Development of a SPR aptasensor: towards a robust tool for detecting traces of lysozyme dimer in oligomeric and aggregated mixtures

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Protein aggregates formed in various steps during the manufacturing or storage of therapeutic proteins affect the quality, safety and efficacy of biopharmaceuticals[1]. While various methods have been developed to assess oligomers and aggregates of different sizes, the methods applicable for detecting aggregates under 50 nm have some limitations. Aggregates’ analysis is complicated by the existence of many of oligomers and conformers with different properties[2], moreover physical aggregates elude separation-based methods. For peptides and proteins undergoing amyloid-type aggregation various antibodies and aptamers have been developed recognizing with high selectivity either the monomeric, the oligomeric or the fibrillar form. Antibody or aptamer-based methods may complement well the current separation-based procedures for aggregate detection, in particular when combined with label-free detection methods such as Quartz Crystal Microbalance[3] or Surface Plasmon Resonance. Working towards developing a sensitive method that will allow detecting low amounts of oligomers in concentrated monomeric protein solutions, we hereby report the optimization of an SPR aptasensor[4] for the detection of lysozyme dimer, chosen here as model of protein oligomers. Lysozyme is a 14.3 kDa enzyme found in the human body as well as in plants, bacteria and animals. Lysozyme was widely used as a model for enzyme catalysis, protein structure, function and interactions, including protein aggregation, moreover was employed as a model in pharmaceutical applications, e.g. drug delivery, novel treatment strategies etc. The method described here exploits the differences in binding kinetics and affinity between the lysozyme monomer and dimer, to an aptamer developed for the monomeric form. In previous works we have optimized an aptasensor for the detection of monomeric lysozyme. The aptasensors design is based on coating the Au interfaces with a self-assembled monolayer of a thiol containing ethylene glycol groups and having carboxylic end groups, that allowed to further immobilize neutravidin by amine coupling. A lysozyme aptamer with the sequence 5’-5'-Biosg_TTT TTT TTT TTT TTT GCA GCT AAG CAG GCG GCT CAC AAA ACC ATT CGC ATG CGG C-3’, biotinylated at the 5’ end was attached next by affinity. The experimental setup included a detection system based on a Spreeta SPR2K23 SPR sensor (Texas Instruments, TX, USA), with a fitted 2-channel PDMS flow cell and a PC interface for signal acquisition. This aptasensor and setup were also used to study the self-association of lysozyme, where high non-specific adsorption of aggregated protein samples was observed[5]. Advancing from this, in this report we show that by changing the ionic strength, pH and composition of testing buffer the non-specific adsorption is minimized and the aptamer’s binding to a covalent lysozyme dimer is exacerbated in certain experimental conditions compared to the binding of monomer (Fig.1). Aiming to achieve quantitative evaluations of aggregates present in the protein sample, we produced intentionally a covalent lysozyme dimer by cross-linking with dimethylsulferimidate, we purified it by size-exclusion chromatography and we used it as standard. The aptasensor detected 1.4 nM lysozyme dimer at pH 7.4. Next, sensorgrams recorded for mixtures of monomer and dimer revealed different features compared to those for either monomer or dimer in individual solutions. Chemometric methods were applied to obtain qualitative and quantitative information regarding the composition of the analyzed mixtures. Seven features of the sensorgrams, representing areas under the curve for specific time intervals were selected as variables to describe the SPR signal for monomer-dimer mixtures. Based on these variables, Principal Components Analysis allowed observing groups of samples with the same dimer content and a relatively good separation between groups with different concentrations of dimer. Additionally, Multiple Regression analysis enabled to establish quantitative correlations between features of the sensorgrams and the dimer concentration in the samples (Fig.2). The amounts of dimer estimated by MR analysis of sensorgrams recorded with the aptasensor were very similar to the theoretical concentrations. By this approach the SPR aptasensor allowed determining 0.1-1% dimer in solutions of lysozyme monomer without any separation. This concentration range corresponds to the typical levels investigated with regards to degradation products and related compounds in pharmaceutical products, as the corresponding maximum allowed limits per compendia or manufacturer specifications are generally set in this range. The aptasensor was furthermore applied to observe the variations in lysozyme oligomer amounts during the aggregation of lysozyme solutions at 60°C and pH 2.
However, the thiol-coated SPR interfaces are not very advantageous for aggregated sampled as tightly binding oligomers mean that the sensor needs to be regenerated in more stringent conditions, which shortens its lifetime. As an alternative to the thiol-coated interfaces we explored graphene-coated surfaces deposited via layer-by-layer and functionalized with lysozyme aptamer[6].

First, clean Au interfaces were coated with poly(allyldiethylammonium) (PDDA), a polycationic polymer, followed by immersion in graphene oxide(GO) solution for 15 minutes. The procedure was repeated to deposit successive layers and the deposition process was followed both by SPR, by following the increase in the SPR angle with the number of PDDA/GO layers and by cyclic voltammetry, using the redox probe \([\text{Fe(CN)}_6^{3-}]\), whose oxidation and reduction current decreased with the increase in the deposited PDDA/GO layers. The optimum number of layers was found to be two and neutravidin was immobilized on the surface by amine coupling with the carboxylic groups on the GO. Finally the biotynilated lysozyme aptamer was bound by affinity on the neutravidin-functionalised surface (Fig.3). The application of this aptasensor for monitoring the aggregation of lysozyme is also described.

The study is a starting point for aptasensors as novel analytical tools for the sensitive detection of small oligomers in therapeutic proteins, for which specific aptamer exists. The versatile aptasensor can be tuned, by simply adjusting the experimental conditions, for the sensitive and specific detection of either the monomer or the dimer, as per the desired application.

References


Figures

Figure 1. Sensorgrams recorded with the aptasensor for lysozyme monomer (3.5 µM, red curves) and lysozyme dimer (35 nM, blue curves), respectively in 20 mM TRIS buffer pH 7.4 with 100 mM NaCl and 5 mM MgCl2 0.05% Tween-20.

Figure 2. Analysis mixtures by Multiple Regression of the SPR data recorded for monomer-dimer mixtures.

Figure 3. The design of lysozyme aptasensors based on (A) thiol-coated interfaces and (B) surfaces coated with PDDA and GO via layer-by-layer. On both interfaces, neutravidin is covalently linked to carboxylic groups on the surface, followed by the binding of a biotynilated lysozyme aptamer.