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Differential scanning fluorescence approach using a fluorescent molecular rotor to detect thermostability of proteins in surfactant-containing formulations

Elisabeth Ablinger[a,b], Stefan Leitgeb[a], Andreas Zimmer[b,*]

[a] Research Center Pharmaceutical Engineering GmbH, Graz, Austria
[b] Institute of Pharmaceutical Sciences, Department of Pharmaceutical Technology, University of Graz, Graz, Austria

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ABSTRACT

A differential scanning fluorimetry (DSF) based high-throughput screening assay with the fluorescent molecular rotor CCVJ (9-(2-carboxy-2-cyanovinyl)julolidine) was developed. CCVJ is mainly sensitive to viscosity and less to polarity in comparison to polarity-sensitive dyes like SYPRO Orange, which was commonly used in DSF measurements. Therefore DSF with CCVJ is a suitable approach for high-throughput screening and stability testing of surfactant-containing protein formulations.

Due to the different detection principles of CCVJ and SYPRO Orange, the midpoint of the fluorescence curve of CCVJ, defined as temperature of aggregation (Tagg), was obtained at a higher temperature than the midpoint of the SYPRO Orange fluorescence curve, defined as temperature of hydrophobic exposure (Th). Granulocyte colony stimulating factor (G-CSF) was used as model protein for all measurements.

Commonly used surfactants in therapeutic protein formulations (polysorbate 20, polysorbate 80 and poloxamer 188) were investigated by DSF with CCVJ and SYPRO Orange. The fluorescence properties of CCVJ were minimally affected by investigated surfactants at concentrations typically used in pharmaceutical protein formulations. SYPRO Orange however, showed high background fluorescence as it also interacts with hydrophobic groups of surfactants. CCVJ was also capable of detecting thermally induced aggregation in the commercial polysorbate 80-containing product Neupogen®.

1. Introduction

The screening method differential scanning fluorimetry (DSF) represents a powerful technique in protein formulation development. DSF is an excellent method to identify solution conditions and excipients that stabilize proteins. It has been previously applied to investigate protein stability under different formulation conditions (He et al., 2010; Cheng et al., 2012; Goldberg et al., 2011). In DSF, the protein is heated by a multiwell RT PCR instrument, in the presence of a fluorescence dye, to monitor thermally induced structural changes of the protein conformation. The fluorescent dyes that are used for DSF are mainly responsive to solvent polarity. They are highly fluorescent in non-polar environment, like exposed hydrophobic groups of unfolded proteins, and are quenched in aqueous solutions. To date, SYPRO Orange is the most favorable dye for DSF due to its high signal-to-noise ratio. SYPRO Orange shows high specificity and sensitivity to surface exposed hydrophobic regions on proteins (Niesen et al., 2007; Nettleship et al., 2008; Samra and He, 2012). The high-throughput format and the low sample volume make DSF an ideal tool for analyzing various formulation conditions simultaneously. In earlier reports DSF with SYPRO Orange was shown to identify formulation conditions that enhance thermal stability of proteins (He et al., 2010; Cheng et al., 2012; Goldberg et al., 2011). The use of DSF with SYPRO Orange is however limited to formulations without surfactants, since interaction with the highly hydrophobic part of surfactants limits the use of SYPRO Orange-based DSF in protein formulation studies (Samra and He, 2012; He et al., 2011). However, another class of fluorophores, fluorescent molecular rotors, was shown in a previous study to be able to detect protein aggregation in the presence of surfactants without high background fluorescence (Hawe et al., 2010). Contrary to SYPRO Orange, fluorescent molecular rotors are mainly sensitive to the viscosity of the environment and to a lesser extent to changes in solvent polarity (Hawe et al., 2008). CCVJ, (9-(2-carboxy-2-cyanovinyl)julolidine), is a molecular rotor containing a nitrogen

Abbreviations: DSF, differential scanning fluorimetry; G-CSF, granulocyte colony stimulating factor; Th, temperature of hydrophobic exposure; CCVJ, 9-(2-carboxy-2-cyanovinyl)julolidine; Tagg, temperature of aggregation; CMC, critical micelle concentration.

* Corresponding author at: Institute of Pharmaceutical Sciences, University of Graz, Department of Pharmaceutical Technology, Humboldtstrasse 46, 8010 Graz, Austria. Tel.: +43 316 380 8881; fax: +43 316 380 9100.
E-mail address: andreas.zimmer@uni-graz.at (A. Zimmer).

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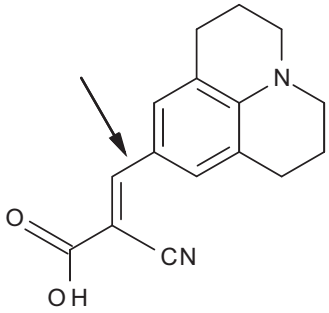

Fig. 1. Structure of the molecular rotor CCVJ. The arrow indicates the bond where internal rotation in the excited state takes place (Hawe et al., 2010).

atom attached to a π-system as electron donor, and a nitrile group, as electron acceptor (see Fig. 1) (Haidekker et al., 2005). Upon photoexcitation, an intramolecular charge transfer from the donor to the acceptor unit takes place. If this nonradiative internal rotation is constrained due to increased viscosity or steric hindrance by binding to protein aggregates, CCVJ loses its excited state energy by an increase in the fluorescence quantum yield (Hawe et al., 2010; Haidekker et al., 2005; Kung and Reed, 1989).

The nonionic surfactants polysorbate 20, polysorbate 80 and poloxamer 188, also known as Tween 20®, Tween 80® and Pluronic F68®, respectively, are commonly added to protein formulations to protect against agitation- and interface-induced aggregation (Randolph and Jones, 2002). They are used in parenteral protein products, because they have low toxicological properties and are well tolerated (Lee et al., 2011; Akers, 2002). Surfactant concentrations used in protein formulations are typically near the critical micelle concentration (CMC), the concentration of surfactants above which micelles form (Jones et al., 1997).

In this study we combined DSF with the fluorescent molecular rotor CCVJ to evaluate thermal stability of the model protein G-CSF in surfactant-containing formulations. In contrast to the commonly used dye in DSF measurements, SYPRO Orange, CCVJ was suitable to detect protein aggregation in the presence of surfactants with DSF for the first time. The three predominantly used surfactants in parenteral protein formulations, polysorbate 20, polysorbate 80 and poloxamer 188, were tested. Furthermore, the suitability of the CCVJ-based DSF approach to detect thermal degradation in the commercial polysorbate 80-containing product Neupogen® was shown.

2. Materials and methods

2.1. Materials

G-CSF was supplied by Sandoz GmbH (Kundl, Austria) in a 10 mM glutamate buffer at pH 4.4 with 5% (w/v) sorbite. Acetic acid glacial and sodium phosphate mono- and di-basic were obtained from Merck (Darmstadt, Germany). Sodium acetate was purchased from Roth (Karlsruhe, Germany). The surfactants polysorbate 20 and polysorbate 80 were obtained from Fluka (Buchs, Switzerland) and poloxamer 188 was provided by BASF Chem Trade GmbH (Burgbernheim, Germany). D-sorbite and the fluorescent molecular rotor 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). A stock solution of CCVJ was prepared in absolute ethanol (AustrAlco Österreichische Alkholhandels GmbH, Spillern, Austria). The SYPRO® Orange dye was acquired from Invitrogen (Eugene, OR, USA) as a dimethyl sulfoxide (DMSO) solution 5000×. The molar concentration and molecular weight of the dye are considered proprietary information and are not released to the public. The commercial product Neupogen® (Expiry date 08-2013, Lot no. 1029442) contained 0.6 mg/ml G-CSF, 0.004% (w/v) polysorbate 80 and 5% (w/v) sorbite in 10 mM acetate buffer at pH 4.0. High-performance liquid chromatography (HPLC) grade water was used throughout the experiments.

2.2. Methods

2.2.1. Sample preparation

Buffer conditions (10 mM acetate at pH 4.0 and 10 mM sodium phosphate at pH 6.0) were achieved via buffer exchange of a concentrated protein stock by illustra NAP™-10 columns (GE Healthcare, Buckinghamshire, UK). After the buffer change, the protein concentration of the eluate was determined on a NanoPhotometer™ (Implen GmbH, Munich, Germany). An extinction coefficient for G-CSF of 15,470 M^{-1} cm^{-1} at 280 nm was identified by using the ProtParam tool of ExPASy (Swiss Institute of Bioinformatics, Basel, Switzerland; http://www.expasy.ch/tools/protparam.html) based on the G-CSF sequence of Ph.Eur. 7.0/2206 (Ph.Eur. 7.0/2206, 2011).

Stock solutions of tested surfactants (polysorbate 20, polysorbate 80 and poloxamer 188) were prepared in 10 mM acetate buffer pH 4.0. Final surfactant concentrations, when adding the fluorescent dye (see below) for DSF measurement, were 0.004%, 0.01%, 0.02%, 0.05%, 0.1% and 0.2% (w/v). All formulations were prepared in a 96-well plate. Surfactant stock solutions were spiked in. Corresponding buffers were added to ensure the buffer concentration.

A corresponding placebo of Neupogen® was prepared: 5% (w/v) sorbite and 0.004% (w/v) polysorbate 80 in 10 mM acetate buffer at pH 4.0.

2.2.2. Differential scanning fluorimetry (DSF)

For measurements with SYPRO Orange, DSF was carried out following the general protocol outlined by Niesen et al. (2007). This method was modified for measurements with CCVJ. To optimize both assays for G-CSF, fluorescent dye concentration and protein concentration were varied. The lowest concentration of dye that generated a strong fluorescent signal was 5× for SYPRO Orange (SYPRO Orange stock solution was diluted 1:1000) and 10 μM for CCVJ with a protein concentration of 0.6 mg/ml.

Immediately before screening, both fluorescence dyes, SYPRO Orange (5000×) and the stock solution of CCVJ in ethanol, were diluted with the corresponding buffer to 25× and 50 μM, respectively. The diluted dye solutions were then added to the protein samples to achieve a 5× SYPRO Orange and 10 μM CCVJ final working concentration. The final concentration of G-CSF was 0.6 mg/ml. 30 μl of prepared samples were added to white, unskirted 96-well PCR plates (Bio-Rad) and sealed with optical foils (Bio-Rad). For measurements with SYPRO Orange, a CFX96 Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was employed. The excitation/emission filter settings were according to the "FRET" channel that is compatible to the SYPRO Orange fluorescence signal. For measurements with CCVJ, an iCycler iQ Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) installed with specific filters for CCVJ (Ex/Em: 435/500 nm) was used. The samples were exposed to a temperature ramp from 25 °C to 95 °C at a heating rate of 1 °C/min and at 0.5 °C increments.

The midpoint of the fluorescence curve of SYPRO Orange, defined as temperature of hydrophobic exposure (Th) and the midpoint of the CCVJ fluorescence curve, defined as temperature of aggregation (Tagg), were calculated by the CFX Manager™ Software (Niesen et al., 2007; Nettleship et al., 2008) using a mathematical second derivative method. The reported Th and Tagg