Supporting Information

Two-photon Macromolecular Probe Based on a Quadrupolar Anthracenyl Scaffold for Sensitive Recognition of Serum Proteins under Simulated Physiological Conditions

Marco Deiana\textsuperscript{a}, Bastien Mettra\textsuperscript{b}, Leszek M. Mazur\textsuperscript{a}, Chantal Andraud\textsuperscript{b}, Marek Samoc\textsuperscript{a}, Cyrille Monnereau\textsuperscript{a, b}, Katarzyna Matczyszyn\textsuperscript{a, *}

\textsuperscript{a}Advanced Materials Engineering and Modelling Group, Faculty of Chemistry, Wroclaw University of Science and Technology, Wyb. Wyspianskiego 27, 50-370 Wroclaw (Poland)

\textsuperscript{b}Univ Lyon, Ens de Lyon, CNRS UMR 5182, Université Lyon 1, Laboratoire de Chimie, F69342, Lyon, France

* to whom the correspondence should be addressed: katarzyna.matczyszyn@pwr.wroc.pl and cyrille.monnereau@ens-lyon.fr

Table of Contents

- Equilibrium fraction p.S2
- Thermodynamic analysis of the Ant-PIm-HSA system p.S3
- Site marker competitive experiments p.S4
- References p.S6
Equilibrium fraction

Additional insights into the Ant-Plm-HSA binding process were provided by analyzing the equilibrium fraction ($f_u$) of the uncomplexed Ant-Plm which is an important pharmacokinetic parameter involved in drug elimination and distribution in the body and can be described by the following equation:\cite{1}

\[
f_u = \frac{Q_0}{Q} = \frac{1 - \frac{n[P]}{[Q]} - \frac{K_d}{[Q]} + [(1 + \frac{n[P]}{[Q]} + \frac{K_d}{[Q]})^2 - 4\frac{n[P]}{[Q]}]^{1/2}}{2}
\]  

(1)

where $P$ and $Q$ are the total protein and ligand concentrations, respectively, $Q_0$ is the uncomplexed ligand concentration and $K_d$ is the equilibrium dissociation constant. A plot of the equilibrium fraction versus different [Ant-Plm] / [HSA] molar ratios is shown in Figure S1.

Figure S1. Plot of the equilibrium fraction of unbound Ant-Plm at different [Ant-Plm] / [HSA] molar ratios at 298 K.

As can be seen from Figure S1, the equilibrium fraction $f_u$ was higher than 45% and increased linearly on increasing the Ant-Plm concentration which supports and anticipates the suitability of Ant-Plm to be used as a fluorescent sensor for HSA detection even at low concentration.
Thermodynamic analysis of the Ant-PIm-HSA system

In order to provide further information on the intermolecular forces involved on the stabilization of the association process between HSA and Ant-PIm, its thermodynamic parameters were determined. Non-covalent forces that dominate ligands and biomacromolecules binding in aqueous solution include electrostatic and hydrophobic interactions, hydrogen bonds and van der Waals forces. If the enthalpy and entropy (ΔH, ΔS) associated to the binding event are assumed not to vary significantly along the temperature range studied, both enthalpy and entropic contribution (TΔS) can be estimated by using the van’t Hoff equation:\(^2,^3\)

\[
\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]  
(2)

\[
\Delta G = \Delta H - T\Delta S
\]  
(3)

As a result, the slope and the intercept of the linear plot between log \(K_a\) and the reciprocal absolute temperature allowed us to establish the values of \(\Delta H\) and \(\Delta S\), respectively (Figure S2).

![Van’t Hoff plot for the binding of Ant-PIm to HSA.](image)

**Figure S2.** Van’t Hoff plot for the binding of Ant-PIm to HSA.

Spontaneity of the process could be ascertained by the negative values systematically measured for \(\Delta G\) (\(-26.2 \pm 0.2, -27.1 \pm 0.1\) and \(-27.9 \pm 0.1\) kJ mol\(^{-1}\) at 298, 304 and 310 K, respectively).
Besides, the positive values of both $\Delta H (14.7 \pm 1.4 \text{ kJ mol}^{-1})$ and $\Delta S (137.5 \pm 12.1 \text{ J mol}^{-1} \text{ K}^{-1})$ indicate that the Ant-Plm-HSA complex is entropically driven, thus stabilized mainly by short-range interactions even though long-range ionic forces mediated by the presence of the polycationic side chains certainly play a stabilizing role. These results can be explained considering the strong hydrophobic character of the anthracenyl derivative and its binding proximity to the hydrophobic binding compartment in which the tryptophan residue is located.

**HSA binding pocket identification through site-marker competitive experiments**

The HSA crystal structure analysis revealed that the main drug binding pockets are located within the hydrophobic cavities of the subdomains IIA and IIIA usually named Sudlow’s sites 1 and 2, respectively. Warfarin, an anticoagulant drug, is a stereotypical Sudlow’s site 1 binder, whereas ibuprofen, a nonsteroidal anti-inflammatory agent, is a well-known Sudlow’s site 2 binder.

To gain information about the Ant-Plm binding site, we monitored the changes in fluorescence of Ant-Plm bound to HSA ($\lambda_{\text{exc}} = 516 \text{ nm}$) upon increasing the site markers concentrations. Unfortunately, no significant displacement of the anthracenyl derivative properties was observed, even at high drug concentrations (data not shown), probably due to the relatively high binding affinity of Ant-Plm to the serum protein and for its extended geometrical configuration which may cause steric hindrances for the insertion of the site markers into the multi-chamber cavity of the protein.

In order to stem this problem, we decided to reverse the approach and carry out further displacement studies, this time keeping constant the HSA-drugs ($\lambda_{\text{exc}} = 280 \text{ nm}$) concentration and...
increasing that of the fluorophore. As depicted in Figure S3, the fluorescence of the HSA-drugs complexes was efficiently quenched by the addition of the Ant-Plm molecules strongly pointing to either a replacement or a co-binding of Ant-Plm onto the protein. Interestingly, the extent of replacing the well-known site markers was greater for ibuprofen than for warfarin.

![Figure S3](image)

**Figure S3.** Effect of Ant-Plm on the fluorescence of HSA-warfarin (black line) and HSA-ibuprofen (red line). $F_1$ and $F_2$ are the fluorescence intensities of HSA-drugs in the absence and presence of Ant-Plm, respectively. [HSA] = [Warfarin] = [Ibuprofen] = 10 µM; [Ant-Plm] ranged from 0 to 18 µM.

In order to quantify the influence of warfarin and ibuprofen on the binding of Ant-Plm to HSA, the Stern-Volmer equation in the presence of site markers was used. The $K_S$ values for the ternary mixture Ant-Plm-HSA-Warfarin and Ant-Plm-HSA-Ibuprofen were found to be $1.1 \pm 0.3 \times 10^4$ M$^{-1}$ and $2.5 \pm 0.2 \times 10^4$ M$^{-1}$, respectively, indicating a relatively higher binding affinity of Ant-Plm towards the subdomain IIA.
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