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On the molecular interaction between albumin and ibuprofen: An AFM and QCM-D study

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ABSTRACT

The adsorption of proteins on surfaces often results in a change of their structural behavior and consequently, a loss of bioactivity. One experimental method to study interactions on a molecular level is single molecular force spectroscopy that permits to measure forces down to the pico-newton range.

In this work, the binding force between human serum albumin (HSA), covalently immobilized on glutaraldehyde modified gold substrates, and ibuprofen sodium salt was studied by means of single molecular force spectroscopy. First of all, a protocol was established to functionalize atomic force microscopy (AFM) tips with ibuprofen. The immobilization protocol was additionally tested by quartz crystal microbalance with dissipation (QCM-D) and contact angle measurements. AFM was used to characterize the adsorption of HSA on gold substrates, which lead to a packed monolayer of thickness slightly lower than the reported value in solution.

Finally, single molecule spectroscopy results were used to characterize the binding force between albumin and ibuprofen and calculate the distance of the transition state (0.6 nm) and the dissociation rate constant (0.055 s^{-1}). The results might indicate that part of the adsorbed protein still preserves its functionality upon adsorption.

1. Introduction

Molecular recognition is involved in many biological events such as antigen-antibody recognition, molecular transport, lectin-carbohydrate interactions, ligands and cellular receptors or cell adhesion. Such interactions are non-covalent, of short range and depend on the recognition site geometry and orientation [1–4].

The binding between proteins and specific ligands is among other things, protein structure dependent. So, any structural rearrangement in proteins might affect the binding with the ligand. Adsorption is one of those events where the protein undergoes structural rearrangements and might cause the loss of its bioactivity [5–7]. Therefore, the quantification of the biological surface activity is very important for the development of bioanalytical techniques and bioimmunosensor surfaces which are of medical and pharmacological interest [8].

Atomic force microscopy (AFM) is not only a suitable technique to obtain high resolution images of the morphology of biological surfaces under physiological conditions [9,10]; by means of single molecular force spectroscopy studies it is possible to measure molecular forces down to few piconewtons as well as to characterize the elastic behavior of biomaterials at the nanoscale [2,11–15]. In particular, force spectroscopy has been used successfully to measure forces between individual molecules (e.g., ligand–receptor complexes). This type of experiments allows understanding the energetic landscape of the interaction by quantifying the distance to the transition state (x_u) and the dissociation rate constant (K_{off}), which is well explained in the work of Bell, Evans and Ritchie [16,17].

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In these experiments, the receptors are usually immobilized on a substrate while the AFM tip is functionalized with the ligand. The next step is to approach the tip towards the surface-bound receptor. This can be done at different approach rates (speed) and at different loads (force exerted on the surface). Once the tip approaches the surface the ligand and receptor complex can be formed. These experiments also permit to vary the ligand-receptor residence time. Then, by pulling the tip away from the surface (with the receptor) the unbinding force of the complex can be measured. An important point to take into account is the binding strength of the receptor and ligand to the substrate and the AFM-tip respectively, which must be stronger than the intermolecular forces between both molecules. This is normally achieved by using self-assembly monolayers (SAMs) or cross linking agents [1].

The importance of this kind of studies has grown in the last 15 years; many biological systems have been investigated by single force spectroscopy, where it has been quantitatively determined the interaction strength of ligands and their receptors.

Among them we can cite, the interactions of avidin–biotin or biotin–streptavidin, digoxigenin–antibody complex or $\alpha_{5\beta1}$ integrin–fibronectin [18–23]. More recent works have demonstrated the utility of single molecular spectroscopy to study importin–nucleoporin molecules involved in nuclear transport, or to develop new strategies for cancer therapy as it is the case of luteinizing hormone releasing hormone (LHRH) and a specific exotoxin, LHRH-PE40 [24,25].

Within the large variety of proteins, there are some that have the ability to interact with several molecules. This is the case of human serum albumin (HSA) which is the most prominent plasma protein involved in the transport of a wide variety of endogenous and exogenous compounds (e.g., bile pigments, fatty acids, drugs, etc.) and it is one of the most widely studied proteins by force spectroscopy [26,27].

HSA (66.7 kDa), that has a globular heart-shaped molecule with a size of $8 \times 8 \times 3\,nm^3$ [3], presents three main domains named I–III, with different grooves where the molecules can be bound reversibly to be transported through the blood [28]. The ligands do not form specific interactions as biotin–avidin or biotin–streptavidin, but they can bind to different binding sites with different binding affinities. This makes HSA a very versatile protein. Furthermore, the ligand may also compete for the same binding pockets which could influence the effectivity of the drug distribution.

In general, human serum albumin is an important factor to determine the pharmacokinetics behavior of many drugs. Therefore, the control of such events is especially relevant for the pharmaceutical industry and medical sciences because albumin is responsible for the binding, the transport and the delivery of a variety of molecules (metabolic products but also drugs) in blood. Among them ibuprofen is one of the most widely used drugs due to its antipyretic, analgesic, and antibacterial activity [29].

In this work, force spectroscopy is used to study HSA adlayer bioactivity by measuring the force between ibuprofen and immobilized HSA on glutaraldehyde. We also show a procedure to immobilize ibuprofen on gold coated AFM-tips. The functionalization of gold surface with Ibuprofen-N$(EG)_6C_{11}$SH has also been investigated with QCM-D measurements, contact angle and ellipsometry. Although a particular case is presented this method can be extended to investigate the interaction of HSA with other ligands, to compare other drugs that can compete for the same binding sites. In addition, the calculation of the dissociation rate constant can serve to complement other thermodynamic quantities, such as the association rate constant, obtained with other techniques in order to achieve a broader spectra of the interactions between protein and ligand.

2. Materials and methods

2.1. Materials

Ibuprofen sodium salt (Mw. 228 g mol^{-1}) and human serum albumin (HSA), 66.4 kDa, were purchased from Sigma–Aldrich. $N_3(EG)_6C_{11}Ac$ (Mw. 535 g mol^{-1}) and $OH(EG)_3C_{11}SH$ (Mw. 336 g mol^{-1}) were supplied by Prochimia. Note that EG stands for ethylene glycol = $(CH_2CH_2O)_n$, C for methylene = $CH_2)_n$, and SAc for thioacetate = $SCOCH_3$. *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) and *N*-hydrohysuccinimide (NHS) were supplied by Sigma–Aldrich. Sodium methoxide (MeONa) used in de-acetylation was purchased from Sigma–Aldrich**.** Trimethilphosphine (PMe_3) and 4-dimethylaminopyridine (DMAP) used as a basic catalyst in the peptidic coupling was provided from Sigma–Aldrich. Amberlite H and sodium sulfate (Na_2SO_4) were purchased from Sigma–Aldrich.

Methanol (MeOH), tetrahydrofuran (THF), dimethylformamide (DMF), ethyl acetate, ethanol (Scharlau), acetone (Sigma–Aldrich) and diethyl ether were used as solvents. Cysteamine hydrochloride and glutaraldehyde (% 25) for gold functionalization were provided by Sigma–Aldrich.

The buffers were prepared with sodium hydroxide (NaOH, Sigma–Aldrich), and sodium dihydrogen phosphate anhydrous (NaH_2PO_4) and sodium phosphate dibasic anhydrous (Na_2HPO_4, Fluka). Finally, mica supports were provided by SPI.

2.2. Surface and sample preparation

Gold film deposition on mica: Gold films were deposited on mica ($1 \times 1\,cm^2$) using the magnetron sputtering technique (ATC 1800, AJA). A 100 nm gold layer was deposited at 150 W, in argon atmosphere at a pressure of 3 mTorr and during the deposition the substrates rotated at 50 rpm for better uniformity. The deposition time was 4 min. The samples were not previously heated. The pressure of the main vacuum chamber was at 1×10^{-8} Torr.

Substrates cleaning procedure: Gold substrates were cleaned with (alternating) ethanol and acetone solutions. The substrates were left in each solvent for 30 min. Then, the substrates were treated with ultraviolet radiation (UV-ozone, Bioforce nanoscience) for 30 min.

HSA immobilization: Gold substrates were immersed in cysteamine hydrochloride water (20 mM) solution overnight which was covered with aluminum foil. The substrates were rinsed with water to remove excess of cysteamine hydrochloride. Afterwards, they were immersed in 1% glutaraldehyde aqueous for 1 h and rinsed gently with water before use for albumin immobilization. Finally, the functionalized gold supports were immersed in a solution of 1 mg mL^{-1} HSA for an incubation time of one hour. The substrates (sensors) were rinsed with water and used immediately after biofunctionalization. All the steps were carried out at room temperature [30,31].

2.3. Synthesis of ibuprofen-N$(EG)_6C_{11}$SH

Ibuprofen-N$(EG)_6C_{11}$SH was synthesized using ibuprofen sodium salt and $N_3(EG)_6C_{11}$SAc as initial compounds.

For the $N_3(EG)_6C_{11}$-SAc thioacetate selective deprotection 50 mg (0.093 mmol) of $N_3(EG)_6C_{11}$SAc were dissolved in 1 mL of MeOH. Then, 155 μL of a 0.25 M MeONa solution were added at 0 °C and left reacting in argon atmosphere at room temperature overnight. When the reaction was finished acid Amberlite-H was added and left for 30 min to neutralize the solution. The product was filtrated and evaporated. 40 mg of $N_3(EG)_6C_{11}$SH (Mw. 493 g mol^{-1}) were obtained (see Fig. 1a,b). Yield: 85%; ^{1}H NMR