Journal of Chromatography A, 1293 (2013) 75–84

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Self-interaction chromatography in pre-packed columns: A critical evaluation of self-interaction chromatography methodology to determine the second virial coefficient

Natalie Rakel, Kristina Schleining, Florian Dismer, Juergen Hubbuch *

Section IV: Biomolecular Separation Engineering, Institute of Engineering in Life Sciences, Karlsruhe Institute of Technology, Engler-Bunte-Ring 1, 76131 Karlsruhe, Germany

ARTICLE INFO

Article history:
Received 7 August 2012
Received in revised form 5 March 2013
Accepted 31 March 2013
Available online 8 April 2013

Keywords:
Self-interaction chromatography
Static light scattering
Protein interactions
Phase behavior

ABSTRACT

The characterization of protein–protein interactions is commonly conducted via self-interaction chromatography to describe magnitude and direction of the interactions with the resulting osmotic second virial coefficient (B_{22}). However, the method is invasive and protein immobilization on the adsorber surface can influence the results obtained. In order to replace batch immobilization procedures followed by a column packing, direct on-column preparation was optimized in terms of protein immobilization under a continuous flow. Surface load was measured applying a novel method based on partial least squares analysis of spectral scans to reduce analytical error when determining the amount of immobilized protein. Subsequently influencing parameters such as the effects of absolute surface load, injected protein concentration and distribution of protein orientation were analyzed and system performance evaluated. The results disprove the consistency of the SIC method regarding the non-random orientation of proteins on adsorber particles. Thus the determined B_{22}-values differ quantitatively from those determined with static light scattering. Furthermore, variations in immobilization conditions influence the results obtained. These results make clear that SIC does not fulfill the theoretical framework of B_{22}-analysis. It is rather a qualitative measure of protein–protein interactions in the respective system used for experimentation.

1. Introduction

Phase behavior of proteins is currently one of the key parameters determining protein purification but even more formulation strategies. Ever higher product titer, the search for alternative processing steps in the mAb industry as well as the drive toward highly concentrated formulations make it mandatory to get a deeper understanding into protein phase behavior, solubility issues and rheological parameters of solutions of high protein concentrations.

A widely used predictive method to describe phase behavior of proteins in solution is the application of the osmotic second virial coefficient (B_{22}). It is a promising fundamental thermodynamic approach to evaluate buffer conditions in terms of protein stability and phase behavior [1–3]. With this approach interactions between molecules of the same type in a diluted solution are characterized. The magnitude and sign of the B_{22}-value indicates whether attraction or repulsion dominates. While a negative value corresponds to attraction, repulsion results in a positive B_{22}. This can be applied to estimate the phase behavior of concentrated protein solutions. George and Wilson [4] postulated that in the so called "crystallization slot", a small range of slight negative B_{22}-values, the probability of crystal growth of various proteins is high. Pjura et al. [5] optimized crystallization conditions by choosing liquid phase compositions with B_{22} within this crystallization slot and could crystallize bovine chymotryspinogen A without any precipitant. Even the crystal structure of a membrane protein could be determined after optimization with B_{22}-screening via X-ray diffraction by Gabrielsen et al. [6]. On the other hand the screening for positive B_{22}-values could be used for a rapid determination of high solubility formulation conditions [1]. Le Brun et al. [7] for example could increase the stability of an IgG1 antibody in solution with a B_{22}-buffer screening. A theoretical explanation for the connection between B_{22} and solubility is published by Haas et al. and Ruppert et al. [8,9].

To determine the B_{22} different colloidal measurement techniques are described in literature such as membrane osmometry [5,10], sedimentation equilibrium measurements [11], self-interaction-chromatography (SIC) [6,11–13] and static light scattering (SLS) [14–16]. Because of the low protein and time consumption SIC is the most commonly applied technique in literature

* Corresponding author. Tel.: +49 721 608 42557; fax: +49 721 608 46240.
E-mail address: juergen.hubbuch@kit.edu (J. Hubbuch).

0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.03.077

and most of the mentioned studies were conducted with this technique. The self-interaction of different kinds of proteins such as membrane proteins [6], antibodies [17,18] or the model proteins [3,19,20] were analyzed either to increase the solubility or to find crystallization conditions. Based on these results García et al. [21] and Deshpande et al. [22] further reduced the required volume by applying the SIC on microchips. Therefore the SIC is object of a thorough investigation in terms of immobilization technique and comparability of the results to static light scattering experiments. SIC is based on the comparison of protein retention behavior in a column with immobilized protein of the same kind to its behavior in an unmodified column. Weak interactions between free protein in the mobile phase and immobilized protein result in a shift in retention volume revealing the nature of their interaction. But covalent immobilization itself is an invasive methodology changing protein properties by eliminating surface charges while leaving only certain parts of the protein accessible to the solvent [23]. The question however arises in how far the immobilization procedure influences the results of a B_{22} measurement? As B_{22} is considered a physical characteristic of protein and buffer systems used, the analytical technique as such should not influence the outcome of analysis. Potential sources of error such as the kind of adsorber material [20], the immobilization degree [3,24] or the injected protein concentration [3,20] have been identified. However the influence of the buffer conditions during immobilization on B_{22}-values and the distribution of protein orientation in the adsorber particles have so far not been addressed.

To shed some light into this, we followed a new approach for preparing a SIC set-up that is based on the treatment of activated adsorber particles in pre-packed columns under continuous flow conditions. This includes immobilization of proteins and blocking of remaining active groups on the adsorber particle surface. The uniformity of protein distribution after immobilization on the adsorber particle surface and along the column was monitored via confocal laser scanning microscopy (CLSM). The overall immobilization degree was determined from the mass balance of remaining protein in the coupling buffer [13,20]. Since a part of the reactive compound on the adsorber particle surface is released during immobilization reaction that shows UV absorption at 280 nm, the time consuming bicinchoninic acid (BCA) assay is mostly used for analytics [1,7,13,20]. In this paper we used an alternative approach that allowed direct quantification of protein in the coupling buffer using its unique absorption spectrum by applying partial least squares (PLS) regression [25].

Following a reproducible and well characterized preparation of a SIC analytical set-up, the following parameters were systematically investigated regarding to their effect on the B_{22}: the injected protein concentration, the adsorber particle surface load and the buffer conditions during the immobilization procedure. Finally, the obtained SIC B_{22}-values were compared to SLS-data.

2. Theory

2.1. Osmotic second virial coefficient (B_{22})

The osmotic second virial coefficient (B_{22}) describes magnitude and direction of non-ideality of the osmotic pressure π in a dilute solution. It is part of the virial expansion of the osmotic pressure π:

$$\prod = RTc_P\left(\frac{1}{M_W} + B_{22}c_P + \cdots\right) \quad (1)$$

Parameters are the universal gas constant R, the temperature T, the protein concentration c_P and the molecular weight of the protein M_W. The reasons for the non-ideality are weak interactions between two molecules of the same kind in a dilute solution. These interactions are based on excluded volume, electrostatic interaction, osmotic potential, hydrophobic and short range interactions (van der Waals, solvation, hydrogen bonding) [26–28]. Interactions between proteins can be described via the potential of mean force $W(r, \Omega_1, \Omega_2)$, which is correlated to the B_{22} [20,29,30]:

$$B_{22} = -\frac{N_A}{M_W^2}\int_{\Omega_1}\int_{\Omega_2}\int_0^\infty \times \left[\exp\left(\frac{-W(r,\Omega_1,\Omega_2)}{k_BT}\right) - 1\right] r^2 dr d\Omega_2 d\Omega_1 \quad (2)$$

where k_B is the Boltzmann constant, N_A is the Avogadro constant, r the center-to-center-distance of two protein molecules in solution and Ω_1, Ω_2 the rotation angles defining the orientation of both molecules toward each other. According to Eq. (2) the B_{22} contains information about apparent intermolecular forces. Repulsive interactions between molecules result in positive, attractive ones in negative B_{22}-values.

The potential of mean force $W(r, \Omega_1, \Omega_2)$ is defined as the work required to bring two indefinitely separated protein molecules to a finite separation r averaged over all possible configurations of the solvent molecule, assumes that the potential of mean force is spherically symmetrical and only accounts for a two body protein–protein interaction [24].

For a chromatographic system where one of the interaction partners is immobilized this relationship does not apply and several studies have correlated the distribution factor K_D to the potential of mean force between the mobile molecule and the stationary phase [3,20,24]. Different approaches in the respective correlations accounted for the experimental differences. Tessier et al. [3] added a separate excluded volume contribution to reach their final B_{22}-value. Teske et al. [24] argued this to be redundant as they measured a retention volume in excess of that for a protein-free stationary phase. Ahamed et al. [20] followed the approach of Teske et al. [24], however, relating the B_{22} to distribution coefficients to K_{SEC} and $K_{overall}$ allowing more flexibility in the experimental set-up. However when comparing data obtaining when measuring B_{22} in free solution addressing Eq. (2) with the chromatographic determination of the B_{22} in SIC several prerequisites are set [3,12,20,24]:

- Adsorber material: usage of inert adsorber material with no interaction of the protein and the adsorber material. A pore diameter which is significantly larger is size than the protein diameter.
- Immobilized protein: random orientation of the immobilized protein and structure conservation. Therefore the free energy change of bringing a protein molecule from the interstitial volume into the pore volume so that it interacts with a single immobilized protein molecule is equal to the potential mean force between two protein molecules free in solution.
- Interaction: one single free protein molecule interacts with one single immobilized protein molecule and does not interact with other free protein molecules. Teske et al. [24] accounted for multipoint interactions indicating, however, that this would lead to deviations between B_{22} measured in free solution and B_{22app} measured in SIC.

If these prerequisites are met the degree of interaction between immobilized protein and the one in solution can be determined by