

## P7 – Interaction of Immunogenic Chlorinated Ovalbumin With Macrophages Receptors Studied by Force Spectroscopy

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Hypochlorite (HOCl/OCl<sup>-</sup>), a product of activated neutrophils, significantly contributes to protein oxidation which occurs at a site of inflammation. Proteins modified by chlorination change their biological activity such as enzymatic activity, resistance to proteolytic cleavage and immunogenicity. While enhanced immunogenicity of chlorinated ovalbumin (OVA-Cl) was already reported its mechanism is still not clear.

In this study we investigated the influence of chlorination of OVA on its recognition (endocytosis) by macrophages – the potential antigen presenting cells (APC). Using AFM-based Force Spectroscopy the strength of interactions of native and chlorinated OVA with a membrane of macrophages was quantified. Proteins were immobilized on gold-coated AFM tips using aqueous solutions of sulfonate derivative of a thiol and the proteins in 100:1 molar ratio. Formation of negatively charged monolayer on the tip dramatically limited its nonspecific interactions with the surface of macrophages. Surface dilution of the proteins ensured the presence of only single or a few macromolecules at the tip surface contact. In such systems so-called adhesion probability (AP) was the measure of the interactions between the proteins and macrophages surface receptors even the measured forces varied significantly for sets of measurements.

The system with native nonimmunogenic OVA showed only negligible AP while application of OVA-Cl gave rise to very high AP (up to 85%), which vary with the contact time of the tip on the macrophage and the loading velocity. Incubation of the cells in OVA-Cl lead to significant reduction of AP that indicates blocking of the receptors by the chlorinated protein. The developed approach based on force spectroscopy measurements was used to study specific interactions between OVA-Cl and macrophages. The method may be widely used for the studies on other ligand-receptor interactions in biological poorly defined systems characterized by large variations of the measured forces.

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## Interaction of immunogenic chlorinated ovalbumin with macrophages receptors studied by Force Spectroscopy

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### INTRODUCTION

Hypochlorite (HOCl/OCl<sub>2</sub>), a product of activated neutrophils, significantly contributes to protein oxidation which occurs at a site of inflammation. Proteins modified by chlorination change their biological activity such as enzymatic activity, resistance to proteolytic cleavage and immunogenicity. Such modified proteins are often recognized as basis for autoimmune disorders, diabetes and other diseases. The enhanced immunogenicity of chlorinated ovalbumin (OVA-Cl) was already reported but the mechanism of the action is still not clear. We report here the studies on the interaction of OVA and OVA-Cl with activated macrophages using Force Spectroscopy. The obtained results indicate specific binding of OVA-Cl to the receptors on the surface of the macrophages.

Classical approach in the analysis of Force Spectroscopy data of biological receptor systems is based on the comparison of the histograms of the rupture forces. It usually suffers from low number of rupture events that originate from purely specific interaction and broad distribution of the measured forces that are often difficult to interpret. Here we propose the approach, which limits non-specific interaction in the system and enables the analysis to be made based on the probability of the observed rupture events.

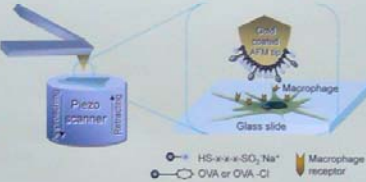


Types of endocytosis in animal cells

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### EXPERIMENTAL SETUP

Gold-coated AFM tips were modified with a mixture of sulfonate-terminated thiol (sodium 2-mercapto-1H-benzotriazole-5-sulfonate) and an appropriate protein (OVA or OVA-Cl) in molar ratio 100:1. Force-distance measurements were performed using Picoforce AFM (Veeco) varying the loading rate and the time tips stay in contact with the surface (contact time). All the measurements were performed in 0.2 M PBS buffer solution. The curves were measured on different spots of the macrophages immobilized on glass and on the glass surface itself as blank experiments. 100-300 curves were collected on each spot; the data were processed and averaged for number of spots. The measurements for a given system were performed at least twice changing the tip and the set of cells.



Legend:  
● HS-CH<sub>2</sub>-SO<sub>3</sub>Na<sup>+</sup>  
● OVA or OVA-Cl  
● Macrophage receptor

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### METHODS

#### Protein chlorination with HOCl

Chicken albumin (OVA) dissolved at a concentration of 20 mg/ml in 0.2 M phosphate buffer (pH 7.4) were incubated with 0.1 mM (OVA-Cl-1) or 1 mM (OVA-Cl-2) HOCl/OCl<sub>2</sub> solution at room temperature for 2 hours. To stop the reaction the samples were treated with stoichiometrical amount of bisulfite and then were dialyzed for 24 hours in 0.2 M phosphate buffer at 4°C.

#### Immunization and macrophages

Mice were immunized intradermally with 200 µg of either native or chlorinated proteins emulsified in complete Freund's adjuvant (CFA). Peritoneal macrophages (MΦ) from Balb/c mice were induced by intraperitoneal injection of 1.5 ml of 2% solution of thioglycollate. Cells were collected 72 h later by washing out the peritoneal cavity with 3 ml of PBS (phosphate buffer solution) containing 3 U heparinase. Before AFM measurement macrophages were adhered on glass slides (1 hour incubation in Hank's Balanced Salt Solution at 37°C in an atmosphere of 5% CO<sub>2</sub>), washed and fixed (5 min. incubation at RT in 1.8% glutaraldehyde in PBS).

### CONCLUSIONS

- The force spectroscopy results indicate specific interactions between OVA-Cl and macrophages.
- The measured rupture forces are in the range of hundreds of pN, which are reasonable values for interaction of single (or several) pairs OVA-Cl receptor AP for the system depends on the loading rate and the contact time due to relatively slow kinetics.
- Usage of sulfonate-terminated monolayer on AFM tip helped to limit nonspecific interactions with macrophages. Blank experiments OVA-Cl vs. glass (no system on a tip, OVA vs. macrophages) show specifically no adhesion.
- "Blocking experiments" show significantly reduced AP only for OVA-Cl, which is able to block the specific receptors on activated macrophages.
- The measured curves for OVA-Cl-1 (see Figure 2A) exhibit mainly single rupture events while for OVA-Cl-2 mostly multiple ruptures are observed (see Figure 2B). For the latter case it may be explained by the presence of many shorter chains (fragmentation) on the AFM tip that form many connections with the macrophage surface within a single contact.
- Elimination of the nonspecific adhesion improves significantly processing of the data - no need of arbitrary selection of "good" curves (non-specific interactions) to be processed. Quantification of the interactions was based exclusively on AP values. Determination of AP seems to be more reliable than determination of sticky forces that are subjected to large variations especially for biological systems.

**Outlook**  
 Future work will address the studies on identification of types of receptors involved in the recognition of OVA-Cl. The proposed here novel approach should also find application in the studies on similar receptor-ligand biological systems.

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### RESULTS

#### ATTACHMENT OF PROTEINS TO GOLD

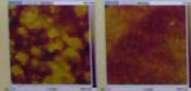


Figure 1. AFM images of gold surfaces decorated with OVA-Cl-1 (A) and OVA-Cl-2 (B) modified proteins.

#### FORCE-SEPARATION CURVES

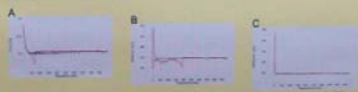


Figure 2. Examples of force-distance curves for (A) OVA-Cl-1 (single rupture event), (B) OVA-Cl-2 (multiple rupture events), (C) blank experiments (no adhesion curve).

#### SELECTIVE BINDING

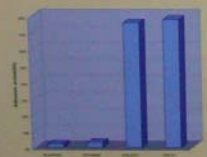


Figure 3. Adhesion probability for different coatings on an AFM tip.

#### SELECTIVE BLOCKING OF RECEPTORS

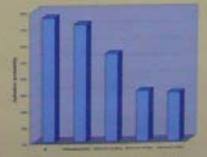


Figure 4. Adhesion probability for competitive blocking experiments. The macrophages were probed the adhesion measurements included in the solutions of

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#### LOADING RATE DEPENDENCE

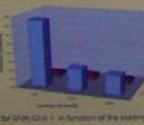


Figure 5. AP for OVA-Cl-1 as function of the loading rate. Blank experiments with OVA are shown for comparison.

#### CONTACT TIME DEPENDENCE

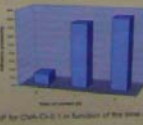


Figure 6. AP for OVA-Cl-1 as function of the time of contact.

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