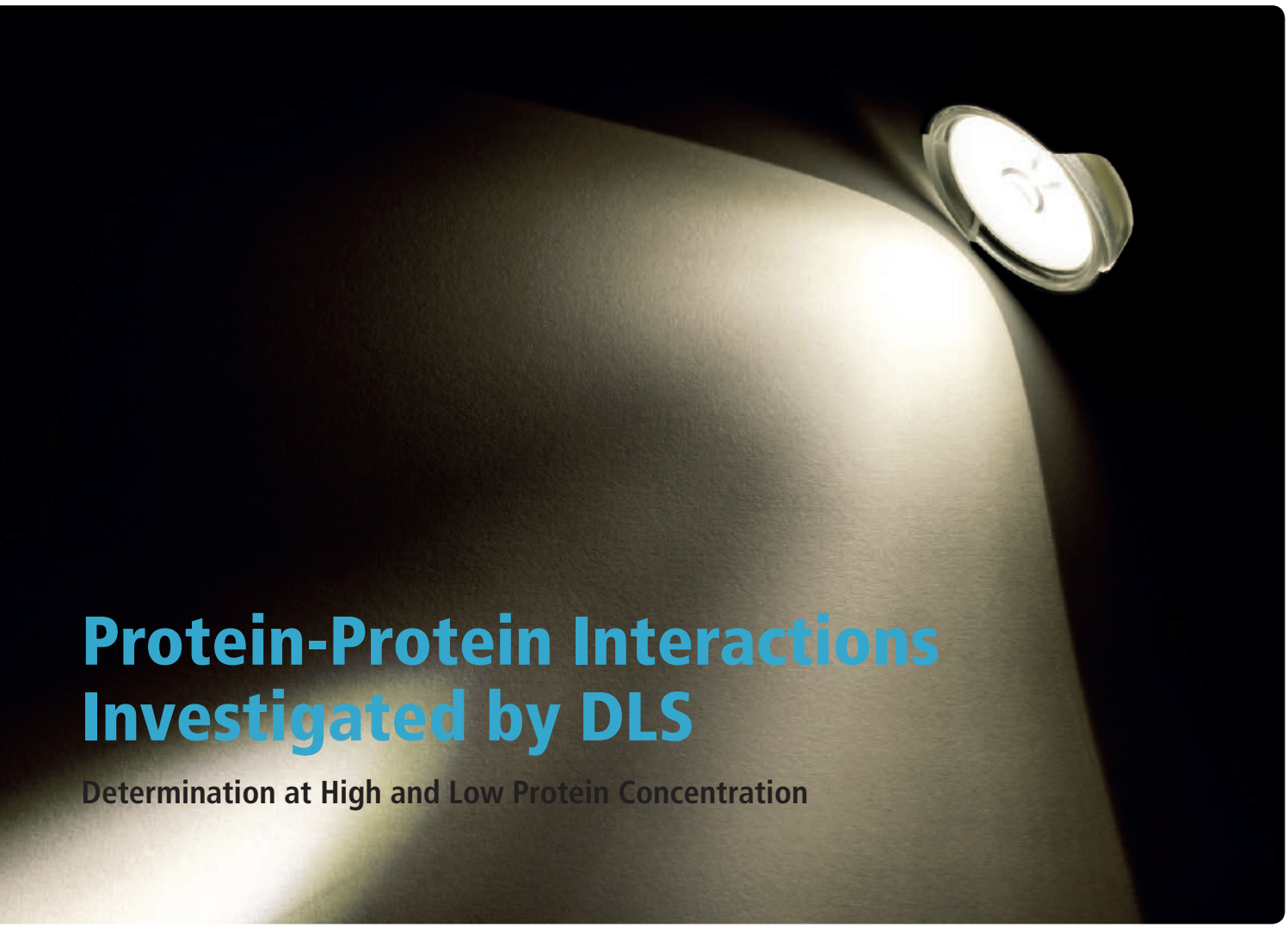

Protein-Protein Interactions Investigated by DLS

Determination at High and Low Protein Concentration

Within the pharmaceutical and biotech industries, therapeutic monoclonal antibodies (mAbs) have attracted significant attention during the last few decades. Their use as therapeutics is steadily increasing, with currently 31 therapeutic mAbs approved worldwide for treatment of a wide range of diseases, including cancer and autoimmune-disorders.

Currently approved mAbs are generally administered through intravenous injection (IV). This requires professional healthcare assistance or hospitalisation. Many of the treated diseases are chronic and require frequent administration; subcutaneous (SC) administration is thus highly preferable, as this would enable patient self-administration. However, mAb treatment requires relatively large doses, often in the range of several mg/kg bodyweight. The volume restrictions (< 1.5 ml) for SC injections, therefore, necessitate the development of high concentration formulations [1].

With the development of high concentration formulations (> 100 mg/kg), the formulation scientist faces a number of significant challenges including: impaired long term stability due to self-association and aggregation; processing, manufacturing and administration difficulties due to high viscosities; and finally, a lack of readily available and applicable analytical methods for the direct characterization of these formulations [2-5].

The increased viscosity is believed to be a result of transient interactions between protein molecules [6]. Protein-protein Interactions (PPI) are traditionally quantified using non-ideality parameters, such as the second viral coefficient (B_{22}) and the interaction parameter (k_D) in dilute solution conditions. The most commonly used technique for determination of B_{22} values is static light scattering (SLS). However, analytical ultracentrifugation (AUC), membrane osmometry, size exclusion chromatography (SEC), self-interaction chromatography (SIC) and more recently dynamic light scattering (DLS) are also available for the determination of B_{22} [7-11].

This article will provide highlights from a study where the apparent radius (R) is measured using dynamic light scattering and compared with the true hydrodynamic radius (R_H) of the molecule to give information about PPI [12]. This novel method to determine PPI, which can be performed at high and low concentration, was compared with k_D values that were also determined by DLS. In addition the relation between PPI and solution viscosity at high protein concentration using three mAbs is also explored.

Materials and Methods

One IgG_1 and two IgG_4s formulated at varying pH and ionic strength were used in the study. The antibodies B72.3 IgG_1 (mAb-A) and IgG_4 (mAb-B) recognise the antigen tumor-associated glycoprotein (TAG-72) and have been developed as a murine antibody of the IgG_1 subclass. The

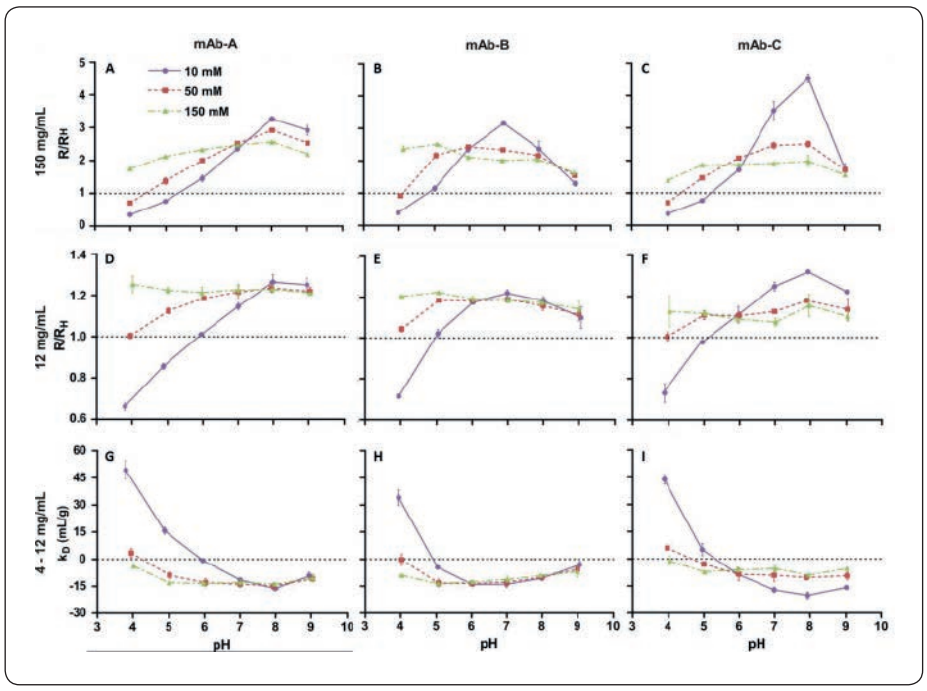

Fig. 1: Nature of PPI at high and low concentration. Attractive interactions above the dashed line horizontal line in A-F and below in G-I. From [12], reprinted with permission from Elsevier.

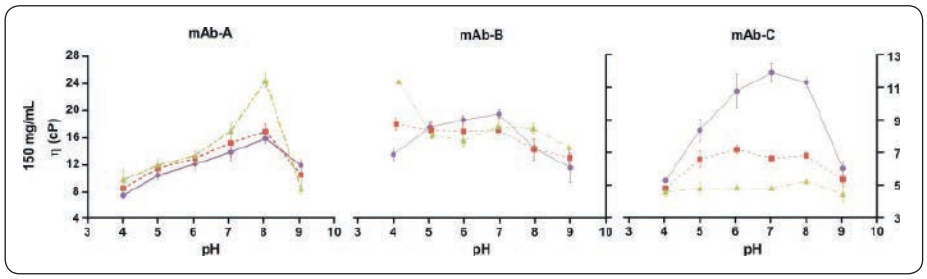

Fig. 2: High concentration viscosity measured by DLS and polystyrene beads. Color codes the same as for Figure 2. From [12]], reprinted with permission from Elsevier.

variable domains were cloned and transferred to human IgG_1 and IgG_4 constant domains. Antibody HzATNP (mAb-C) is a humanized IgG_4 antibody against the hapten trinitrophenyl (TNP). Both mAb-A and mAb-B have kappa (κ) light chains and the sequence identity in the light chain is therefore 100 %. Sequence identity in the constant region of the heavy chain is approximately 95 %. mAb-C, being an IgG_4, has a similar constant region as mAb-B, whereas the variable region shares only 45 % sequence identity (including 9 inserts) with mAb-B. All antibodies were purified using Protein A affinity chromatography followed by concentration and buffer exchange (10 mM histidine buffer pH 6.5) using tangential flow-filtration (TFF).

Dynamic Light Scattering – Determination of k_D and R/R_H

All DLS studies were performed at 25 °C, using a DynaPro Plate Reader equipped with an 830 nm laser source (Wyatt, Santa Barbara). The software used was Dynamics V7, Version 7.1.0 (Wyatt). The samples were measured by adding 35 µl to a 384 Well UV-Star Clear Microplate (781801). The plate was covered with a clear disposable tape to avoid evaporation during measurement. Prior to measurement all plates were centrifuged at 3000 rpm for 5 minutes using an Eppendorf Centrifuge 5810 R. 10 acquisitions of each 5 seconds were measured for each sample with auto adjustment of attenuator and laser power. Three wells of each sample were averaged and all samples were measured in triplicate. Water was chosen as a standard solvent during all measurements.

For the determination of k_D, high concentration samples were diluted with the buffer of interest to obtain a concentration of 12 mg/ml. 5M NaCl was spiked in during dilution to adjust solution ionic strength to 50 and 150 mM respectively. 12 mg/ml samples were filtered through 0.22 µm filters and diluted in filtered buffers (with desired pH and ionic strength) to obtain lower concentration samples, which were then added to the microplate. The k_D value was then determined by a linear fit of the measured (mutual) diffusion coefficients (D_m) as a function of concentration (c) (Eq. 1).

$$D_m = D_0 (1 + k_D c)$$

Where D_0 is the self-diffusion coefficient (diffusion coefficient as $c \rightarrow 0$) and k_D is the interaction parameter.

For the determination of the relative size, the apparent hydrodynamic radius of the antibodies was determined at 150 and 12 mg/ml directly from the Stokes-Einstein equation without any corrections for solution viscosity. The true hydrodynamic radius was calculated from D_0 using the Stokes-Einstein equation. The relative size was then calculated (apparent radius divided by hydrodynamic radius) to show the nature of the PPI. Values < 1 correspond to repulsive PPI, whereas values > 1 relate to attractive interactions.

Results and Discussion

Protein-Protein Interactions

At pH 8.0, mAb-A demonstrated a maximum relative size irrespective of ionic strength (Fig. 1). As the ionic strength is increased, the PPI-pH profile gradually becomes more flat. At pH 4.0 at 10 and 50 mM ionic strength and at pH 5.0 at 10 mM ionic strength repulsion was observed. All other pH and ionic strength combinations showed attractive PPI. Almost no change in relative size was observed with increasing ionic strength at pH 7.0. At pH 8.0 and 9.0 the interactions became less attractive with increasing ionic strength.

At pH 7.0, mAb-B showed a maximum size. The maximum shifted towards lower pH (pH 6.0 at 50 mM and pH 5.0 at 150 mM) upon increasing ionic strength. At low pH (pH 4.0 and 5.0), there was a sharp increase in size. The size is increased to a size larger than that at the higher pH values at high ionic strength, which indicates the presence of strong attractive PPI. The effect of increasing ionic strength at pH 6.0 and pH 8.0 is minimal, and only a slight decrease in relative size is observed. At pH 9.0 a small increase is observed.

The relative size-pH profile of mAb-C is somewhat different compared to mAb-A and B; the maximum is at a similar pH as that of mAb-A (pH 8.0), but the relative size is considerably larger than mAb-A. A sharp decrease in relative size is observed when increasing the pH from 8.0 to 9.0. The effect of increasing ionic strength is strong at pH 7.0 and 8.0 and shows a large decrease in relative size, whereas almost no change in relative size is observed at pH 6.0 and 9.0. Large relative size increases were observed at low pH (4.0 and 5.0), where interactions change from being repulsive at low ionic strength to attractive at high ionic strength.

Generally there was a good correlation between the relative size (R/R_H) and the interaction parameter (k_D) for all three mAbs, especially in the low concentration range. The differences between low and high concentration are not very large, but may well have a direct physical meaning and be a better indication of the real PPI at high concentration.

The novel methodology using relative radius is a simple and rapid alternative to determine relative PPI directly under formulation conditions. Also, using the plate reader approach the method can serve as a high-throughput screening tool in formulation development.

Viscosity

The PPI measurements were correlated with solution viscosity (measured by DLS using polystyrene nanospheres and ultrasonic shear rheology) as a function of pH (4 to 9) and ionic strength (10, 50 and 150 mM). Measurements show that the highest solution viscosity was observed under conditions with the largest negative magnitude of k_D, the largest relative radius (R/R_H) and the lowest net charge. An increase in ionic strength resulted in a change in the nature of the PPI at low pH from repulsive to attractive (Fig. 1) with a corresponding increase in viscosity (Fig. 2). In the neutral to alkaline pH region the mAbs behaved differently with respect to increase in ionic strength. Two mAbs (A and B) showed little or no effect of increasing ionic strength, whereas mAb-C showed a marked decrease in attractive PPI (Fig. 1C,F,I) and viscosity (Fig. 2C).

A larger negative magnitude of k_D for mAb-C corresponded to a larger relative size, which again correlated with higher viscosity at high protein concentration.

The effect of increasing ionic strength was remarkably different between mAb-A and mAb-B compared to mAb-C. mAb-C showed an almost flat PPI profile at 150 mM ionic strength. Both k_D and R/R_H showed that the intermolecular interactions were less attractive for mAb-C compared to mAb-A and B at high ionic strength. The lower negative magnitude of k_D and lower R/R_H also correlated with a lower solution viscosity for mAb-C under these solution conditions.

Previous studies have mainly investigated mAbs of the IgG_1 and IgG_2 subclass. The study described here demonstrates that mAbs of the IgG_4 subclass behave similar to the other subclasses. By comparison of the three tested mAbs with mAbs investigated in other studies, a clear linear trend emerges between the pH of strongest attractive PPI and highest solution viscosity. The determination of PPI using either k_D or relative radius (R/R_H) is thus a useful prediction tool in the determination of solution conditions that favor low solution viscosity at high protein concentration of therapeutically used mAb molecules.

Conclusion

PPI measurements are of great importance to predict the viscosity of a high concentration protein formulation as a function of pH and addition of excipients. The classical parameters to quantify PPI, k_D and B_{22}, require significant dilution of the high concentration mAb solution to allow their measurements. Thus, although they have predictive strength, they are not performed under true formulation conditions. In the study it was observed that the relative size of the mAb, as measured by DLS directly on the high concentration solutions, was an equally strong predictor of the PPI. These measurements can also be performed at lower concentration, within the same range of the k_D determination, but require only a single concentration if the real hydrodynamic radius of the mAb is known. Note that the latter is effectively determined when doing the k_D analysis. The relative size measurements are significantly simpler than the other methods to determine PPI and can be used in a high throughput format using a plate reader at the actual concentration of interest. Further studies are required to assure its robustness towards varying formulation conditions and its predictive nature of the PPI for other proteins.

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