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Optimization of high-concentration endostatin formulation: Harmonization of excipients' contributions on colloidal and conformational stabilities

Shujing Wang, Xinyi Zhang, Guoliang Wu, Zhou Tian, Feng Qian*

School of Pharmaceutical Sciences & Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Tsinghua University, Beijing 100084, China

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ABSTRACT

Recently, increasing research efforts have been devoted into developing high-concentration protein drugs for subcutaneous injection, especially for those with short half-lives and high-dose requirement. Proteins at high concentrations normally present increased colloidal and structural instability, such as aggregation, fibrillation and gelation, which significantly challenges the high-concentration formulation development of protein drugs. Here we used endostatin, a 20 kD recombinant protein, as a model drug for high-concentration formulation optimization. The colloidal and conformational stability of endostatin at high concentration of 30 mg/mL were investigated in formulations containing various excipients, including saccharides (mannitol, sorbitol and sucrose), salts (ArgHCl and NaCl), and surfactants (tween 20 and 80). Protein fibrillation was characterized and semi-quantified by optical polarized light microscopy and transmission electron microscopy, and the amount of fiber formation at elevated temperature of 40 °C was determined. The soluble protein aggregates were characterized by dynamic and static light scattering before and after dilution. The conformational stability were characterized by polyacrylamide gel electrophoresis, fluorescence, circular dichroism, and differential scanning calorimetry. We observed that the soluble aggregation, fibrillation and gelation, induced by conformational and colloidal instabilities of the protein solution, could be substantially optimized by using suitable stabilizers such as combinations of saccharides and surfactants; while formation of gel and soluble aggregates at high protein concentration (*e.g.*, 30 mg/mL) and elevated temperature (40 °C) could be prevented by avoiding the usage of salts. It's worth emphasizing that some stabilizers, such as salts and surfactants, could show opposite contributions in conformational and colloidal stabilities of endostatin. Therefore, cautions are needed when one attempts to correlate the colloidal stability of high-concentration proteins with their conformational stability, and the colloidal and conformational protein stabilities must be harmonized by a balanced selection of various types of excipients.

1. Introduction

With the ever increasing use of therapeutic recombinant proteins, stable protein solutions are highly desired for long-term storage, efficacious delivery, and free of adverse immunogenicity from protein derivatives or their assemblies (Bee et al., 2012; Frokjaer and Otzen, 2005). High-concentration (typically >15 mg/mL) protein formulations amendable for subcutaneous injection are much more patient friendly compared with traditional intravenous infusion, especially for those with short pharmacokinetic half-lives, thus have drawn increasing amount of research efforts from the industry and academia (Jackisch et al., 2014; Wang et al., 2015b). Proteins at high concentrations often demonstrate increased colloidal instability, such as aggregation, fibrillation and gelation. Aggregation has been reported on a vast of protein therapeutics and consuming great efforts in protein drug development (Frokjaer and Otzen, 2005; Lange and Rudolph, 2009; Mahler et al., 2010; Morris et al., 2009; Sahin et al., 2012; Shire et al., 2004; Wang et al., 2010). Fibrillation, another prominent issue of protein physical stability, has been extensively reported to be associated with *in vivo* amyloidogenic disorders such as Alzheimer's, Parkinson's, mad cow disease, cystic fibrosis, diabetes type II, *etc.* (Chiti and Dobson, 2006; Morris et al., 2009). Protein insoluble aggregation/fibrillation is also widely reported *in vitro* on model proteins such as lysozyme (Choudhary

* Corresponding author.
E-mail address: qianfeng@biomed.tsinghua.edu.cn (F. Qian).

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and Kishore, 2014) and protein drugs such as insulin (Choudhary et al., 2015), IgG2 (Sahin et al., 2012), α-chymotrypsinogen (Barnett et al., 2015), *etc*. Gelation has been observed on a variety of proteins such as myofibrillar protein (Brewer et al., 2005), β-lactoglobulin (Blake et al., 2015), ovalbumin (Xiong et al., 2016), *etc*. Although these colloidal properties highly depend on the intrinsic structural properties of proteins, they could be enhanced or inhibited by various environmental factors, such as additives, temperature, interfaces and mechanical stresses, *etc*. (Nielsen et al., 2001; Wang et al., 2010). The additives commonly used include saccharides/polyols, salts, surfactants and amino acids.

The enhancement of both colloidal and conformational stability of proteins is the essential goal of protein formulation optimization. Therefore, abundant researches have been conducted on the optimization and characterization of both kinds of stabilities and their possible correlations, especially on mAbs (Brader et al., 2015). Although protein conformation largely dictates the protein function and properties, the conformational stability of protein is not necessarily in accordance with the colloidal stability of the protein solution, especially at high concentrations of proteins and when the contributions of multiple environmental factors are involved. Also, it worth pointing out that, the colloidal instability of concentrated protein solutions could be caused by the specific or non-specific interactions (*i.e.*, H-bonding, hydrophobic, ionic, electrostatic, dipole-dipole, *etc*.) between protein molecules or between protein and environment, it could also be caused by or enhanced by the conformational instability of proteins. To simplify our discussion in this study, we use the term "colloidal instability" broadly without considering the exact cause at molecular level. Therefore, such colloidal instability could be due to the intermolecular interactions between native protein with intact protein conformation, or between denatured proteins that have already lost their conformational stabilities due to intramolecular protein unfolding. Excipients with diverse biophysical-chemical properties are included in formulations to stabilize the protein drugs from unfolding, aggregation, fibrillation, and gelation through different mechanisms (Ghosh et al., 2016; Kamerzell et al., 2011; Mahler et al., 2010; Roberts, 2014; Singh and Singh, 2003). Generally, stabilization of proteins through the addition of excipients, such as sugars/polyols, amino acids or salts, is believed to be realized dominantly by the thermodynamics of weak protein-excipient-water interactions (Timasheff, 1993), which is hard to be directly characterized with current technologies. Furthermore, the unfolding, aggregation, fibrillation and gelation behaviors of proteins are mostly thermodynamic and kinetic processes involving intramolecular protein folding/unfolding and intermolecular protein–protein association/dissociation. Although it has been reported that B_{22} value obtained at low concentrations could be of predictive value to the protein viscosity and aggregation propensity under limited conditions (Saito et al., 2012), the stability prediction of high-concentration protein formulations is still quite challenging, even with the molecular details and stability characteristics of the static protein at low concentrations.

Endostatin, a 20 kD C-terminal fragment from collagen XVII, displays potent inhibition on endothelial cell migration, proliferation and angiogenesis (Boehm et al., 1997; Dhanabal et al., 1999; Fu et al., 2009; O'Reilly et al., 1997; Yamaguchi et al., 1999). Modified recombinant endostatin (Endostar) was approved by the State Food and Drug Administration in China in 2005 for the treatment of clinical non-small cell lung cancer (NSCLC) in combination with cisplatin or other chemotherapy drugs (Folkman, 2006; Han et al., 2011; Zhou et al., 2011). The recombinant protein endostatin requires daily administration due to its short *in vivo* half-life of around 10 h. Therefore, a highly concentrated endostatin formulation for subcutaneous injection is highly desired to save daily clinic visits for intravenous administration and to significantly improve patient compliance.

There are several challenges in developing high-concentration therapeutic proteins, including solubility, viscosity and stability (Shire et al., 2004). Formulation optimization by selection of excipients with suitable physical-chemical properties provides the most effective and practically way for many protein drugs (Bhambhani et al., 2012). Therefore, we hereby use endostatin as a model protein and focus on its formulation optimization at high concentration of 30 mg/mL with various additives. We investigated both the colloidal and the conformational stability of endostatin in various formulations, in attempt to propose potential mechanisms behind the aggregation, fiber formation and gelation of endostatin (Zheng, 2009; Zhou et al., 2011). Furthermore, we aimed to identify and illustrate the consistency and discrepancy on the contributions of various additives to the colloidal and conformational stabilities of endostatin formulations.

2. Materials and methods

2.1. Materials and sample preparation

2.1.1. Protein

The endostatin protein, of 183 amino acid and with 9 extra residues (MGGSHHHHH, known as a zinc-binding peptide) at the N-terminus, (Fu et al., 2009; Jiang et al., 2009) was kindly provided by Simcere (Jiangsu Simcere Pharmaceutical Co. Ltd., China). The protein was recombinantly expressed from *E.coli*, purified and successfully refolded into the correct conformation. The endostatin was supplied in the buffer of 30 mM HAc-NaAc buffer at pH 5.5. This buffer was herein treated as the primary buffer unless specifically mentioned.

2.1.2. Reagents

Reagents for preparing excipients were of analytical grade and obtained from commercial vendors as follows: mannitol and sorbitol (Amresco), dextrose and sucrose (Beijing Chemical works), Tween 20 and 80 (polysorbate 20 and 80, Xilong Chemical), L-histidine and L-histidine-HCl (J&K chemical), L-arginine-HCl and trehalose (Sigma), guanidine-HCl and urea (Amresco), β-cyclodextrin (Zibo Qianhui Biotech), L-glycine, glycerol, NaCl, and NaAc (SinoPharm Chemical Reagent).

2.1.3. Containers

Containers used for stability test included 2 mL screw top glass vials (Agilent Technologies, INC.), microcentrifuge tubes (Axygen Eppendorf, also called EP tubes) and prefilled glass syringes (RTF, Gerresheimer Bunde GmbH).

2.1.4. Solution/formulation preparation

All buffers were filtered through 0.22 μm nitrocellulose membranes before usage (Millipore). Samples were concentrated using centrifugal filter units with the molecular weight cutoff of 10 kD (Amicon Ultra, Merck Millipore Ltd., Germany) and filtered through 0.45 μm nitrocellulose membranes before usage (Millipore). Formulations were prepared by mixing high concentration stocks of the endostatin, excipients and buffers to target concentrations.

2.2. Concentration measurement

The concentration of endostatin in solution was determined with UV–vis spectrophotometer (UV-4802, UNICO, China) by measuring the UV absorbance at 280 nm and 288 nm with diluted