Characterization and Binding Capacity Studies of PEGylated Lysozyme

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Introduction

- PEGylation of proteins is a well established method to increase in vivo clearance rate and reduce toxicity and immunogenicity of therapeutic proteins. This polymeric modification changes the biochemical and physical properties of the protein and will therefore influence the behavior during a chromatographic purification. Resin manufacturers such as TOSOH are interested to know how a covalent PEG modification influences the performance and binding capacity of a protein on prepacked HPLC columns and chromatographic bulk media.

- Lysozyme is a well known standard protein, which is generally used to determine the dynamic binding capacity of Ion Exchange Chromatography (IEC) resins; therefore we decided to use PEG-lysozyme as a model protein in our study.

- PEGylated lysozyme is produced out of methoxy-PEG-aldehyde (with a MW of 5kDa, 10kDa and 30kDa) and hen white egg lysozyme in a phosphate buffer (0.1M, pH 6.0) in the presence of sodium-cyano-borohydrid ($NaCNBH_3$) as a reducing agent. The product mixture is analyzed by a TSKgel G3000SW$_{xl}$ SEC HPLC column, SDS-PAGE, IEC (TSKgel SP-5PW (20)) and subsequent MALDI-TOF.

- Dynamic binding capacity (DBC) tests on different cation exchange media were performed with purified mono-PEGylated lysozyme.

Methods

- **PEGylation of egg white lysozyme:**
  - **5, 10, 30kDa methoxy PEG-aldehyde**
  - **100mmol/L Phosphate buffer (Na_2HPO_4, NaH_2PO_4) pH 6.0**
  - **PEGylation by reductive alkylation**
  - **20mmol/L $NaCNBH_3$ to reduce a Schiff base**
  - **100mmol/L HCl to stop PEG-reaction**

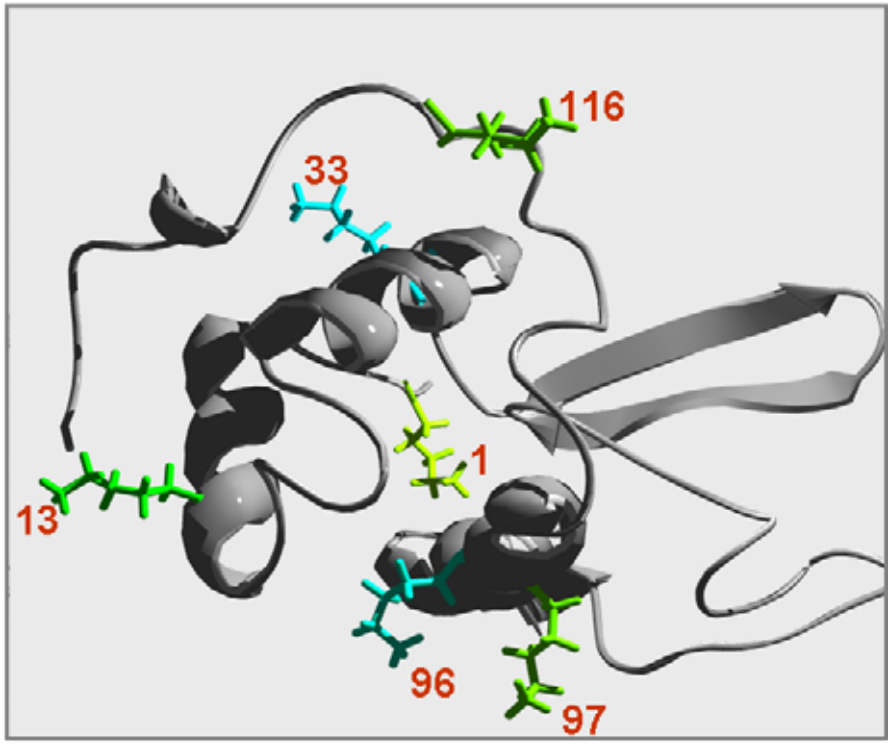
- **SEC-HPLC:**
  - **Column:** **TSKgel G3000SW_{XL}, 5µm, 7.8mm ID x 30cm**
  - **HPLC-System:** **Shimadzu Prominence**
  - **Flow rate:** **1mL/min**
  - **Mobile phase:** **0.1mol/L Phosphate buffer; 0.1mol/L Na_2SO_4, pH 6.7**
  - **Detection:** **UV@280nm**
  - **Injection volume:** **20µL**

Methods, Continued

- **IEC-FPLC:**
  - **Column:** TSKgel SP-5PW, 20µm, 6.6mm ID x 22cm, 1000Å
  - **Flow rate:** 0.85mL/min
  - **Mobile phase:** Buffer A: 25mmol/L Phosphate buffer; 0.1mol/L Na_2SO_4, pH 6.0
    Buffer B: A + 0.5mol/L NaCl
  - **Detection:** UV@280nm
  - **Injection volume:** 100µL

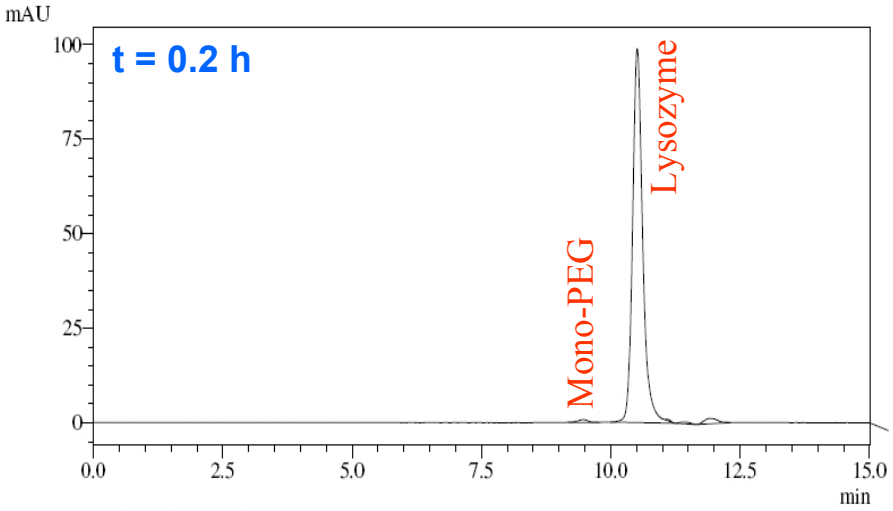
- **SDS-PAGE:**
  - Precast gradient 4-15% Tris-HCl SDS-polyacrylamide gel
  - **Sample buffer:** Laemmli
  - **Running buffer:** Tris/glycine/SDS
  - **Staining:** Silver staining

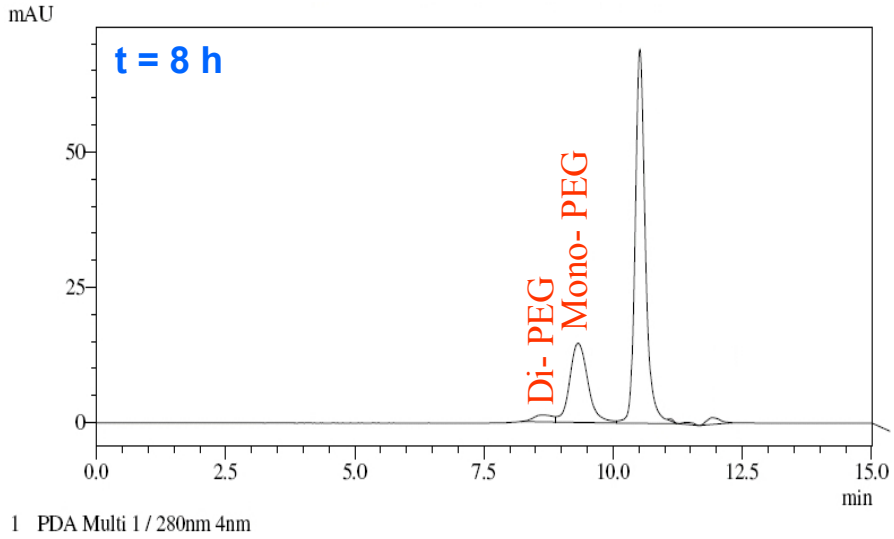
Schematic Diagram of Lysozyme

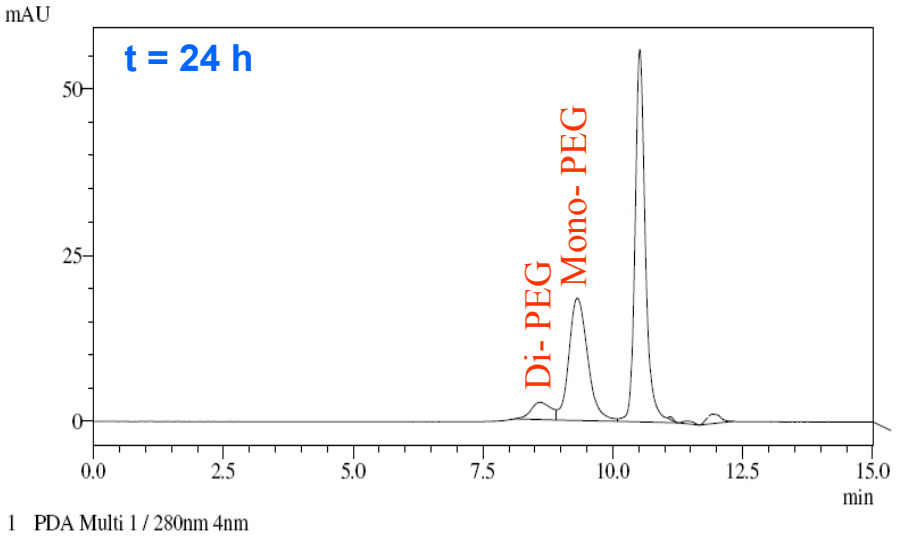


Lysozyme has six lysine residues as possible PEGylation reaction sides.

Time Course of Lysozyme PEGylation Reaction (5kDa PEG) Monitored with TSKgel G3000SW$_{XL}$ SEC Column

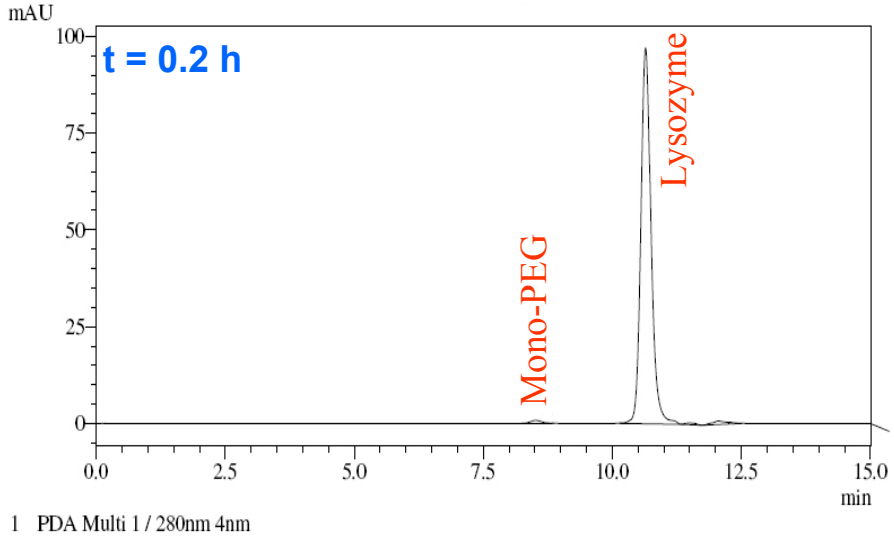


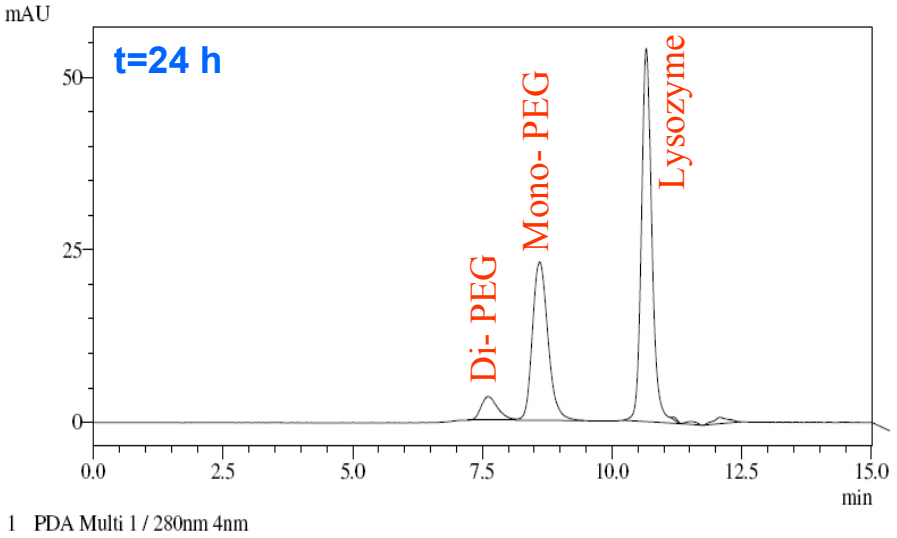


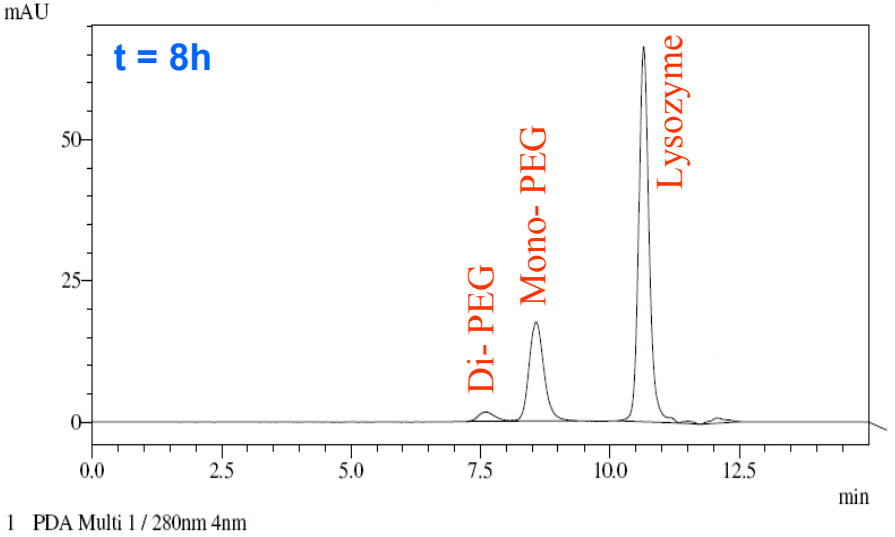


Di-, monoPEG and unPEGylated lysozyme species can be resolved by SEC-HPLC analysis.

Time Course of Lysozyme PEGylation Reaction (10kDa PEG) Monitored with TSKgel G3000SW$_{XL}$ SEC Column

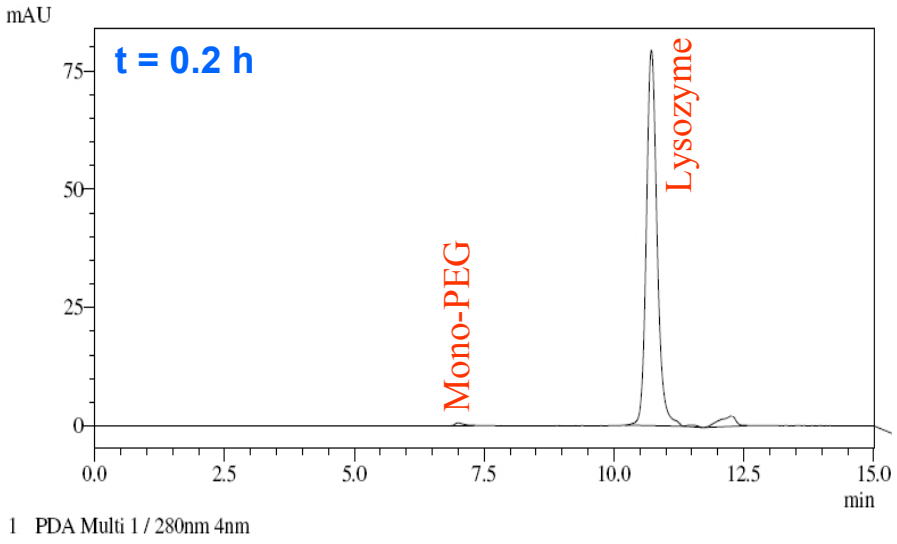


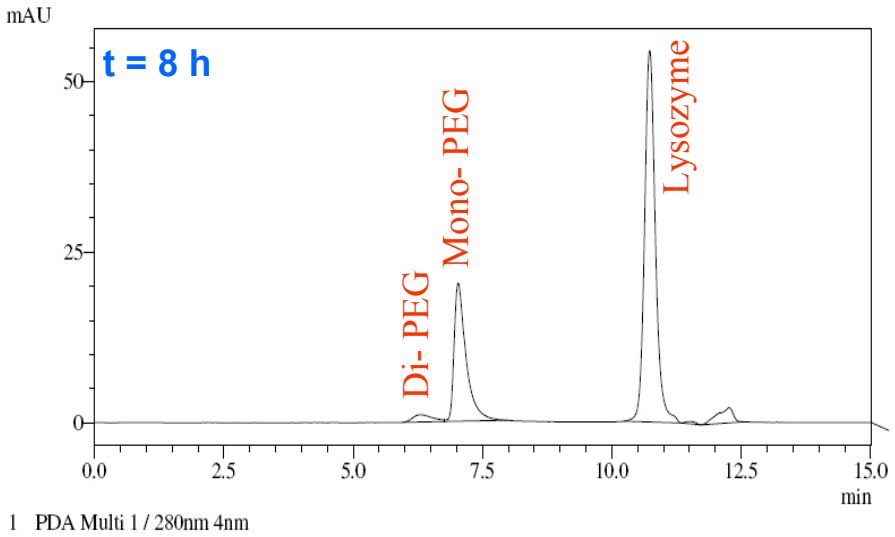


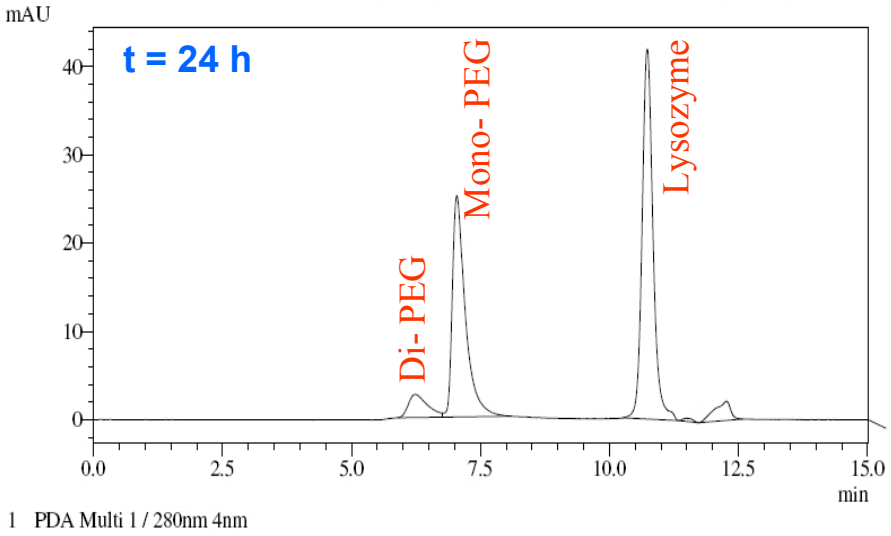


Di-, monoPEG and unPEGylated lysozyme species can be resolved by SEC-HPLC analysis.

Time Course of Lysozyme PEGylation Reaction (30kDa PEG) Monitored with TSKgel G3000SW$_{XL}$ SEC Column

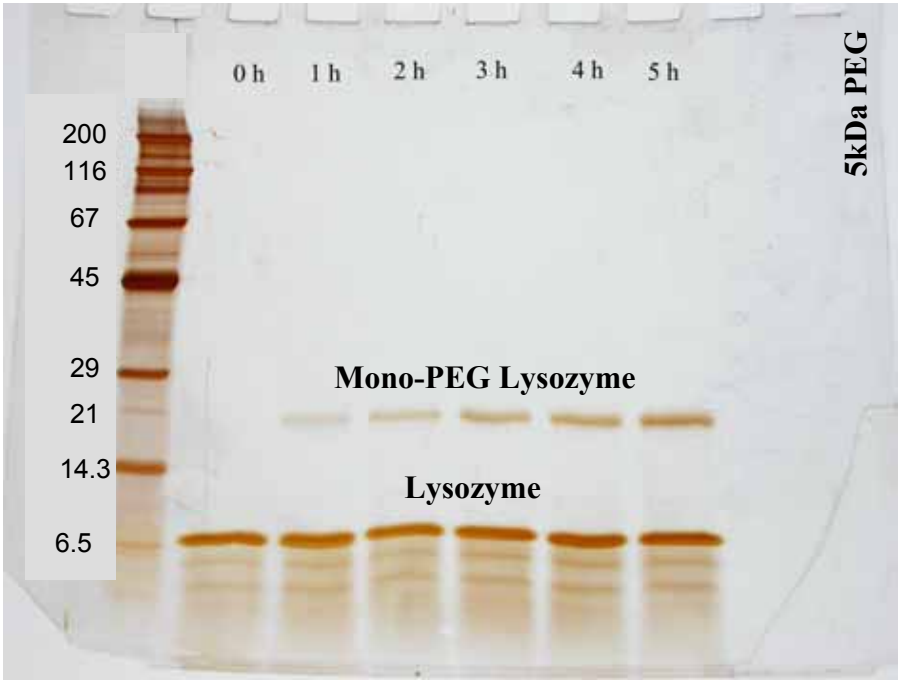


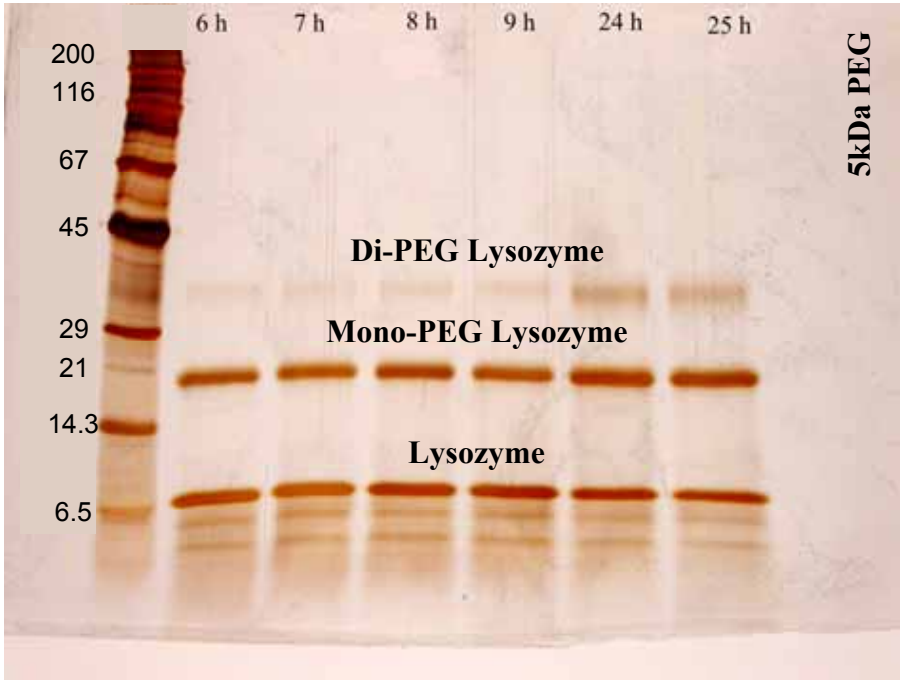




Di-, monoPEG and unPEGylated lysozyme species can be resolved by SEC-HPLC analysis.

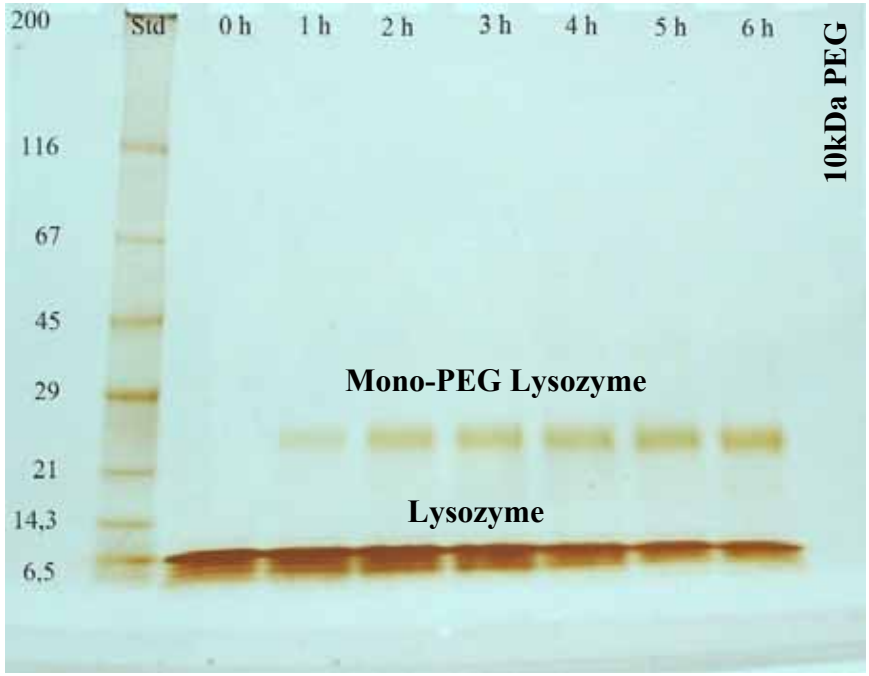
SDS-PAGE Analysis of Lysozyme PEGylation Assay

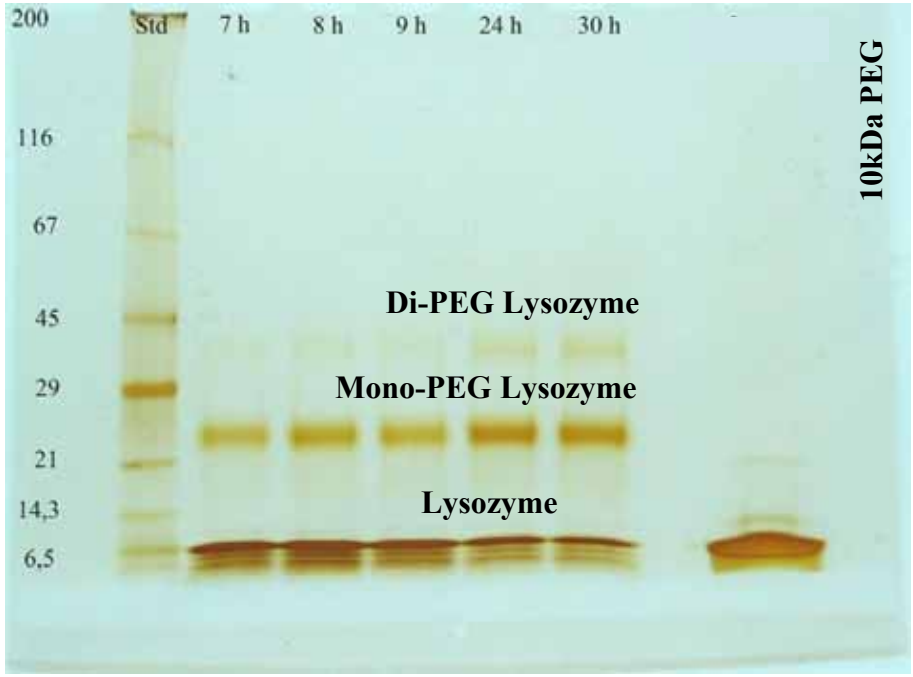




Besides diPEG-lysozyme, monoPEG-lysozyme and unPEGylated lysozyme, a 30kDa triPEG species can be detected after 24 hours.

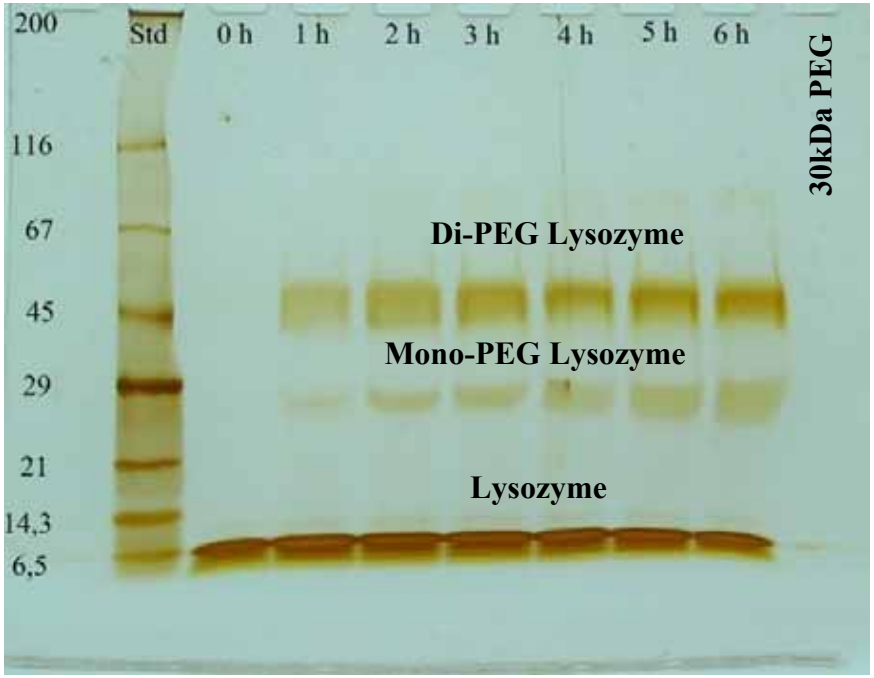
SDS-PAGE Analysis of Lysozyme PEGylation Assay

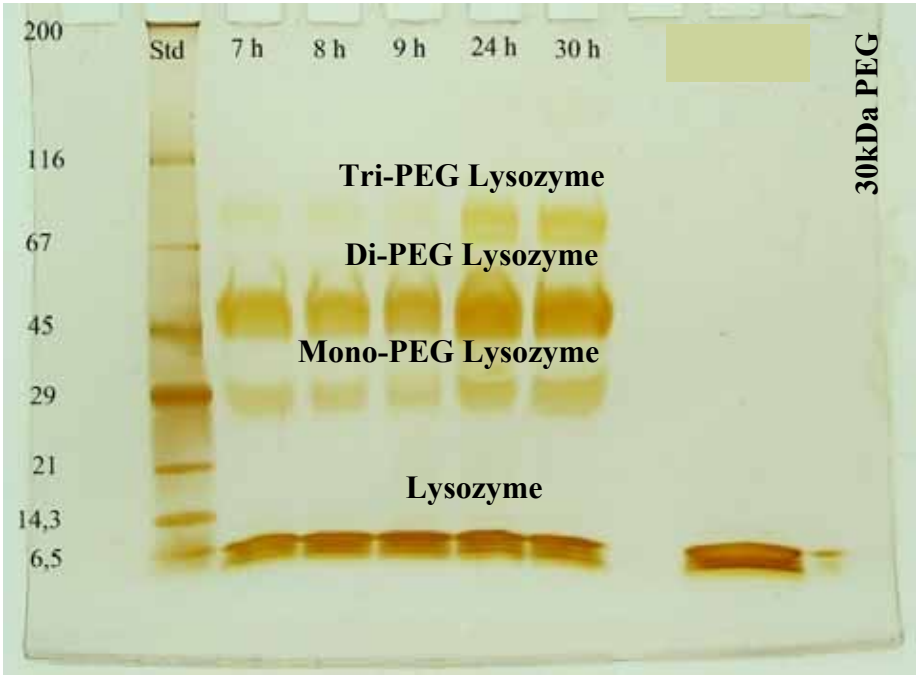




Besides diPEG-lysozyme, monoPEG-lysozyme and unPEGylated lysozyme, a 30kDa triPEG-lysozyme species can be detected after 24 hours.

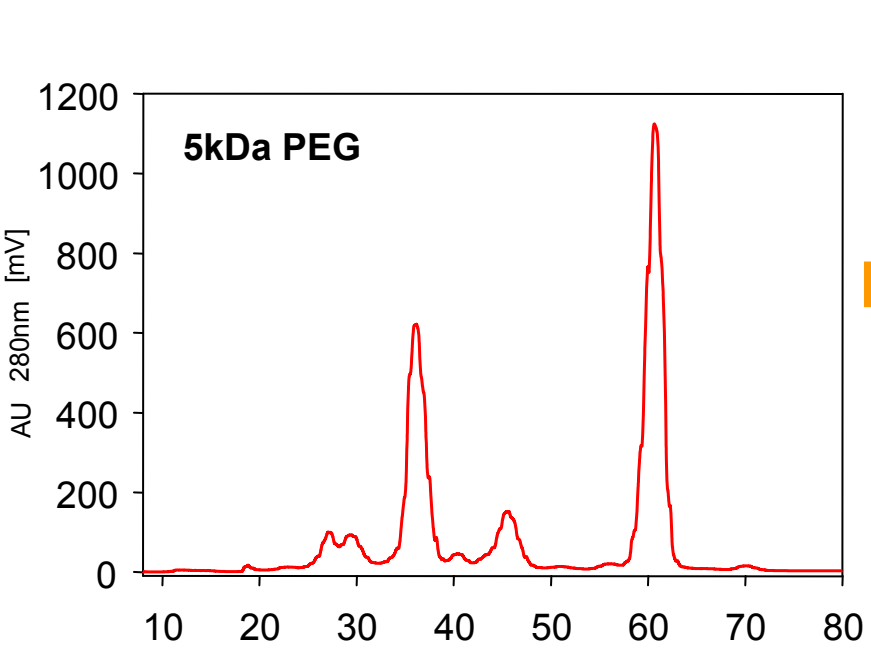
SDS-PAGE Analysis of Lysozyme PEGylation Assay

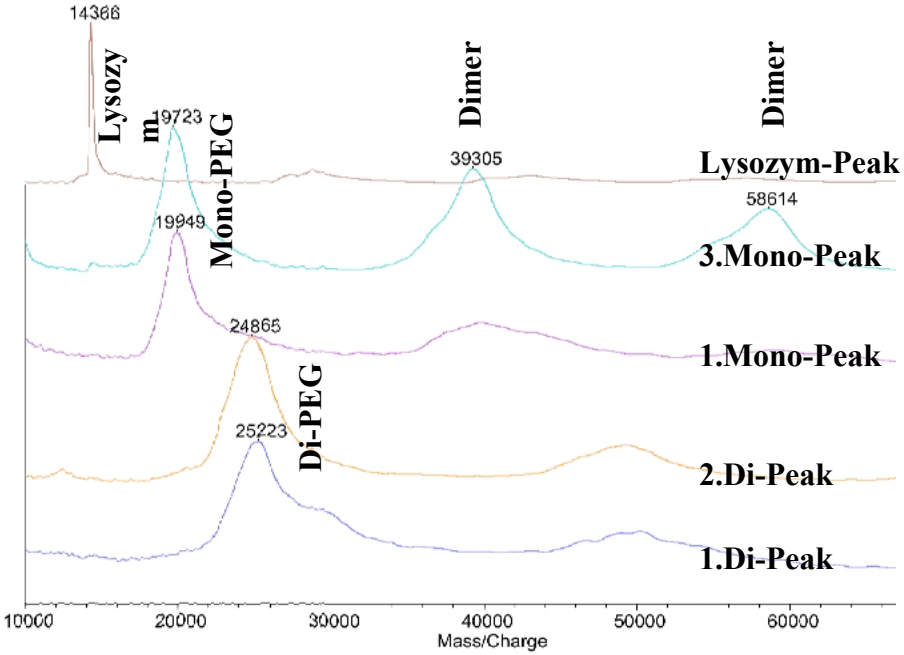




Beside Di-, monoPEG and unPEGylated lysozyme, a 30kDa triPEG species can be detected after 24 hours.

Cation Exchange Assay of Lysozyme PEGylation: 5kDa PEG

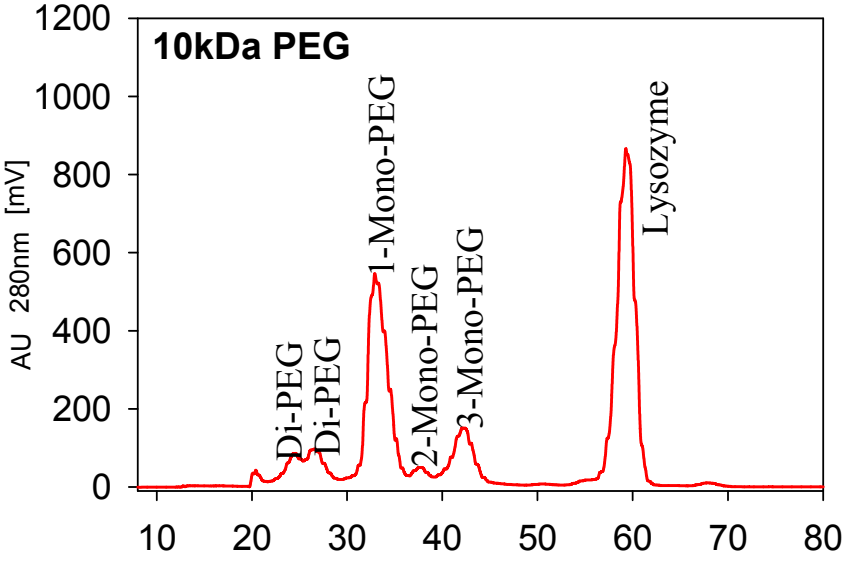


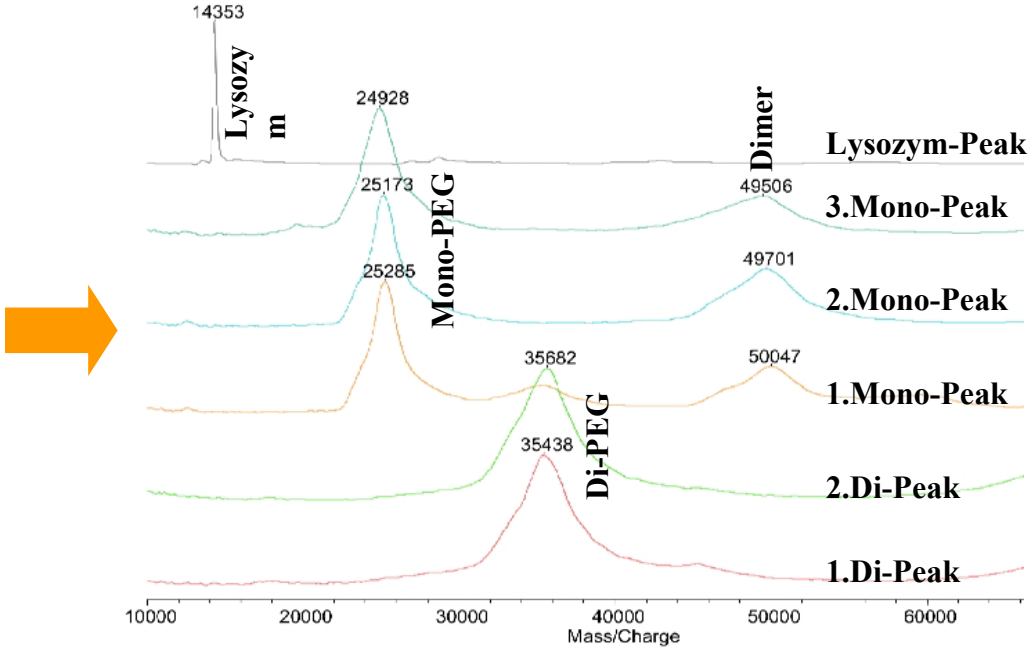


Cation exchange assay of lysozyme PEGylation on TSKgel SP-5PW (20) resin and subsequent MALDI-TOF spectra of collected peaks.

With thanks to Dr. Harald Lange and Kai Darsow, Institute of Bioprocess Engineering, University of Erlangen, for the MALDI-TOF results.

Cation Exchange Assay of Lysozyme PEGylation: 10kDa PEG

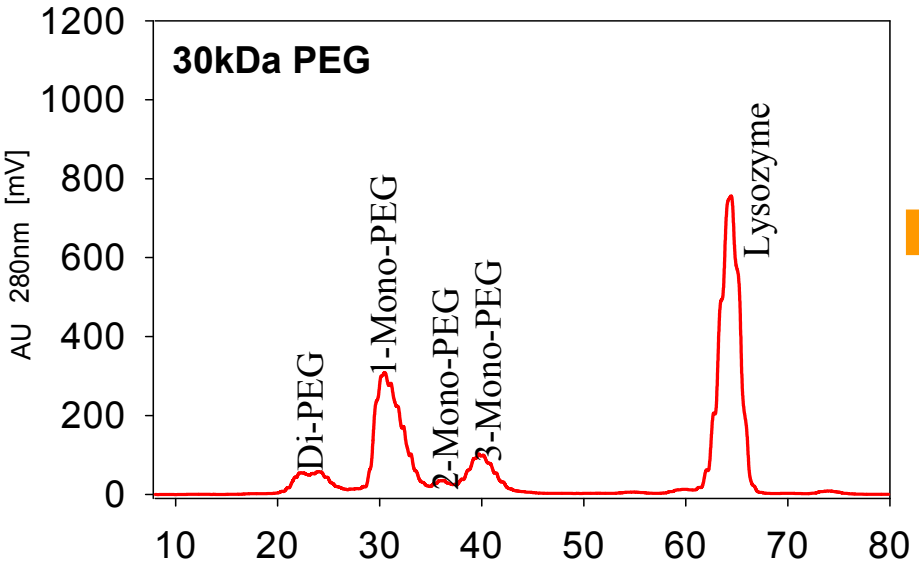


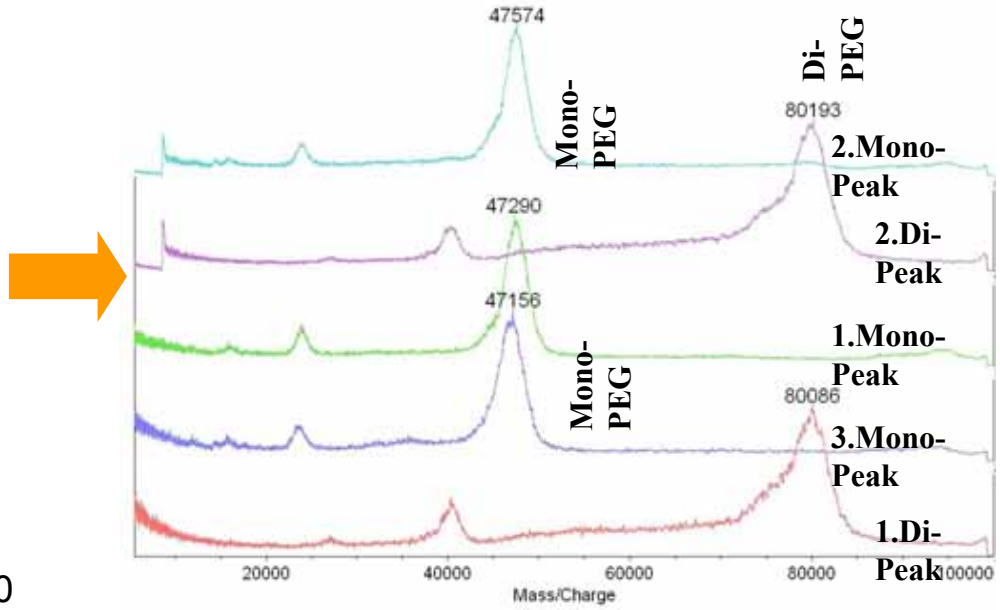


Cation exchange assay of lysozyme PEGylation on TSKgel SP-5PW (20) resin and subsequent MALDI-TOF spectra of collected peaks.

With thanks to Dr. Harald Lange and Kai Darsow, Institute of Bioprocess Engineering, University of Erlangen, for the MALDI-TOF results.

Cation Exchange Assay of Lysozyme PEGylation: 30kDa PEG





Cation exchange assay of lysozyme PEGylation on TSKgel SP-5PW (20) resin and subsequent MALDI-TOF spectra of collected peaks.

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Dynamic Binding Capacity of 1-monoPEG and Native Lysozyme on Different Cationic Exchange Resins

mg/mL					
	SP-5PW	SP-650M	SP-550C	SP-550EC	GigaCap S-650M
Particle Size	20µm	65µm	100µm	150µm	75µm
Nominal Pore Size	1000Å	1000Å	500Å	500Å	1000Å
Mono-PEG lysozyme	29	18	53	28	95
Lysozyme*	33 (87%)	33 (55%)	69 (77%)	51 (54%)	121 (79%)

* Figures in brackets indicate relative % of lysozyme capacity that is accessible to mono-PEG lysozyme.

Conclusions

- SEC chromatograms show different elution volumes for monoPEG-lysozyme, diPEG-lysozyme and native lysozyme respectively for 5kDa, 10kDa and 30kDa PEG. HPLC-SEC using a TSKgel G3000SW$_{XL}$ column is a fast tool to monitor time, concentration and temperature-dependent synthesis of PEGylated lysozyme species. An SDS-PAGE control experiment verified the SEC results.

- Cation exchange resin TSKgel SP-5PW (20) separates mono-PEGylated and di-PEGylated lysozyme isoforms, which is confirmed by MALDI-TOF analysis.

- Dynamic binding capacity studies (see TABLE 1) of 1-monoPEG on different cation exchange resins show significant differences. Best results regarding capacity are achieved with Toyopearl GigaCap S-650M resin; as for resolution, TSKgel SP-5PW has the best performance.

- Long term objective is to optimize LC resins (IEC and HIC) regarding high binding capacity in combination with high resolution of PEG-isoforms.