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ARTICLE

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A case study and use of sedimentation equilibrium analytical ultracentrifugation as a tool for biopharmaceutical development

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Abstract Analytical ultracentrifugation (AUC) has re-emerged as a powerful technique for protein characterisation. We report the pivotal role sedimentation equilibrium AUC has played in the development of macrophage inflammatory protein-1α (MIP-1α) as a protein therapeutic. MIP-1α has potential clinical applications in cancer but its clinical use is limited, since it associates to form large insoluble aggregates in physiological buffers. Using AUC as a screening technique, we have produced a biologically active variant of MIP-1α, BB-10010, which has a reduced tendency to aggregate in physiological buffers. The aggregation of protein based pharmaceuticals is routinely monitored by size exclusion chromatography (SEC). Comparison of the data acquired by SEC and AUC, demonstrates that owing to the complexity of BB-10010, AUC analysis is required in addition to SEC to provide a rigorous characterisation of molecular association. This work has been extended to include the use of AUC as an analytical tool to monitor the quality of BB-10010 during formulation and stability studies.

Key words Analytical ultracentrifugation · Biopharmaceutical · Protein · Chemokine

Introduction

The re-emergence of analytical ultracentrifugation (AUC) as a technique for the characterisation of protein size, shape and interactions is a well documented phenomenon (see e.g. Hensely 1996). This been driven by advances in instrumentation and data analysis and by the interest of using proteins derived from biotechnology as pharmaceutical products. At British Biotech we use AUC during manufacture, formulation and quality control to provide information concerning aggregation and biologically important molecular interactions. This paper illustrates some of our work in the application of modern AUC in protein pharmaceutical development by highlighting the key role it has played in the development of BB-10010, a variant of the protein macrophage inflammatory protein-1α (MIP-1α).

Macrophage inflammatory protein-1α (MIP-1α, 7700 $g \cdot mol^{-1}$ molecular weight) is a member of the β-chemokine family. Its ability to act as an inhibitor of haematopoietic stem cell cycling (Lord et al. 1992) has led to interest in the potential clinical use of the product in protecting stem cells against the deleterious effects of cancer chemotherapy. More recently the discovery that MIP-1α prevents HIV proliferation (Cocchi et al. 1996) has heightened expectations for the molecule. The clinical use of MIP-1α, is compromised however, because at psychological ionic strength the monomeric molecule associates to form heterogeneous, multimeric complexes of mass ranging from 100,000 to more than 250,000 $g \cdot mol^{-1}$ (Patel et al. 1993; Graham et al. 1994). Characterisation of MIP-1α including sedimentation equilibrium AUC analysis showed that high ionic strength buffers or low pH substantially reduced association (Patel et al. 1993). This indicated that electrostatic interactions are important for the association of higher molecular weight complexes. To avoid the production and formulation problems associated with the aggregation of MIP-1α, a library of mutants were produced with the aim of improving the solution characteristics of the protein whilst retaining its biological activity. All mutants were screened for biological function and association properties. From this work a variant of MIP-1α termed BB-10010, which contains the single amino acid substitution of Asp26>Ala was identified as having significant changes in solution properties whilst retaining biological activity indistinguishable from that of the wild type (Hunter et al. 1995). The study presented here exemplifies the use of AUC in the development of protein based drugs from early experimental studies through to supporting the development and product characterisation required for clinical evaluation.

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Experimental

Materials

All proteins were purified from culture supernatants derived from recombinant yeast strains expressing a 69 amino acid protein based upon MIP-1α (Hunter et al. 1995). Variants of MIP-1α were prepared according to the method of Patel et al. (1993). The identity and purity of all protein samples used in this work was confirmed by matrix assisted laser desorption time of flight mass spectroscopy and N-terminal sequencing. All solvents and other chemicals used in this work were of HPLC grade or equivalent. Samples were stored in phosphate buffered saline (PBS) at –70 °C or freeze dried from ammonium bicarbonate and stored at –20 °C prior to use.

Analytical ultracentrifugation

Sedimentation equilibrium AUC measurements were performed using a Beckman XLA analytical ultracentrifuge (Beckman Instruments Inc, Palo Alto CA, USA). All measurements were performed on protein in phosphate buffered saline unless stated. Details of rotor cells, rotor speeds, scanning parameters and protein concentrations are described in the figure legends. To ensure equilibrium had been achieved all scans were repeated 3 hours after the first scan. All data were analysed using MicroCal Origin software for the XLA ultracentrifuge (MicroCal Software Inc, Northampton MA, USA). A value for partial specific volume of 0.725 $ml \cdot g^{-1}$ was used in all calculations which was derived from the amino acid sequence of BB-10010 (Laue et al. 1992). The apparent weight average molecular weight (M_w) was determined from the equilibrium distribution using a least squares fitting procedure IDEAL 1 (Beckman Instruments) that assumes the presence of a single ideally behaving species and that the region of data selected for analysis represents the entire macromolecular distribution in the ultracentrifuge cell (Cölfen and Harding 1997). The software was also used to plot ln concentration vs radius shared (ln A vs r^2) and the apparent point weight average molecular weight [$M_{w,app}(r)$] vs concentration [A(r)].

Size exclusion chromatography (SEC)

SEC analysis was performed using a Pharmacia Superdex G-75 HR 10/30 column (Pharmacia, Uppsala, Sweden) pre-equilibrated in phosphate buffered saline (PBS). 50 µl of sample was loaded onto the column and eluted at 1 mL/min in PBS. The absorbance of the eluate was monitored at 280 nm. Where required molecular weight was estimated by comparison of the elution position to those of molecular weight markers (BioRad Laboratories, Hercules CA, USA).

Results

Variant selection

Mutagenesis of MIP-1α was initially directed at the neutralisation of charged groups on the surface of the molecule. This was followed by a more extensive strategy which made no assumptions as to the residues which were involved in polymerisation. These mutants were screened by a variety of techniques to identify variants with desirable solution properties which retained biological activity.

We first assessed molecular size using size exclusion chromatography (SEC, Fig. 1). The MIP-1α peak is asymmetric with the bulk of the protein excluded from the column (limit 75,000 $g \cdot mol^{-1}$) and the remainder trailing to positions equivalent to lower molecular masses. This elution profiles indicates a heterogeneous mixture of soluble multimeric complexes ranging in mass from approximately 20,000 to >75,000 $g \cdot mol^{-1}$. A number of mutants including BB-10010 (Fig. 1) were observed to elute later than MIP-1α, indicating that they are dissociated relative to the wild type protein. Most of these mutants eluted as an asymmetric peak indicating that although smaller than the wild type, these proteins also consist of a heterogeneous mix-

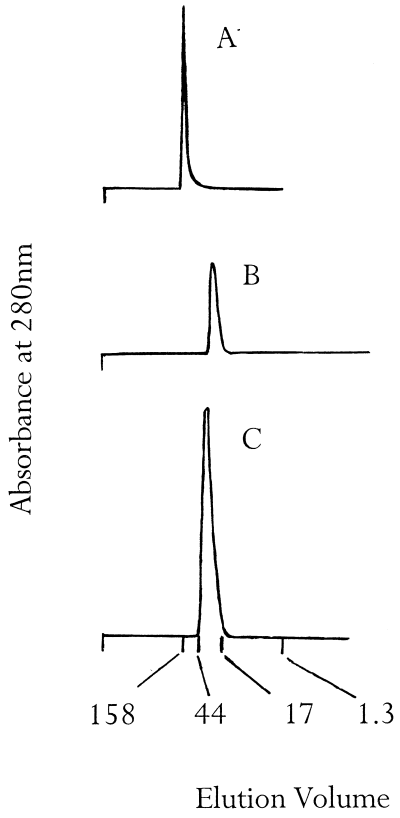

Fig. 1A–C Size exclusion chromatography analysis of MIP-1α and BB-10010. Elution profiles of (**A**) 2.0 $mg \cdot mL^{-1}$ MIP-1α, (**B**) 2.0 $mg \cdot mL^{-1}$ BB-10010. (**C**) 10 $mg \cdot mL^{-1}$ BB-10010. The elution positions of molecular weight standards with individual molecular weights (in kDaltons) marked are shown on chromatogram (**C**)

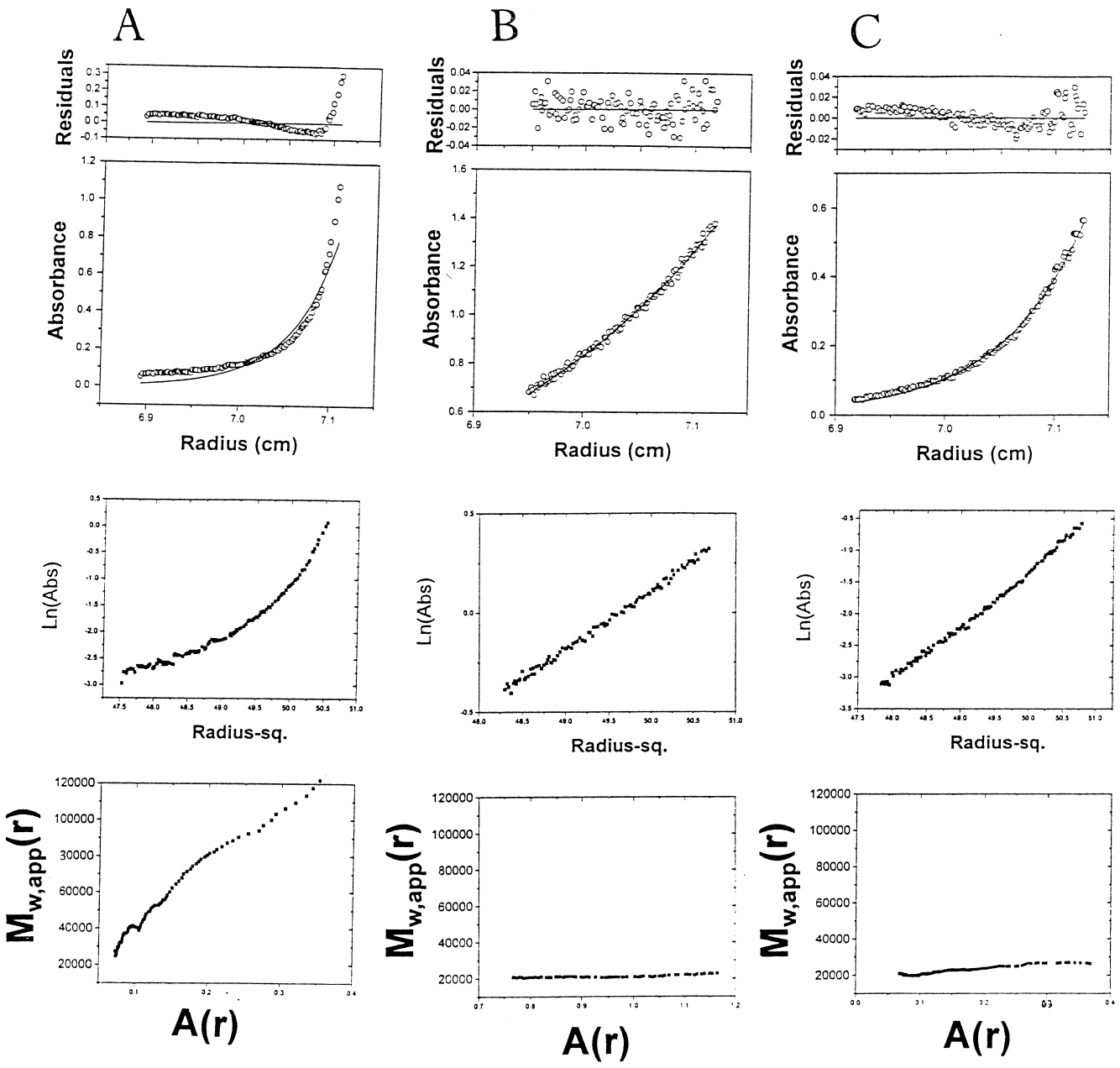

Fig. 2A–C Sedimentation equilibrium AUC of 0.1 mg · mL^{-1} MIP-1α and 0.1 mg · mL^{-1} and 1.0 mg · mL^{-1} BB-10010. Equilibrium profiles of (**A**) 0.1 mg · mL^{-1} MIP-1α, (**B**) 0.1 mg · mL^{-1} BB-10010. (**C**) 1.0 mg · mL^{-1} BB-10010 with single component (Ideal 1) fits. Differences between data and the IDEAL 1 fit are presented as residuals. L_n absorbance vs. radius squared and apparent point weight average molecular weight [$M_{w,app}(r)$] vs. concentration [A(r)] plots are also shown for each set of data. BB-10010 in PBS was spun at 15,000 rpm for 20 h to attain equilibrium. The cells were scanned in increments of 0.001 cm at 225 nm (0.1 mg · mL^{-1}) or 290 nm (1.0 mg · mL^{-1}). Each data set is an average of 4 scans

ture of sizes. This work demonstrates the utility of using SEC in the screen of mutants, since it is relatively fast and is able to provide an estimation of molecular size and degree of heterogeneity. Comparison of the SEC profiles of disaggregated mutants showed clearly that their association properties were not all the same. Sedimentation equilibrium AUC was therefore used to analyse all the mutants with the aim of defining the association properties of these molecules in more detail. The results of sedimentation equilibrium analysis of wild type MIP-1α and BB-10010 are shown in Fig. 2. Each example is fitted to a model which assumes an ideally behaving single component (Ideal 1). The differences between the data and the best fit model are shown as residuals. Diagnostic plots of log concentration vs radius shared (ln A vs r^2) and apparent point weight average molecular weight [$M_{w,app}(r)$] vs concentration [A(r)] are also shown for each sample. The wild type sample is a poor fit to the model and has residuals and a ln A vs r^2 plot that are consistent with a high degree of self-association (McRorie and Voelker 1993). The molecular weight versus concentration plot is indicative of the presence of a range of molecular sizes from 20,000 g · mol^{-1} to

>100,000 g · mol^{-1}. The weight average molecular weight (M_w) of wild type MIP-1α is in excess of 100,000 g · mol^{-1}. The MIP-1α variant BB-10010 at the same concentration (0.1 mg · mL^{-1}) has random residuals and a linear ln A vs r^2 plot demonstrating a distinct lack of self association. At this concentration the M_w of the system is approximately 20,000 g · mol^{-1}. At 1 mg · mL^{-1} BB-10010 has an M_w of approximately 40,000 g · mol^{-1} and displays the characteristics of a self-associating system. The degree of self association in this sample as estimated from both diagnostic plots appears dramatically less than that of the wild type protein: for example the apparent weight average molecular weights vs concentration vary only between 15,000 and 40,000 g · mol^{-1}. The concentration dependent association of BB-10010 is confirmed by SEC analysis at 2 mg · mL^{-1} and 10 mg · mL^{-1} indicating that molecular weight increases with concentration.

Association properties of BB-10010

Using SEC and sedimentation equilibrium AUC, mutants of MIP-1α were identified which had a reduced tendency to associate. This data together with an assessment of biological activity and other analytical techniques lead to the selection of BB-10010 as the molecule most suitable for clinical development. Part of this work entailed a more detailed analysis of the association properties of the molecule.

The data acquired in the initial screening of the mutants (Figs. 1 and 2) indicate that BB-10010 in PBS is a self-associating system. At concentrations above 0.1 mg · mL^{-1} the protein associates in a concentration dependent way to give a mixture of sizes. The lack of discrete peaks in the SEC indicates that the species present are interchanging at a rate significantly faster than the time period of an SEC experiment (30 min). Figure 3 compares the molecular weights estimated from SEC and AUC. There is a discrepancy between the molecular weight estimations from the two techniques that is more pronounced at higher concentrations. This is due to the dilution that occurs during the SEC process which causes the BB-10010 to dissociate leading to an underestimation molecular weight. SEC is therefore unable to give a direct estimate of the molecular weight. This is one of the key reasons we choose AUC as the principal technique for the investigation of the association properties of the molecule. This was done by determining the weight average molecular weight of BB-10010 at a range of protein concentrations and rotor speeds. The results of one of these experiments are shown in Fig. 4. At each of the three rotor speeds the apparent weight average molecular weight is dependent upon protein concentration. At higher rotor speeds. the concentration dependence of $M_{w,app}$ is less pronounced so that curves at different rotor speeds are not superimposable, this is a result of different association states sedimenting to different equilibrium distributions as the rotor speed is varied and as such reflects the heterogeneity of the system. At low concentrations the curves converge at an apparent molecular weight of

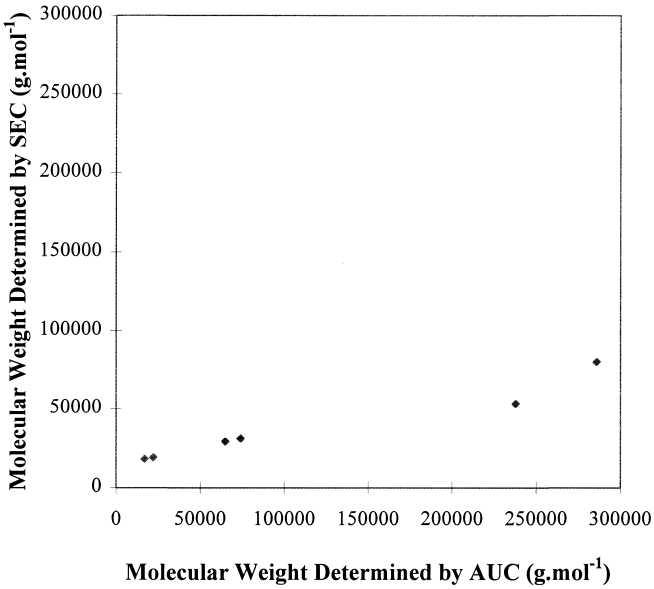

Fig. 3 Comparison of molecular weights of BB-10010 estimated from sedimentation equilibrium AUC and SEC. Molecular weight was estimated at a range of BB-10010 concentrations by comparison with molecular weight standards in SEC analysis and by AUC using a single component fit (IDEAL 1)

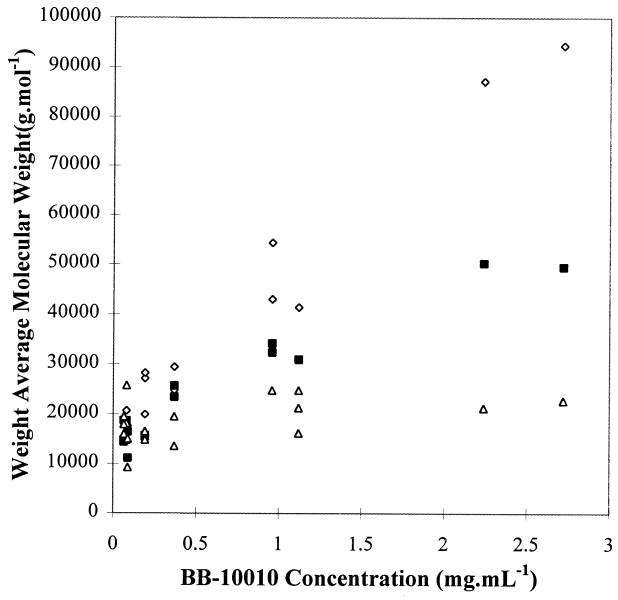

Fig. 4 The Association properties of BB-10010. Sedimentation equilibrium analysis of BB-10010 in PBS as a function of concentration and rotor speed. BB-10010 was spun at (◇) 15k rpm, (■) 25k rpm and (△) 35k rpm. Weight average molecular weight was calculated by fitting the data to a single component fit (IDEAL 1). BB-10010 in PBS was spun at 15,000 rpm for 20 h to attain equilibrium. The cell was scanned in increments of 0.001 cm. Each data set is an average of 4 scans

15,000 g · mol^{-1} which is the expected molecular weight for a dimer. This convergence confirms that dimers are key units in the association pathway of the molecule. We have also attempted to model the association pathway of BB-10010 using this data. This has provided difficult owing to

the complexity of the system at high concentrations. At low concentrations (<0.2 mg · mL^{-1}) however, the data are best fitted to a model that assumes that only dimers and tetramers are present. This assumption is consistent with the distribution of sizes estimated from a $M_{w,app}(r)$ vs A(r) plot. The dissociation constant for the dimer ⇄ tetramer interaction obtained from this fit is of the order of 5 ± 2 μM. In a separate experiment based upon high sensitivity SEC we estimate that the dimer to monomer dissociation constant is of the order of 10 nM (results not shown).

Pharmaceutical development

The results described above demonstrate the usefulness of AUC in the characterisation of the association properties of BB-10010. We have also used AUC to provide key information during the production, formulation and stability testing of the molecule. Examples of this are detailed below.

To optimise a filtration step of the BB-10010 production process the molecular size as a function of NaCl concentration was investigated. The results (Fig. 5) show that at concentrations of >0.2 M NaCl BB-10010 is primarily dimeric. This information has enabled process parameters such as type of filter and ionic strength of the product stream to be designed to optimise product recovery and quality.

AUC has also been used to assess the suitability of candidate formulations of BB-10010. In certain formulations the time dependent generation of an apparent higher molecular weight species was observed by SEC analysis (Fig. 6). The additional higher molecular weight species (designated M′) is not excluded from the column (limit 75,000 g · mol^{-1}) and appears to consist of more than one species. M′ could be related to larger species from the BB-10010 association pathway. To investigate if this is the case M′ was purified to homogeneity by preparative SEC and analysed by sedimentation equilibrium AUC. Figure 7 shows the apparent weight average molecular weight of purified M′ compared to unaffected BB-10010 as a function of protein concentration. The molecular weight of M′ appears to be less dependent upon concentration than normal BB-10010. Molecular weight estimates from the SEC and AUC indicate that M′ has a molecular weight of around 60,000–100,000 g · mol^{-1} which is equivalent to 8–12 monomer units (or to 2–3 tetramer units). AUC analysis of unaffected BB-10010 confirms that species of this size are present at the high product concentrations which give rise to M′. M′ is therefore probably not a result of aggregation in the sense normally applied to proteins but is a species that cannot dissociate as it is diluted. BB-10010 has therefore undergone some alteration that causes higher affinity interactions between molecules of BB-10010 so that they become effectively irreversibly associated.

Discussion

The increased use of sedimentation equilibrium AUC for the characterisation of protein interactions has been stim-

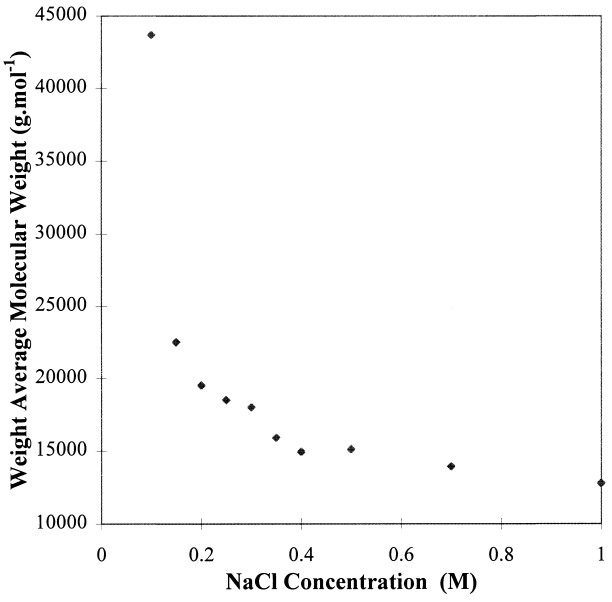

Fig. 5 Sedimentation AUC analysis of BB-10010 at increasing NaCl concentrations. All samples were in 50 mM phosphate, pH 7.4. Weight average molecular weight was estimated by fitting the data to a single component fit (IDEAL 1). BB-10010 in PBS was spun at 15,000 rpm for 20 h to attain equilibrium. The cell was scanned in increments of 0.001 cm at 230 nm. Each data set is an average of 4 scans

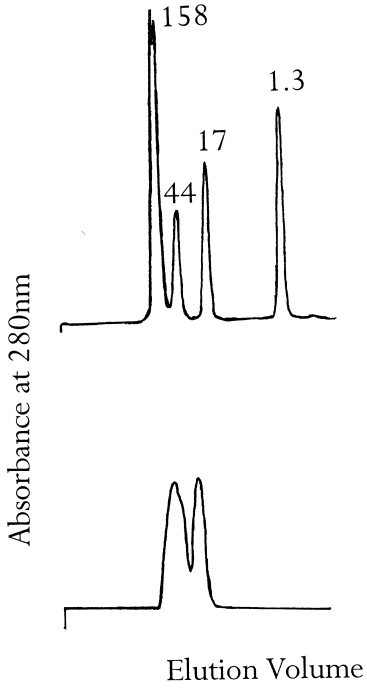

Fig. 6 SEC analysis of a BB-10010 Formulation. Elution profile of a sample of BB-10010 after storage at 25 °C for 9 months (*bottom*). The chromatogram is compared to molecular weight markers with molecular weights marked in kDaltons (*top*)

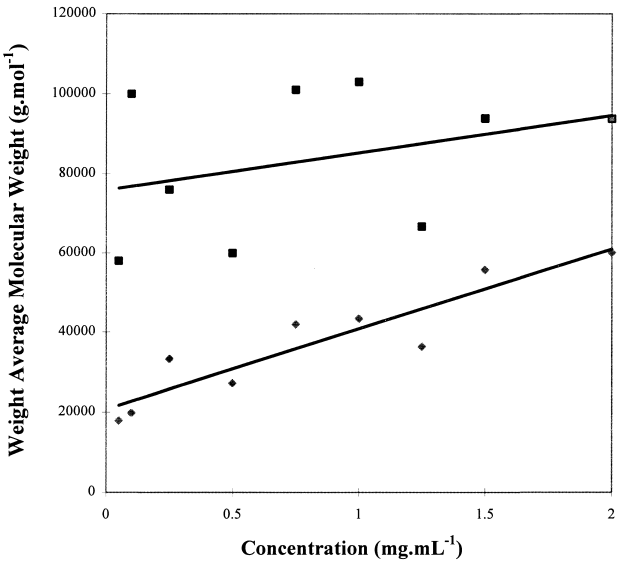

Fig. 7 Sedimentation equilibrium AUC analysis of BB-10010 and purified M′ (BB-10010) degradation product. (◆) normal BB-10010 and (■), M′ derived from BB-10010. Weight average molecular weight was estimated by fitting the data to a single component fit (IDEAL 1). BB-10010 in PBS was spun at 15,000 rpm for 20 h to attain equilibrium. The cell was scanned in increments of 0.001 cm at 230 nm. Each data set is an average of 4 scans

ulated by the advent of modern instrumentation. In addition, the Regulatory Authorities stipulate the quality of recombinant protein based pharmaceuticals must be ensured by the provision of a highly detailed description of all structural aspects of the molecule including its association properties (Richardson 1996). This requirement has resulted in AUC increasing in significance as our BB-10010 project has progressed in to clinical trials. AUC has been particularly important for this project because of the relative complexity of the association properties of the product. The work presented in this paper demonstrates that this technique can be applied to the characterisation of biotechnology products in general.

Important issues in the evaluation and development of any analytical method must include: 1) What new or additional information does the technique provide?; 2) Is the technique suited for routine analysis?; 3) How can the data be analysed and interpreted to provide meaningful information? These issues have been considered during the development of BB-10010 and have been used to demonstrate the utility of AUC as an analytical tool for all stages of protein pharmaceutical development.

Traditionally SEC is the technique of choice for the analysis of the association properties of protein based pharmaceutical products. This is particularly true in the screening for unwanted aggregation and the relative quantitation of association states e.g. dimer vs. monomer. In this project SEC proved particularly useful in screening mutants for lack of aggregation. SEC was, however, unable to provide more detailed information because of the kinetics of the association pathway of the molecule. The dynamic equilibrium that exists between various species at high concentrations is perturbed by the dilution that occurs during SEC analysis. This makes precise estimates of molecular size impossible. Since AUC does not involve dilution this is not a problem. Furthermore AUC is able to provide a semi-quantitative description of the heterogeneity of the system. Although AUC is particularly useful in this project we believe that it has a role to play in the characterisation of most recombinant biopharmaceuticals. This is especially true when used to expand upon or supplement SEC analysis, which has advantages over AUC in terms of resolution and sensitivity. For example analysis of the BB-10010 degradation product M′ by AUC and SEC was able to yield more information than either technique could do if used solely.

The ability of analytical ultracentrifugation to provide a rigorous description of the association properties of proteins and their interactions is the key strength of the technique. The complexity of AUC relative to other techniques has however meant that in the past it has not been well suited to routine analysis. With modern instrumentation there are several reasons why this is no longer the case. Firstly, the experiments are easy to perform and the results are reproducible. Secondly, the use of rotor cell with six sectors allows the simultaneous analysis of up to nine samples in a four cell rotor. This provides the ability to perform analysis with a relatively high throughput. Data are typically collected overnight which further reduces the time required to analyse a sample. This has proved useful when screening large numbers of mutants for their association properties or for investigating the behaviour of BB-10010 in a wide range of formulations and processing conditions. Thirdly, data analysis is relatively simple. The work presented here mainly deals with fitting data with a single component. This is normally considered to be only the preliminary stage of a detailed hydrodynamic evaluation of a self associating system (McRorie and Voelker, 1994). It does however provide a simple way of indicating molecular size and heterogeneity. Although this analysis provides information that may not be considered rigorous compared to what is achievable with the AUC, the data is informative particularly when combined with SEC analysis.

When applying the use of AUC as a routine tool to compare consistency between batches, formulations etc., the definition of what constitutes a significant change is critical. To be of any use any analytical technique must have meaningful acceptance or pass/fail limits. For analysis of proteins by AUC this is a challenging question which is made even more difficult in our case by the complexity of the system under study. On a routine basis we simplify the analysis by diluting to a uniform low concentration where self association is less prevalent. Analysis under these conditions has been used to evaluate the repeatability of the analytical method and to set limits on the results. The parameters we routinely evaluate are M_w and deviation from linearity of the ln A vs r^2 plot. These are simple to determine and interpret but are able to detect relatively small changes in the association properties of a molecule on a routine basis. This approach is especially well suited to

comparative studies between closely related samples for example mutants or different batches of product.

The results of the mutagenesis program demonstrate that it is possible to manipulate the self-association pathway of MIP-1α. BB-10010 was chosen for clinical evaluation on the basis of many parameters including biological activity and association characteristics. AUC and SEC played a key role in providing this information and have been subsequently used to define the association behaviour of the molecule. The complexity of the system at higher concentrations precludes a detailed description of the larger associated species. At lower concentrations we are using the technique in an ongoing effort to define the association pathway and have already confirmed that dimers and tetramers are key to the association process. AUC has also been able to evaluate the strength of interaction between these species. Finally, AUC has been able to provide valuable information concerning the behaviour of BB-10010 during production and formulation which has facilitated its development as a pharmaceutical product.

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