

Probing the Sudlow Binding Site with Warfarin: How Does Gold Nanocluster Growth Alter Human Serum Albumin?

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The search for new fluorescent molecules is vital to the advancement of molecular imaging and sensing for the benefit of medical and biological studies. One such class of new fluorescent molecule is fluorescent gold nanoclusters encapsulated in Human Serum Albumin (HSA-AuNC). In order to use this new fluorescent molecule as a sensor or fluorescent marker in biological imaging both in vitro and in vivo it is important to understand whether/how the proteins function is changed by the synthesis and presence of the gold nanoclusters inside the protein. Natural HSA acts as the main drug carrier in the blood stream, carrying a multitude of molecules in two major binding sites (Sudlow I and II). To test the effects of gold on the ability of HSA to act as a drug carrier we employed warfarin, an anticoagulant drug, as a fluorescent probe to detect changes between natural HSA and HSA-AuNCs. AuNCs are found to inhibit the take up of warfarin by HSA. Evidence for this is found from fluorescence spectral and lifetime measurements. Interestingly, the presence of warfarin bound to HSA also inhibits the formation of gold nanoclusters within protein. This research provides valuable insight into how protein function can change upon synthesis of AuNCs and how that will affect their use as a fluorescent probe.

Introduction

The search for new fluorescent molecules is vital to the advancement of molecular imaging and sensing for the benefit of medical and biological studies. Intensive research has been carried out in the development of nanoscale sensing and imaging using fluorescence based techniques based on newly developed fluorophores¹⁻⁸. One such class of new fluorophore are fluorescent noble metal nanoclusters. A number of methods of creating these fluorophores have been developed; of interest to this study is the protein-directed synthesis of gold nanoclusters (AuNCs) using Human Serum Albumin (HSA) as the template protein. Much work has been carried out studying the fluorescent properties of protein encapsulated gold nanoclusters^{2,9-11}. Interestingly they have been shown to possess unusually long fluorescence lifetimes in the microsecond regime, for a variety of protein, e.g., human serum albumin, bovine serum albumin and lysozyme, used to stabilize the AuNCs¹². These nanoclusters have also been shown to be highly stable over a long period of time and highly resistant to photobleaching¹³. These properties add on their water solubility, biocompatibility, large stoke shift, sensitivity to metal ions as well as protein conformational changes¹⁴⁻¹⁶, making protein encapsulated AuNCs a potentially ideal probe for the

tracking of long-term biological processes both in vitro and in vivo. To exploit this potential, it is essential to understand whether/how the protein function is changed by the synthesis and internal presence of the gold nanoclusters. Natural HSA acts as the main drug carrier in the blood stream carrying a multitude of molecules in two major binding sites (Sudlow I and II). Warfarin is traditionally used to thin the blood in patients with heart complications¹⁷. It is also a fluorescent molecule; with an absorption and emission maximum of 320nm and 390nm respectively. Warfarin is a non-rigid molecule, as seen in Figure 1, transported around the body via HSA, binding to the major drug Sudlow I binding site¹⁷. Upon binding to the Sudlow site the drug's fluorescence is enhanced due to the molecule becoming stabilised, resulting in reduced energy dissipation due to non-radiative heat transfers to the surrounding solvent. To test the effects of gold on the ability of HSA to act as a drug carrier, in this work, we employed warfarin as a fluorescent probe to detect changes between natural HSA and HSA-AuNCs by comparing the interaction of warfarin with natural HSA and HSA-AuNCs using steady state and time-resolved fluorescence spectroscopy.

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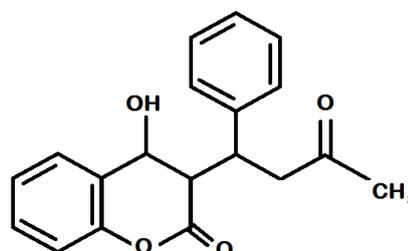


Figure 1: Chemical structure of the warfarin molecule unbound to HSA.

Materials and Methods

The synthesis of HSA-AuNCs was carried out using Xie's method¹⁸. All samples were then dialysed into PBS buffer solution with a concentration of 40 μ M, using 10kDa dialysis cassettes. All chemicals used were purchased from Sigma-Aldrich. All dilutions of the sample were carried out using PBS buffer. Fluorescence emission spectra were measured on a Horiba Fluorolog 3. Fluorescence lifetimes were measured using the Time Correlated Single Photon Counting (TCSPC) technique on a Horiba Deltaflex. For all experiments a TAC range of 100ns was used and a NanoLED pulsed light source with an excitation wavelength of 303nm¹⁹. Analysis was carried out using DAS6 software from Horiba. The method of fitting used was the non-linear least-squares method using the following exponentials model to describe the decay kinetics:

$$I(t) = \sum_{i=1}^n b_i \exp\left(-\frac{t}{\tau_i}\right) \quad [1]$$

Where τ_i are the decay times with amplitudes b_i and $\sum b_i = 1$.

Equation [2] describes the normalised pre-exponential factors:

$$\alpha_i = \frac{b_i}{\sum_{i=1}^n b_i} \quad [2]$$

Results and discussion

Figure 2 shows the normalised fluorescence spectra of warfarin (free and bound to HSA), HSA and HSA-AuNCs. Free warfarin has an emission peak centred on 393nm under 320nm excitation, while warfarin bound to HSA has an emission peak at 370nm. The 340nm emission from HSA under 290nm excitation was due to fluorescent amino acid tryptophan in HSA. A red emission of 670nm and a shoulder around 440nm from HSA-AuNC under 290nm excitation was consistent with previous reports²⁰. The emission at 440nm from HSA-AuNCs has been reported before either as a result of intermediate, smaller AuNCs forming within the protein or the result of oxidised tryptophan present in the protein²¹⁻²³.

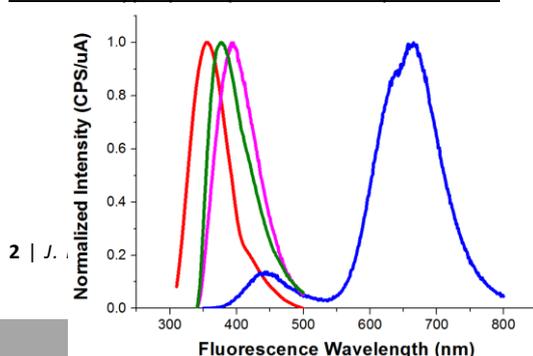


Figure 2: Fluorescence Emission Spectra of warfarin free in solution (pink), warfarin bound to HSA (green), tryptophan from HSA (red) and HSA-AuNCs (blue).

To compare the binding of warfarin to HSA-AuNC and HSA, we introduced warfarin to both solutions in the same manner and compared the intensity of warfarin's fluorescence in both solutions with warfarin alone in buffer solution. The HSA and HSA-AuNCs concentrations were kept the same at 40 μ M while increasing the concentration of warfarin from 0 to 15 μ M. All samples were excited at 320nm. The peak of warfarin fluorescence was observed at 372nm for warfarin with HSA and 393nm for warfarin with HSA-AuNC or on its own, implying different bound state of the warfarin molecule. Figure 3 shows the fluorescence intensities of warfarin as a function of warfarin concentration.

Fluorescence intensity of warfarin was enhanced by 6 times when 15 μ M of warfarin was mixed with HSA in comparison with that from the same warfarin solution alone. This compares well with previous studies undertaken with warfarin binding to HSA under the same conditions²⁴, indicating the binding of warfarin with native HSA.

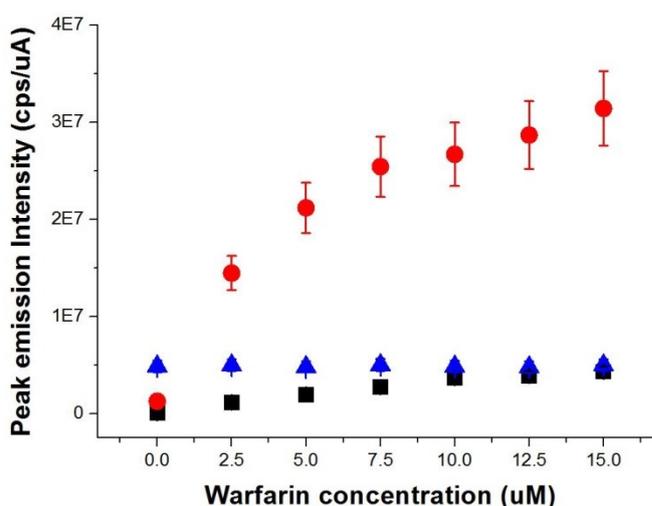


Figure 3: Fluorescence intensity of warfarin emission against warfarin concentration in native HSA (red, emission 372nm), HSA-AuNCs (blue, emission 393nm) and pure warfarin solution (black, emission 393 nm) (excitation 320nm).

In contrast to the significant increase of warfarin emission in Warfarin-HSA, no apparent change in the fluorescence intensity

of warfarin in HSA-AuNC solution was found. The relatively low fluorescence intensity and emission peak position at 393nm were comparable with free warfarin. The slightly higher initial intensities at low warfarin concentration were due to the overlapping of warfarin emission with the weak emission from HSA-AuNC around 440nm. Nevertheless, the significant difference between the behaviour of warfarin with HSA and HSA-AuNC indicates much reduced binding of warfarin to HSA-AuNC.

The fluorescence lifetime of warfarin has been shown to be a powerful indicator of the local environment of the molecule in previous studies¹³. TCSPC measurements of the fluorescence lifetimes of warfarin were taken under the various conditions as used in above intensity studies. The fluorescence intensity decay curves were fitted to a multi-exponential model using non-linear least-squares analysis and the results are shown in Table 1. It was found that warfarin alone in solution had a very short, single lifetime of 0.12 ± 0.01 ns. This result compares well with previous publications²⁵, and was explained by the molecule losing rotational and vibrational energy to the surrounding solvent²⁶. Upon binding to pure HSA in solution the fluorescence decay was best fitted with 3 lifetime components. The shorter lifetime is no longer present indicating that there is no warfarin in solution. The longer lifetimes of 0.34 ± 0.02 , 1.75 ± 0.06 ns and 6.63 ± 0.15 ns can be explained by the stability the warfarin gains as a result of binding to the HSA protein and are again consistent with previous reports²⁷.

Sample	Warfarin	HSA-War	HSA-AuNCs + War
τ_1 (ns)	0.12 ± 0.01	0.34 ± 0.02	0.16 ± 0.02
τ_2 (ns)	-	1.75 ± 0.06	1.86 ± 0.31
τ_3 (ns)	-	6.63 ± 0.15	4.98 ± 0.28
b_1	0.026 ± 0.001	0.054 ± 0.001	0.169 ± 0.001
b_2	-	0.036 ± 0.001	0.094 ± 0.001
b_3	-	0.002 ± 0.001	0.030 ± 0.001
α_1	1.00	0.58	0.93
α_2	-	0.39	0.05
α_3	-	0.03	0.02
χ_2	1.37	1.30	1.30

Table 1: Lifetime analyse data from warfarin alone, warfarin with HSA and warfarin with HSA-AuNC.

Interestingly warfarin fluorescence lifetimes had different values when in solution with HSA-AuNCs. The decay is again best described by a 3-exponential function with similar lifetime values of 0.16 ± 0.02 ns, 1.86 ± 0.31 ns and 4.98 ± 0.28 ns. However

the shortest lifetime, τ_1 is the dominant fluorescent species with a corresponding α_1 value of 0.93. This short lifetime compares well with the lifetime of free warfarin. This result suggests that the majority of warfarin molecules do not bind to HSA when AuNCs are present in HSA. The existence of longer lifetime components is possibly due to warfarin binding to free HSA in solution which does not contain AuNCs²⁶.

In order to show that most of the warfarin still remains unbound in solution with HSA-AuNCs, additional native HSA was added to the solution. Any large increases in warfarin fluorescence intensity can be attributed to the binding of free warfarin to the native HSA. The fluorescence intensity of warfarin was seen to increase dramatically upon adding HSA to the HSA-AuNCs + warfarin solution, as shown in Figure 4. Fluorescence anisotropy decay studies proved to be inconclusive in comparison to the fluorescence intensity, spectral and lifetime evidence. In order to rule out the influence of intrinsic fluorescence from HSA, control tests were carried out by adding further amounts of HSA to HSA-AuNCs (without warfarin) and to HSA with warfarin already attached. It can be seen that the fluorescence intensity of warfarin remains stable with additional HSA up to $40 \mu\text{M}$ and are close to the intensity from HSA-AuNCs –warfarin solution with higher additional HSA concentrations. It was also found that the emission at 393nm remained low in HSA-AuNCs solution without warfarin with a very small increase in the intensity at high HSA concentration due to the intrinsic fluorescence of HSA.

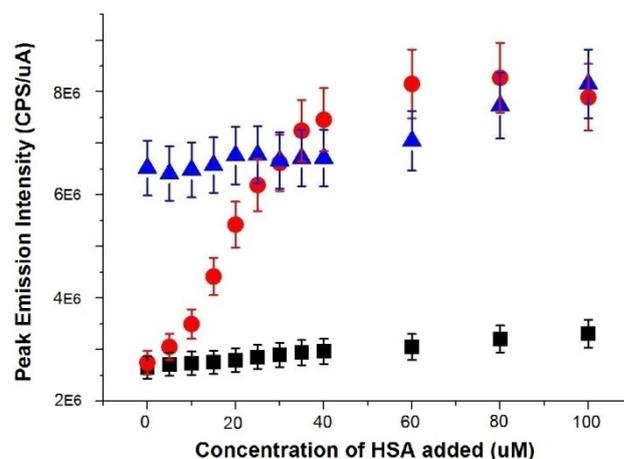


Figure 4: Fluorescence intensity of warfarin upon adding additional free HSA to solution of HSA-AuNCs ($40 \mu\text{M}$) + warfarin ($10 \mu\text{M}$) (red), as well as solution of HSA ($40 \mu\text{M}$) + warfarin ($10 \mu\text{M}$) (blue) and HSA-AuNCs ($40 \mu\text{M}$) solution (black) as references.

This suggests that: firstly, in the case of HSA-warfarin, most warfarin is bound to HSA and the increase found here is due to intrinsic HSA emission; secondly, the large fluorescence intensity increase when adding additional HSA to HSA-AuNCs + warfarin is not due to intrinsic HSA, but binding of free warfarin to newly added HSA; thirdly, the final emission intensity of HSA + warfarin and HSA-AuNC + warfarin + HSA are very close suggesting that all previously unbound warfarin in the HSA-AuNC solution was bound to HSA. These findings confirm that warfarin in HSA-AuNC solution is free and unbound in solution and the previous low fluorescence intensity of warfarin was due to a lack of warfarin binding rather than quenching processes or alterations to the fluorescence nature of warfarin. It is clear that the existence of AuNCs in HSA affects the binding of warfarin to protein. This may be due to the nucleation site of AuNCs being in close proximity to the warfarin binding site²⁰.

If this were true, the growth of AuNC in HSA would also be affected by the binding of warfarin with HSA. To test this idea, we synthesized AuNCs in HSA with pre-bound warfarin in comparison with native HSA. The formation of AuNCs was investigated by monitoring the fluorescence emission from AuNCs. Figure 5 shows the emission of spectra taken after 0, 1 and 6 hr of synthesis. It can be seen that initially there was a strong emission at 440nm, implying a large amount of intermediate product (e.g., small gold clusters), while fluorescence at 640nm dominated the spectrum after 6hrs, indicating the formation of large AuNCs.

Figure 6 depicts the time evolution of these two peaks from solution with HSA and HSA with pre-bound warfarin. In the case of native HSA, the emission at 440nm decreases overtime, while the emission at 640nm increases. This indicates the decrease of intermediate products as they merging into larger gold nanoclusters, as reported before²⁸.

The lack of fluorescence at either 440nm or 640nm for the synthesis using HSA with pre-bound warfarin indicates that neither intermediate products nor large AuNCs are present.

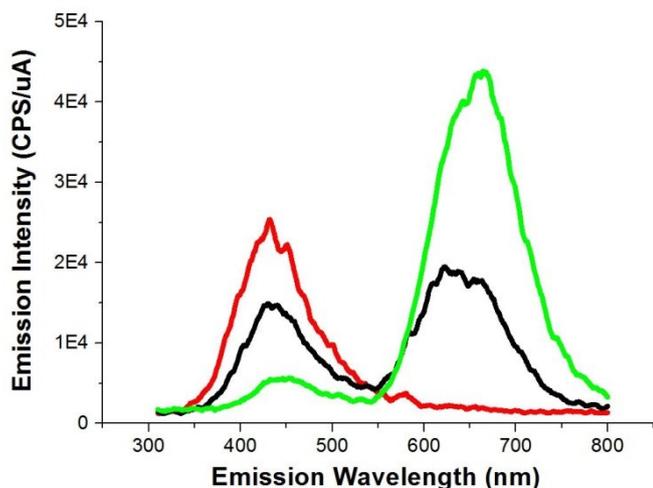


Figure 5: Fluorescence Emission spectra of HSA-AuNC taking at different periods during synthesis; 0 hr – red, 1 hr – black, and 6 hrs - green (excitation 280nm).

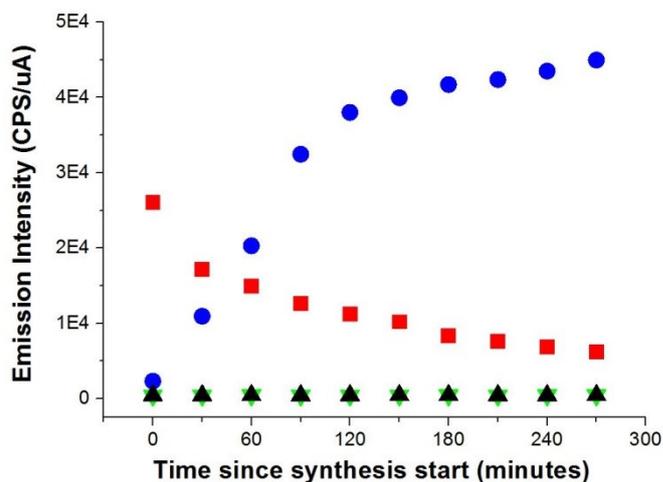


Figure 6: Time evolution of fluorescence intensity of emissions at 440nm and 640nm from HSA-AuNC (red and blue) and HSA-warfarin-AuNC (black and green) during the synthesis (excitation 280nm).

It is thus clear that the bound warfarin affects the ability of HSA to interact and bind with the gold salt present in solution during synthesis.

From previous molecular dynamics simulation work²⁰ it was seen that Au atoms interacted with the Sudlow I binding site for a short amount of time before transporting to a final AuNC nucleation site. Tyrosine has been shown to play a critical factor in the reduction of gold salt; of which an accessible residue resides within the Sudlow I binding site. It is possible that this Tyrosine residue (Position 149 for BSA, position 148 for HSA in the polypeptide chain) plays a critical role in the reduction of the gold salt molecules during synthesis.

Warfarin binding to the amino acid residues: arginine, lysine and may block the accessibility of this tyrosine molecule to gold salt while it is bound to HSA. This explains the lack of small intermediates formation in the case of HSA with pre-bound warfarin. This also explains why warfarin cannot bind to HSA-AuNCs; it is possible that excess gold salt or by-products of the reduction reaction block the Sudlow I binding site. Chloride ions have been previously reported to have a high affinity for histidine²⁹. The Chlorine bond to the positively charged nitrogen centres of these residues through hydrogen bonding. If the leftover chlorine ions from the gold salt reduction bind to the above residues present in the Sudlow I binding site access could be physically hindered for warfarin. Due to the very low concentration of excess chlorine ions it is not possible that protein conformation is drastically altered. It is suggested that the warfarin binding affinity decreases with chlorine ion concentration increase due to both competing for the same binding site³⁰. The presence of chlorine ions would also change the steric attraction between warfarin and the Sudlow I binding site lowering the overall binding affinity. Another factor to consider is the formation of dimers and aggregates of protein after AuNC nucleation in HSA; the formation of aggregates may physically block Warfarin to access binding at Sudlow site I. Alternatively increases to the negative surface charge of HSA

upon AuNC formation may be responsible for the lack of warfarin-HSA binding. Further studies are required for revealing the detail mechanisms.

Conclusions

We have successfully used warfarin as a fluorescent probe to study the influence of AuNCs in HSA on protein's functionality. It was found that warfarin was unable to bind to HSA-AuNC, implying the Sudlow I binding site of HSA is directly affected by the presence of AuNCs. It was found that AuNCs cannot be formed in HSA with a warfarin molecule already bound. This suggests that the Sudlow I binding site may have an important role in the gold atom transport and cluster nucleation. We suggest that the tyrosine present in the Sudlow I binding site plays a critical role in the reduction of gold salt and subsequent formation of AuNCs. These findings should be taken into consideration in the future if protein-encapsulated proteins are to be used as fluorescent probes in the study of biological process where the Sudlow I binding is important. It is not clear if other molecules binding to the Sudlow I binding sites of HSA are affected by the presence of AuNCs and further studies would be required.

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