest to use changes in the ligand binding as a diagnostic tool, e.g. for early diagnosis of different types of tumors. The fatty acid binding sites in HSA have been identified and the crystal structure of HSA co-crystallized with six fatty acids is known [3]. Recently, continuous-wave electron paramagnetic resonance (CW EPR) on fatty acids bearing paramagnetic moieties (nitroxide spin label) has been used to detect changes in the fatty acid binding behavior of HSA in tumor patients [4].
Despite the numerous studies on HSA, the affinity of each binding site to take up a fatty acid and the order of binding of the individual ligands to the respective binding sites is surprisingly little studied. NMR studies on the competition of drugs binding to the ligand binding sites have recently shed light on the affinity of fatty acids to bind to mutants of HSA [5]. Nevertheless, we aim at characterizing the affinity of the fatty acid binding sites by exposing wild-type HSA in different ratios to fatty acids that bear EPR-active spin labels. Together with the standard method of CW EPR, we employ a modern method of pulse EPR, called double-electron-electron resonance (DEER) that can measure the dipolar (through-space) coupling between individual spin labels. Finally, through the known $r^{-3}$-dependence of the dipolar coupling the distance $r$ between the spin labels can be calculated [6, 7].

## Material and Methods

Human serum albumin (HSA, molecular weight~66,000 g/mol) and 16-doxyl stearic acid (16-DSA) were purchased from Sigma-Aldrich (Germany) and used as received. Samples were prepared in mixtures of completely de-ionized water and ethanol or glycerol and while the HSA concentration was kept constant at 0.6 mM, the ratios of HSA/16-DSA was varied from 0.5 to 6.
CW EPR spectra in solution ($T$=293 K) at X-band (~ 9.4 GHz) were measured on a Magnettech MS200 benchtop spectrometer (Magnettech, Berlin, Germany). Four-pulse DEER time-domain signals were obtained on on a Bruker ELEXSYS 580 spectrometer with a Bruker Flexline split-ring resonator (ER 4118X-MS3) with over-coupling to Q ≈ 100 at a temperature of 20 K. The pulse sequence is $(\pi/2)_{\nu A}$-$\tau_1$-$(\pi)_{\nu A}$-$t'$-$(\pi)_{\nu B}$-$(\tau_1+\tau_2-t')$-$(\pi)_{\nu A}$-$\tau_2$-*echo*, with a phase cycle [(+*x*)-(-*x*)] was applied to the first pulse, and $\tau_1$ was averaged over 8 increments ($\Delta\tau_l$= 8 ns) to suppress proton modulations [6]. Here, $\nu_A$ is the observer frequency (local maximum at the low-field edge of the EPR absorption spectrum) and $\nu_B$ the pump frequency (global maximum of the EPR absorption spectrum, $\nu_B = \nu_A$- 66 MHz). The time $t'$ after the first π-pulse was incremented, the pulse lengths and delays were as follows: $(\pi/2)_{\nu A}$=32 ns, $(\pi)_{\nu A}$=32 ns, $(\pi)_{\nu B}$ =12 ns, $\tau_1$ =200 ns, and $\tau_2$ was set to the longest possible time (at least 2 μs), $\Delta t'$ = 8 ns, $t'_0$ = 80 ns. The pump pulses were generated by feeding the output of a Magnettech OSC 101/1 sweep oscillator to one microwave-pulse-forming unit of the spectrometer. The repetition time was 2 ms. DEER time traces were analyzed either by direct integral transformation or assuming a distribution of Gaussian-shaped distance peaks [6, 7].

## Results

Our approach is graphically represented in Fig. 1. We have used the published crystal structure of HSA co-crystallized with six fatty acids ([3], no. 1Bj5 in the Cambridge database), to model approximate distances between fatty acid spin labels (shown as blue ellipses (fatty acid) and magenta circles (attached spin label)). The 15 distances prevalent in the system range from 1.2 to 6 nanometers and almost all of them fall into the realm of distances detectable with DEER (~1.5 nm to ~8 nm). The complex data analysis of the measured DEER time traces for all the ratios of HSA/16-DSA and comparison with the distances obtained from the crystal structure we could identify the most likely distances and hence the most likely binding sites (data not shown).

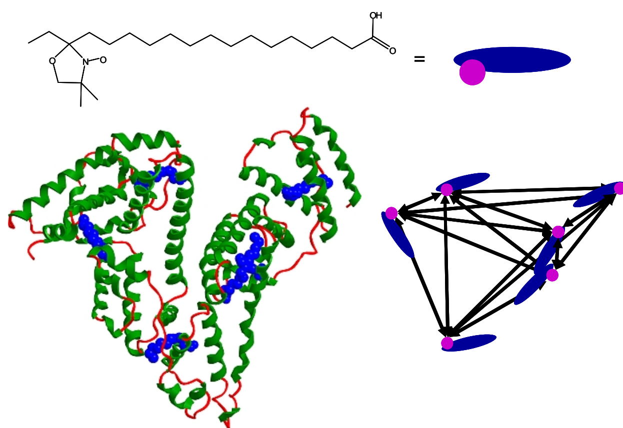

**Fig. 1**:
Graphical representation of the approach used in this study. By comparison of the measured and the projected (from the crystallographic data, [3]) distances the affinities of binding for the different sites are characterized.

## Discussion

Our results demonstrate that we are able to distinguish at least several groups of sites by their inter-site distances. Our findings are largely consistent with the recently published relative affinities for the fatty acid binding sites [5] that were obtained by a complex mutation strategy combined with NMR spectroscopy. The analysis of the data is so far in an experimental stage but we aim at standardizing the method and developing this method into a diagnostic tool by expanding the studied samples from simple HSA in solution to actual blood serum samples from patients with different disease backgrounds. This may finally allow diagnoses based on the identification of competitive binding of endogenous ligands to the binding sites.

## References

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