Bio-Works

TECHNICAL NOTE

Split peaks as a phenomenon in liquid chromatography

What is the cause of peak splitting?

Peak splitting can occasionally be observed in liquid chromatography but why does it happen?

Split peaks can be caused by many different factors and can sometimes be easily corrected. The root cause must however be identified. First, it must be determined if it is one distorted peak, which may be due to packing issues/compromised bed integrity, or if there are two partially resolved compounds. The analysis of all peaks in the chromatogram will generate knowledge about their identity.

Common causes for peak-splitting, distorted peaks and peak tailing are non-optimal packing of the resin which can result in channeling or a void at the top of the packed bed (Fig. 1). Another cause relating to the column hardware can be partially blocked flow path or clogging of inlet filter/frit (Fig. 1). Some resin properties, such as unspecific hydrophobicity has been shown to promote peak-splitting under certain conditions of some molecules. Lastly, the target itself may be the cause of peak splitting due to aggregation or charge differences, for example. A good rule of thumb is if only one peak is splitting, then the issue is most likely method or chemistry related, *i.e.* not due to packing or hardware defects. Changing the injected mass of sample to study ratio of split peaks/distorted peaks can also indicate if it is one or two partially resolved compounds.

Resin integrity/hardware dependence

- A bed packed too densely might crack, which can lead to channelling and early breakthrough. If some of the target molecules diffuse in the cracks and others in the beads it might result in split peaks.
- A bed packed too loosely can further compress causing a liquid gap, a void at the top of the column where mixing can occur. This induced mixing chamber will generate an uneven diffusion of the target molecule into the resin resulting in less defined peaks.
- The frit and/or spreader in the top adaptor of the column protects the resin from large contaminants and provides an even spread of the loaded sample onto the resin. A clogged or damaged frit/spreader will affect the uniformity of the sample spreading. If the clogged frit is due to target aggregation for example, it may partially release from the frit over time which will also distort the peaks and may lead to split peaks. Dead volumes (cavities) in the system flow path (such as mixer/t-connections) may also negatively affect the separation of the molecules.

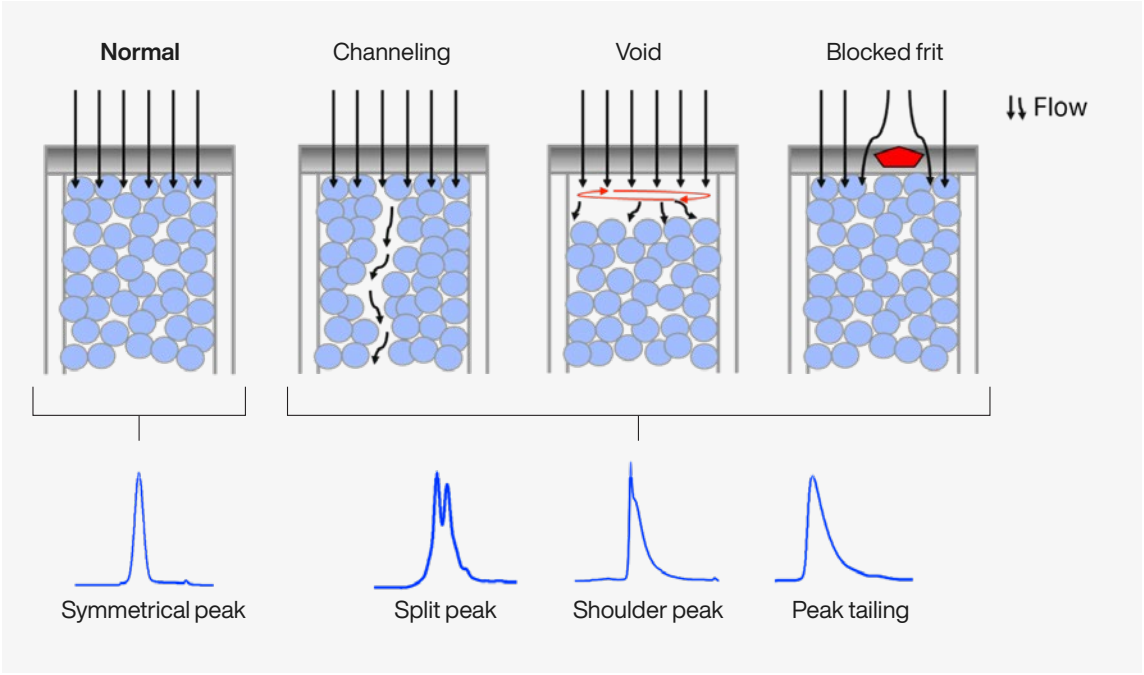

Figure 1. Peak splitting or distorted peaks depending on resin integrity or hardware issues.

Sample dependence

Complex samples such as monoclonal antibodies are sometimes prone to show split peak behaviors, especially in cation exchange (CIEX) steps. These molecules which have many different types of intra- and inter-molecular interactions, are highly affected by pH and tend to form dimers or bigger aggregates under acidic conditions.

- Protein aggregation is undesired interactions between protein monomers and can be caused by factors such as pH, ionic strength, protein concentration, freeze-thaw operations and temperature. The protein may undergo conformational changes or have surface interactions that promotes aggregation, which will generate two closely related peaks, often seen as split peaks in techniques such as CIEX. Generally, the formation of aggregates, so-called high molecular weight species (HMWS), are shown to be product or process related. On-column aggregate formation may be dependent on the resin type and on the resin's interactions with the loaded mAb.
- A difference in the charge of the target molecule may also cause peak splitting. This charge difference may originate from the feed or be promoted by the running conditions during the purification.

Identification of causes and solutions

Above we described some common causes of the peak splitting phenomena. The first thing to do is to identify the nature of the peaks and determine which are the contributing factors.

Running a purification in reverse mode and studying the elution profile may tell you if the resin integrity is compromised (void or channeling) or if there is clogging in the frit in the top adaptor since these issues appears in the top part of the column. The resin can also be cleaned operating in reverse flow to efficiently clean the frit and upper part of the column. The wash buffer may include organic solvents such as acetonitrile or methanol to remove hydrophobic contaminants, check the resin's specifications. If the resin integrity is poor, it can simply be repacked, and split peaks may be diminished.

Dimeric or bigger aggregations of the target may be identified with size exclusion chromatography and charge differences of targets may be identified with isoelectric focusing, for example.

It has been reported that the proportion of on-column aggregate formation partly depends on the resin pore sizes, pore size distributions, different surface extenders and also interactions between the mAbs and the resin's structure (Guo and Carta 2015, Guo et al. 2016, Reck et al. 2017). WorkBeads™ 40S shows a very low proportion of on-site mAb aggregation (Fig. 2).

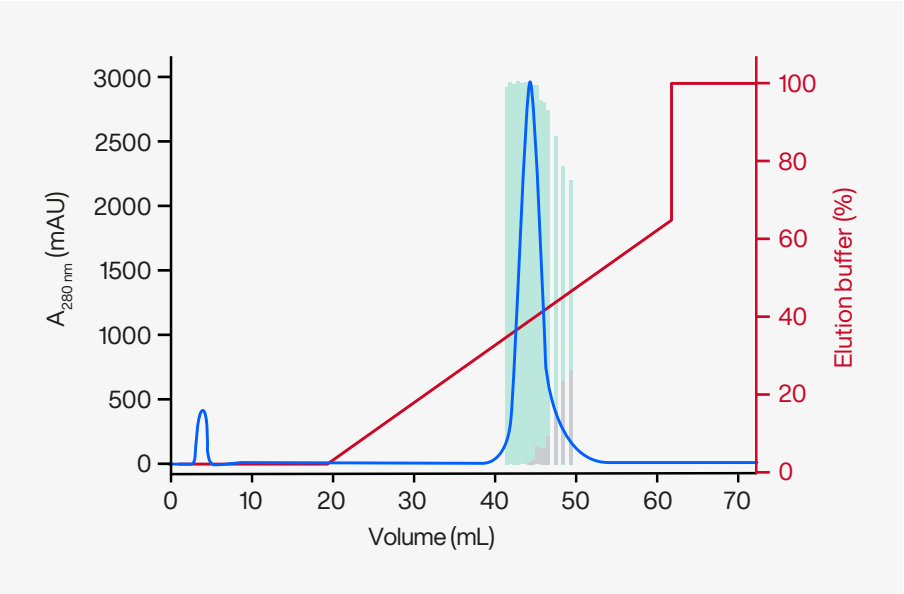

Figure 2. mAb polishing on WorkBeads 40S. Main peak consists of monomers (green bars) and aggregates (grey bars). Since there is a partial overlap between monomers and formed aggregates, the peak is partially distorted. After removal of aggregates the peak consists of <1% HMWS and is symmetrical.

A major question is also if the charge variants and aggregations must be removed or not.

Peak-splitting behavior has commonly been diminished at larger scales when purifying monoclonal antibodies on CIEX. This indicates that a more robust method can be obtained at larger scale, regardless of if the observed split peaks seem to originate from the packing quality or the stability of the target.

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