Reactions of Auranofin and its Pseudohalide Derivatives with Serum Albumin Investigated through ESI-Q-TOF MS

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Supporting Information Placeholder

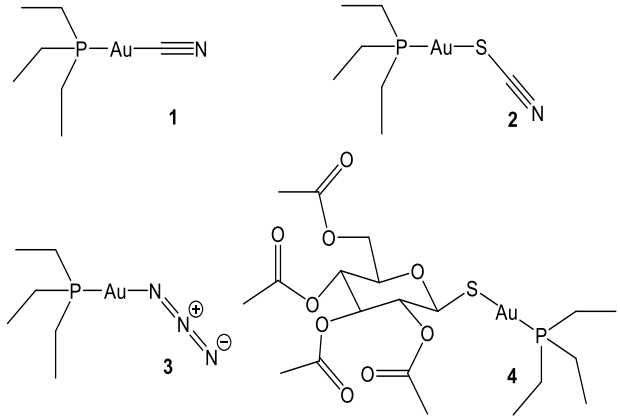
ABSTRACT: The reactions of auranofin and three pseudohalide derivatives with bovine serum albumin were explored by ESI-Q-TOF mass spectrometry; a detailed molecular description of the resulting adducts is achieved revealing even subtle differences in reactivity within this series of gold(I) complexes. Our study shows that this kind of investigative approach, formerly applied to the interactions of metal-based drugs with small model proteins of MW 10-15 kDa, *e.g.* cytochrome c and lysozyme, may now be extended with success to far larger proteins such as serum albumin (MW 66 kDa).

Auranofin (AF hereafter) is an established gold(I) drug for the treatment of severe rheumatoid arthritis first approved for clinical use in 1985. In recent years, owing to the implementation of so called "drug repurposing strategies", a great therapeutic potential was disclosed for AF;[1-3] AF is now being evaluated as an experimental drug for several parasitic and microbial diseases, and also for various types of cancers, with encouraging results.[4-6]

Conversely, serum albumin (MW 66429 Da) is one of the most widely studied mammalian proteins being the most abundant protein in the plasma with a concentration of about 0.3 mM. The aminoacidic chain of serum albumin contains 583 residues; it is characterised by the presence of 17 disulphide bonds and one free cysteine residue (Cys34).[7] Serum albumin is the major carrier for AF in the blood; the interactions of AF with this protein already attracted much attention in the past. In particular, this topic was studied in depth by Frank Shaw and co-workers in the late 80s/early 90s: owing to the application of various independent biophysical methods these authors could demonstrate that AF binds tightly and specifically (bovine) serum albumin through gold(I) coordination to the free thiol group of Cys34 following the release of the 1-thio-beta-D-glucose-2,3,4,6-tetraacetate ligand.[8]

Taking advantage of the considerable technological progresses recorded in the last decade in the field of mass spectrometry, we have now revisited the reaction of AF with serum albumin using a Sciex TripleTOF 5600+ MS instrument, that represents the cutting-edge technology in high resolution mass spectrometry. In addition, we have explored comparatively the reactions of serum albumin with three AF analogues bearing triethylphosphine and a pseudohalide as ligands.[9]

Chart 1. The four investigational Au(I) compounds: AFCN (1), AFSCN (2), AFN_3 (3) and AF (4).



Quite surprisingly, until a few years ago, the scientific literature was virtually devoid of any relevant mass spectrometric study describing metallodrug-protein interactions apart from a few relevant exceptions.[10,11] However, during the past few years, our research group has gained a considerable expertise in the investigation of metallodrugs' reactions with proteins relying on extensive and systematic ESI-MS studies of the adducts that metallodrugs form with various small proteins such as lysozyme, cytochrome c and ribonuclease A.[12-14] Thanks to some recent research efforts,[15-17] ESI mass spectrometry has emerged as an important tool to characterise the metallodrugs' interactions with biomolecules and potential targets at the molecular level and also to predict their probable reactivity and mode of action within complex biological samples.[18] In spite of that, the study of the interactions of metallodrugs with larger proteins in their intact form still posed too severe challenges to be faced with the instrumentation then available and remained largely unaddressed. It is just worth reminding that an original MS study concerning the reactions of a few gold drugs with albumin, was

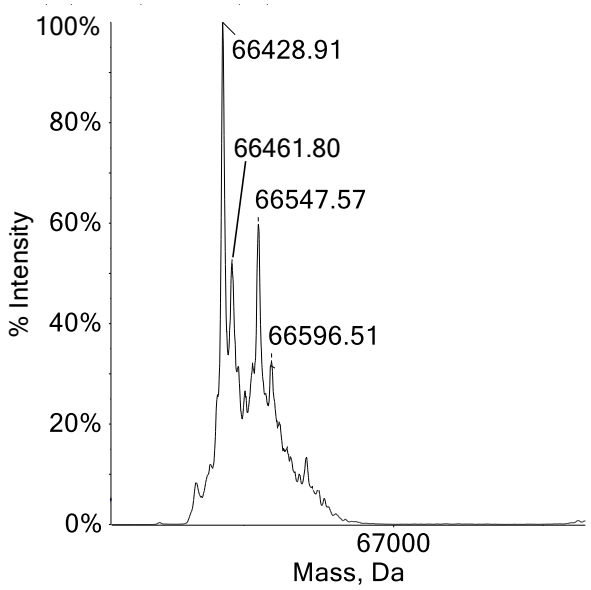


Figure 1. Deconvoluted ESI-MS spectrum of BSA solution 10^{-5} M in 20 mM ammonium acetate buffer, pH 6.8.

reported in 2006 by Ralph and co-workers: a rather satisfactory description of the metallodrug-protein adducts was obtained but details were missed due to the lower analytical performances of the instrumentation available at the time.[19] Taking advantage of the newly available MS instrumentation, we are now in the conditions to analyse in detail the interactions of metallodrugs with proteins as large as albumins, obtaining in a very short time good-quality and highly informative high-resolution MS spectra on very diluted samples (10^{-6} M). The panel of gold compounds that we have used for this study is shown in chart 1; the panel includes, beyond AF (**4**), three of its analogues where a pseudohalide, namely cyanide (AFCN, **1**), thiocyanate (AFSCN, **2**) or azide (AFN_3, **3**), replaces the 1-thio-beta-D-glucose-2,3,4,6-tetraacetate ligand. These gold(I) compounds were prepared by simple synthetic procedures as previously reported.[9] Notably, these compounds are sufficiently soluble and stable in aqueous solutions for the time needed to perform the described experiments.[20] The interactions occurring between the four gold compounds and bovine serum albumin (BSA) were investigated according to the well-established ESI-MS protocol previously developed in our laboratory to study the reactions between metal based drugs and small model proteins.[21-23] First of all, the ESI-MS spectrum of a control sample of native and commercially available BSA was recorded (Figure 1). The main peak at *m/z* 66428.91 Da corresponds to the native free protein while the other major peak at 66547.57 Da, with a mass shift of +118.66 Da, is perfectly consistent with an additional cysteine (+119 Da) bound to Cys34. Indeed, both human and bovine serum albumin normally show characteristic post-translational modifications (PTMs), such as cysteinylation, involving the unique cysteine residue normally present as a free thiol.[19,24] The signal at 66461.80 Da is tentatively assigned to a protein species bearing oxidized Cys34 (Cys-SO_2H) in agreement with previous suggestions. In fact, about one-fourth of circulating albumin is known to be present as mixed disulphides with cysteine, while in the remaining fraction the thiol may be oxidized to higher oxidation states including sulfinic acid (RSO_2H).[25,26] These observations are well consistent with the presence of significant amounts of reactive oxygen species (ROS) in cells and tissues; indeed, it is well-known that the free thiol group of BSA plays a key role in several ROS scavenging mechanisms undergoing progressive oxidation.[25] Lastly, the signal at 66596.51 Da belongs to a glycated form of BSA, also in this case present in native serum proteins.[27]

Afterwards, the three investigational Au(I) compounds, *i.e.* AFCN (**1**), AFSCN (**2**) and AFN_3 (**3**), were individually challenged with BSA in aqueous solutions at physiological pH, at a metal to protein ratio of 3:1; aliquots of each sample were then taken at increasing time intervals after mixing (namely at 1, 24, 48, 72 h) and the respective ESI-MS spectra recorded in the positive ion mode. In all cases, extensive metalation of the protein was soon apparent already after 1 h incubation, suggesting the establishment of rapid and specific interactions. The spectrum depicted in Figure 2A, acquired after only 1 h of incubation, shows that in the case of AFCN (**1**) the main adduct (66742.20 Da) well corresponds to a single $AuPEt_3^+$ moiety bound to the protein. This is perfectly consistent with the presence of one specific gold binding site on BSA having a much greater affinity than any other binding site. A similar behaviour was also found for AFSCN (**2**) (Figure 2B) with the formation of the same type of mono-adduct; yet, in the latter case, we noticed a larger reactivity that is indicated by the peculiar formation of a bis-adduct with BSA, corresponding to the BSA + $2AuPEt_3^+$ species (67056.24 Da). Despite the presence of only one reactive cysteine in BSA, the formation of a bis-gold adduct is not in contrast with previous reports: as a matter of fact, a similar reactivity was already proposed and documented in the literature for similar Au(I) compounds that were reported to form a thiolate-bridged bis-gold complex on Cys34 in BSA.[8]

The greater reactivity of AFSCN (**2**) compared to AFCN (**1**) may well be explained by the stronger nucleophilic character of CN^- compared to SCN^-, making the first compound far more reactive toward the thiol group.[28] To this regard, the reactivity of AFN_3 (**3**) is similarly emblematic; due to the greater propensity of the azido group to leave gold(I) coordination and produce the $AuPEt_3^+$ moiety, the bis-adduct with BSA (67056.34 Da) was the only species formed in the same kind of reaction (Figure 2C).

Since AFCN (**1**), AFSCN (**2**) and AFN_3 (**3**) are structurally-related to auranofin (AF, **4**), this led us to repeat the same type of experiment with AF. Compound **4** was incubated with BSA under identical solution conditions and the mass spectrum depicted in Figure 2D obtained after 1 h. A single main adduct at 66742.95 Da is observed corresponding, once again, to the binding of the $AuPEt_3^+$ moiety to the free thiol group of Cys34. This finding is again in nice agreement with literature data where the thiosugar ligand, present in AF (**4**), is described as the "*leaving group*" in this molecule and the $AuPEt_3^+$ portion is reputed as the biologically-active molecular fragment.[29-31] The three other signals present in the spectrum at 66429.40, 66460.38 and 66546.61 Da are assigned to unreacted BSA and its PTMs, as previously described. When the incubation time was extended to 24, 48 and 72 h, some appreciable changes in the adducts distribution progressively occurred (See ESI-MS spectra in SI). In particular, for AFSCN (**2**) the equilibrium between mono- and bis-adduct moved backward with time toward a net preponderance of the monoadduct. However, at this stage of the research, we are

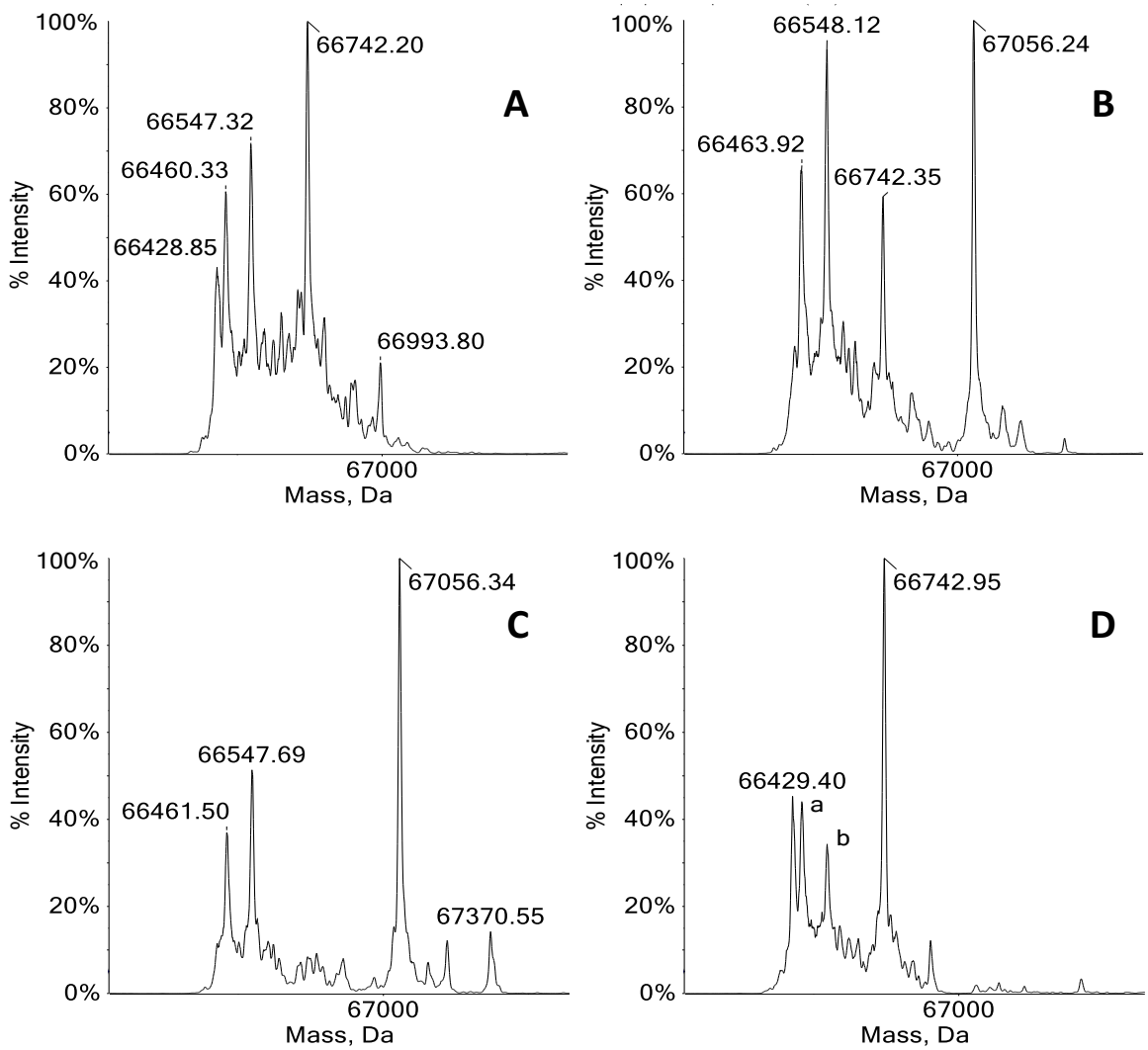


Figure 2. Deconvoluted ESI-MS spectrum for AFCN (**1**) (panel A), AFSCN (**2**) (panel B), AFN_3 (**3**) (panel C) and AF (**4**) (panel D) incubated for 1 h with bovine serum albumin 10^{-4} M (3:1 metal to protein molar ratio) at 37 °C in 20 mM ammonium acetate buffer, pH 6.8. Peak (a) is 66460.38 Da, peak (b) is 66546.61 Da.

not able to understand in full the reasons for this behavior; probably the observed shift in the mono/bis-adduct equilibrium is due to the greater thermodynamic stability of the mono-adduct and to its diminished steric hindrance. On the other hand, for AFCN (**1**) the major protein complex still was the mono-adduct, while for AFN_3 (**3**) the presence and the relative abundance of the bis-adduct remained unchanged with time. As expected, in the case of AF (**4**), BSA + $AuPEt_3^+$ was the only species observed in the ESI-MS spectra recorded over 72 h (Figure 2D). As mentioned above, a couple of PTMs of native BSA are detected in all mass spectra recorded after 1 h of incubation with unchanged relative abundances. It is interesting to note as for incubation times greater than 24 h the signals belonging to these PTMs progressively disappear. Probably, the higher stability of the Au-S bond compared to S-S, and the presence of an excess of the free gold compounds in solution determined the progressive decrease of these PTMs with a consequent increase in gold-BSA adduct formation.

It is remarkable that in all cases the phosphane ligand remains coordinated to the gold(I) center while the pseudohalide ligand is replaced by the thiol group, in agreement with previous observations by Shaw[8] and Bierbach.[32] Also, a rather clear correlation between the chemical features of the three pseudohalide leaving groups (the order of nucleophilicity being $CN^- > SCN^- > N_3^-$) and their reactivity with the thiol group could be established. In fact, depending on the nature of the pseudohalide, the reactivity of the various gold complexes towards this biological target varied accordingly.[33] In other words, a sort of fine-tuning of the reactivity, and then of the typology of metal/protein adducts formed, was easily and uniquely revealed through ESI-MS.

In conclusion we have shown here that high resolution ESI-Q-TOF MS experiments, owing to the latest technological advancements, offer today a straightforward and excellent tool to analyse the reactions of metal-based drugs with proteins as large as serum albumin. Remarkably, these experiments are carried out through direct sample infusion with minimal sample manipulation and minimal sample consumption. Detailed information is gained on protein speciation and adduct formation. In addition, as ESI-MS spectra are obtained in a very short time, detailed time course experiments may be carried out easily. The method is so informative that in spite of the occurrence of roughly the same kind of reactivity between the various gold(I) complexes and BSA, subtle differences in the

kinetics of adduct formation and in the nature and distribution of the formed adducts could be highlighted. This kind of approach will now be extended to characterize the adducts that this important serum protein does form with other metal-based drugs of even greater biological and medical relevance.

ASSOCIATED CONTENT

Supporting Information

Listing of experimental details and ESI-MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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Table of Contents Graphic and Synopsis

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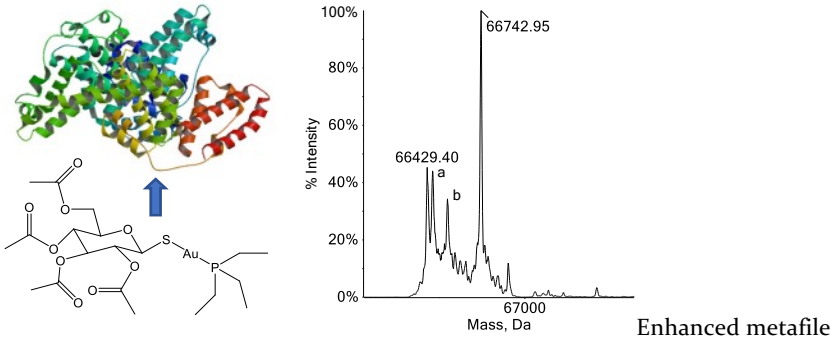

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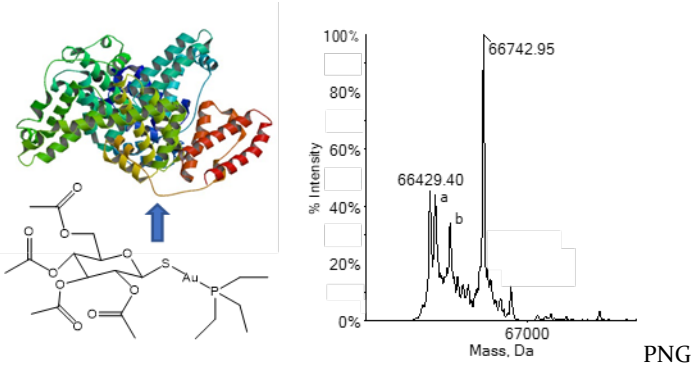

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