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Research Paper

Protein–polyelectrolyte interactions: Monitoring particle formation and growth by nanoparticle tracking analysis and flow imaging microscopy

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ABSTRACT

The purpose of this study was to investigate the formation and growth kinetics of complexes between proteins and oppositely charged polyelectrolytes. Equal volumes of IgG and dextran sulfate (DS) solutions, 0.01 mg/ml each in 10 mM phosphate, pH 6.2, were mixed. At different time points, samples were taken and analyzed by nanoparticle tracking analysis (NTA), Micro-Flow Imaging (MFI) and size-exclusion chromatography (SEC). SEC showed a huge drop in monomer content (approximately 85%) already 2 min after mixing, while a very high nanoparticle (size up to 500 nm) concentration (ca. 9×10^8/ml) was detected by NTA. The nanoparticle concentration gradually decreased over time, while the average particle size increased. After a lag time of about 1.5 h, a steady increase in microparticles was measured by MFI. The microparticle concentration kept increasing up to about 1.5×10^6/ml until it started to slightly decrease after 10 h. The average size of the microparticles remained in the low-μm range (1–2 μm) with a slight increase and broadening of the size distribution in time. The experimental data could be fitted with Smoluchowski's perikinetic coagulation model, which was validated by studying particle growth kinetics in IgG:DS mixtures of different concentrations. In conclusion, the combination of NTA and MFI provided novel insight into the kinetics and mechanism of protein–polyelectrolyte complex formation.

1. Introduction

The interaction between proteins and polyelectrolytes has been receiving increasing attention in pharmaceutical sciences because of the growing importance of protein drugs [1]. The latter is mainly related to their specificity and the lack of toxic metabolites, resulting in considerably less interference with untargeted biological processes and, hence, less adverse effects and increased clinical efficiency [2]. Successfully developing protein drugs, however, requires the availability of highly pure protein batches as well as suitable formulations that guarantee the physical and chemical stability of the protein [3–5] until its delivery at the target site.

Polyelectrolytes are a major group of the macromolecules that have shown to offer advantages in purification [6,7], stabilization [8] and delivery of therapeutic proteins [9,10]. Polyelectrolytes are suitable as component of protein delivery systems because they can be selected with specific hydrophilicity, versatile charge properties, biodegradability, natural origin, and roles in preventing aggregation and denaturation of proteins [11–14]. Moreover, polyelectrolytes have been used to increase the amount of protein loaded onto the surface of solid microneedles and microparticles via layer-by-layer deposition of oppositely charged proteins and polyelectrolytes [15,16]. Furthermore, polyelectrolyte-mediated precipitation methods have been used in protein purification processes [17] to decrease the number of isolation steps at a low cost. This approach is considered to be more selective than the use of other precipitants, such as ammonium sulfate or organic solvents [18]. In addition, another advantage of polyelectrolyte-assisted precipitation along with protein co-precipitation techniques [19] is that these methods do not require organic solvents that could be harmful to the protein as well as the environment. The molecular interaction involves electrostatic interactions between charged surfaces of the protein and oppositely charged groups of the polyelectrolyte [20]. The onset of complexation depends on several parameters, such as pH, ionic strength, protein/polyelectrolyte ratio and physico-chemical characteristics of the protein and the polyelectrolyte (e.g., charge, size) [21,22]. These interactions create insoluble complexes, which then aggregate further to form larger particles that will eventually precipitate from the

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solution [23]. The process of particle growth depends not only on the nature of the particles, but also on external factors, such as temperature, stirring and sedimentation [24].

A great challenge in the direct assessment of protein–polyelectrolyte interactions has been the lack of techniques that are able to simultaneously detect, characterize and quantify (sub)visible particles that form upon complexation of protein and polyelectrolyte. Emerging particle analysis techniques [25,26], however, may provide reliable ways to monitor protein–polyelectrolyte complex formation and growth. In the nanometer range, nanoparticle tracking analysis (NTA) is a valuable technique that counts and sizes particles in a suspension. In the flow-cell of NTA, the particles scatter a beam of laser light, which is detected through a microscope and recorded into a video exhibiting the movement of particles in the suspension. The displacement of individual particles, or the Brownian motion, in a plane is tracked in time to deduce the individual particle size [27–30]. In the micrometer size range, flow imaging microscopy techniques, such as, Micro-Flow Imaging (MFI), are currently gaining ground as established methods for micron-size particle sizing and counting [31–34]. The principle of detection is based on the change in the light intensity passing through a particle compared to the background. Based on the captured images the particle size and count are derived. The same images can be used to assess several morphological aspects of individual particles, such as aspect ratio and transparency.

The aim of this study was to develop a method based on the combination of NTA and MFI to monitor and characterize the process of particle formation and growth during protein–polyelectrolyte complexation, assisted by size-exclusion chromatography (SEC) to quantify the amount of unbound protein monomer. A monoclonal antibody was used as a model protein and dextran sulfate as a model polyelectrolyte. The experimental data for a few different experimental conditions were fitted with Smoluchowski's perikinetic coagulation model [35,36].

2. Materials

Dextran sulfate (from Leuconostoc spp., Mw = 5000), sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, sodium azide and sodium sulfate (p*K*a < 2) were obtained from Sigma (Sigma–Aldrich, Steinheim, Germany). Ultrapure water (18.2 MΩ cm) was dispensed by using a Purelab Ultra water purification system (ELGA LabWater, Marlow, UK). A monoclonal human IgG1 subclass (IgG; pI = 8.4), formulated at 65 mg/ml in 10 mM sodium citrate buffer containing 5% sucrose at pH 6.0, described before [28,37,38], was used as a model protein. Stock solutions of 0.01 mg/ml of IgG in aqueous solution of 10 mM phosphate buffer, pH 6.2 (filtered by using a 0.22-μm polyethersulfone-based syringe driven filter unit (Millex GP, Millipore, Ireland)), was prepared. The same buffer was used to prepare stock solutions of 0.01 mg/ml dextran sulfate. In preliminary studies we found that a low buffer concentration and a pH value lower than 7 are beneficial for the formation of IgG-dextran sulfate complexes. Addition of the protein or polyelectrolyte had no effect on the pH of 6.2.

3. Methods

3.1. Mixing, incubation and sampling procedure

A volume of 13 ml of the IgG stock solution was poured into a graduated glass cylinder (Duran®, Hirschmann, Eberstadt, Germany), with an inner diameter of 1.4 cm and a height of 14.9 cm. 13 ml of the dextran sulfate stock solution was added to the IgG solution. Subsequently, the IgG/dextran sulfate mixture was homogenized by gentle pipetting up and down 15 times, and then incubated for a period of 14 h. In order to avoid unwanted movements and temperature fluctuations (as these might affect the kinetics of the particle growth), the glass container was kept on a sturdy bench where the analytical instruments were located. The first sample was taken immediately after mixing. The sampling was continued for 840 min after preparation according to the scheme shown in Fig. 1.

Samples were directly used for the different analyses, unless otherwise stated. The experiment was performed twice. During the experiment the room temperature in the laboratory was monitored (23 ± 0.8 °C). In order to prepare samples for quantification of the free protein monomer content, 1 ml of the sample was centrifuged in a 1.5 ml Eppendorf tube at 18,000*g* for 15 min. A hundred μl from the top part of the liquid was taken and immediately used for measurement of the monomer content by using SEC. The supernatant was analyzed by NTA to confirm that it was free of particles (results not shown).

In order to check the applicability of the method to other formulations and to validate whether Smoluchowski's perikinetic coagulation model (see below) describes the particle formation process, additional mixing experiments were performed with two different concentrations of IgG and dextran sulfate in the starting materials, namely 0.005 and 0.02 mg/ml (instead of 0.01 mg/ml). The experimental procedure was kept the same, except that the particle formation process was monitored for only 270 min.

3.2. Size-exclusion chromatography

High pressure size-exclusion chromatography (SEC) was performed to quantify the amount of free IgG monomer in the solution in the absence and presence of dextran sulfate. This was executed on an Agilent 1200 chromatography system (Agilent Technologies, Palo Alto, California) combined with a Wyatt Eclipse (Wyatt Technology Europe GmbH, Dernbach, Germany). A Yarra 3 μm SEC-2000 column (300 × 7.8 mm) coupled with a Yarra Security Guard precolumn (Phenomenex, Torrance, CA, USA) was used. Centrifuged (18,000*g* for 15 min) samples (100 μl) were injected and separation was performed at a flow rate of 0.5 ml/min. The mobile phase consisted of 100 mM sodium phosphate, 100 mM sodium sulfate, and 0.05% w/v sodium azide at pH 7.2. Ultraviolet absorption detection was performed at 280 nm. In order to calculate the monomer decrease after complexation, the areas under the curve (AUC) of the UV signal were used.

3.3. Nanoparticle tracking analysis

NTA was performed at room temperature (23 ± 0.5 °C) with a NanoSight LM20 (NanoSight Ltd., Amesbury, United Kingdom) equipped with a 640 nm laser and operating at an angle of 173° with respect to the flow cell (100 × 80 × 10 μm). Samples were taken from the mixture vessel by using a sterile 1 ml syringe (BD Discardit II, New Jersey). The contents of the syringe were injected into the chamber by an automatic pump (Harvard apparatus, Catalog no. 98-4362). For each sample a 90 s video was captured with shutter set at 1495 and gain at 400. The video was analyzed

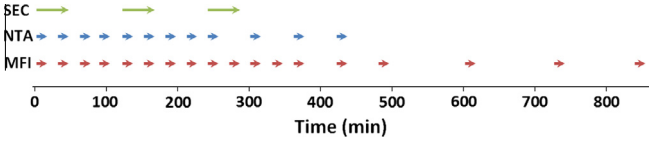

Fig. 1. Schematic overview of the sampling time points for each type of measurement performed in this study. The length of each rod indicates the approximate analysis time per sample, including sample pretreatment and handling.

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