Review Article

Chromatographic Removal of Endotoxins: A Bioprocess Engineer's Perspective

Clarence M. Ongkudon,^{1,2} Jia Han Chew,¹ Boyin Liu,¹ and Michael K. Danquah¹

¹ Bio Engineering Laboratory, Department of Chemical Engineering, Monash University, Wellington Road, Clayton, VIC 3800, Australia

² Biotechnology Research Institute, University Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia

Correspondence should be addressed to Clarence M. Ongkudon, clarence@ums.edu.my

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Gram-negative bacteria are widely used for the production of gene-based products such as DNA vaccines and bio-drugs, where endotoxin contamination can occur at any point within the process and its removal is of great concern. In this article, we review the structures of endotoxin and the effects that it causes *in vivo*. The endotoxin removal strategies are also discussed in the light of the different interaction mechanisms involved between endotoxins and bioproducts particularly plasmid DNA and proteins. For most cases, endotoxin removal is favoured at a highly ionic or acidic condition. Various removal methods particularly chromatography-based techniques are covered in this article according to the relevant applications.

1. Introduction

Gram-negative bacteria are widely used in the biotechnology industry for recombinant DNA production, where endotoxin contamination can occur at any point within the processes [1]. Endotoxins must be removed from proteins prepared from Gram-negative bacteria prior to its administration into the human and animal bodies to avoid any adverse side effect [1]. Many purification methods have been developed for endotoxin removal, including LPS affinity interactions, two-phase extractions, ultrafiltration, affinity chromatography and anion exchange chromatography [2]. The use of tailor-made endotoxin-selective adsorbent matrices for endotoxin removal is reported elsewhere [3]. The selection of a suitable endotoxin removal system is based on the properties of the bioproducts being purified. The interaction between the anionic phosphate in LPS and the cationic ligands on the sorbents are mostly utilised as the mechanism of endotoxin removal [4]. Anion exchange and affinity chromatography are based on cationic functional ligands such as diethylaminoethanol, histidine, polymyxin B, poly (ϵ -lysine), and poly (ethyleneimine) [4]. Hydrophobic interactions between the lipid A portion and sorbent are also considered to

be important attributes that removal techniques can take advantage of [4]. Endotoxin molecules tend to form micelles or vesicles in aqueous solution [5]. Due to the difference in sizes of endotoxins and water as well as salt and other small molecules in protein-free solutions, ultrafiltration can be employed. In the presence of proteins, affinity chromatography and two-phase extraction methods can take advantage of the physical-chemical interaction between endotoxin and protein to completely remove endotoxin [6]. Detergents can be used to separate endotoxin from a protein surface, however an additional step is required to remove the surfactant from the product [5].

2. Structure of Endotoxins

Endotoxins, also known as lipopolysaccharides (LPS), are mostly found in the outer membrane of Gram-negative bacteria [1]. They are the integral part of the outer cell membrane and are responsible for the organization and stability of the bacteria [6]. The general structure of all endotoxins is a polar heteropolysaccharide chain, with three distinct domains: the O-antigen region, a core oligosaccharide part and a Lipid A part (Figure 1).

FIGURE 1: Structure of bacterial lipopolysaccharides (Source: <http://en.wikipedia.org/wiki/Lipopolysaccharide>, accessed on 10 Jan 2012).

Lipid A is the most conserved part which is responsible for the toxicity of endotoxins [1], while, the effect of polysaccharides is negligible. The Lipid A structures were first studied based on Enterobacteria [1]. The common architecture of Lipid A is a disaccharide, with glucosamine being the monomer. The two glucosamine monomers are linked between position 1 and 6, and both of them are phosphorylated to produce bisphosphorylated β -(1-6)-linked glucosamine disaccharide. Furthermore, there are fatty acids ester-linked at positions 3 and 3' and amide linked at positions 2 and 2' [7]. The position 6' is attached to the oligosaccharide region [7].

The oligosaccharide moiety is the core unit of LPS. Enteric bacterial LPS cores typically consist of 8–12 sugar units [7]. Alternative structures are reported for the inner core where the heptose may be substituted by a phosphate, pyrophosphate, or phosphorylethanolamine group [8]. The phosphate groups and charged sugar residues in the inner core and Lipid A are responsible for the stability of LPS by interactions with cations. Moreover, a diversity of negatively charged components is also reported, such as one to three units of α -3-deoxy-D-manno-oct-2-ulonic acid (Kdo) and hexuronic acid [7].

The O-specific chain is composed of repetitive subunits and only exists in smooth-type Gram-negative bacteria [7]. There may be up to 50 identical subunits in an O-chain unit, and each subunit consists of up to eight sugar units. Unlike the inner core region, the frequent components in O-chain structures are deoxysugars [9]. There are various O-chain structures, including linear or branched backbones which are substituted by many kinds of aglycones [10]. The O- and N-acetyl phosphate and phosphorylethanolamine are common substitutes found. Some non-stoichiometric substitutes may also exhibit, such as amino acids, acetamidino groups as well as formyl groups [9].

3. Endotoxins-Bioproducts Molecular Interactions

Modern bioprocess technologies enable numerous methods to produce proteins. Common methods include microbial bioprocesses for the expression of human proteins, such as growth hormones and interferons [11]. In addition, culture techniques are also important processes where mammalian cells, yeast and fungi are utilised for proteins exhibiting post-translational modifications, such as monoclonal antibodies [11]. Endotoxin contamination has become an important issue in biomanufacturing. It is very common to find high levels of endotoxin in albumin, collagen or gelatin [12]. Endotoxin may also be present in an antibody or peptide [12].

Endotoxin contamination in a therapeutic biomolecules production process usually occurs when it is released from within the process, or introduced by non-sterile process condition [11]. Endotoxins have very strong biological effects and are responsible for causing fever and shivering, hypotension, adult respiratory distress syndrome, disseminated intravascular coagulation and endotoxin shock [11]. As a result of diversity in the negatively charged groups and hydrophobic character of endotoxin, the interactions usually occur with positively charged substances as well as molecules with hydrophobic moieties. Endotoxins exhibit a significant capability to interact with biomolecules, including proteins. Various proteins such as anti-endotoxin antibodies and proteinaceous endotoxin receptors have been reported to have interactions with endotoxins due to electrostatic driving force [12]. Basic proteins such as lysozyme, lactoferrin and transferrin are also involved [11]. In cases of haemoglobin transferring interactions, the effect is not caused by binding but by deaggregation of supramolecular endotoxin structures [13]. Although it is considered that hydrophobic interactions are involved, the details of the mechanisms require further studies. It is hypothesised that the protein-endotoxin complex may be stabilised by the calcium bridges resulting from the competition between the protein-bound carboxylic groups and endotoxin-bound phosphoric acid groups for calcium ion [11]. The micellar aggregates formed by LPS are considered as biologically active, which indicate multiple protein interaction with LPS molecules [1]. Studies have shown that the oligomeric interactions result in a globular complex consisting of self-assembly lipophorin particles and a protein that serves as pro-coagulant [1]. These interactions result in the masking of endotoxin, making removal procedures hard [11].

It has been reported that endotoxins can activate the complement system which is part of the innate immune system that is responsible for eliminating pathogens from organism [14]. The endotoxins can also affect the inflammation and coagulation processes by interacting with the kinin system [14]. LPS also contributes greatly to effects *in vivo* by releasing cytokines and by expressing Tissue Factor [12]. Examples of proteins that show strong affinity towards endotoxins include lipopolysaccharide-binding protein (LBP), bacterial/permeability-increasing protein (BPI), amyloid P component and cationic protein 18 [13]. Anion-cation

complexes of great stability could be formed by endotoxin-bioprocess interaction if the structure of the bioproducts is flexible [13]. To study the effects of endotoxin contamination on biomaterials, researchers have used purified endotoxins containing the lipid and polysaccharide portions only [12]. The purified endotoxins were more potent than environmental endotoxins. The study has also shown an increase of TNF α , IL-1 β , IL-6 and nitric oxide production in macrophages, while the osteoclast differentiation was also induced [12]. A significant increase in osteolysis was reported within 7 days of endotoxin *in vivo* implantation; however, the effect vanished after 21 days.

As discussed above, the endotoxins have both positive and negative effects. They can stimulate particular immune systems but can also affect the functionalities of proteins. Therefore, in order to avoid the adverse effects, the endotoxin removal strategies are essential.

4. Chromatography-Based Endotoxin Removal Strategies

Significant amount of studies and research have been conducted in the last decade concerning the development of a novel endotoxin removal method. The growing demand for highly purified plasmid DNA for therapeutic usage has prompted a greater effort in this field. Lab scale endotoxin removal technique involves cycles of washing with alkali ethanol, nitric acid and 70% ethanol performed in an ultrasonic bath [12, 15–18]. However, this method does not apply to all contaminated solutions. The difficult destruction and removal of endotoxin from particles such as proteins was due to its stable structure brought about by strong electrostatic interactions between phosphoric acid section of endotoxin and carboxylic part of protein [5, 19]. Commercial purification steps generally include ultracentrifugation, enzymatic digestion and chromatographic methods [20–25].

Chromatography is likely the most reliable and widely applied method due to its ability to exploit the size, charge, hydrophobicity, nucleotide accessibility and affinity of a biomolecule (Table 1) [26]. Chromatographic methods which are currently being studied involve affinity, size-exclusion, membrane ultrafiltration, membrane microfiltration, slalom, anion-exchange, cationic-exchange, hydroxypatite, hydrophobic interaction, reverse-phase and thiophilic adsorption. Microfiltration and ultrafiltration are based on membrane adsorbers [3, 27, 28]. They are based on convective mass transport and have shorter path length which translates to shorter residence time [13]. Both filtrations show relatively good endotoxin clearance [13]. The choice of a stationary phase material is based on the chemical nature, physical characteristics and chromatography performance indicators [29]. Other important factors to be considered are chromatographic affinity of endotoxin and protein, affinity of endotoxin for protein, temperature, pH, types of detergent and solvent [1, 30].

4.1. Stationary Matrices. One of the latest chromatographic adsorbent materials being finetuned is poly (glycidyl

methacrylate) or known as PGMA. Dispersion, emulsion and suspension polymerisations involving glycidyl methacrylate (GMA) and ethylene glycol dimethylacrylate (EDMA) make PGMA [31]. PGMA has large pores, which translate to rapid mass transfer, high adsorption kinetics and negligible low-flow resistance [29]. Past researchers have functionalised PGMA with urea, ammonia and DEAE-Cl as well as covalent coupling with imminodiacetic acid-metal (IDA-metal) chelator for the purification of plasmid DNA [29, 31]. Adsorption isotherm was determined as Langmuir due to the strong plasmid molecules-ligands interactions. During the functionalisation of the monolith, temperature reduction will increase the reaction time and reduce the ligand densities [29]. DEAE-Cl ligand has a lower energy cost when compared to ammonia and it displays higher efficiency and capability of plasmid DNA (pDNA) adsorption when compared to urea [29]. PGMA functionalised with IDA-metal chelator has been used in Immobilised Metal Affinity Chromatography (IMAC) for protein adsorption and has shown favourable results. Addition of Ethylenediaminetetraacetic Acid (EDTA) provides for the regeneration of metal-chelated particles with no morphology damage or protein adsorption capacity loss [31]. Chelating agents destroy the bridging effect of calcium ions leading to the prevention of endotoxin aggregation [5]. However, only low levels of endotoxin removal could be achieved.

Other chromatographic matrices studied are namely poly (γ -methyl-L-glutamate) bead [13, 51] and N, N-dimethylaminopropylacrylamide (DMAPAA) spherical particles [13, 32]. Poly (γ -methyl-L-glutamate) beads are small in size thus have greater contact areas for endotoxin adsorption. This enables high selectivity of molecules for example BSA (protein). However, the effective adsorbent might experience structural changes caused by chemical instability of ester bonds [13]. On the other hand, DMAPAA is produced through DMAPAA and N-allylacrylamide (AAA) copolymerisation [32]. Endotoxin and protein adsorption takes place under a low salt condition [33]. To improve endotoxin removal at high-salt conditions, aminated poly (γ -methyl-L-glutamate) is suggested [52]. The pore size and charge density of DMAPAA can also be manipulated [13]. Moreover, it is stable under cleaning-in-place (CIP) conditions [13]. Being completely regenerative, DMAPAA is worth the continuous study for practicality.

4.2. Affinity-Based Chromatography. Affinity chromatography consists of triple helix (THAC), protein-DNA, immobilised metal (IMAC), boronate, polymyxin B, histamine, arginine and histidine affinities [34]. This method uses synthetic ligands for specific elution and involves the impregnation of poly(ϵ -lysine) into cellulose beads [35]. Being a clean and natural polymer, cellulose base beads provide greater endotoxin selectivity [35]. Pore size plays a major role in particle selectivity. For instance, high endotoxin retention is promoted using small pore size based on size-exclusion effects while large pore size reduces ionic interactions for negatively charged proteins [35]. A

TABLE 1: Summary of endotoxin removal methods.

Method	Main features	References
Direct washing	with alkali ethanol, nitric acid and 70% ethanol in ultrasonic bath	[12, 15–18]
Microfiltration and ultrafiltration based on membrane adsorbents	Convective mass transport and have shorter path length which translates to shorter residence time. Both filtrations show relatively good endotoxin clearance.	[3, 13, 27, 28]
Monolith	(i) PGMA has large pores which translate to rapid mass transfer, high adsorption kinetics and negligible low-flow resistance (ii) Addition of Ethylenediaminetetraacetic Acid (EDTA) provides for the regeneration of metal-chelated particles with no morphology damage or protein adsorption capacity loss	[29, 31]
Particle based adsorbents	Endotoxin and protein adsorption using DMAPAA takes place under a low salt condition	[13, 32, 33]
Two phase micellar system	(i) Involves separation of aqueous surfactant solution into micelle-rich and micelle-poor regions through excluded-volume interactions (ii) External agent notably Triton X-114 is needed to maintain the inherent biological activity of protein while reducing endotoxin level by 100-fold (i) Poly(ϵ -lysine) derived cellulose beads provide greater endotoxin selectivity (ii) High endotoxin retention is promoted using small pore size based on size-exclusion effects (iii) The high selectivity eliminates the need for multiple purification steps and reduces production costs (iv) Reproducible, scalable and capable of specifically recognising and purifying supercoiled pDNA in arginine affinity chromatography (v) Arginine being non-immunogenic avoids interference with endotoxin assays	[1]
Affinity chromatography	(i) It can be applied for RNA, pDNA and endotoxin removal (ii) There are situations involving special affinity interaction such as pure plasmid DNA binding specifically only to Fe ³⁺ charged chelating compound	[5, 34–43]
Immobilised metal affinity chromatography (IMAC)	(i) It uses composite polyacrylamide as the column which is highly porous (ii) Ultrafiltration is used if protein is not present. This method is capable of removing large endotoxin aggregate with alkanediol as one of the many agents used for effective endotoxin removal	[44, 45]
Size-exclusion chromatography (SEC) and ultrafiltration	(i) It uses composite polyacrylamide as the column which is highly porous (ii) Ultrafiltration is used if protein is not present. This method is capable of removing large endotoxin aggregate with alkanediol as one of the many agents used for effective endotoxin removal	[1, 34, 46, 47]
Anion-exchange chromatography (AEC)	It has rapid separation, wide selection of AEC media, sodium hydroxide (NaOH) sanitisation and does not require any solvents (i) Is thought to be more efficient than anionic exchanger in terms of endotoxin removal	[1, 6, 48]
Cation-exchange chromatography (CEC)	(ii) Polycationic ligands offer extremely strong attraction/binding for endotoxins (iii) PEI as a hydrophilic polymer has superior biocompatibility and exhibits hydrophobic interactions with endotoxin while PLL works well for protein recovery and still usable after binding capacity exhaustion	[1, 6, 13]
Immobilized hydrophobic ligands and reverse-phase chromatography (RPC)	Interact with non-polar protein surfaces through van der Waals forces for high endotoxin removal	[49, 50]

combination of electrostatic, hydrophobic and hydrogen bond interactions are present in the affinity chromatography [36]. Bound substances can be recovered or removed through desorption process. The high selectivity of particles achieved using this method eliminates the need for multiple purification steps and reduces production costs of therapeutic products [36]. The current drawbacks of affinity chromatography are low yield and high salt concentration requirement for substance elution. THAC is the formation of oligonucleotide-ligand triplex and pDNA duplex [37, 38]. It is time consuming and thus not a wise selection for affinity chromatography [39]. On the other hand, histidine affinity provides relatively low yield and involves high binding buffer concentration while histamine affinity displays decontamination potential but could not work independently at the moment [40, 41]. Some studies have also been carried out on arginine affinity recently and have shown promising results. Arginine being non-immunogenic avoids interference with endotoxin assays [5]. The ligands interact with aromatic sections of protein and dissociate their interactions with endotoxins. Its binding and elution behaviour is highly influenced by ionic strength of buffers [41]. Being reproducible, scalable and capable of specifically recognising and purifying super-coiled pDNA due to greater nucleic acid base exposure, arginine affinity chromatography has great potential and should be explored further [5, 42].

Another interesting and potential affinity chromatography worth noting is IMAC. It can be applied for RNA, pDNA and endotoxin removal. The type and state of metal ions dominate its binding behaviour. Metal ions are categorized into soft, intermediate and hard [44]. Soft metal ions consist of Cu^+ , Hg^+ and Ag^+ . Intermediates are made up of Cu^{2+} , Ni^{2+} , Zn^{2+} and Co^{2+} while hard metal ions are Fe^{3+} , Ca^{2+} and Al^{3+} . The affinity capture is affected by the combination of metal ion-chelating compound [45]. The chelating compounds include IDA (tridentate), NTA (tetra dentate) and CM-Asp (tetra dentate). The additional metal ion coordination site offered by tridentate can lead to a stronger binding affinity in IDA [44]. Ionic strength increase motivates greater RNA binding on IMAC and is attributed to electrostatic and hydrophobic interactions [44]. There are situations involving special affinity interaction such as pure plasmid DNA binding specifically only to Fe^{3+} charged chelating compound [44]. The binding dependency on DNA molecular weight and conformation is not neglected [53]. It is interesting to note that pDNA does not bind in the presence of RNA due to steric hindrance [44]. Immobilised metal ions accessibility of RNA is more promising than pDNA leaving pDNA uncaptured. Apart from IMAC, boronate affinity interaction provides an option for RNA purification [34]. Another affinity-based ligand, Polymyxin B works by disorganizing bacteria wall and interacting hydrophobically with endotoxin [43]. Unfortunately, it has slow binding kinetics leaving the purification procedure time consuming. Furthermore, polymyxin B cannot effectively recover proteins and remove endotoxins at the same time as both shares the same charge.

4.3. Size Exclusion Chromatography. For a simple, inexpensive and reproducible pDNA or protein purification, size-exclusion chromatography (SEC) can be considered [34]. It uses composite polyacrylamide as the column which is highly porous [34]. Among the commercial polyacrylamides available are Superose 6B (Pharmacia, Sweden), Sephacryl S-1000 (GE Healthcare, UK), Sepharose 6 Fast Flow (GE Healthcare, UK) and Zorbax GF250 (Agilent Technologies, USA). Sephacryl S-1000 is most widely utilized and displays optimum performance in resolving pDNA isoforms [54]. For faster mass transport, Superose 6B would be a better option since the beads have a higher pressure resistance [55]. Zorbax GF250 is a SEC media that requires RNase pre-digestion, which is time and energy consuming [46]. Out of all the options, Sepharose 6 Fast Flow is currently considered the best media with better pDNA selectivity over RNA and experiences compacting effect from ammonium sulphate elution buffer [56]. Agarose gel electrophoresis and restriction analysis are then used for pDNA purity evaluation [34]. Compared to the other chromatographic methods, size-exclusion has limited pDNA capacity and selectivity. Both size-exclusion and ultrafiltration chromatography require product and contaminant to have large size difference for effective endotoxin removal [47]. Ultrafiltration is used if protein is not present. This method is capable of removing large endotoxin aggregate with alkanediol as one of the many agents used for effective endotoxin removal [1].

4.4. Anion Exchange Chromatography. One of the most currently used chromatographic techniques for endotoxin removal is anion-exchange chromatography (AEC). It has rapid separation, wide selection of AEC media, sodium hydroxide (NaOH) sanitisation and does not require any solvents [48]. Salt gradient is vital in AEC as it is used for different nucleic acids elution according to charge density [1]. High salt concentration (high ionic strength) is maintained to avoid low charge density impurities adsorption [1]. For protein purification, competing interactions at the binding sites may occur. Presence of both charges of protein will give rise to co-adsorption [13]. The negatively charged proteins compete with endotoxins for binding sites which eventually exhaust the ligand binding capacity. Presence of only net positively charged proteins will cause protein molecules to experience repulsion from the ligands and compete with ligands to capture endotoxin. Endotoxins captured by proteins will be dragged out of the column and diminish the endotoxin removal efficiency of the ligand. Therefore, AEC is only suitable for purification of positively charged protein as biological product loss may occur when negatively charged protein is used [6]. For pDNA purification, negatively charged phosphate group on DNA interacts with positively charged ligands.

Exhibition of poor selectivity towards pDNA due to non-specific binding is the downside [34]. Furthermore, slight conductivity level change will disrupt affinity of protein to column [19]. Though, this situation can be improved with modified AEC media such as Q-Sepharose (GE Healthcare, UK), Fractogel DEAE (Merck, Germany), Sephacryl

S-500 HR (GE Healthcare, UK), Qiagen/DEAE modified (Qiagen, Germany), Poros QE (Applied Biosystems, USA) and expanded bed. Q-Sepharose is the most widely used AEC media. Incorporation of alcohols in Q-Sepharose could reduce the dielectric constant of buffers and improve pDNA and RNA binding selectivity [57]. Qiagen/DEAE modified consists of defined silica beads. It is a strong pDNA binder and elutes pDNA only at high-salt conditions [34]. However, silica beads are unstable under extreme pH condition [31]. Sephacryl S-500 HR has a high selectivity towards pDNA over RNA through implementation of selective access of molecules into pores and allows passing through of RNA and proteins but bans pDNA [58]. Fractogel DEAE has recently been identified as potentially the best commercial media for therapeutic pDNA production attributing to its excellent pDNA purification, recovery, reproducibility, robustness and dynamic capacity [34]. The use poly (GMA-EDMA)-based AEC column for the direct purification of plasmid DNA from lysed bacterial cell lysates has been reported [59]. In that study, chromatography conditions such as pH, flow rate, gradient elution rate, as well as matrix pore size were specifically optimised and resulted in high endotoxin removal and plasmid recovery.

Expanded bed AEC is another alternative media which has no other distinct advantages but the capability of purifying highly viscous solutions with ease [34]. For denatured pDNA, Poros QE provides an extra moderate and nonspecific binding between hydrophobic moieties of Poros and base of pDNA [34]. Cation-exchange chromatography is lesser known compared to AEC. However, several researchers claimed that cationic exchanger is more efficient than anionic exchanger in terms of endotoxin removal [1]. Polycationic ligands offer extremely strong attraction/binding for endotoxins. This is proven by the fact that desorption of endotoxins is still low even at high-salt conditions [2]. Known agents for cationic exchanger are PEI, zirconia-immobilised PLH and Poly-L-lysine (PLL). Zirconia-immobilised PLH is not viable as it is expensive and unstable under alkaline conditions [13]. PEI as a hydrophilic polymer has superior biocompatibility and exhibits hydrophobic interactions with endotoxin while PLL works well for protein recovery and still usable after binding capacity exhaustion [6].

4.5. Hydrophobic Interaction Chromatography. Hydrophobic interaction chromatography (HIC) explores differences in hydrophobicity for plasmid purification and captures pDNA under high-salt conditions due to the predominant hydrophilic feature of pDNA [49, 60]. Immobilized hydrophobic ligands interact with nonpolar protein surfaces through van der Waals forces for high endotoxin removal [49]. Protein and endotoxin are adsorbed onto the ligands and later separated using salt addition based on gradient elution [35]. Binding buffers commonly applied are ammonium sulphate and sodium citrate. Though, sodium citrate is preferred due to its lesser environmental impact compared to the former [49]. HIC also functions as an analytical tool for pDNA quality control and monitoring [34].

Other chromatographic method that is dominated by hydrophobic interactions is reverse-phase chromatography (RPC). RPC is divided into liquid (RPLC) and ion-pair (RPIPC) [34]. For RPLC, hydrophobic interaction occurs between the ligands and bases while hydrophobic ligands in RPIPC interact with hydroorganic eluents. Retention time in RPLC depends on the molecular structure and size of nucleic acids. RPIPC is reproducible and effective for commercial production of therapeutic pDNA. However, RPC is toxic and requires organic solvents for operation [50]. Thiophilic adsorption chromatography (TAC) involves interactions between thioether ligands and pDNA molecules [34]. Elution of impurities is based on ammonium sulphate concentration whereas elution of pDNA is based on sodium chloride (NaCl) gradient [34]. Similar to HIC, TAC displays optimum performance under a high-salt environment. Additional chromatographic methods worth noting are slalom chromatography (SLC) and hydroxyapatite chromatography (HAC). SLC is only applicable to large DNA molecules and is dominated by hydrodynamic effects [34]. Impurity and product elutions are influenced by molecular size. HAC is made up of mixed-mode ion exchanger involving both charge moieties [34]. Phosphate groups of pDNA compete with other nucleic acids for positively charged ligands. As a whole, HAC discriminates pDNA from other impurities fairly effectively.

4.6. Endotoxin Removal Using Paramagnetic Particles. A novel magnetic endotoxin removal resin containing silica-based MagneSil paramagnetic particles is incorporated to clear lysate and bind plasmid while a guanidine/isopropanol wash is introduced to remove RNA, protein and endotoxin levels [61]. In general, their additions have led to significant improvement in transfection performance through greater assay-to-assay transfection reliability and plasmid DNA purity without affecting plasmid yield [61]. Researchers successfully achieved 90% reduction in endotoxin levels when paramagnetic particles were used as an additional step of endotoxin lipopolysaccharide removal [61]. Furthermore, the modified MagneSil Tfx method results in impurities level similar to those achieved by anion exchange methods [61].

5. Conclusion

The endotoxin removal methods reviewed in this paper include laboratory scale direct washing, microfiltration and ultrafiltration based on membrane adsorbers, stationary monolith, particle based adsorbents, two phase micellar system and several chromatography techniques. Chromatography methods involve affinity, size-exclusion, slalom, anion-exchange, cationic-exchange, hydroxyapatite, hydrophobic interaction, reverse-phase and thiophilic adsorption. The choice of endotoxin removal method depends on the level of purity, rapidness, difficulty of operation, availabilities of chemicals and the cost. Affinity chromatography and IMAC are generally not recommended due to high time consumption and low yield whilst SEC has limited capacity and selectivity. AEC is a preferred technique over other

chromatography modes due to its high selectivity, rapid separation and reproducibility. The additional improvement resulting from the introduction of modified AEC media has significantly improved the selectivity towards plasmid DNA.

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