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A novel method to determine residual detergent in biological samples post endotoxin reduction treatment and evaluation of strategies for subsequent detergent removal

Anne Serdakowski London [a,*], Chietara Japutra [a], Kyle Planck [a], Michael Lihon [a], Andrew Anh Nguyen [b]

[a] Novartis Biologics Center, Novartis Institutes for Biomedical Research, Cambridge, MA, USA
[b] Department of Ophthalmology, Novartis Institutes for Biomedical Research, Cambridge, MA, USA

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ABSTRACT

Endotoxin removal using detergent washes and extractions are well-established, efficient, and cost-effective methods; however, removing residual detergent post treatment has been shown to be a challenge. In this communication, we show a simple and fast method for determining the detergent concentration in a protein solution post treatment and highlight strategies for detergent removal to achieve levels below the critical micelle concentration (CMC), the minimum concentration at which detergent micelles form.

1. Introduction

The preclinical biological protein production process involves multiple steps that can affect the desired metrics for downstream application. In addition to yield and purity, it is critical that the recombinant proteins have low endotoxin levels as endotoxin can interfere with cellular assays and in vivo animal studies. Endotoxins are lipopolysaccharides (LPS) derived from the outer cell wall of Gram negative bacteria and are known to have a pyrogenic effect [1,2] on mammalian systems as small amounts of endotoxin can elicit significant immunological response.

Unfortunately, in a preclinical, non-GMP grade setting, exposure to endotoxins is unavoidable since they are present in air, water, lab ware, and equipment. Hence, it is critical to ensure minimum exposure to endotoxin at various steps, from preparation of plasmid DNA from Gram negative *Escherichia coli* for transfection to processing of conditioned expression supernatant and subsequent purification. The unique structural components of endotoxin (Fig. 1A), the non-polar lipid component, called Lipid A, the core oligosaccharide, and a heteropolysaccharide representing the surface antigen (*O*-antigen) allow for various mechanisms to be utilized to selectively bind and subsequently remove endotoxin from biological solutions [3]. This can be loosely be grouped into two approaches. One approach is based on the size exclusion of endotoxin such as gel filtration chromatography [4,5], ultrafiltration [6], or sucrose gradient centrifugation [7]. The second approach targets possible electrostatic or hydrophobic interactions of the endotoxin molecules. The commonly used methods for this approach include ion-exchange chromatography [8,9], affinity adsorbents, such as immobilized L-histidine, poly-L-lysine, poly (γ-methyl L-glutamate) and polymyxin B [10–12], or extraction with detergents [13,14]. Detergents have also been used in chromatography wash steps [15–18] to successfully reduce endotoxin in biological samples. Despite the number of techniques that have been developed for endotoxin removal, currently no single method is applicable for endotoxin removal for all biological solutions without compromising protein recovery and/or potentially affecting structural and biological activity of the protein. Therefore, the selected approach for successful endotoxin remediation is frequently tailored to the target protein properties and adaptability in the purification processes.

One popular method, commonly known as detergent extraction, is a phase separation facilitated by non-ionic detergent such as Triton X-114 (tertiary-octylphenol poly (ethyleneglycolether)) to form a two-phase micellar system. Extraction has been shown to be a robust technique capable of removing 99% of contaminating endotoxin from a biological solution [13,19,20] and is "gentle", preserving the immunoactivity, physical and biological activity of the protein [14]. This technique utilizes the unique structure of endotoxin by drawing endotoxin molecules into the micellar structure of detergents through non-polar interactions

Abbreviations: CMC, critical micelle concentration; LPS, lipopolysaccharides; GMP, good manufacturing practices; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; LAL, Limulus Amebocyte Lysate; PBS, phosphate buffered saline; mM, millimolar; CHO, Chinese hamster ovary; RPM, revolutions per minute; kDa, kilo-daltons; HPLC, high performance liquid chromatography; mL, milliliter; CV, column volume; CSE, control standard endotoxin.

* Corresponding author.
E-mail address: anne.london@novartis.com (A.S. London).

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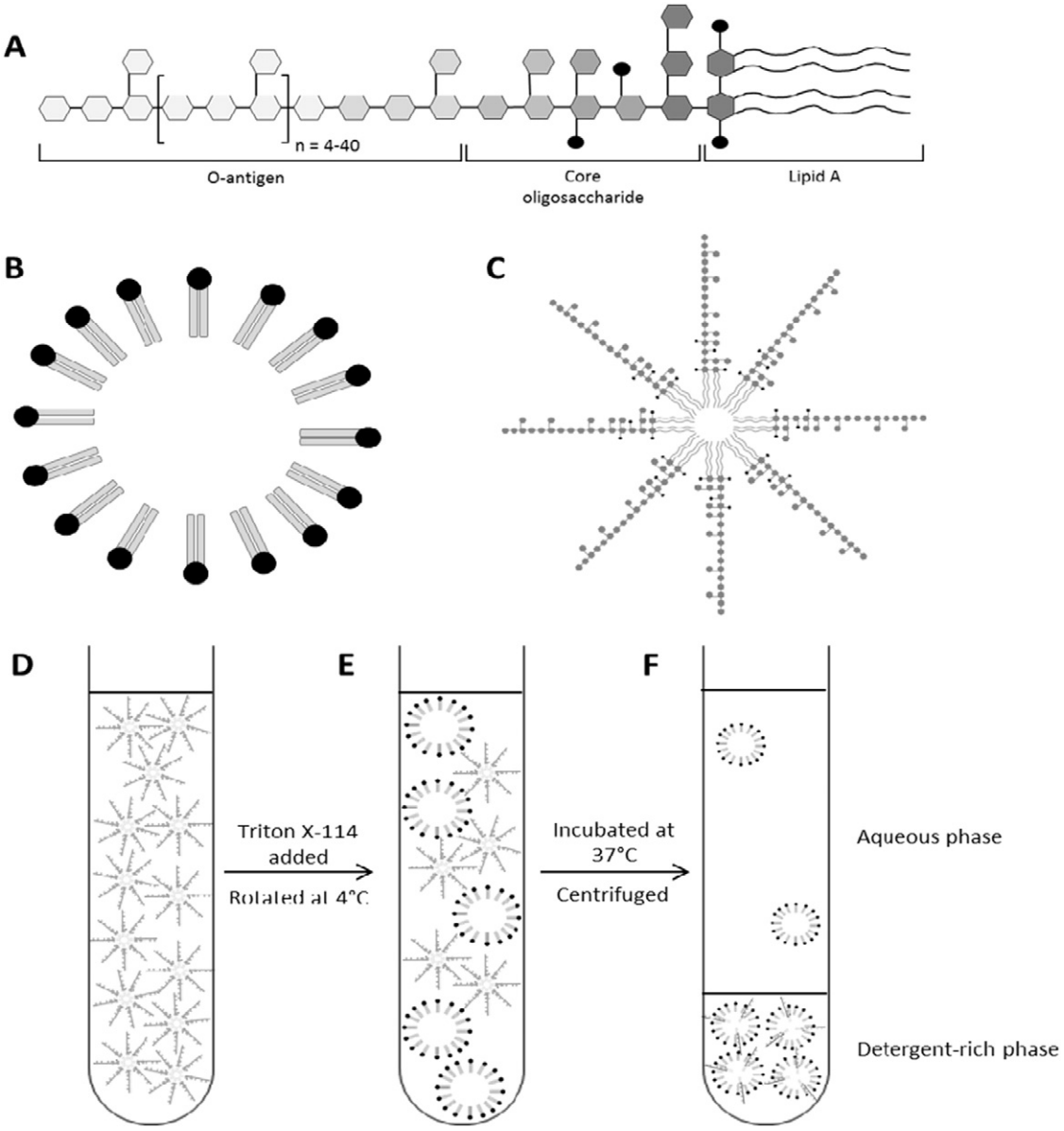

Fig. 1. A, Structure of typical lipopolysaccharides (LPS) monomer, which consists of hydrophobic lipid region and hydrophilic *O*-antigen. B. Two-dimensional rendering of detergent micelle with hydrophobic core when present at concentrations higher than critical micelle concentration (CMC). C, Two-dimensional rendering of endotoxin micelle with hydrophobic core. Detergent extraction process highlighted in panels D–F. Panel D illustrates a solution with high endotoxin concentration. After addition of detergent, the solution is cooled to 4 °C to form a homogenous solution, as shown in panel E. The solution is then heated briefly at 37 °C and centrifuged to separate the now extracted endotoxin and detergent from the aqueous phase, as the detergent micelles have internalized the endotoxin monomers.

of alkyl chains of lipid A with the surfactant's tail groups, separating the endotoxin from the aqueous phase of a protein solution (Fig. 1). This phase separation phenomenon is dependent on the cloud point of the detergent, a temperature at which detergent micelles aggregate into droplets with low water content, forming a new detergent-rich phase separate from the aqueous phase containing the protein. Triton X-114 is an ideal detergent since its cloud point is at 22 °C, a temperature compatible with most recombinantly expressed proteins and antibodies that has minimal effect on their stability and activity. Due to its homogenous nature at low temperature, Triton X-114 detergent is typically added to the protein solution and incubated at 4 °C (Fig. 1E) to enable the detergent to be fully soluble with the protein and the contaminating endotoxin. The temperature of the mixture is then raised to 37 °C (above the detergent cloud point), which induces a partitioning into an aqueous phase containing the hydrophilic proteins and a detergent phase containing the captured endotoxin. The mixture is then centrifuged to separate the insoluble detergent and endotoxin from the aqueous solution containing the protein, resulting in a low endotoxin aqueous solution.

Although the detergent washes and extractions methods are very successful in removing endotoxin from the protein solution, residual detergent may remain and is difficult to remove. Presence of excess unbound detergent in the biological sample can have interference on many downstream bioanalytical applications such as enzyme-linked immunosorbent assays (ELISA) and mass spectrometry. In addition, many detergents have overlapping absorbance at 280 nm wavelength, which will result in overestimation of target protein concentration in spectroscopic analysis. It is critical to remove as much unnecessary detergent as possible from protein solutions prior to characterization or in vivo use due to the possible effects it may have on the stability or activity of the system. Detergents are known to bind to biological membranes at low concentrations, however the full characterization of the binding effects are still unknown [21]. Triton X-100 has been shown to dissociate soluble proteins such as hemocyanin [22], alkaline