Investigation of Non Covalent Interactions between Paramagnetic Complexes and Human Serum Albumin by Electrospray Mass Spectrometry

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Running title: Study of affinity of contrast agents for serum albumin.

Abstract

Stable gadolinium (III) chelates are nowadays routinely used as contrast agents for magnetic resonance imaging (MRI). Their noncovalent binding to Human Serum Albumin (HSA) has shown to improve their efficacy. Noncovalent interactions lead to complex formation that can be quantified by several techniques usually tedious and time consuming. In this study, electrospray-ionization (ESI) mass spectrometry was used to investigate the interaction between HSA and several gadolinium (III) complexes. The results were
compared to those obtained in liquid phase. Four gadolinium complexes were investigated: Gd-DTPA 1, Gd-C₄Me-DTPA 2, Gd-EOB-DTPA 3, and MP-2269 4. The relaxometry study shows that complexes 1 and 2 have no significant affinity for HSA, while 3 and 4 show increasing affinities for the protein. By ESI-MS, 1:1 and 1:2 complexes between HSA and MP-2269 were detected for a twofold excess of the contrast agent, whereas a ligand/protein molar ratio of 4/1 was necessary to observe a 1:1 stoichiometry for Gd-EOB-DTPA, an observation which is in good agreement with the known weaker affinity of the contrast agent for the protein. At a 4-fold molar excess, no supramolecular complex was observed for Gd-DTPA 1 and Gd-C₄Me-DTPA 2. A tenfold molar excess was necessary to detect a 1:1 complex, confirming the very weak affinity of these contrast agents for HSA.

Introduction

Contrast agents for magnetic resonance imaging (MRI) are compounds which are able to enhance the contrast of the image by altering the water proton longitudinal (T₁) and/or transverse (T₂) relaxation times of the surrounding tissues (¹,²). Stable gadolinium chelates are widely used because of the physicochemical properties of this lanthanide (seven unpaired electrons, nine coordination sites and favorable electronic relaxation time). The complexation of this rare earth ion with an organic ligand like a polycarboxylic acid is necessary to minimise its toxicity but reduces to some extent its efficacy as the active core of the contrast agent. Through their action on the relaxation time T₁, these paramagnetic complexes induce an increase of the MR signal intensity;
consequently, areas of the body containing those contrast agents will appear brighter in the MR image.

When properly designed, these complexes are able to bind noncovalently to endogenous macromolecules. Such interactions reduce the molecular mobility of the complex, leading to a better modulation of the magnetic interactions between the unpaired electrons of the paramagnetic ion and the water protons and therefore to an improvement of the efficiency (relaxivity) of the agents\(^3\). On the other hand, binding to blood proteins prolongs the vascular residence time of the contrast agents, a situation which is favorable to angiography and perfusion imaging\(^4\).

Human serum albumin (HSA, calculated molecular weight from the sequence 66,437 Da\(^5\)) is one of the proteins targeted by magnetic resonance agents because it is a large globular protein known to constitute about 4.5% of the plasma and to bind a large variety of molecules. The binding of a drug to this protein reduces its renal excretion rate and extends its blood half-life.

The commercial contrast agent Gd-DTPA 1 or Magnevist\(^\circledR\) (Schering, Germany) has no significant affinity for HSA but some of its derivatives can bind noncovalently to the protein. For example, the Gd (III) complex of 4-pentylbicyclo[2.2.2]octane-1-carboxyldi-L-aspartyllysine-derived DPTA, known as MP-2269 4, (figure1) shows by proton relaxometry a high affinity for serum albumin and by imaging an excellent vascular enhancement\(^6,7,8\). Gd-(S)-EOB-DTPA 3 [(4S)-4-(4-ethoxybenzyl)-3,6,9-tris(carboxymethyl)-3,6,9-triazaundecanedioic acid, gadolinium complex] designed as a hepatocyte agent is also known for its moderate noncovalent binding to HSA\(^9\).
Specific noncovalent binding is a driving force in the context of tissue-specific molecular imaging. It is therefore highly important to estimate the affinity of a contrast agent with respect to its target molecule. Such noncovalent interactions can be quantified by several techniques which are usually tedious and time consuming, like equilibrium dialysis, ultrafiltration, chromatography, ultracentrifugation\(^{10}\) and relaxometry. Today, mass spectrometry is emerging as a powerful tool for studying noncovalent interactions including protein interactions with inhibitors, cofactors, metal ions, or other peptides\(^{11,12,13}\). From the measurement of the molecular mass of the entire complex, and knowing the mass of the individual binding partners, the stoichiometry can be derived. Electrospray ionization mass spectrometry (ESI-MS) can thus provide complementary data to those obtained by more traditional techniques. In this work, we apply ESI-MS to study the noncovalent interactions between human serum albumin (HSA) and the contrast agents 1-4, known for their diverse affinities for the protein. It has to be mentioned that among those complexes, only 1 and 3 are commercially available for clinical use.

**Materials and Methods**

Electrospray mass spectra were obtained on a Q-tof 2 (Micromass, Manchester, UK). The nanospray source was operated in the positive ion mode at a capillary voltage of 1.4 kV. Samples, dissolved in ammonium acetate (100 mM), were injected at a flow rate of a few nL/min with needles. Each spectrum is the sum of approx. 400 scans. The raw spectra were then baseline corrected before deconvolution, which was performed using the program MaxEnt\(^{1}\) TM.
Non defatted HSA A-1653, purchased from Sigma (Bornem, Belgium), was desalted by five dilution-concentration steps using Microcon YM-10 from Millipore (Brussels, Belgium). The protein concentration was measured spectrophotometrically (UV 280 nm) (8452A diode array spectrophotometer Hewlett-Packard, Brussels, Belgium). The concentration of albumin samples injected in the mass spectrometer was 5 µM.

Gd-C₄-Me-DTPA 2 was prepared as described recently(14), Gd-DTPA 1 and Gd-EOB-DTPA 3 were provided by Schering AG (Berlin, Germany), and MP-2269 4 was provided by Mallinckrodt (Saint-Louis, USA) (figure 1). All experiments were done with a fixed HSA concentration (5 µM), while the concentration of the ligand was varied between 5 and 50 µM, depending on its affinity towards the protein.

**Results and Discussion**

Human serum albumin, the target protein in our study, is the major proteinic component of blood plasma with a molecular weight of about 66437 Da. It contains 585 aminoacids, 35 of which are cystein residues involved in 17 disulphide bridges. Some publications concerning the applications of electrospray ionisation mass spectrometry to biological compounds have shown spectra of human and bovine serum albumin recorded on positive mode with very high charge states indicating a partial denaturation of the protein(15-19). In order to use mass spectrometry as a complementary technique to study the affinity of contrast agents towards human serum albumin, it is important to find experimental conditions that keep the protein in its native state and limit the formation of adducts with the macromolecule. The protein was thus dissolved in
a solution of ammonium acetate. In this solution, four charge states ranging from 15+ to 18+ were observed, in agreement with a molecular weight of 66,540 Da. The molecular weight of HSA, computed from SwissProt entry P02768 using the ExPASy tool “pI/MW calculator” (assuming no modifications) is 66472.21. Thus, ammonium acetate disturbs only slightly the conformation of human serum albumin. However, it has to be mentioned that the protein shows a very weak mass spectrometric response.

We explored the effect of the source temperature and the cone voltage on the spectra of HSA in ammonium acetate. From 65°C to 125°C at a high cone voltage (180V), the mass spectrum does not show an additional peak but the signal intensities are slightly increased at high temperature due to a better desolvation. The peak with the maximum intensity is always the one corresponding to the charge state 16+. As expected, a decrease of cone voltage causes a significant increase in the width of each peak, from about 30 m/z for a cone voltage of 180 volts to about 100 m/Z for a cone voltage of 60 volts at low temperature. We decided thus to record all spectra at a cone voltage of 180 volts and a source temperature of 80°C in order to obtain a good signal intensity as well as a good spectral resolution.

The first contrast agent tested, MP-2269 4, is known to interact by noncovalent binding with HSA in solution. When the concentrations of HSA and MP-2269 were equal (5 µM), signals corresponding to a complex between the protein and one molecule of contrast agent were observed, in addition to the peaks corresponding to free HSA. When the MP-2269 4 concentration was twice the concentration of the target, a new signal corresponding to a stoichiometry 2:1
clearly appeared. When the concentration of contrast agent was increased until 20 µM, this stoichiometry was preserved and the intensities of the peaks of the free protein decreased. For each massive, the intensity of the peak of the complex between the protein and one ligand was the most important (figure 2). Different to the spectrum of HSA alone, the charge state showing the highest intensity was 17+.

For Gd-EOB-DTPA 3, which is known to have a moderate affinity for HSA, a fourfold molar excess was necessary to observe a signal corresponding to a complex with a 1:1 stoichiometry. When the concentration of Gd-EOB-DTPA 3 was brought to a ten molar excess, the stoichiometry reached a maximum of three molecules of contrast agents bound to the protein (figure 3). As observed above with MP-2269, the charge state showing the highest intensity was 17+.

In order to confirm that the detection in the gas phase reflects the behaviour observed in solution, we studied the affinity of Gd-DTPA 1, a contrast agent showing no affinity for HSA in solution. With ESI-MS, at least a tenfold molar excess was necessary to observe some association between Gd-DTPA and the protein (figure 4).

Similarly, for Gd-C₄Me-DTPA 2 a complex corresponding to one ligand bound to the protein was observed for a tenfold molar excess of ligand (figure 4). At lower ligand concentrations no complex peak could be detected.

The relative intensities of the multiply charged ions from the various noncovalent complexes were compared. An excellent qualitative correlation was found with the binding constants known from solution studies, indicating that the results of the titration experiments carried out with the electrospray mass
spectrometry technique can be used for qualitative assessment of such interactions.

In order to obtain quantitative information about the affinity of Gd-EOB-DTPA 3 and MP-2269 4 for HSA, the peak heights of the various multiply charged ions of the free protein and of the protein-contrast agent complex were summed to evaluate the respective concentrations. This treatment was performed by using the program MaxEnt1™ (figure 5) which uses the maximum entropy method to reconstruct singly charged mass spectra from multiply charged ones. With this treatment, we could obtain the concentrations of the different species in gas phase (equations 1 and 2).

\[
\frac{C_1}{(C_1 + C_2 + C_3 + \ldots + P_f)} [P_0]
\]

concentration of complex 1

\[
\frac{P_f}{(C_1 + C_2 + C_3 + \ldots + P_f)} [P_0]
\]

equation 1 concentration of free protein

equation 2

Where \(C_1, C_2, C_3, \ldots\) are the peak intensities of the different complexes, \(P_f\) is the peak intensity of the free protein and \([P_0]\) is the total concentration of protein in solution.

Using the concentration values obtained by this procedure, an apparent association constant was calculated from the data of each solution assuming two identical binding sites, in agreement with earlier relaxometry studies. Apparent association constants of about 27000 M\(^{-1}\) and about 5000 M\(^{-1}\) were obtained (table 1) for MP-2269 4 and Gd-EOB-DTPA 3 respectively, following the same trends as the constants determined by relaxometry.
Conclusion

The advantages and disadvantages of ESI-MS are often discussed and compared to other biophysical methods for the investigation of noncovalent interactions. At present, soft ionisation mass spectrometry is increasingly used to analyse noncovalent complexes. The present study of the interaction between HSA and MRI contrast agent offers a previously unreported system confirming the versatility of electrospray mass spectrometry for the characterisation of noncovalent complexes. In fact, the study of interactions between HSA and MRI contrast agents by ESI-MS allowed a confirmation of their affinities for HSA. Thus, MP-2269 4 is the contrast agent which has the best affinity for the protein, followed by Gd-EOB-DTPA 3.

For Gd-DTPA 1 and Gd-C4Me-DTPA 2, the titration experiment showed that it was necessary to reach a tenfold excess of ligand to observe peaks corresponding to a complex. These contrast agents have thus very low, if any, affinity for HSA. Another advantage of mass spectrometry is the direct information on stoichiometry. The number of ligands noncovalently bound to the macromolecule is easily determined. This certainly represents important information for the evaluation of the association constant that is not easily obtained by classical biophysical techniques. Thanks to its speed and sensitivity, electrospray mass spectrometry is a technique able to compete with usual techniques.
Acknowledgements

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Figure 1: Structures of Gd-DTPA 1 (MW=591), Gd-C₄Me-DTPA 2 (MW=605), Gd-EOB-DTPA 3 (MW=725), and MP-2269 4 (MW=1142).

Figure 2: Electrospray spectra of HSA (5 µM) with MP-2269 at A: 5 µM, B: 10 µM, C: 20 µM. D: Electrospray spectra of HSA without contrast agent. ▼ free HSA, • HSA+1ligand, ⊘ HSA+2 ligands.
Figure 3: Electrospray spectra of HSA (5 µM) with Gd-EOB-DTPA at A: 20 µM, B: 50 µM. ▽ free HSA, • HSA+1ligand, ⊗ HSA+2 ligands, * HSA+3 ligands.

Figure 4: Electrospray spectra of HSA (5 µM) with Gd-DTPA and Gd-C₄-Me-DTPA at A: Gd-DTPA 20 µM, B: Gd-DTPA 50 µM, C: Gd-C₄Me-DTPA 20 µM, D: Gd-C₄Me-DTPA 50 µM. ▽ free HSA, • HSA+1ligand.
Figure 5: Procedure used for the quantitative processing of the mass spectra (example: HSA 5 µM / MP-2269 10 µM).
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Table 1: Association constants calculated from spectra in real mass.
References


