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Light scattering coupled with reversed phase chromatography to study protein self-association under separating conditions

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ABSTRACT

An on-line method, coupling reversed phase chromatography with static light scattering, was developed to determine the association state of freshly eluted proteins. Under downstream process conditions, human insulin desB30 and human insulin AspB28 were tested at concentrations up to 8.5 mg/mL. The refractive index increment (dn/dc) for insulin was found to depend strongly on the solvent used. A refractive index increment of 0.184 ± 0.003 mL/g was found in an aqueous buffer, pH 7.4, whereas the value was 0.155 ± 0.003 mL/g in 30%, w/w ethanol. The methodology combines on-line SLS and UV measurements with the pre-determined refractive index increment values. The developed on-line method was verified by standard off-line measurements establishing the association state at concentrations between 0.2 and 6.0 mg/mL. The equipment was calibrated utilizing insulin under conditions reported to ensure either monomer or hexamer forms. The self-association of human insulin desB30 was found to be strongly suppressed in 30%, w/w ethanol at pH 7.4 in which the monomer predominates. When stabilized by zinc ions in 30%, w/w ethanol at pH 7.4, an average association number of 3.7 was found. These data demonstrate the effect of ethanol to lower strongly the energy advantage by protein self-association. Potassium chloride and/or calcium chloride in the eluents were found to be of no consequence to the association state.

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1. Introduction

The scope of the work presented here was to develop an analytical method for monitoring on-line the immediate association state of a protein at the outlet of a reversed phase chromatographic (RPC) column. Knowledge of the current association state of any active pharmaceutical ingredient can be of great importance to the design of an efficient downstream process. For proteins, this is particularly true when separating target proteins from undesired product-related impurities, as these may participate indiscriminately in the formation of oligomers and thus hamper the separation due to formation of oligomers of mixed composition.

Establishing the association state under production conditions would also assist in building accurate mathematical models for a downstream step.

In order to prove the methodology, insulin was chosen as a model protein for two reasons. Firstly, highly selective liquid chromatography purification protocols already exist. Insulin has commonly been purified using RPC on e.g. C18 phases with pore sizes of 10 nm [1] and 30 nm [1,2]. Secondly, the protein's well-described self-association behavior can be utilized to prepare stable internal size standards for the experimental work.

Human insulin is a double-chained polypeptide consisting of 31 hydrophilic and 20 hydrophobic amino acids [3]. In water, the shielding of hydrophobic residues by dimerization is thermodynamically favorable [4,5]. This shielding of hydrophobic surfaces is part of the reason for the relatively high solubility of insulin in polar solvents. Insulin exhibits a complex equilibrium of mono-, di-, tetra-, and hexamers with the dimer as the stable subunit of all insulin oligomers [3,6]. Two dimers form the tetramer, and the hexamer forms upon binding of a third dimer [7]; i.e. no trimers or pentamers exist [3]. Hydrodynamic diameters of the mono-, di-, and hexamer are 2.6, 3.6, and 5.6 nm, respectively [2,8]. The insulin hexamer is highly favored by the binding of two zinc ions in solutions above pI (pH 5.4) [3,8].

The association state of insulin has been studied extensively under a large number of conditions [9–11] and using many different techniques. Direct measurement of the oligomers' physical

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properties can be done via e.g. mass spectrometry [9], sedimentation [12,13], or light scattering [8,14–17]. Indirect measurement is based on indication of insulin's tertiary structure found by either light absorbance [17–20], nuclear magnetic resonance [21,22], or circular dichroism [6,12,18,23].

Both non-aqueous and aqueous solutions of insulin have been studied at different pH values with or without inorganic salts [23]. Looking at published data for the association state of insulin in aqueous solutions, the most important factors seem to be protein concentration [8,14,15,22,24], pH [3,4,10,24,25], ionic strength [13,15,24], and whether or not zinc ions are present [8–10,26]. Relatively few results have been published on aqueous mixtures of ethanol [4,17,23,27], which may be utilized for preparative reversed phase chromatography [27,28], and none at all for a 30%, w/w ethanol/water mixture buffered at neutral pH. However, experiments utilizing indirect size determination have indicated some effect of ethanol on insulin's self-association [29].

In this work, static light scattering (SLS) was used to determine the concentration dependent association state of human insulin desB30 (HI-desB30) in aqueous solutions and in hydro-organic solutions with and without high salt concentrations. HI-desB30 and human insulin show the same concentration-dependent tendency to form oligomers [12]. As a comparative measure, human insulin AspB28 (HI-AspB28) was also examined as this insulin analog has been engineered to exhibit a suppressed association tendency compared with human insulin [7,11,12]. HI-AspB28 is also known under the name "insulin aspart". Bovine serum albumin (BSA) was used as molecular mass reference [16,30].

The experiments presented here used a novel setup featuring a reversed-phase column directly coupled on-line to an SLS detector and a UV detector. A standard SLS setup would encompass SLS detection and measurement of the differential refractive index (RI) of the eluate. For RPC, using a hydro-organic solvent to elute the proteins from the column would make it impractical, if not impossible, to use RI as a measure of solute concentration. For silica-based resins, contact with the resins' highly polar residual silanol groups would cause eluent heterogeneity [31]. The RI reading would become very unstable opposite the standard RPC UV reading, which is unaffected by fluctuations in the ethanol concentration. Hence, this on-line setup utilized the SLS flow cell for measuring the scattered light and the UV flow cell for protein concentration determination. Elimination of the RI instrument from the on-line setup demanded that the refractive index increment (dn/dc) had to be established off-line for the solvent used in order to calculate the molecular masses from the SLS and UV measurements. dn/dc values between 0.18 and 0.20 mL/g [30] are reported for most proteins in aqueous solutions, including insulin [2,8]. However, no solid information was found published for proteins in aqueous mixtures of salt and 30%, w/w ethanol.

2. Materials and methods

2.1. Chemicals and protein samples

All chemicals were of analytical grade. BSA was purchased from Sigma Corp. Novo Nordisk A/S, Denmark supplied HI-AspB28 and HI-desB30 as freeze-dried powders.

2.2. Static light scattering (SLS)

SLS was measured using a Dawn EOS instrument from Wyatt Technology, Santa Barbara, CA, USA, with a K5 flow cell.

2.3. Refractive index

The differential refractive index of solutions was measured offline by a differential refractometer, RID-10A, Shimadzu, Japan. All refractive index measurements were done at a constant temperature of 40.0 °C, the internal temperature of the refractometer. Although the light scattering measurements were done at ambient temperature, the change in refractive index increment was estimated as being in the range 0.2–1% higher at the lower temperature and thus insignificant to the present investigation [32,33]. The Astra 4.73.04 software package was used to control the instrument and partly to analyze data. However, most of the data analysis was done using Microsoft Excel. The refractive index increment (dn/dc) values for HI-desB30 and HI-AspB28 in the different solvents were found by linear regression as the slope of differential refractive index values plotted versus insulin concentration. For the calibration with BSA, a monomer molecular mass of 66400 g/mol and a dn/dc value of 0.185 mL/g were assumed. Molar masses of 5682 and 5819 g/mol were assumed for HI-desB30 and HI-AspB28, respectively.

2.4. Static light scattering data treatment

In principle, light scattered by a solution of molecules with a molecular mass M, and a mass concentration c, is described by Eq. (1) [34], where R_θ is the excess Rayleigh ratio at the scattering angle θ.

$$\frac{Kc}{R_\theta} = \frac{1}{MP(\theta)} + 2Bc \text{ with } K = \frac{4\pi^2 n_0^2 (dn/dc)^2}{N_A \lambda_0^4} \quad (1)$$

The instrument presented the excess Rayleigh ratio as a difference ΔU between the voltage signal from the solution and that of the pure solvent. The two are connected through an instrumental constant, i.e. $R_\theta = k_{\text{instr}}\Delta U$. K is the optical contrast constant dependent on n_0, the refractive index of the pure solvent, and $\lambda_0 = 690$ nm, which is the vacuum wavelength of the light source. The form factor of the molecules, $P(\theta)$, is set to 1 for all scattering angles for the molecules in this investigation (deviations from 1 would require the molecules to be larger than about $\lambda_0/20$; in this case larger than approx. 30 nm). B is the second virial coefficient, describing non-ideality of the solution and is negative if the dissolved molecules attract, i.e. tend to aggregate, and positive if their interaction is repulsive.

Although the light-scattering instrument used is capable of measuring at 16 different angles at the same time, only the signal from the detector at a scattering angle of 90° was used.

Referring to Eq. (1), Eq. (2) defines the apparent molecular mass M_{app} as:

$$\frac{1}{M_{\text{app}}} = \frac{1}{M} + 2Bc \quad (2)$$

Eq. (1) can, through the assumptions made (see above), be rewritten as Eq. (3), where k_{cal} is a new instrumental calibration constant:

$$M_{\text{app}} = k_{\text{cal}} \frac{\Delta U}{(dn/dc)^2 c} \quad (3)$$

If several molecular species (i.e. different oligomeric states) with molecular masses M_i and corresponding mass concentrations c_i are present in the solution, the apparent molecular mass found under ideal conditions by light scattering is the weight average molecular mass, see Eq. (4) [34]. If the species are i-mers of a monomer, the equation can be rewritten as shown:

$$\bar{M}_w = \frac{\sum_i c_i M_i}{\sum_i c_i} = \frac{\sum_i c_i (iM)}{\sum_i c_i} = M \times \frac{\sum_i c_i \times i}{\sum_i c_i} \quad (4)$$

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