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Factors influencing mAb aggregation in mammalian cell culture

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Introduction and Purpose

Monoclonal antibodies (mAbs) are important biopharmaceuticals for the treatment of many diseases. During manufacturing the proteins tend to form **aggregates**, which reduce product yields, influence drug performance and safety. Environmental conditions during production in **mammalian cell culture** influence the formation of **high molecular weight (HMW) species**. In this report, we show how mAb aggregates can be detected directly in the cell culture supernatant using **size exclusion chromatography (SEC)** in a high pressure liquid chromatography (HPLC) system. We have investigated the impact of batch cultivation in different culture vessels, the addition of Valproic acid (VPA) as small molecule enhancer of protein production and the influence of the cell culture environment itself on the formation of mAb aggregates in **Chinese hamster ovary (CHO)** cell culture. Our results prove that aggregate formation can occur already during **upstream processing (USP)** due to **intracellular** and **extracellular mechanisms** and is not only a problem in downstream processing (DSP).

Methods

Cell culture

A CHO DG44 cell line producing an aggregation-prone mAb was used as model system. The cells were cultivated at 37° C, 5% CO_2, 80% humidity at 140 rpm in a ISF1-XC shaking incubator (Kühner) and seeded at $4x10^5$ viable cells/mL in SFM4CHO medium (Thermo scientific) supplemented with 10 g/L glucose and 4 mM glutamine. To study the influence of VPA on mAb aggregation, the histone deacetylase (HDAC) inhibitor was added in different concentrations. To ensure typical growth and protein production, culture parameters were measured during cultivation. Viable cells and viability were measured using Cedex XS (Roche), substrate and metabolite concentrations were measured using Konelab Arena 20 (Thermo Scientific) and product concentrations were measured using Protein A HPLC.

SDS-PAGE

Unfiltered CHO supernatants were either incubated with DTT at 95° C for 5 min (reduced) or were analyzed untreated (non-reduced). 35 µl of each sample were loaded to the gel using a protein concentration of 88 µg/mL adjusted with phosphate-buffered saline (PBS). Non-reducing SDS-PAGE was performed at 160 V for 90 min, reducing SDS-PAGE for 70 min.

SE-HPLC

Formation of soluble aggregates was analyzed using an Agilent 1100 HPLC (Agilent Technologies) system and an UltiMate 3000 (Thermo Scientific) system, equipped with a MAbPac SEC-1 (Thermo Scientific) or Yarra S4000 (Phenomenex) SEC columns. SEC separation was performed at room temperature isocratically, at a flow rate of 0.3 and 0.8 mL/min using a mobile phase consisting of PBS, which was adjusted to pH 7.2 and filtered 0.1 µm prior to use. The respective amounts of mAb monomer and aggregates were quantified by calculation of the peak areas detected by the UV detector. For aggregate analysis, cells were separated (10.000 g for 5 min) and the supernatants were pre-filtered (0.2 µm).

Conclusions

MAb aggregates formed in cell culture

- CHO supernatants contain mAb monomer, aggregates and cell culture components
- Quantification of mAb aggregate content directly in CHO supernatant feasible, since culture medium and host cell components are smaller than mAb monomer and aggregates
- HMW species are product-related

Factors influencing mAb aggregation

- *Culture vessel*
  - Cells cultivated in agitated systems (shake flasks and TPP tubes) contained more aggregates than static cultivation in T-flasks
- *Protein production enhancer VPA*
  - Above a VPA concentration of 2 mM more mAb aggregates were observed
  - **Intracellular mAb aggregate formation**
- *Cell culture environment*
  - MAb aggregation kinetic continues in cell culture supernatant over time
  - Equilibrium reached after 96 h
  - No effect of VPA in cell-free supernatant
  - **Extracellular mAb aggregate formation**

Results

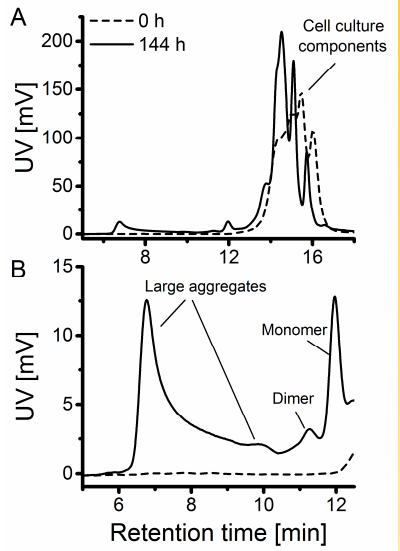


Figure 1: MAb aggregation in cell culture.

SE-HPLC analysis of mAb-producing CHO cell supernatant. Cells were cultured in 125 mL shake flasks using a working volume of 25 mL. Supernatant was analyzed directly after inoculation and after 144 h of cultivation. At the beginning of cultivation only host cell and medium components were visible. After 144 h of cultivation **mAb monomer, dimer and large aggregates were detected** in the culture supernatant. **SFM4CHO culture medium and host cell proteins eluted later** (>13 min) than mAb monomer and aggregates (before 13 min) **allowing aggregate quantification directly in the supernatant**. To ensure that the HMW species were product-related, SDS-PAGE of CHO cell culture samples was performed under reducing and non-reducing conditions. (B) is an enlargement of (A).

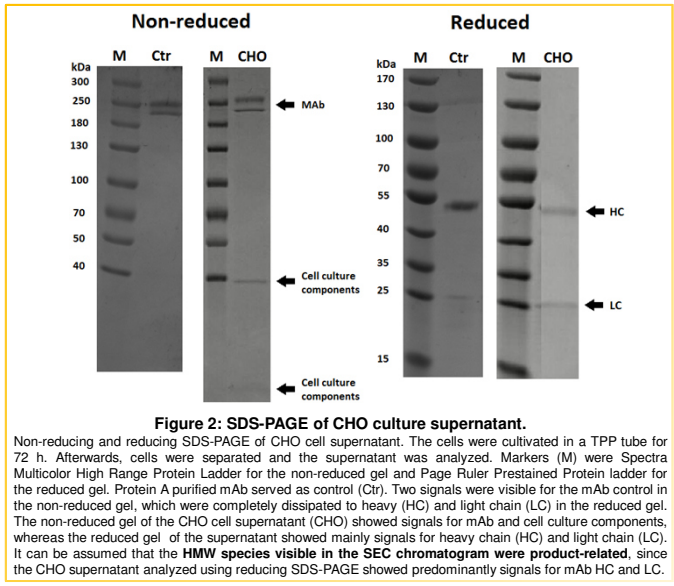
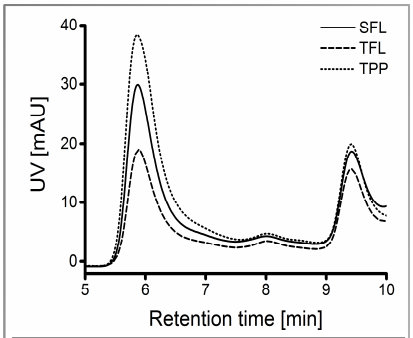


Figure 2: SDS-PAGE of CHO culture supernatant.

Non-reducing and reducing SDS-PAGE of CHO cell supernatant. The cells were cultivated in a TPP tube for 72 h. Afterwards, cells were separated and the supernatant was analyzed. Markers (M) were Spectra Multicolor High Range Protein Ladder for the non-reduced gel and Page Ruler Prestained Protein ladder for the reduced gel. Protein A purified mAb served as control (Ctr). Two signals were visible for the mAb control in the non-reduced gel, which were completely dissipated to heavy (HC) and light chain (LC) in the reduced gel. The non-reduced gel of the CHO cell supernatant (CHO) showed signals for mAb and cell culture components, whereas the reduced gel of the supernatant showed mainly signals for heavy chain (HC) and light chain (LC). It can be assumed that the **HMW species visible in the SEC chromatogram were product-related**, since the CHO supernatant analyzed using reducing SDS-PAGE showed predominantly signals for mAb HC and LC.



	Aggregates (%)	Standard deviation (%)
Shake flask	71.8	± 1.7
T-flask	66.9	± 0.7
TPP flask	73.2	± 0.2

Figure 3: Influence of culture vessel.

MAb-producing CHO cells were cultured either in shake flasks (SFL), T-flasks (TFL) or TPP tubes (TPP) using a working volume of 20 mL. After 120 h of cultivation, cells were separated, the supernatant was filtered (0.2 µm) and analyzed using SE-HPLC**. Large mAb aggregate species were formed in all culture vessels**. The supernatant of **TPP tubes contained more mAb aggregates** (73.2% ±0.2%) **than** the supernatants of **T-flasks** (66.9% ± 0.7%) **and shake flasks** (71.8% ±1.7%). The higher content of aggregates was mainly caused by the formation of large aggregates (< 7.5 min), since mAb monomer and small aggregates (> 7.5 min) were marginally effected. Reasons for higher aggregate content in the TPP tubes and the SFL flasks could be higher shear forces or higher protein concentrations in agitated systems. The experiment was performed in replicates (n=3), whereof one chromatogram of each vessel was depicted as example. The mean values with standard deviations were summarized in the table.

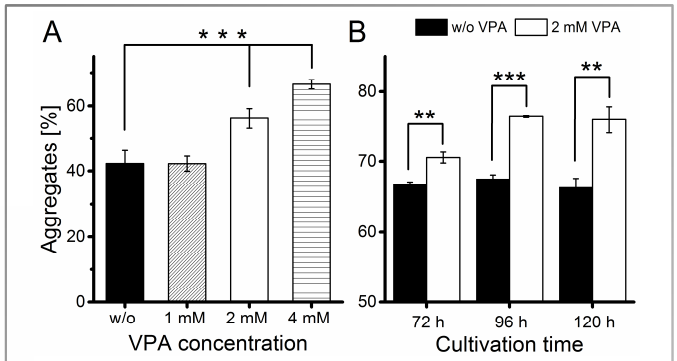
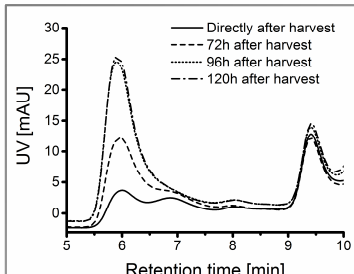


Figure 4: Influence of VPA.

(A) MAb-producing CHO cells were cultured in a 12-well suspension plate with different VPA concentrations using a working volume of 2 mL. After 96 h cultivation, the supernatant was analyzed**. VPA concentrations above 1 mM significantly induced the formation of mAb aggregates**. Cells cultured with 2 and 4 mM VPA resulted in 56.1% ±3.1% and 66.5% ± 1.4% mAb aggregates, respectively, whereas with 1 mM and without VPA only around 42% mAb aggregates were formed. (B) MAb-producing CHO cells were cultured without and with 2 mM VPA in a TPP tube with a working volume of 10 mL. The amount of **mAb aggregates increased over cultivation time** when VPA was present, whereas without VPA the amount of aggregates only changed marginally. *P<0.05, **P<0.01, ***P<0.001 for one-way ANOVA (A) and two-tailed t-test (B).



	Aggregates (%) w/o VPA ± SD (%)	Aggregates (%) 2 mM VPA ± SD (%)
Directly after harvest	57.7	57.7
72 h after harvest	70.4 ± 1.1	72.5 ± 0.3
96 h after harvest	74.4 ± 06	74.6 ± 0.4
120 h after harvest	74.1 ± 0.6	75.4 ± 0.1

Figure 5: Influence of cell culture environment.

CHO cells producing a mAb were cultivated in a TPP tube with a working volume of 10 mL. After 96 h cultivation, cells were separated and the supernatant was analyzed for aggregate content. The cell-free supernatant was further incubated at 140 rpm under culture conditions in a 12-well suspension plate with 2 mL end volume without or with 2 mM VPA. Samples were re-analyzed for mAb aggregate formation after 72 h, 96 h and 120 h. Astonishingly, **mAb aggregation kinetic continued in the cell-free culture supernatant** resulting in the formation of large aggregates, whilst mAb monomer and small aggregates were hardly effected. Directly after harvest the CHO supernatant contained only 57% mAb aggregates. After incubation in the cell culture environment the mAb aggregate content increased to 74% **reaching an equilibrium after 96 h**. Addition of VPA did not significantly effect mAb aggregate formation in the cell culture supernatant. Supernatants incubated with 2 mM VPA contained only 1% more mAb aggregates than supernatants incubated without VPA. This indicates that **VPA induced an intracellular aggregate formation** independent of extracellular aggregate formation caused by the cell culture environment. The mean values with standard deviations were summarized in the table.