Application Note

Monitoring detergent removal from biological samples using the Direct Detect® spectrometer

Introduction

In biological science, detergents are primarily used to disrupt the cell membrane in order to release and solubilize lipid-associated proteins. Some detergents are used to ensure solubilization of recombinant proteins during the purification process, while others can facilitate protein stabilization, crystallization or denaturation. Detergents are also used as additives to prevent non-specific binding and/or protein precipitation in various immunoassays[1,2]. Additional applications of detergents include extraction of nucleic acids and preparation of liposomes. While highly useful in initial sample preparation, detergents can interfere with many downstream applications, such as enzyme-linked immunosorbent assays (ELISAs), isoelectric focusing (IF), nuclear magnetic resonance (NMR) and mass spectrometry (MS), and therefore require removal prior to the analysis[3,4].

General Detergent Properties

Detergents are surface-active agents (surfactants) distinguished by their amphipathic structure. Each detergent molecule contains a hydrophilic portion (head) and a hydrophobic group (tail), a composition that permits aggregation in aqueous media and formation of micelles. Detergents are characterized by the critical micelle concentration (CMC), defined as the maximum monomer concentration above which a given detergent exists in larger non-covalent complexes (micelles), where hydrophobic tails cluster in the center of the complex while the hydrophilic head groups are exposed to the aqueous media. The CMC value is also a useful guide to solubilization potency; lower CMC values indicate that less detergent is required in order to form micelles and subsequently disrupt membrane organization[1-5]. A detergent's aggregation number is the average number of detergent monomers in a micelle. The molecular weight of the micelle, indicating relative micelle size, represents another useful parameter when evaluating detergents for downstream removal. Smaller micelles with low aggregation numbers are more easily removed and are usually preferred for separations based on the molecular size of the investigated protein.

Overview of Detergent Removal Methods

Methods used for detergent removal take advantage of general properties like hydrophobicity, CMC, aggregation number and the charge. The most commonly used detergent removal methods[6-7] include:

- **Dialysis:** removes detergent based on size (high MW species retained)
- **Centrifugal ultrafiltration:** removes detergent based on size (high MW species retained)
- **Gel filtration:** removes detergent based on size exclusion (low MW species retained)
- **Hydrophobic adsorption:** exploits the ability of detergents to bind to hydrophobic resins. The resin with the bound detergent can be removed by centrifugation or filtration.
- **Ion-exchange chromatography:** exploits the differences in charge between protein-detergent micelles and protein-free detergent micelles. Method is applicable to nonionic and zwitterionic detergents.

EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

Overview of Detergent Removal Monitoring Methods

Various colorimetric[8,9] and MS[8,10] methods are traditionally used to determine detergent removal efficiency. Absorbance at 275 and 280 nm is frequently used to estimate concentration of detergents like Triton® X agent (100 and 114) and NP-40[8]. This method is highly limited because it requires subtraction of protein contribution to the signal, which is impossible to pre-estimate in lysates and other complex biological samples. Concentration of SDS is typically measured using a colorimetric assay, such as Stains-All dye[9]. Another colorimetric method, using concentrated sulfuric acid and phenol[10], is frequently used to determine the concentrations of glycosidic and bile salt-based detergents like octyl glucoside and CHAPS. Use of tandem liquid chromatography and mass spectrometry (LC-MS) or MALDI-MS has been reported in case of monitoring removal of Tween® 20 agents and BRIJ-35[8]. Total organic carbon (TOC) analysis has also been employed in the monitoring of detergent removal efficiency. However, to be accurate, the TOC method requires analysis of flow-through (removed detergent), because the remaining sample contains other organic compounds, like proteins and lipids, which inflate TOC results.

Here, we report a novel mid-infrared (MIR) spectrometry-based method that permits fast and impartial analysis of detergent removal from biological samples.

Materials and Methods

2% CHAPS (EMD Millipore Cat. No. 220201), 0.5% deoxycholic acid sodium salt (EMD Millipore Cat. No. 264101), 2.5% CTAB (hexadecyl-trimethyl-ammonium bromide, Sigma Cat. No. H-9151), 1% NP-40 (Sigma Cat. No. N-6507) and 1X RIPA lysis buffer (EMD Millipore Cat. No. 20-188) prepared with 1X Dulbecco's phosphate-buffered saline (PBS, EMD Millipore Cat. No. BSS-1006-A) were used to evaluate the application of MIR spectrometry to monitoring detergent removal. All concentration estimations were performed using Direct Detect® assay-free sample cards (EMD Millipore Cat. No. DDAC00010-GR) and the Direct Detect® spectrometer (EMD Millipore Cat. No. DDHW00010-WW), applying 2 µL of sample solution per membrane position.

The spectrometer was calibrated with each of the analyzed detergents and the RIPA lysis buffer. Sample concentrations were determined in reference to the corresponding calibration method. A series of ten concentration points from 4% to 0.031% was used to generate the CHAPS calibration curve. Nine concentration points from 4% to 0.031% were used in order to generate the deoxycholic acid sodium salt calibration curve. A series of seven concentration points from 2.5% to 0.039% and from 2% to 0.031% were used to prepare the CTAB and NP-40 calibration curves, respectively. Seven concentration points from 1X to 0.016X were used to generate calibration curve for RIPA buffer.

Size separation using centrifugal filters with various nominal molecular weight limits (NMWL) is a common detergent removal method of choice. Amicon® Ultra 0.5 mL filters containing Ultracel®-10 Membrane (10 kDa NMWL, EMD Millipore Cat. No. UFC501096), Ultracel®-30 Membrane (30 kDa NMWL, EMD Millipore Cat. No. UFC503096) and Ultracel®-100 Membrane (100 kDa NMWL, EMD Millipore Cat. No. UFC510096) were used in the study. Each investigated detergent or buffer solution (500 µL per sample) was placed in the Amicon® Ultra devices. Detergent removal was analyzed using three devices for each NMWL (10 kDa, 30 kDa and 100 kDa). The devices were spun at 14,000xg for 5 minutes. The retentate in each device was adjusted with PBS back to 500 µL followed by MIR spectrometry-based estimation of the remaining detergent concentration. Each measurement was performed in triplicate. The devices were spun again, volume readjusted to 500 µL and the concentration was measured again (in triplicate). Data were collected for a total of three spin-removal cycles; however, in order to ensure sufficient signal-to-noise ratio in the last (most dilute) analysis, the volume recovered from the last spin cycle was adjusted to 250 µL only.

Amicon® Ultra 2.0 mL filters containing Ultracel®-10 Membrane (EMD Millipore Cat. No. UFC201024), Ultracel®-30 Membrane (EMD Millipore Cat. No. UFC203024) and Ultracel®-100 Membrane (EMD Millipore Cat. No. UFC210024) were also used to monitor removal of NP-40 and RIPA buffer. Similarly to the analysis performed using Amicon® Ultra 0.5 mL devices, detergent removal was analyzed using three devices for each NMWL (10 kDa, 30 kDa and 100 kDa). Each detergent solution sample (500 µL) was placed in an Amicon® Ultra 2.0 mL device and the volume was adjusted by addition of 1.5 mL of PBS. The device was spun at 7,500xg for 5 to 15 minutes, depending on the device NMWL. The retentate was adjusted with PBS back to 500 µL followed by MIR spectrometry-based estimation of the remaining detergent concentration. Each measurement was performed in triplicate. The volume in the device was increased by addition of 1.5 mL of PBS and the device was spun again, followed by volume readjustment to 500 µL and concentration measurement (in triplicate). Data were collected for total of three spin-removal cycles with the volume recovered from the last spin cycle adjusted to 250 µL only.

Removal of RIPA buffer was also monitored using Amicon® Pro purification systems (EMD Millipore Cat. Nos. ACS501024, ACS503025 and ACS510024). The above described protocol for monitoring of detergent removal using Amicon® Ultra 0.5 mL devices was modified to include dilution with 9.5 mL PBS (10 mL total volume) for each spin-removal step. Centrifugal force was reduced to 4,000xg and the centrifugal time was extended to allow concentration to volumes less than 500 µL. Data (in triplicate) were collected for a total of three spin-removal cycles, with the volume recovered from the last spin cycle adjusted to 250 µL only.

Results and Discussion

To enable downstream analysis of many biological samples, it is often crucial to successfully remove detergents used in the original sample preparation. Detergent removal can be achieved applying variety of available techniques. At the same time, proper monitoring of the removal process presents challenges and, until now, a universal approach applicable to a majority of detergents used in biological science was not available.

A nonionic (NP-40), anionic (sodium deoxycholate), cationic (hexadecyltrimethyl ammonium bromide (CTAB)) and a zwitterionic (CHAPS) detergent, as well as a commonly used lysis buffer, containing both nonionic and anionic detergents, were used to verify efficient application of a MIR spectrometry-based approach in monitoring of detergent removal. The CMCs of detergents used in the study ranged from 0.29 mM (0.0179% w/v) to 6 mM (0.3073% w/v). Micelle sizes of the detergents chosen covered a wide range, from ~1.2 kDa to ~90 kDa (Table 1).

The Amicon® Ultra centrifugal filters and the Amicon® Pro purification system are efficient laboratory tools that can be used for detergent removal from biological samples. As already mentioned, the chemical nature of most detergents allows for micelle formation above the CMC. Also, the CMC value for given detergent is highly influenced by the temperature and presence of other buffer components, like salts, potentially making detergent behavior (and removal) an unpredictable process. Micelle formation results in aggregation of the detergent and leads to gross changes in molecular structure. As a consequence, the majority of detergents concentrated above their CMC will behave more like large, globular proteins. This affects the amount of the detergent that can be removed from a biological sample solution by size-based devices with specific NMWLs. Therefore, only proper monitoring of detergent removal efficiency can ensure that a sample is sufficiently free of detergents to enable successful downstream analysis.

In the set of detergents investigated, a zwitterionic CHAPS is characterized by the highest CMC and relatively low micelle size (Table 1). At 2%, a concentration commonly used for biological sample preparation, this detergent exists in micelles. However, the micelles formed by CHAPS are relatively small (6,150 Da) and should be removable using most commercially available size exclusion devices. Amicon® Ultra 0.5 mL devices were used to remove CHAPS from 0.5 mL buffered solution containing 2% of the detergent. Efficiency of the removal was monitored using the Direct Detect® spectrometer.

Table 1.
Properties of detergents used for this study.

Detergent	Type	Agg. #	MW	MW	CMC	CMC
			mono [Da]	micelle [Da]	[mM] at RT	[%] at RT
NP-40	Nonionic	149	617	90,000	0.29	0.0179
CHAPS	Zwitterionic	4-14	615	6,150	6	0.3073
CTAB	Cationic	170	365	62,000	1	0.0365
Sodium Deoxycholate	Anionic	3-12	415	1,200-5,000	2	0.0830

Figure 1 shows that use of the 10 kDa membrane allowed for removal of around 60% of the detergent in three centrifugal spins, the 30 kDa membrane was highly successful in removing 100% of detergent after the third spin, and removal performed using the 100 kDa device required only two spins in order to deplete the detergent below instrument detection level (0.015% in the case of CHAPS).

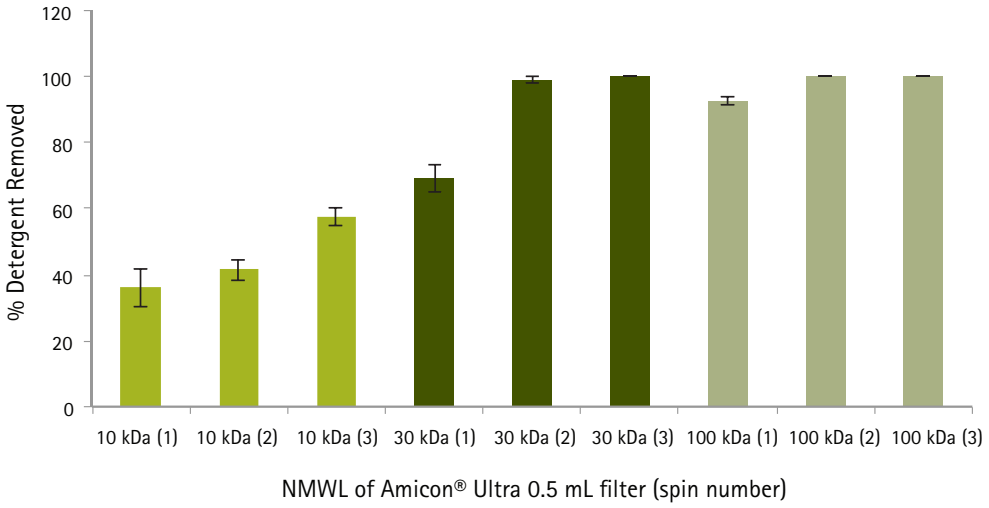

Figure 1.
MIR-based monitoring of CHAPS removal by Amicon® Ultra 0.5 mL devices. First three columns show results obtained in three consecutive spins using 10 kDa devices; middle columns represents results from three spins using 30 kDa devices and last three columns show removal using 100 kDa devices. Error bars represent standard deviation of three measurements each using three replicate devices per NMWL.

Anionic sodium deoxycholate shows the second highest CMC in the investigated series. Also, the micelles formed by this detergent have the smallest size within the investigated set (Table 1) and were expected to be removed by all of the devices used. Indeed, the depletion of 0.5% sodium deoxycholate from 0.5 mL of buffered solution by size exclusion spin devices proved to be successful regardless of the device NMWL (Figure 2).

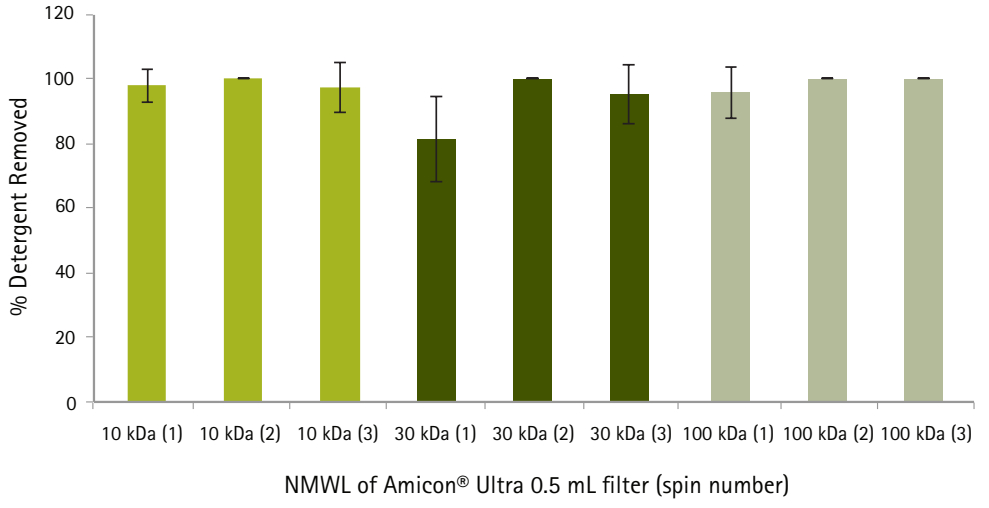

Figure 2.
MIR-based monitoring of sodium deoxycholate removal by Amicon® Ultra 0.5 mL devices. First three columns show results obtained in three consecutive spins using 10 kDa devices; middle columns represents results from three spins using 30 kDa devices and last three columns show removal using 100 kDa devices. Error bars represent standard deviation of three measurements each using three replicate devices per NMWL.

Cationic CTAB and nonionic NP-40 are characterized by relatively low CMCs, but their micelles, due to the size, are typically difficult to remove by size exclusion approaches. Figure 3 confirmed that removal of 2.5% CTAB and 1% NP-40 from 0.5 mL of buffered solution could only be successfully achieved after two to three consecutive spins using the device with NMWL higher than micelle size (100 kDa). Because RIPA lysis buffer at 1X concentration contains 1% NP-40 and 0.25% sodium deoxycholate, effective RIPA buffer removal using the Amicon® Ultra 0.5 mL filter could be achieved only with the 100 kDa membrane, similar to the membrane NMWL requirement for removal of NP-40.

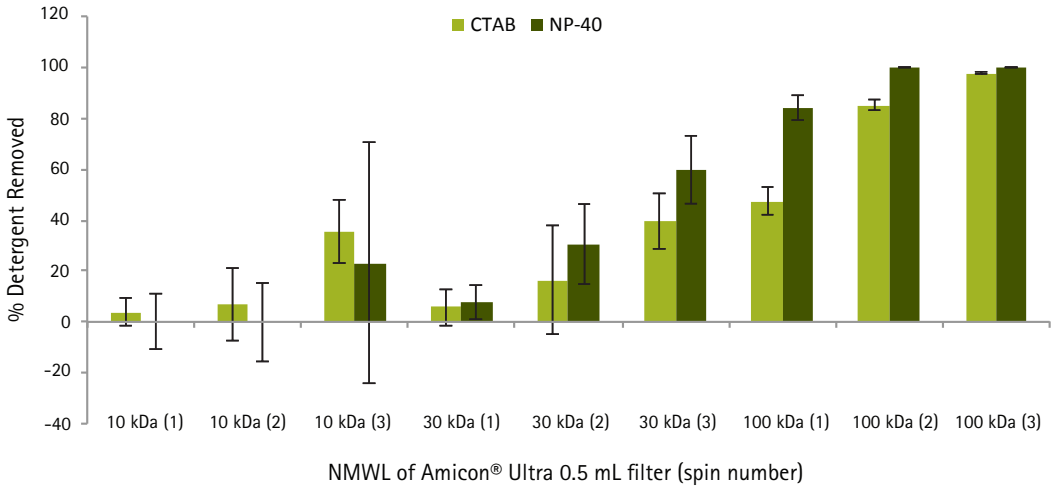

Figure 3.
MIR-based monitoring of CTAB (green) and NP-40 (blue) removal by Amicon® Ultra 0.5 mL filters. First three columns show results obtained in three consecutive spins using 10 kDa devices; middle columns represents results from three spins using 30 kDa devices and last three columns show removal using 100 kDa devices. Error bars represent standard deviation of three measurements each using three replicate devices per NMWL.

As shown above, removal of detergents from biological solutions requires careful planning and proper monitoring. Unfortunately, because of micelle size, the size-based centrifugal depletion of many detergents requires using membranes with NMWL as high as 100 kDa, resulting in loss of the proteins of interest in the sample. One way to first reduce micelle formation prior to detergent removal is by sample dilution. To this end, we modified the depletion protocol to allow for sample dilution and subsequent detergent removal all in one centrifugal size exclusion device, significantly improving the efficiency of detergent removal without excessively high NMWL membranes.

RIPA lysis buffer, Amicon® Ultra 2.0 mL and Amicon® Pro purification systems were used to show that detergent diluted below its CMC could be easily removed by a size-based method. Figure 4 shows results of detergent removal from 0.5 mL of 1X RIPA solution. Even a four-fold sample dilution (achieved with the Amicon® Ultra 2.0 mL device) was insufficient to bring the concentration of NP-40 present in RIPA below its CMC. Consequently, only a 100 kDa device was able to deplete the detergent below the instrument detection level (0.008% in case of NP-40). However, a twenty-fold dilution (achieved using the Amicon® Pro system) did permit efficient detergent removal with all NMWL membranes used.

Similar to the measurements for CTAB and NP-40 depletion (Figure 3), the analysis of RIPA removal using Amicon® Ultra 0.5 mL filters exhibited substantial error (Figure 4). Poor mixing of the analyzed sample is the most probable reason for the observed error. For measurement consistency, the Amicon® Ultra 0.5 mL filter was filled with PBS up to original 500 µL after each spin. In order to avoid any loss of the analyzed solution, it was mixed using a regular pipette. Consequently, the highly viscous retentate was not evenly dispersed throughout the analyzed volume at the time of assay-free card preparation.

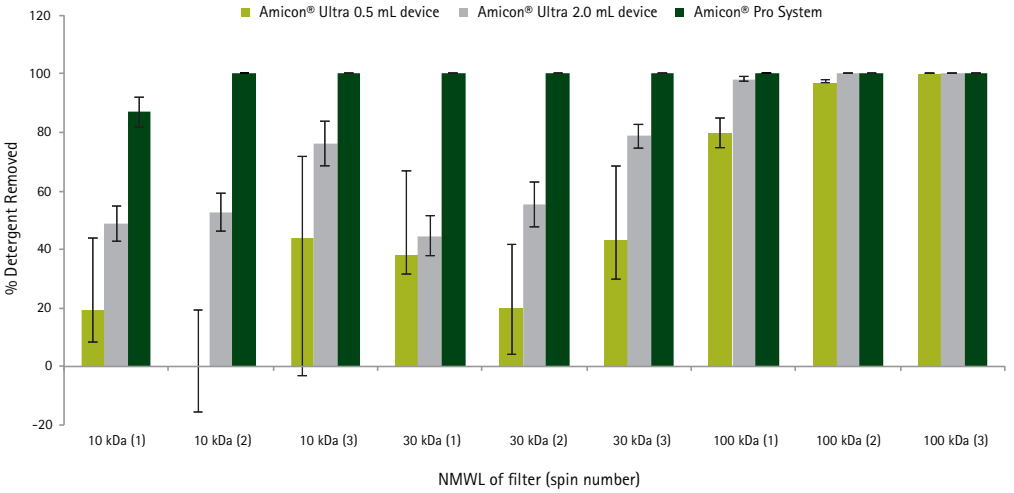

Figure 4.
The Amicon® Pro purification system enables efficient detergent removal, even using small (10 kDa) NMWL membrane devices. Data reflect MIR-based monitoring of RIPA buffer depletion by Amicon® Ultra filters and Amicon® Pro devices, Consecutive spins using 10 kDa, 30 kDa and 100 kDa devices are grouped in sets of columns.

Conclusion

We have demonstrated the success of a novel, centrifugal, size-based detergent removal method that minimizes the risk of protein loss. The Amicon® Pro purification system, with its large volume reservoir, enables twenty-fold sample dilution, reducing the detergent concentration of most samples to below detergent CMC. As a result, detergents can be removed with low-NMWL centrifugal ultra filters, promoting high recovery of proteins of interest.

Further, we demonstrate that the mid-infrared (MIR) spectrometry-based method for monitoring detergent removal presents a convenient, rapid, universal and highly effective technique. The method requires only single-time instrument calibration using the detergents of interest and can be combined with majority of known detergent removal techniques.

References

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