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The influence of impurity proteins on the precipitation of a monoclonal antibody with an anionic polyelectrolyte

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ABSTRACT

In order to meet the growing demand for monoclonal antibodies (mAbs), many research studies focus on the development of new purification technologies to overcome disadvantages of conventional methods. The precipitation of mAbs with anionic polyelectrolytes is a promising alternative offering advantages like e.g. low additive consumption. The antibody–polyelectrolyte interaction depends on numerous factors. While the influence of factors like pH value, ionic strength and polyelectrolyte concentrations was already examined in different studies, this study focuses on the influence of different properties of impurity proteins on the precipitation of a monoclonal antibody with the anionic polyelectrolyte polyanetholesulfonic acid. For this purpose, five model impurity proteins, bovine serum albumin, ovalbumin, α-Lactalbumin, β-Lactoglobulin, and myoglobin, were selected with regard to their isoelectric points and molecular weights. Additionally, the surface charge distribution of these proteins was simulated. The experimental results provide a better insight in the electrostatic interactions taking place between proteins and polyelectrolyte molecules.

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1. Introduction

The increasing demand for monoclonal antibodies (mAbs) for therapeutic and analytical applications led to an intensified development of cell cultures with high expression levels. With these enhanced expression systems, antibody titers up to 5–10 g/L or even higher can be achieved [1–3]. The downstream process (DSP) still represents the bottle neck of the production process of mAbs [1,4,5] and simultaneously makes up 50–80% of the total production costs [6,4]. As a consequence, the demand for more efficient purification processes, which can handle these high antibody titers, increases continuously [7,5]. One promising alternative to replace traditional purification methods is the precipitation of mAbs with anionic polyelectrolytes [8–11]. Polyelectrolytes (PEs) are polymers consisting of macro molecules which cover primarily ionic or ionizable groups [12,13]. Despite their hydrophobic backbone, PEs dissociate in polar solvents like water into a polycharged backbone and a corresponding number of low molecular counter ions. They can form complexes with oppositely charged molecules, due to their anionic or cationic groups. This ability can be exploited for the isolation of proteins. Cooper et al. [14] and Kayitmazar et al. [15] give an extended overview of different PEs used for the isolation of proteins. Depending on the pH value of the surrounding solution, proteins wear charged groups on their surface, which can interact with the oppositely charged polyelectrolyte molecules due to electrostatic attraction. As a consequence of these interactions they form complexes, which can aggregate to insoluble particles. These particles can be separated mechanically from the liquid supernatant. Afterwards, the solid precipitate can be re-dissolved by adding a solution of high pH value or with high salt concentration [10,11]. The main advantages of this method are the small quantity of additive (polyelectrolyte) consumption, the simplicity of technical feasibility and the reversible and gentle complex formation [10]. Some research studies focused on the influence of polyelectrolytes on the secondary structure and stability of monoclonal antibodies and proteins. Based on different analysis methods like Fourier transform infrared spectroscopy [8] or circular dichroism [16] no influence of polyelectrolytes on secondary structure was detected.

One of the main factors influencing the interaction of proteins with polyelectrolytes is the pH value [e.g., 17,10,11], as it mainly influences the charge of a protein and thus the electrostatic interaction of proteins and polyelectrolytes. Theoretically, above their isoelectric point (pI), proteins can interact with anionic polyelectrolytes due to their positive net charge. But the interaction does not depend on the net charge only [18,19]. Moreover, proteins of

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Nomenclature

Abbreviations

α-Lact	α-Lactalbumin
β-Lacto	β-Lactoglobulin
BSA	bovine serum albumin
HPLC	high performance liquid chromatography
iPF	initial precipitate formation
mAb	monoclonal antibody
Myo	myoglobin
Ova	ovalbumin
PASA	polyanetholesulfonic acid
PE	polyelectrolyte
pI	isoelectric point

nearly equal isoelectric points can show different precipitation behaviors depending on the distribution of charges on their surface. The surface charges of proteins can be distributed over the protein surface in two different ways [20]. While for some proteins positive and negative charges are evenly spread over their surface, other proteins show charge patches on their surface. In the latter case one side of a protein can be charged nearly totally negative while another side of the protein is mainly positively charged. This results in a different complexation behavior. Xu et al. [19] compared the coacervation behavior of BSA and β-Lactoglobulin with the cationic polyelectrolyte poly (diallyldimethylammonium chloride). Despite their similar isoelectric points of 4.9 (BSA) and 5.2 (β-Lacto), they show different coacervation behaviors, which can be attributed to the different surface charge distribution [19].

Besides pH value, factors like the polyelectrolyte concentration, molecular weight as well as kind of polyelectrolyte influence the polyelectrolyte precipitation and are variably adjustable. Factors which are given by the system are the salt concentration, the impurity content and the spectrum of impurities. In our last paper we showed that the model impurity BSA has a strong influence on the precipitation of a monoclonal antibody with the anionic polyelectrolyte polyanetholesulfonic acid (PASA) [11]. While the influence on mAb precipitation of the other factors mentioned was examined in various studies [8–11], the influence of impurities with different specific properties has not been studied in detail yet.

Therefore, in the present study we investigated the precipitation behavior of proteins with different properties like the molecular weight, the isoelectric point and the surface charge distribution. In addition to these pure protein experiments we investigated the influence of different impurity protein properties on the precipitation of a monoclonal antibody depending on the pH value as well as the polyelectrolyte concentration. The range of isoelectric points selected was oriented to the isoelectric points of the main proteins detected in Chinese hamster ovary cells culture supernatant [21]. For this purpose we chose five commercially available model impurities, Bovine serum albumin (BSA), ovalbumin (Ova), α-Lactalbumin (α-Lact), β-Lactoglobulin (β-Lacto), and myoglobin (Myo), which either differ in their molecular weights or isoelectric points.

α-Lactalbumin, β-Lactoglobulin and myoglobin were selected having comparable molecular weights, but strongly differ in their isoelectric points, to determine how the isoelectric point influences the precipitation behavior. Bovine serum albumin (BSA) and ovalbumin (Ova) were selected having comparable isoelectric points but different molecular weights to determine trends of the influence of molecular weight on the precipitation behavior. Additionally, to interpret the influence of the surface charge distribution, we simulated the electrostatic potential in dependence of pH value for all proteins investigated.

2. Materials and methods

2.1. Materials

Polyanetholesulfonic acid sodium salt with a molecular weight of 30 kDa was obtained from Sigma–Aldrich and was used without further purification. The monoclonal antibody (kindly provided by Merck Millipore) has a molecular weight of around 145 kDa and an isoelectric point (pI) of 8.5. Bovine serum albumin (BSA), α-Lactalbumin (α-Lact), β-Lactoglobulin (β-Lacto), myoglobin (Myo), and ovalbumin (Ova) were purchased from Sigma–Aldrich. Sodium chloride (NaCl) ($\geqslant$99.8%), sodium acetate (anhydrous, $\geqslant$99.0%), acetic acid ($\geqslant$99.8%), and Tris-hydrochloride ($\geqslant$ 99.0%) were obtained from Roth. Water, used for experiments or analytics, was ultra-filtered by a Milli-Q Water purification system (Millipore, 0.05 μS).

2.2. Polyelectrolyte precipitation

Experiments were carried out comparable to the procedure described in [11]. An acetic acid acetate buffer was used to realize a constant pH value. Stock solutions of 20 g/L mAb, 5 g/L of different impurity proteins, and various concentrations of polyanetholesulfonic acid (PASA) were prepared to achieve the desired concentrations in the precipitation mixture. All stock solutions were adjusted to the desired pH value with 1 M HCl. Precipitation was carried out in 1.5 mL Eppendorf tubes. 200 μL of the specific impurity protein stock solution and 50 μL of mAb stock solution were added to 700 μL of buffer solution and mixed on a Vortex agitator (VWR). In pure impurity protein experiments, 50 μL of water were added to the system instead of mAb stock solution. Samples were shortly centrifuged (2 min at 1200 × *g*, Centrifuge 5415 R, Eppendorf) for sample residue removal from the cap to avoid sample loss. Precipitation was induced by adding 50 μL of PE stock solution to the protein buffer mixture. The precipitation components were first mixed for 5 min at 1000 rpm in order to ensure a homogeneous mixture and then for another 90 min at 300 rpm and 20 °C in a temperature-controlled mixer (Thermomixer comfort, Eppendorf) to realize complete precipitation. Afterwards the precipitation tubes were centrifuged for 10 min in a fixed-angle rotor centrifuge (Centrifuge 5415 R, Eppendorf) at 1200 × *g* to achieve a complete phase separation. 950 μL of supernatant was removed and the precipitate was washed two times with 950 μL of the corresponding precipitation buffer. Afterwards the precipitate was re-dissolved by adding 950 μL of the running buffer of HPLC (20 mM Tris–HCl, pH 8.7) and mixing for 30 min at 600 rpm. Samples of 800 μl from re-dissolved precipitate were taken, filtered with syringe filter units (Rotilabo®-Spritzenfilter, 0.2 μm, PTFE membrane, Roth) and transferred into HPLC-vials for analysis. Each experiment was realized three times.

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