

## Convenient separation of GCSF using Asymmetrical Flow Field-Flow Fractionation

Recombinant granulocyte colony stimulating factor (rh-GCSF, 18.8kDa) is a cytokine of remarkable therapeutic relevance that has a strong tendency to aggregate. As a direct consequence, there is a great interest in powerful and convenient separation techniques in order to characterize it more completely under a variety of conditions.

Our aim was to develop an Asymmetrical Flow Field-Flow Fractionation (AF4) method that provides separation and quantification of the monomer's soluble and insoluble aggregates as well as the determination of the molecular weights ( $M_w$ ).

RhGCSF samples containing low molecular weight aggregates (*lmwa*) and high molecular weight aggregates (*hmwa*) were produced by applying shear stress or high temperature.

An Eclipse AF4 system was equipped with a standard channel (25 cm) or the new small channel (18cm), 350  $\mu$ m or 490  $\mu$ m spacer and regenerated cellulose membrane (5 kDa cutoff). The samples were analyzed by a DAWN and a UV detector. Gel Permeation Chromatography (GPC) experiments were also carried out using the same hardware equipment, but running in a GPC mode using a Tosoh TSKGel® G3000SW<sub>xl</sub> column.

For the determination of optimal separation conditions, different channels, spacers and channelflow to crossflow ratios were tested. Figure 1 demonstrates that 0.6 mL channel flow with 1.6 mL cross flow (small channel, 490  $\mu$ m spacer) rendered the best separation. In comparison to the standard channel, the run time could be reduced from 58 minutes to 28 minutes and the eluent volume from 251 mL to 42 mL. Finally,  $M_w$  were determined more accurately with the small channel. For mechanically stressed samples, the  $M_w$  of the two major fractions could be assigned to monomer (18.3kDa) and dimer (34.4kDa).

Applying the same separation conditions to thermally-treated samples, the detection of *hmwa* was feasible in the void peak via the steric hyperlayer mode. Filtered samples which showed a *diminished* void peak proved this peak to represent *hmwa* (Figure 2). In contrast, size exclusion separation of *hmwa* could not be achieved. However, good agreement for *lmwa* was observed.

The successful development of a fast AF4 separation method suitable for a small pharmaceutical model protein and its aggregates was presented. In contrast to the standard channel, the new, shorter channel technology allowed the determination of the respective  $M_w$  due to sharper peaks and stronger detector signals. These are but further steps towards establishing AF4 as standard analytical tool, even for low molecular weight proteins (e.g. cytokines).

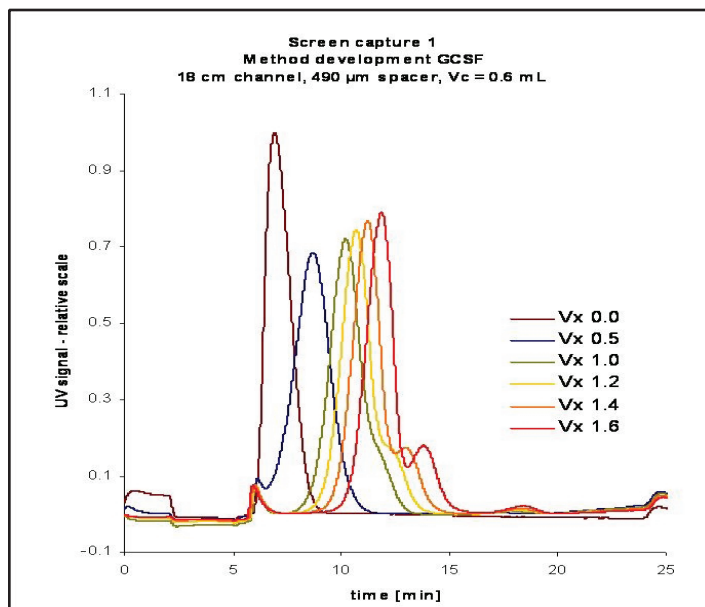


Figure 1. The 0.6 mL channel flow with 1.6 mL cross flow (small channel, 490  $\mu$ m spacer) rendered the best separation.

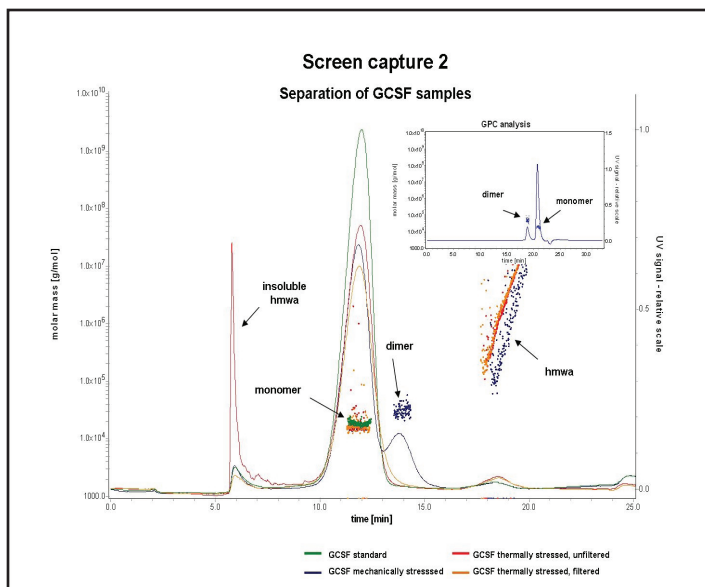


Figure 2. Separation via SEC contrasted with the AF4 separation shows that the *lmwa* separates well, but the *hmwa* does not.

