APPLICATION NOTE

Quantifying Oligonucleotides Binding to Human Serum Albumin with MST: A Faster and Precise Approach for Drug Candidates ADME Profiling

Tatjana Grasser[1], Ingo Röhl[1], Hans-Peter Vornlocher[1], Nuska Tschammer[2]

[1]Axolabs GmbH, Germany

[2]NanoTemper Technologies , Germany

Abstract

The binding of therapeutic drug candidates to human serum albumin (HSA) and other plasma proteins needs to be monitored and profiled during the development of drug candidates as it affects their adsorption, distribution, metabolism, and excretion (ADME).

Antisense oligonucleotides are an emerging therapeutic option to treat diseases with known genetic origin. The interaction of these oligonucleotides with proteins like HSA determines their pharmacokinetics (transport and distribution in target tissues) and pharmacodynamic (binding to the mRNA target) properties and hence their eventual pharmacology. Fast characterization of their protein-binding properties requires a simple, robust and reliable method for the quantification of oligonucleotide binding properties to HSA.

In this work, we illustrate the versatility of MST to determine micromolar binding affinities of Cy5-labeled oligonucleotides for HSA. Importantly, these Cy5-labeled oligonucleotides can be used as a tracer to determine the EC50 of an unlabeled oligonucleotide in a competition assay.

Introduction

Human serum albumin (HSA) is the most abundant protein in plasma — typically present at concentrations of 35 – 50 mg per mL of plasma — and displays an extraordinary ligand-

binding capacity, providing a depot and carrier for many endogenous and exogenous compounds[1,2]. It represents the main carrier for fatty acids, affects pharmacokinetics of many drugs, provides the metabolic modification of some ligands, renders potential toxins harmless, accounts for most of the anti-oxidant capacity of human plasma, and displays pseudo-enzymatic properties[2]. HSA appears to be formed by three homologous domains and each domain is made up by two separate helical domains that are connected by random coil[3] with a variety of binding sites. The conformational adaptability of HSA involves more than the immediate vicinity of the binding site(s), this being the root for the observed ligand-dependent allosteric conformational transitions[3,4]. Notably, HSA undergoes pH- and allosteric effector-dependent reversible conformational isomerization[3] that affects its binding to drugs. For example, although Warfarin and Ibuprofen bind to sites distinct from the sites for fatty acid binding, they compete with fatty acid binding to HSA. Allosteric interactions have been reported to affect binding equilibria between HSA and Ibuprofen, Warfarin and Lorazepam[3].

Aside of mentioned natural and synthetic ligands, HSA also binds to phosphorothioates and oligonucleotides conjugated to ligands like Ibuprofen[5], which is of importance when developing oligonucleotide-based drugs. The interaction of antisense oligonucleotides with HSA determines their pharmacokinetics (transport and distribution in target tissues) and pharmacodynamic (binding to the mRNA target) properties and hence their eventual pharmacology[5]. To develop safer and more effective oligonucleotide drug candidates, it would be valuable to enhance the interaction of these molecules with proteins involved in transport and absorption and to minimize the interaction with proteins responsible for undesirable side effects.

Currently, the most commonly used method for the quantification of interaction between the oligomers and HSA is the electrophoretic mobility shift assay (EMSA)[6,7]. In this assay a solution of protein and nucleic acid are combined, and the resulting mixtures are subjected to electrophoresis under native conditions through polyacrylamide or agarose gel. After electrophoresis, the distribution of species containing nucleic acid is determined, usually by autoradiography of ^{32}P-labeled nucleic acid. EMSA is often considered to bear number of limitations and disadvantages like the fact that dissociation can occur during the electrophoresis since samples are not at equilibrium during the run[7], the use of radioactive material (^{32}P), problematic waste disposal and inconvenient detection and subsequent quantification. The MST assay on the other hand, offers an alternative approach with excellent sensitivity, the advantage of detecting the interaction in equilibrium

and in solution with only minute amounts of fluorescently-labeled oligonucleotides or proteins. And unlike other approaches, a K_d can be measured in less than 10 min using Monolith NT.115, or less than 2 min using the Monolith NT.Automated.

Results and discussion

In order to avoid modification of HSA by labeling with a fluorophore that could allosterically influence the binding of the oligonucleotides, we decided to label the oligonucleotide with Cy5 instead. This approach also means one can use the labelled oligonucleotide in a competition assay with any unlabeled oligonucleotide. Phosphate buffer saline (PBS) was used as the buffer for the MST assays. Because Tween-20 interacts with HSA[6], we performed the binding experiments with and without 0.05% Tween-20 in the assay buffer. The addition of Tween-20 to PBS resulted in over three-fold worsening of the K_d value for the Cy5-modified phosphorothioate oligonucleotide. Consequently, all experiments were performed in the absence of Tween-20. The K_d value determined was 64.9 ± 2.7 µM (Figure 1, left panel). Interestingly, no binding of the Cy5-cholesterol-conjugated oligonucleotide could be determined in the absence of Tween-20. Upon the addition of 0.05% Tween-20, the binding of this oligomer to HSA could be determined with the K_d value of 28.4 ± 3.8 µM (Figure 1, right panel). We hypothesize that Tween-20 binds to the fatty acid binding pocket[8] causing the conformational change, which in turn allosterically enhances the binding of the Cy5-cholesterol-conjugated oligonucleotide to HSA.

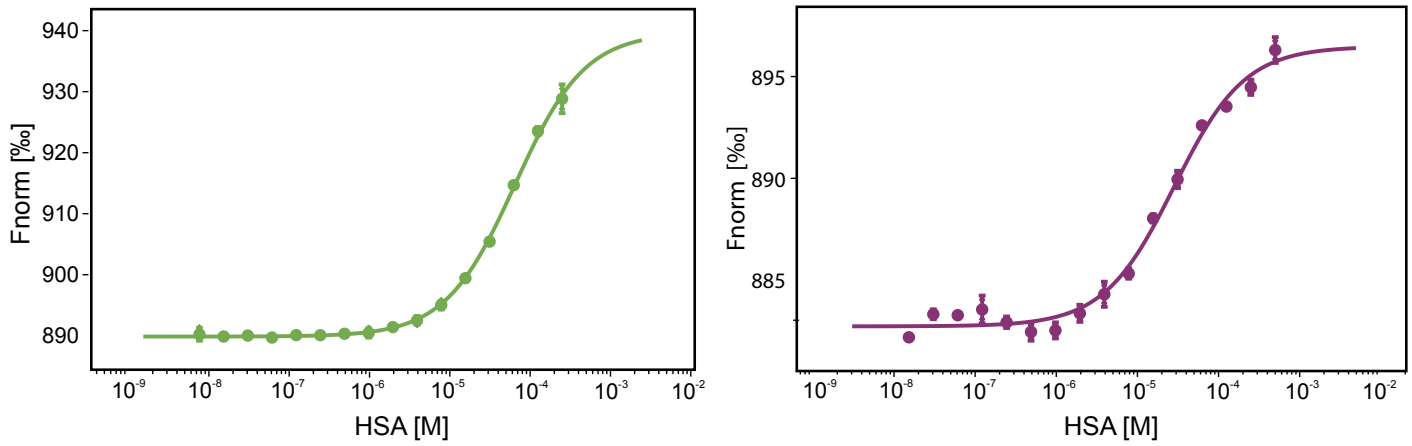
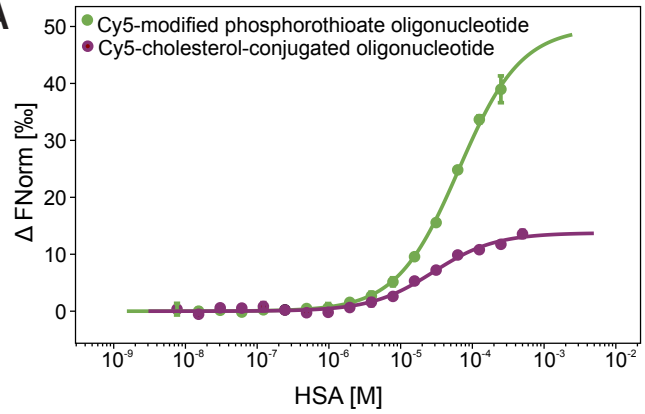


Figure 1: The Cy-5 labeled nucleotides bind to HSA. For the experiments 20 nM of labeled nucleotide was used and HSA titrated at indicated concentrations. Left panel: Cy5- modified phosphorothioate oligonucleotide, K_d = 64.9 ± 2.7 µM; right panel: Cy5-cholesterol-conjugated oligonucleotide, K_d = 28.4 ± 3.8 µM.

The comparison of binding profiles of the Cy5-labeled oligonucleotides is shown in Figure 2. Since the Cy5-modified phosphorothioate oligonucleotide offers a nearly four-fold increase in signal amplitude and better signal-to-noise ratio compared to the Cy5-cholesterol-conjugated oligonucleotide it was chosen for further competition experiments.

For the competition assay HSA (100 µM) was prelabeled with the Cy5-modified phosphorothioate oligonucleotide (50 nM) for 15 min. Then, either the unlabeled phosphorothioate modified oligonucleotide or cholesterol-conjugated oligonucleotide were added in increasing concentrations and incubated for an additional 15 min to displace the labeled oligonucleotide from the binding pocket in PBS without Tween-20. Both unlabeled oligonucleotides readily displaced the Cy5-modified phosphorothioate oligonucleotide (Figure 3). This is reflected by the EC50 values: 8.6 ± 1.0 µM for the phosphorothioate modified oligonucleotide, and 3.8 ± 0.4 µM for the cholesterol-conjugated oligonucleotide.

A



B

Cy-5 labeled nucleotide	Amplitude	Signal-to-noise
Phosphorothioate oligonucleotide	49.8	137.9
Cholesterol-conjugated oligonucleotide	13.7	28.1

Figure 2: Comparison of the dose-response curve of both Cy5-labeled nucleotides (A). The Cy5- modified phosphorothioate oligonucleotide yields nearly four-fold larger amplitude and better signal-to-noise ratio than the Cy5-cholesterol-conjugated oligonucleotide (B).

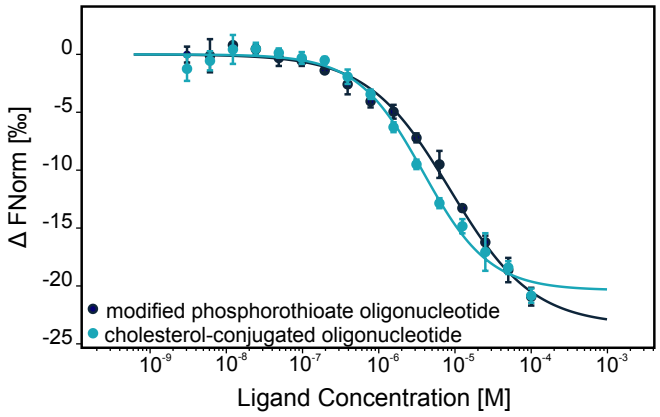


Figure 3: Competition assay between the Cy5-modified phosphorothioate oligonucleotide and the ligands modified phosphorothioate and cholesterol-conjugated oligonucleotides. HSA (100 µM) was prelabeled with the Cy5-fully modified phosphorothioate oligonucleotide (50 nM) and the unlabeled ligand titrated at indicated concentrations. The EC50 values determined were 8.6 ± 1.0 µM for the modified phosphorothioate oligonucleotide, and 3.8 ± 0.4 µM for the cholesterol-conjugated oligonucleotide.

Overall, the use of Cy5-labeled oligonucleotide as a tracer is an excellent approach to quantify the oligonucleotide binding properties to HSA. All MST experiments described here are characterized by an excellent signal-to-noise ratio that enables reliable determination of affinities between the oligonucleotides and HSA.

Conclusions

Antisense oligonucleotides are an emerging therapeutic option to treat diseases with known genetic origin. Their pharmacokinetics and pharmacodynamics are dependent on their interaction with plasma proteins like HSA. We have shown that MST offers unprecedented advantages over classical assay like EMSA for the quantification of oligonucleotide binding properties to HSA. It delivers fast and precise K_d values. The high information content of MST data enables straightforward assay development including the selection of the optimal assay buffer. For this particular interaction we recommend the use of Cy5-labeled oligonucleotide as a tracer. This tracer can be used for the screenings of a variety of different oligonucleotide constructs providing flexibility and most convenient assay setup.

Materials and Methods

Oligonucleotide Synthesis

All nucleotides were provided by AxoLabs GmbH (Kulmbach, Germany) and were produced by automated chemical solid-phase synthesis using commercially available building blocks. After cleavage from the solid support, the synthesized oligos were purified by high performance liquid chromatography (HPLC).

MST experiments

For the MST experiments the following oligonucleotides were tested: cholesterol-conjugated oligonucleotide, fully phosphorothioate modified oligonucleotide, Cy5 coupled to a fully phosphorothioate modified oligonucleotide and Cy5 coupled to a cholesterol-conjugated oligonucleotide. HSA (Cat# A9731, Lot# SLBC7527V, Sigma Aldrich) was used as a binding partner. Buffer phosphate buffered saline (PBS, pH 7.4) with or without 0.05% Tween-20 was used as assay buffer. The Cy5-labeled oligomer was prepared at 20 nM and working concentration solutions and dilution series were prepared either in Protein LoBind Eppendorf Tubes (Cat# 003 108.094, Eppendorf) or non-binding surface 384-well assay plate (Cat# 4513, Corning). For the competition assays 100 µM of HSA was preincubated for 15 min with 50 nM of Cy5-labeled oligomer in PBS. The probes were filled in Monolith NT.115 Premium Capillaries (Cat# MO-K025, NanoTemper Technologies).

Instrumentation and data analysis

Measurements were performed on a NanoTemper Monolith NT.115 instrument. Final Cy5-oligonucleotide concentration of 20 nM yielded fluorescence intensities of about ~400 counts at an LED power of 20%. The samples were measured at high MST power using MO.Control software to acquire the data. The parameters were deduced after 1.5 s MST on-time and the resulting dose-response curves fitted with MO.Affinity Analysis either to a one-site binding model to extract K_d values or, in the case of the competition assays, fitted by Hill model to determine the EC50 values.

References

1 Varshney, Ankita, et al. "Ligand binding strategies of human serum albumin: how can the cargo be utilized?" Chirality: The Pharmacological, Biological, and Chemical Consequences of Molecular Asymmetry 22.1 (2010): 77-87.

2 Fanali, Gabriella, et al. "Human serum albumin: from bench to bedside." Molecular aspects of medicine 33.3 (2012): 209-290.

3 Fasano, Mauro, et al. "The extraordinary ligand binding properties of human serum albumin." IUBMB life 57.12 (2005): 787-796.

4 Manoharan, Muthiah, et al. "Improving antisense oligonucleotide binding to human serum albumin: Dramatic effect of ibuprofen conjugation." ChemBioChem 3.12 (2002): 1257-1260.

5 Crooke, Stanley T. "Basic principles of antisense therapeutics." Antisense research and application. Springer, Berlin, Heidelberg, 1998. 1-50.

6 Hellman, Lance M., and Michael G. Fried. "Electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions." Nature protocols 2.8 (2007): 1849.

7 Alves, Carolina, and Celso Cunha. "Electrophoretic mobility shift assay: Analyzing protein-nucleic acid interactions." Gel Electrophoresis-Advanced Techniques. InTech, 2012.

8 Garidel, Patrick, Claudia Hoffmann, and Alfred Blume. "A thermodynamic analysis of the binding interaction between polysorbate 20 and 80 with human serum albumins and immunoglobulins: A contribution to understand colloidal protein stabilisation." Biophysical chemistry 143.1-2 (2009): 70-78.