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A novel affinity adsorbent for the capture and removal of endotoxin

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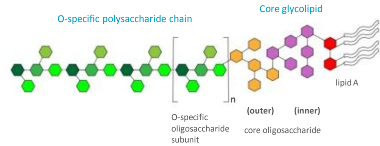
Introduction

Escherichia coli is a Gram-negative bacteria that is a popular host organism for the expression of recombinant protein bio-therapeutics. Lysis of the outer membrane of Gram-negative bacteria, a requirement to release internalised target bio-molecules, also releases substantial quantities of lipopolysaccharides (LPS – also known as endotoxin) into the process solution.

LPS (Figure 1) elicits a variety of pathophysiological effects on the body and its removal to acceptable levels, particularly in the presence of proteins with which it often associates, continues to be a major challenge.

FIGURE 1

Schematic representation of Gram-negative bacterial endotoxin (lipopolysaccharide).



The currently accepted maximum endotoxin dose limit is 5 EU per kg body weight per hour . 1 EU/mL is equivalent to 0.1 - 0.2 ng of endotoxin so purification processes need to be very effective in removing LPS from the product. Frequently there is reliance on established purification steps such as ion-exchange chromatography to remove endotoxin though separating LPS from proteins can be very challenging and is not always achievable by this approach.

Here we describe a novel, non-toxic and highly stable synthetic affinity adsorbent (PBL-1071) identified for the capture and removal of endotoxin from biological products.

PBL-1071 comprises a specific affinity ligand attached to near mono-disperse 6% cross-linked agarose bead (PuraBead® 6XL) which is resistant to high pH and can be regenerated (detoxified) easily with NaOH.

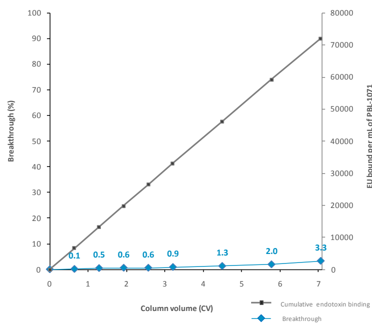
Binding Capacity

PBL-1071 has a high binding capacity for endotoxin, even for short residence times (1.2 minutes with minimal LPS breakthrough). Both dynamic (Figure 2) and static binding capacity were determined.

Platform	Automated chromatography workstation
Column parameters	2 cm bed height x 1 cm Ø (1.56 mL CV)
Equilibration buffer	50 mM sodium phosphate, pH 7.2
Load	11 mL of buffer containing 10,400 EU/mL
Wash buffer	50 mM sodium phosphate, pH 7.2
Clean in Place (CIP)	0.5 M NaOH

FIGURE 2

Cumulative binding capacity and breakthrough of endotoxin from the PBL-1071 adsorbent.



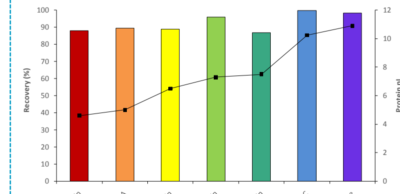
Endotoxin breakthrough of 3.3% was observed following loading with 72,000 EU per mL of PBL-1071 (40,000 EU/mL resin at 1% breakthrough). The static binding capacity was determined to be **>855,000** EU per millilitre of the PBL-1071 adsorbent.

Protein Binding

PBL-1071 exhibits low protein binding and a wide range of proteins can be processed regardless of their iso-electric point. High protein recoveries were obtained regardless of protein pI. Figure 3 indicates that >80% recovery is achieved for various model proteins spanning the pI spectrum.

FIGURE 3

Graphic representation of protein recovery following passage through PBL-1071.

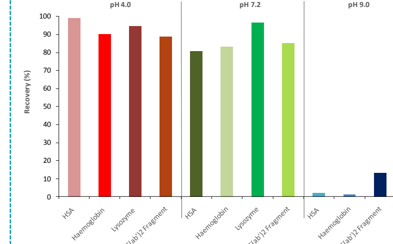


Buffer pH

PBL-1071 can operate in acidic to neutral conditions (pH 4.0 to pH 7.2) without a reduction in endotoxin clearance and maintaining >80% recovery of various proteins (up to 5 mg/mL). At pH 9.0 both endotoxin and target protein co-bind to the PBL-1071 adsorbent (Figure 4).

FIGURE 4

Graphic representation of protein recovery between pH 4.0 and pH 9.0.

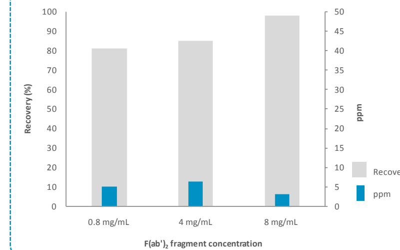


Effect of protein concentration on endotoxin removal and protein recovery

The impact of target protein concentration on endotoxin binding and protein recovery is shown in Figure 5. Increasing F(ab')$_2$ concentration resulted in increased target protein recovery (pH 7.2). Increasing protein concentration had no significant effect on the level of endotoxin clearance obtained.

FIGURE 5

Graphic representation of the recovery of F(ab')$_2$ fragments at varying concentrations (grey) and endotoxin clearance (blue). Initial endotoxin concentration was 24,000 EU/mL for all samples. Note 1 ppm endotoxin is equivalent to ~7.5 EU per mg protein.



The effect of various additives on target protein (F(ab')$_2$ fragment) recovery and endotoxin binding were also investigated. It was observed that additives such as high levels of salt (0.5 M NaCl) and metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}) detrimentally affected performance, however, EDTA (up to 20 mM) positively promoted protein recovery from ~85% to 95% without affecting endotoxin clearance.

Endotoxin removal from purified antibody fragment

PBL-1071 was used to capture endotoxin from an antibody fragment in an *E. coli* lysate partially purified using Fabsorbent™ F1P HF as an initial capture step (Figure 6 & Table 1). Clarified cell lysate was loaded onto Fabsorbent™ F1P HF and the F(ab')$_2$ fragment eluted at pH 5.0. The eluate was loaded directly onto the PBL-1071 adsorbent.

Platform	Automated chromatography workstation	
Step	Capture	Polish
Adsorbent	Fabsorbent™ F1P HF	PBL-1071
Column parameters	18.4 cm bed height (37 mL CV); 2.6 cm Ø column	3 cm bed height (2.4 mL CV); 1.0 cm Ø column
Equilibration / Wash buffer	50 mM Tris, pH 8.0	50 mM sodium phosphate, pH 7.2
Load	1 CV of *E. coli* cell lysate containing antibody fragment	Fabsorbent™ F1P HF eluate
Elution buffer	50 mM sodium citrate, pH 5.0	n/a

FIGURE 6

Non-reduced SDS-PAGE of the flow through (FT) sample for the F(ab')$_2$ protein samples post PBL-1071 endotoxin removal step.

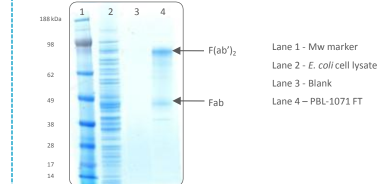


TABLE 1

Endotoxin levels determined using a chromogenic endotoxin assay kit.

Sample	Endotoxin (EU/mL)
Cell lysate	192,000
Fabsorbent™ F1P HF eluate	46,000
PBL-1071 flow through	19

Fabsorbent™ F1P HF produced a high purity antibody fragment, followed by a 4 log reduction of endotoxin achieved across the process (3.4 log clearance was observed for the PBL-1071 adsorbent column step).

Endotoxin removal of dAbs expressed in *E. coli*

Domain Antibodies (dAbs) are the smallest binding units of antibodies (~13 kDa). Two different dAbs produced in *E. coli* (post initial capture step) were loaded onto equilibrated PBL-1071. Results presented in the table below show high protein recovery and significant endotoxin clearance.

	pI	Load [Protein] mg/mL	Load EU/mg protein	Protein recovery (%)	EU/mg protein in non-bound	Endotoxin clearance
dAb 1	7.3	0.70	7200	85	110	1.8 log
dAb 2	4.6	0.34	22000	94	440	1.7 log

Conclusions

- PBL-1071 is a synthetic affinity ligand adsorbent that removes endotoxin from a wide range of proteins across the pI spectrum with recoveries of >80%, and can operate in a range of conditions from acidic to neutral pH.
- PBL-1071 has a high capacity for endotoxin and binds up to 40,000 EU/mL of adsorbent at 1% breakthrough with a 1.2 minute residence time. A total endotoxin binding capacity of >855,000 EU/mL of adsorbent is achievable with low protein binding (>80% recoveries).
- Protein recoveries can be improved by increasing the target protein concentration without detrimental impact on endotoxin binding making the PBL-1071 ideally suited to final polishing applications.
- Purification of an antibody fragment can be performed using Fabsorbent™ F1P HF followed by PBL-1071 to provide a 4 log clearance of endotoxin.
- PBL-1071 gave an endotoxin clearance of ~1.8 log from purified Domain Antibody expressed in *E. coli*.

Acknowledgements

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