Advances in Protein Sample Preparation: Centrifugation in Pharmacology Research

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Centrifugal devices are frequently used as an alternative to chromatographic sample preparation processes, for applications in a wide variety of industries. Such devices can be used in protein concentration, buffer exchange, and fractionation of proteins and nucleic acids. The sample reservoir design of many centrifugal devices induces formation of a concentration gradient during centrifugation which leads to excessively high sample concentrations at the bottom of the reservoir. This excessive concentration at the bottom of the reservoir drastically increases the risk of protein aggregation and precipitation, which affects the biological activity of a protein and thereby significantly reducing or potentially eliminating the ability for it to be used for its intended analytical purpose. Innovative spin device designs have been developed to minimise the incidence of aggregation, and are critical to the ongoing research of many laboratories.

Protein Purification

Protein aggregation can occur during upstream operations and downstream processing, when a protein denatures and unfolds. This exposes previously concealed hydrophobic sites which bind to hydrophobic regions on other molecules, or when native proteins form aggregates via hydrophobic regions on their outer surface, making chromatography difficult. The formation of aggregates depends on the solution conditions, temperature, and protein concentration. Avoidance of these conditions to reduce the occurrence of protein aggregation often demands extra protein purification steps, leading to increased costs.

Many laboratories use centrifugal devices to support their research, particularly in cancer, pharmacology and biochemical research. In pharmacology applications, scientists study how to improve the efficacy of drugs, and how to deliver them to the target site without causing systemic side effects. Downstream processing for the recovery and purification of proteins is achieved by exploiting the physiochemical properties of molecules and aggregates, such as size, charge and shape. There are various methods used to do this, such as chromatography, centrifugation, filtration and precipitation.

The first step after protein extraction is protein purification. Conventional methods require centrifugation to pellet the sample, followed by removal of the buffer and recovery of beads.

Here it is important to minimise sample loss. Before downstream analysis, many proteins must be concentrated for high quality data output and reproducible protein assay results.

The protein must be in its native, soluble form, dissolved in the buffer at an appropriate concentration, without compromising the yield, even if the protein is low in abundance.

Industry Requirements

Western blotting is an important tool for the large-scale study of proteomes. This technique is used in basic science research, proteomics research and diagnostics to detect the presence of proteins in unknown or complex sample mixtures, in addition to determining the relative abundance of certain proteins. Once proteins have been separated from a sample via gel electrophoresis, they are transferred to a specific membrane, which immobilises the proteins for downstream analysis. After incubation with labelled antibodies to identify the protein of interest, the membrane is washed to remove any unbound antibodies, and is further analysed using immunodetection methods.

The need to analyse specific proteins from increasingly small samples has driven the requirement for innovative centrifugal devices. Researchers have a key interest in identifying and characterising trace amounts of carry over host proteins contaminating a therapeutic protein of interest, and to optimise each step of purification process to meet regulatory requirements.

Many academic laboratories perform primary Western blots multiple times per week, in order to visualise the protein on a membrane and to find out how far the proteins express. Concentrating proteins with a higher input can be challenging. Centrifugal devices for difficult to concentrate proteins, such as membrane proteins, can be used to quantify and compare expression amongst experimental groups using a small sample size.

Purification Challenges

The ultimate goal of clinical and proteomics research laboratories is to reduce the number of steps in the protein purification process. Sample preparation is hugely important in proteomic research, and ineffective sample preparation can lead to the loss of valuable samples and time and compromise chromatography analysis, while effective steps can facilitate the discovery of diagnostic and pharmaceutical products. Most chromatography experiments require only a small sample volume, so the protein concentration of this sample must therefore be high. As samples get smaller and more numerous, the need for novel methods to purify proteins and improve assays, such as affinity chromatography and reverse phase chromatography, has led to the production and development of innovative centrifugal devices.

In pharmacological research, patient samples frequently lack sufficient protein for Western blot analysis. The greatest limitation of Western blots is that it can be difficult to visualise rare-expressing proteins. To avoid the complications of a small sample volume, experiments can begin with a higher amount of protein. In real life applications, however, researchers can rarely request a larger patient sample once it has been obtained, because they often are taken during surgery or clinical visits. For example, tumour samples are usually between 2 - 3 mg before protein extraction, but there is not enough expression the protein of interest. A larger tissue sample cannot be obtained once the procedure is complete, but the sample preparation step can be adapted so that it is compatible with the experimental design. For example, Western blot is not sensitive enough to detect 1 µg for every 25 µg of total protein, but concentrated protein can be used with the correct molecular weight cut-off (MWCO) and eliminate all the uninterested proteins and salts to counter this. Smaller centrifugal devices are therefore useful.

Alternatively to Western blot, ultrafiltration is a membrane separation technique, used to separate extremely small particles and dissolved molecules in fluid. Molecule size, determined by molecular weight, is the primary basis for separation. Molecules larger than the membrane pores are retained by the membrane surface and are concentrated. Molecule shape and charge can also play a role in membrane retention. For example, linear molecules such as DNA will be able to pass more easily though pores which are designed to retain globular shaped proteins of the same molecular weight.

Ultrafiltration allows the removal or exchange of salts and the removal of detergents (diafiltration) and is popular because it is usually relatively rapid, and does not adversely affect the protein sample.

The Importance of Device Architecture

Protein purification is a complex process, which involves a number of intricate sequential steps. The separation step usually creates the need for the sample to be desalted or concentrated to prepare the biomolecule sample for the next step of purification. Using ultrafiltration, molecules are separated based on molecular size, so laboratories can choose the appropriate device based on the MWCO. Most centrifugal devices are suitable for 4 mL - 15 mL samples, but many laboratories are realising the benefits of smaller (1.5 mL - 2 mL) devices, as this is more practical in the basic science laboratory, especially for Western blot applications, where concentrated protein is not always available. By using concentrated proteins at smaller sample sizes, the experiment does not need to be repeated as it would with non-concentrated proteins. This saves the laboratory valuable time, and allows results to be visualised quicker.

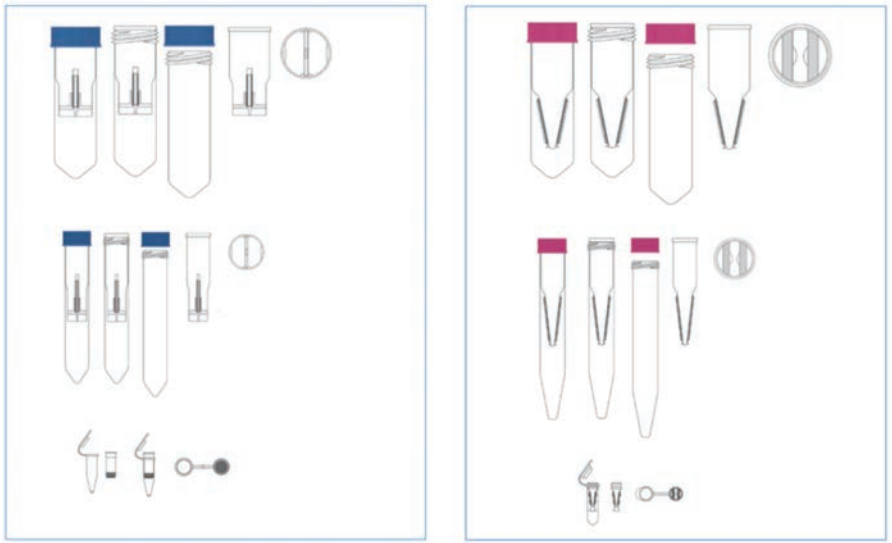

Figure 1: (Left) 1 kDa molecular weight cut off (MWCO) concentrators with 'U'-shaped sample reservoir. (Right) Industry standard peptide concentrators with 'V' shaped reservoir.

The majority of spin device designs excessively concentrate the protein solution where the filters are located, due to the 'V' shape of the funnel insert. Excessive concentration creates a protein gradient that is very dense at the bottom of the filter, thereby drastically increasing the risk of aggregation and precipitation and, ultimately, affecting the biological activity of a protein. Some devices have a circular ('U'-shaped) sample reservoir and include the filter unit. This provides a wider area to disperse the concentrated protein in a more efficient manner. The traditional conical shape of the device (*Figure 1*, right) can make it challenging to resuspend samples. This is made easier by the flat shape of more innovative, smaller devices (*Figure 1*, left), which also reduce the chance of damaging the membrane with the pipette tips.

Future developments

Funding is the key contributing factor to the direction of laboratory work. In most basic science laboratories, research requirements tend to answer one small question at a time. This is considered during experimental design, and is carried forward when pursuing a certain technique. When advanced, innovative solutions are needed to solve a specific problem, researchers look to their collaborative partners to help progress the work.

Two case studies for academic institutions are available to illustrate these devices in use. Please visit *http://go.pall.com/centrifugal-devices-cancer-research.html?utm-source* and *http://go.pall.com/centrifugation-pharmacology-research.html*