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Reducing endotoxin contamination in chitosan: An optimized purification method for biomedical applications

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ABSTRACT

Chitosan, a natural biopolymer, is widely used in biomedical applications due to its biocompatibility. It also possesses immunostimulatory properties and is notably studied and used as a vaccine adjuvant. However, endotoxin contamination, an often-overlooked extrinsic factor, may compromise its safety and confound the interpretation of its biological or immunostimulatory effects. Despite its widespread use, data on endotoxin levels in commercially available chitosan remain limited, potentially contributing to inconsistent immunological outcomes.

In this study, we developed a simple four-step purification process that effectively reduces endotoxin levels without altering key chitosan properties, namely the degree of deacetylation (DDA) and molar mass (MW). We characterized the endotoxin content of various commercial chitosan samples using the Limulus Amebocyte Lysate (LAL) assay and evaluated their immunostimulatory properties in vitro, both before and after purification. A 1 N sodium hydroxide treatment for 48 h reduced endotoxin levels by up to 100-fold, lowering the most contaminated sample from 62,000 EU/g to approximately 600 EU/g, while preserving DDA and MW. In vitro immunoassays showed that proinflammatory cytokine responses (IL-1β, TNF-α, IL-6) in J774A.1 macrophages were primarily driven by endotoxin contamination, which was significantly reduced following purification. Structural properties of chitosan showed no measurable impact on cytokine profiles.

This study underscores the critical need for endotoxin assessment and removal in chitosan intended for biomedical applications.

Abbreviations

CD	Cluster of differentiation
DDA	Degree of deacetylation
EU	Endotoxin unit
IL	Interleukin
LPS	Lipopolysaccharide
Mn	Number average molecular weight
MW	Molar mass
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
PDI	Polydispersity index
SD	Standard deviation
SEC-MALS	Size Exclusion Chromatography Multi-Angle Light Scattering
TFF	Tangential flow filtration
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor

1. Introduction

Chitosan, a biopolymer derived from chitin, has garnered increasing attention for biomedical applications due to its bioavailability, non-toxicity, immunomodulatory ability and biocompatibility (Alemu et al., 2023). Fields of application include tissue engineering (Chevrier et al., 2018; Croisier & Jérôme, 2013), drug delivery (Jeandupeux et al.,

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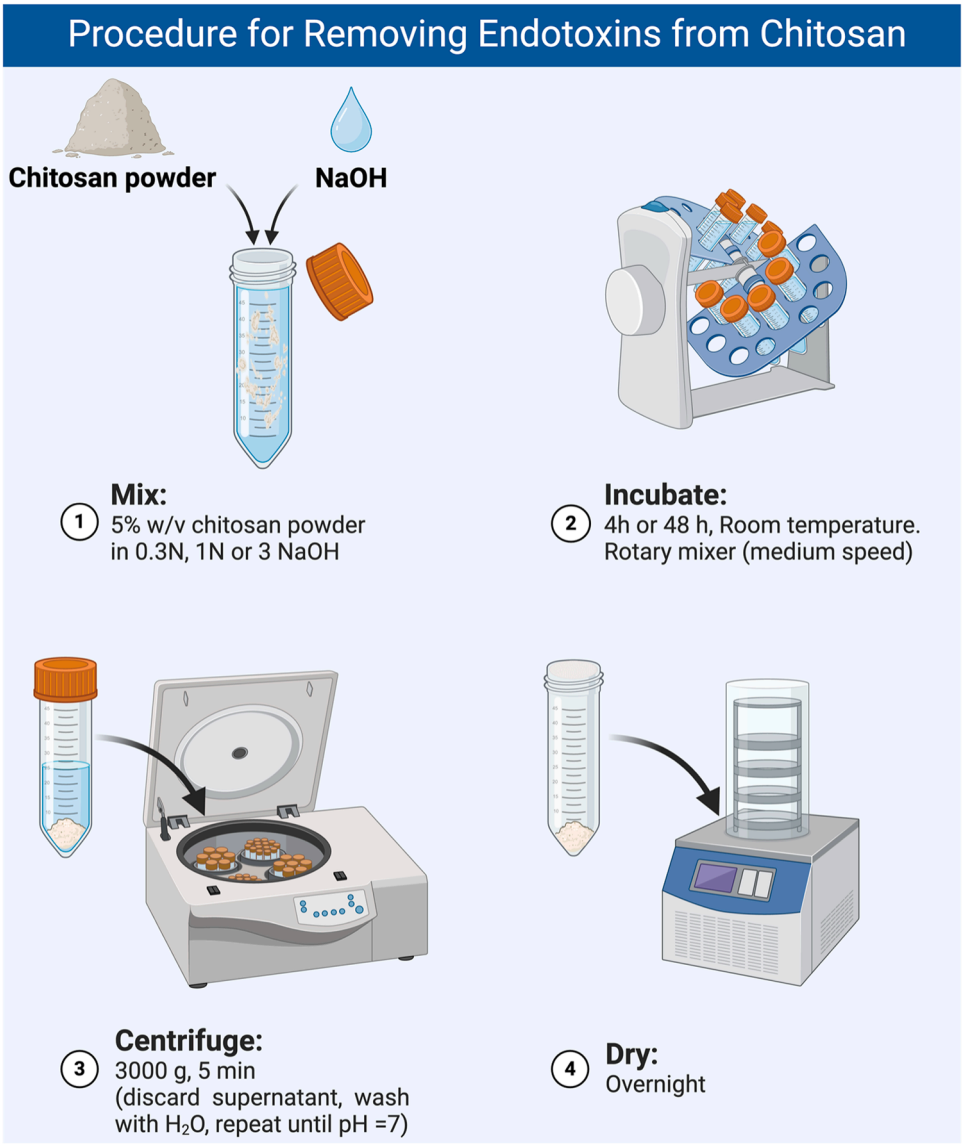

Fig. 1. Schematic representation of the conditions tested for endotoxin removal from chitosan.

2021; Padhi et al., 2022), wound healing (Chevrier et al., 2022; Rajinikanth et al., 2024), and vaccination, particularly as a vaccine adjuvant (Carroll et al., 2016; Kumar et al., 2024). The term "chitosan" refers to a broad class of polymers with varying physicochemical properties, such as the degree of deacetylation (DDA, the fraction of glucosamine monomers) and molar mass (MW), which influence their biological effects (Aranaz et al., 2021). Notably, chitosan possesses immunomodulatory properties, including the ability to enhance immune cell activity and stimulate the production of pro-inflammatory cytokines, as highlighted in several studies (Bueter et al., 2014; Carroll et al., 2016; Fong et al., 2017; Guzmán-Morales et al., 2011; Oliveira et al., 2012). However, inconsistencies reported in the literature and may arise from variability in, and/or limited characterization of, physicochemical properties (DDA, MW), as well as the presence of contaminants (Ghattas et al., 2025).

One major potential contaminant of chitosan is endotoxin. Endotoxins are pyrogenic lipopolysaccharide (LPS) structures associated with the outer membrane of Gram-negative bacteria. The LPS structure, comprising a polysaccharide core, an O-antigen and a lipid A component, triggers immune responses via TLR-4 signaling (Akira et al., 2006; Bryant et al., 2010; Holst et al., 1996; Kim et al., 2007), leading to the release of proinflammatory cytokines such as IL-6, IL1β, and TNF-α (Srivastava et al., 2017). When degraded, the structure of LPS disassembles, rendering it unrecognizable to its receptor (Lu et al., 2008; Sandle, 2013). Chitosan may become contaminated with LPS (Ravindranathan et al., 2016). The formation of chitosan-LPS complexes arises from the cationic nature of the polymer, which enables electrostatic interaction between its amine groups and the negatively charged groups of LPS (Davydova et al., 2000). The phosphate and carboxylic substitutions on the lipid A fragment of LPS, contributing to its overall negative charge, likely serve as the primary binding sites with chitosan (Davydova et al., 2000; Sandle, 2013). Given the potential for endotoxin-induced immune responses, failure to adequately assess endotoxin levels in chitosan products could compromise its safety and efficacy in biomedical interventions (USP, 2022). Additionally, limited or inconsistent characterization and reporting of key physicochemical parameters, such as DDA and MW, may limit the understanding of their role in modulating chitosan's biological activity.

While many studies report chitosan's immunostimulatory effects, few account for the role of endotoxin contamination. Existing purification methods are often complex and involve harsh treatments, such as chloroform extraction, CO_2 solubilization, or high-temperature sterilization (Banach-Kopeć et al., 2022; Lebre et al., 2019; Reay et al., 2023). In this study, we hypothesized that commercial chitosan products might

Table I
Physicochemical characteristics of chitosans used in this study.

Manufacturer	Physicochemical characteristics				
	Provided by manufacturer		Results of characterization tests		
	DDA (%)	Mn (kDa)	DDA (%)	Mn (kDa)	PDI
Spectrum Chemicals	99	N/A	91.6	60 ± 1	2.31 ± 0.02
MP biomedicals	≥92	N/A	94.5	73 ± 1	2.00 ± 0.02
AK scientific	>90	N/A	97.6	97 ± 1	2.26 ± 0.01
Sigma MMW	≥75	190–310	72.4	184 ± 2	2.37 ± 0.03
92–138 (in-house)	–	–	91.9	138 ± 1	1.30 ± 0.02

Table II
Quantification of endotoxins in chitosans prior to purification.

Manufacturer	Endotoxin content (EU/g)	
	Provided by manufacturer	Results of LAL tests
Spectrum Chemicals	N/A	1230 ± 55
MP Biomedicals	N/A	62,160 ± 1790
AK Scientific	N/A	259 ± 3
Sigma MMW	N/A	486 ± 11
92–138 (in-house)	–	20 ± 3

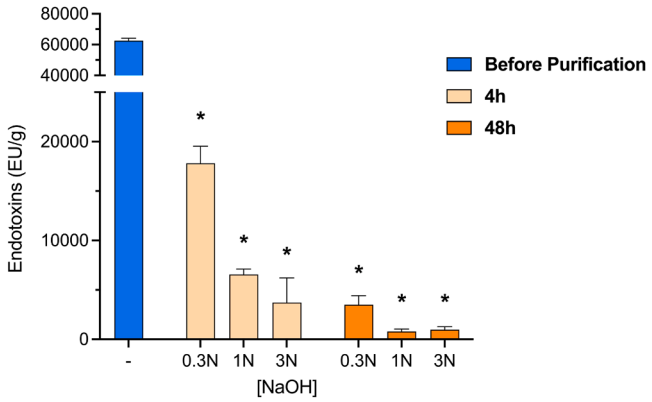

Fig. 2. Effect of time and NaOH concentration on endotoxin levels in chitosan solutions. The most contaminated chitosan sample was selected for purification using three different NaOH concentrations (0.3 N, 1 N, and 3 N) over two time periods: 4 and 48 h. Data are presented as the mean ± SD from three independent experiments each performed with two technical replicates. *P*-values: *denotes $p < 0.05$ compared to control (before purification).

be contaminated by endotoxin, thereby confounding their observed immunostimulatory properties. To test this hypothesis, we developed a four-step chitosan's depyrogenation process and conducted in vitro assessments of its immunostimulatory properties both prior to and following purification.

2. Materials and methods

2.1. Chitosan

Medical-grade chitosan was produced in-house from a raw chitosan (BioSyntech, Québec, Canada). It was heterogeneously deacetylated in concentrated sodium hydroxide to achieve the targeted degree of deacetylation of 92 % and depolymerised using nitrous acid to achieve a

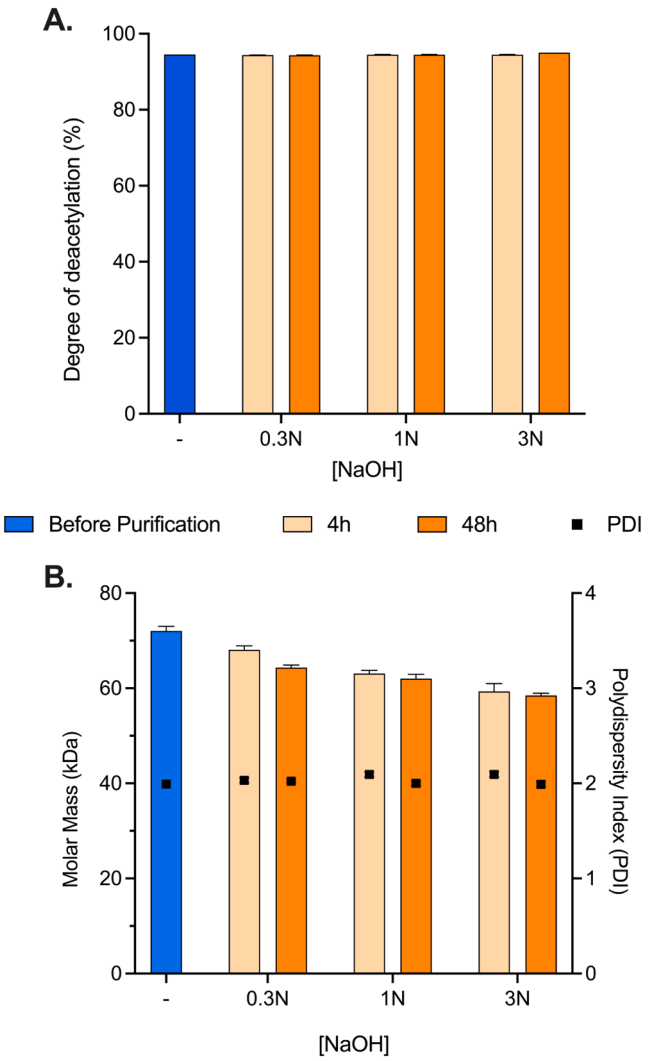

Fig. 3. Variation in physicochemical properties of commercial chitosan following different purification conditions. The most contaminated chitosan sample was selected for purification with three NaOH concentrations (0.3 N, 1 N, and 3 N) for two-time durations: 4 and 48 h. DDA values are shown in (A), Mn and PDI are shown in (B). Data are presented as the mean ± SD from three independent experiments.

specific number-average molar mass (Mn) target of 140 kDa, as previously described (Lavertu et al., 2006). Commercially available chitosans were purchased from MP biomedicals (Cat# 150,597), AK scientific (Cat# K638), Spectrum Chemicals (Cat# C1569), and Sigma (Cat# 448, 877).

The molar mass distribution of chitosan was determined by Size Exclusion Chromatography Multi-Angle Light Scattering (SEC-MALS), as described in Nguyen et al. (Nguyen et al., 2009). The system was composed of a Shimadzu Prominence-i 2030C (LC2030C Plus) HPLC instrument, a Wyatt Optilab TrEX dRI (WTREX-13) refractive index detector and a Wyatt miniDAWN TREOS light scattering detector. The light scattering detector was calibrated using toluene, and the refractometer was calibrated using an aqueous sodium chloride solution. Two SEC columns connected in series (Column 1: TSK Gel G6000PW XL-CP (13 μm), 7.8 mm x 30 cm, Tosoh Bioscience Part No.0021875 and Column 2: TSK Gel G5000PW XL-CP (10 μm), 7.8 mm x 30 cm, Tosoh Bioscience Part No.0021874) were used with a guard column (TSK Gel Guard Column PW XL -CP (13 μm), 6.0 mm x 4 cm, Tosoh Bioscience Part No 0021,876). Briefly, chitosan solutions were prepared at 1 mg/mL by dissolving in 0.1 M sodium acetate, 0.4 mM sodium azide (pH

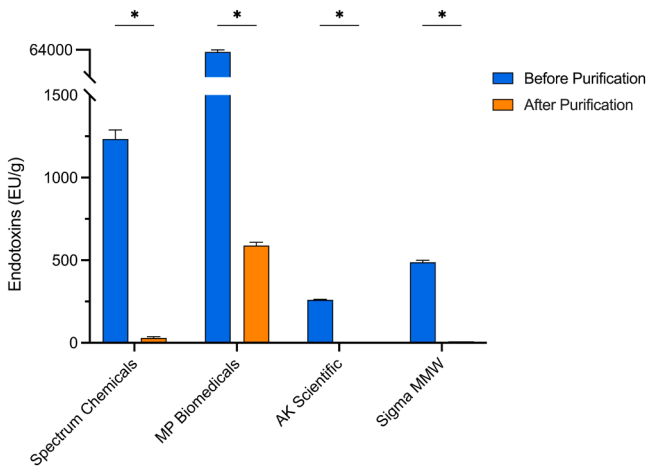

Fig. 4. Comparison of endotoxin amount before and after chitosan purification of commercial chitosan. The level of endotoxin contamination for each of the four commercial chitosans analyzed in this study was measured as described, both before and after purification with 1 N NaOH for 48 h. Data are presented as mean ± SD from three independent experiments each performed with two technical replicates. *P*-values: *denotes $p < 0.05$.

= 4.5) mobile phase. Samples were filtered through a syringe filter (0.45 µm, 13 mm hydrophilic Durapore® membrane, Millipore). Analyses were performed at room temperature at a flow rate of 0.8 mL/min, using an injection volume of 100 µL. Data were recorded and analyzed using Astra V software (Wyatt).

The DDA was assessed using ^{1}H NMR spectroscopy, as outlined by Lavertu et al. (Lavertu et al., 2003), using a Brucker NMR 800 MHz spectrometer equipped with a Brucker TCI Cryoprobe (800S4). Briefly, 10 mg of chitosan was dissolved in a solution comprising 1.96 mL D_2O and 0.04 mL DCl at room temperature for 30 min. Subsequently, approximately 0.4 mL of this solution was transferred to a 3 mm NMR tube and heated to 70 °C for 10 min prior to spectrum acquisition. DDA was determined by integration of the H1 proton peak of the deacetylated monomer (H1D) and the peak representing the three protons of the acetyl group (HAc), using Eq. (1):

$$DDA\ (\%) = \left(\frac{H1D}{H1D + \frac{HAc}{3}}\right) \times 100 \quad (1)$$

2.2. Endotoxin quantification

Chitosan was dissolved overnight in 0.02 % v/v acetic acid (Fisher, 296,355,100) in LAL Reagent water (MSJ Biolynx, SKW020P). Samples were then tested using a chromogenic Limulus amebocyte lysate (LAL) assay (Food & Drug Administration, 2012; Munson, 1985; USP, 2022) (Sensitivity 0.001 EU/mL) by Associates of Cape Cod, Inc. (Massachusetts, USA).

2.3. Chitosan purification

For purification from endotoxins, commercial chitosans were weighed into depyrogenized vials under aseptic conditions. Samples were then incubated at 5 % w/v in 0.3 N, 1 N, or 3 N sodium hydroxide (NaOH) solution (VWR, Cat# 1.09137.1000) for 4 h or 48 h on a rotary mixer (Fig. 1). Following incubation, the chitosan suspensions were centrifuged at 3000 g for 5 min at room temperature. The supernatant was discarded to remove NaOH solution. The chitosan pellet was then washed multiple times with DNase/RNase-free, protease-free, and endotoxin-free distilled water (UltraPure™ Invitrogen, Cat#10,977,015) to ensure complete removal of residual NaOH.

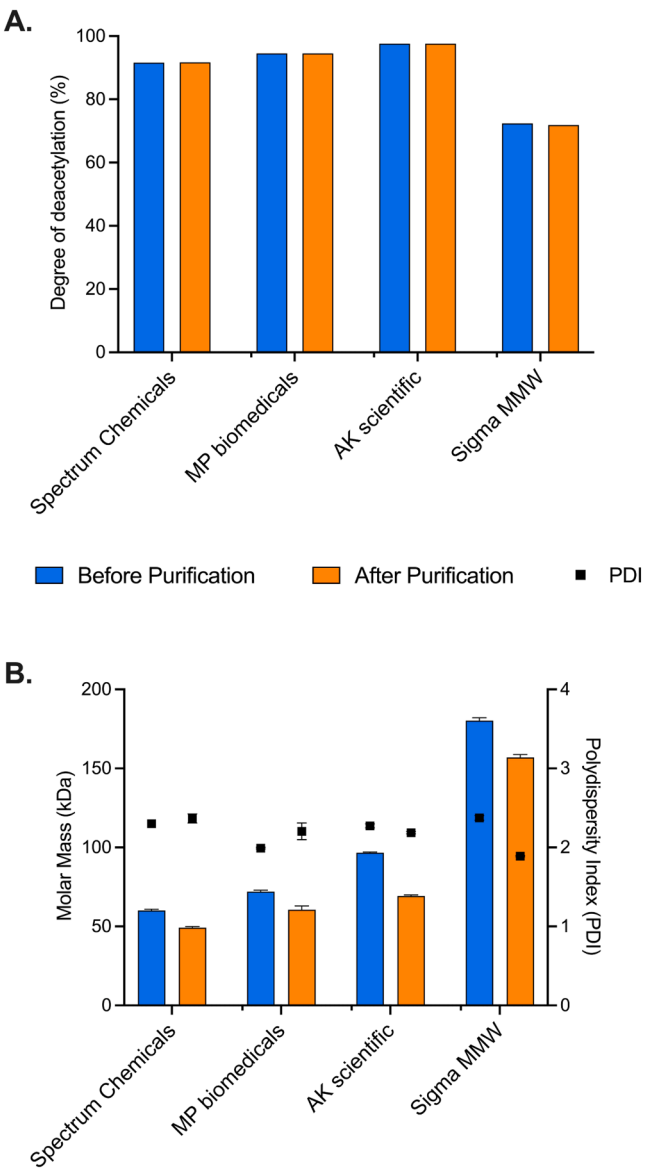

Fig. 5. Comparison of physicochemical properties variation from commercial chitosan after purification. The figure shows the DDA (A), number average molecular weight (Mn) and Polydispersity Index (PDI) (B), of four different commercial chitosan before and after purification. Data presented are mean ± SD from three independent measurements.

Washing steps were repeated until the supernatant reached a pH of 7. The purified chitosan samples were then freeze-dried and stored at room temperature until use.

2.4. Cell culture

J774A.1 murine macrophages (ATCC TIB-67), purchased from ATCC (Manassas, VA, USA), were used to assess the immunostimulatory properties chitosans before and after purification. This murine macrophage model is widely used to study immune responses, including cytokine production and inflammatory signaling (Lam et al., 2009). The macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and plated at a density of 1×10^5 cells in 1 mL in 24-well plates. The cell cultures were maintained in a controlled environment, with a humidified CO_2 incubator set at 37 °C and 5 % CO_2.

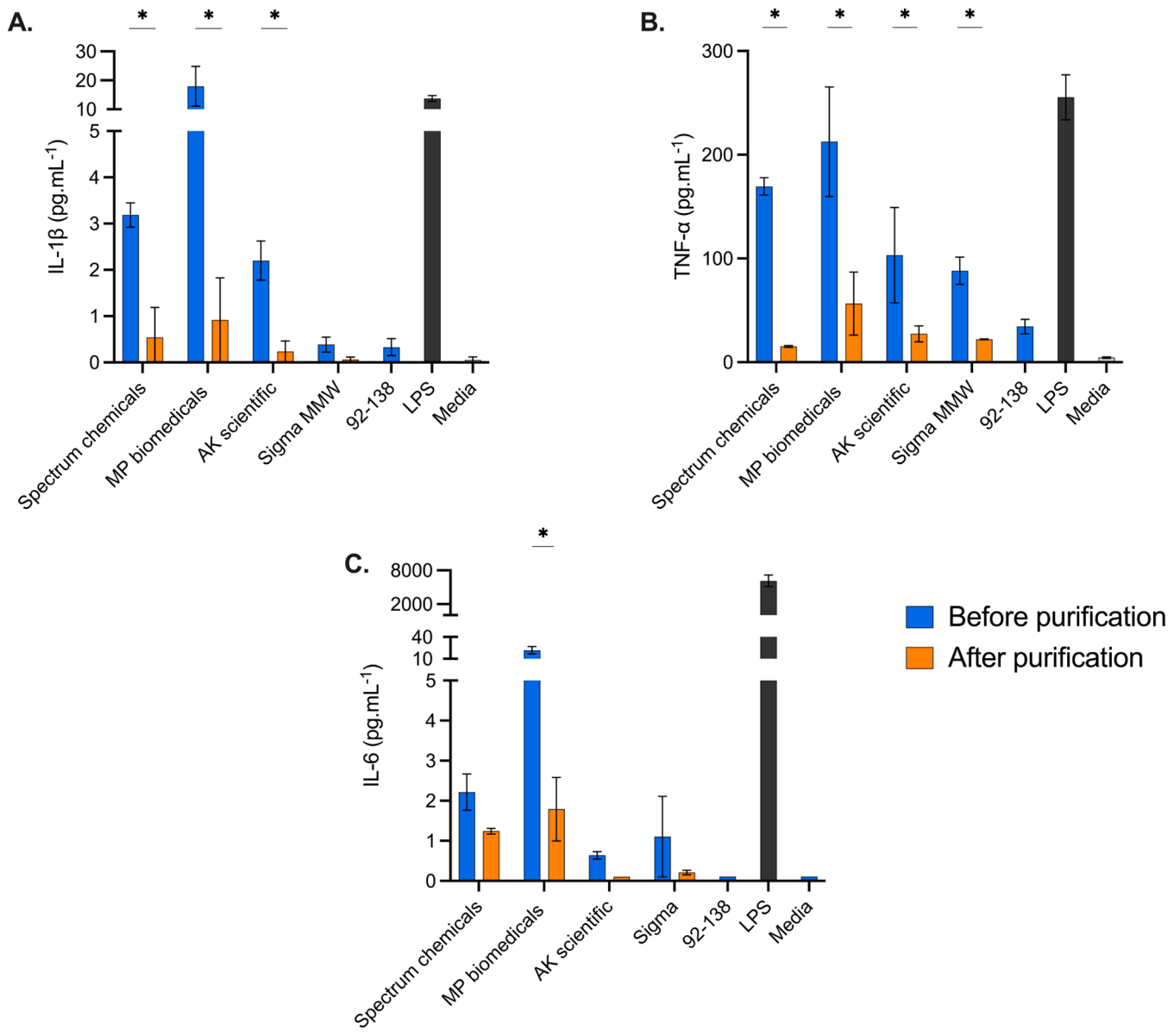

Fig. 6. Proinflammatory cytokine levels in J774A.1 murine macrophages stimulated with chitosan before and after purification. Macrophages were stimulated with 0.1 mg/mL chitosan acquired from various suppliers, 0.1 μg/mL lipopolysaccharide (LPS), or media alone (control). Secreted IL-1β is represented in (A), TNF-α in (B), and IL-6 in (C). The presented data represent mean ± SD from three independent experiments each performed with two technical replicates. *P*-values: * denotes $p < 0.05$.

2.5. Cell stimulation

Chitosans (Table I) were dissolved in hydrochloric acid (Cat# H9892, Sigma-Aldrich) at a concentration of 0.5 % w/v overnight on a rotary mixer, using a glucosamine:HCl ratio of 1:1. The resulting solutions were filter-sterilized through 0.2 μm filters (Millex™ SLGV004SL, Millipore), divided into small volumes in sterile microcentrifuge tubes (Cat# MTCC200, Sigma-Aldrich). Chitosan solutions were stored at 4 °C until use for in vitro immunoassays. Subsequently, the macrophages were subjected to a 24-hour stimulation period using either 0.1 mg/mL of each chitosan or 0.1 μg/mL of LPS isolated from *Salmonella enterica* (Sigma Aldrich, Cat# L6511). Following the stimulation period, the macrophage culture media was harvested, centrifuged at 500 g for 10 min, and then stored at −80 °C until further analysis.

2.6. Measurement of in vitro cytokine response

The levels of interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) in the J774A.1 macrophages cell culture supernatants were quantified using the Luminex™ 200 system (Luminex, Austin, TX, USA). Samples were analyzed at Eve Technologies (Calgary, Canada) using Mouse Cytokine Proinflammatory Focused 10-Plex Discovery Assay Array (Eve Technologies, MDF10: Immunology Multiplex Assay MD10 (Sigma, Cat# MCYTOMAG-70 K)). The measurement of these markers was performed in accordance with the manufacturer's protocol (MilliporeSigma, Burlington, Massachusetts, USA). Additionally, cells treated with media alone were included as negative controls.

2.7. Statistical analysis

Data are presented as mean ± SD. To compare either two or multiple groups, Student's *t*-test or one-way analysis of variance (ANOVA)

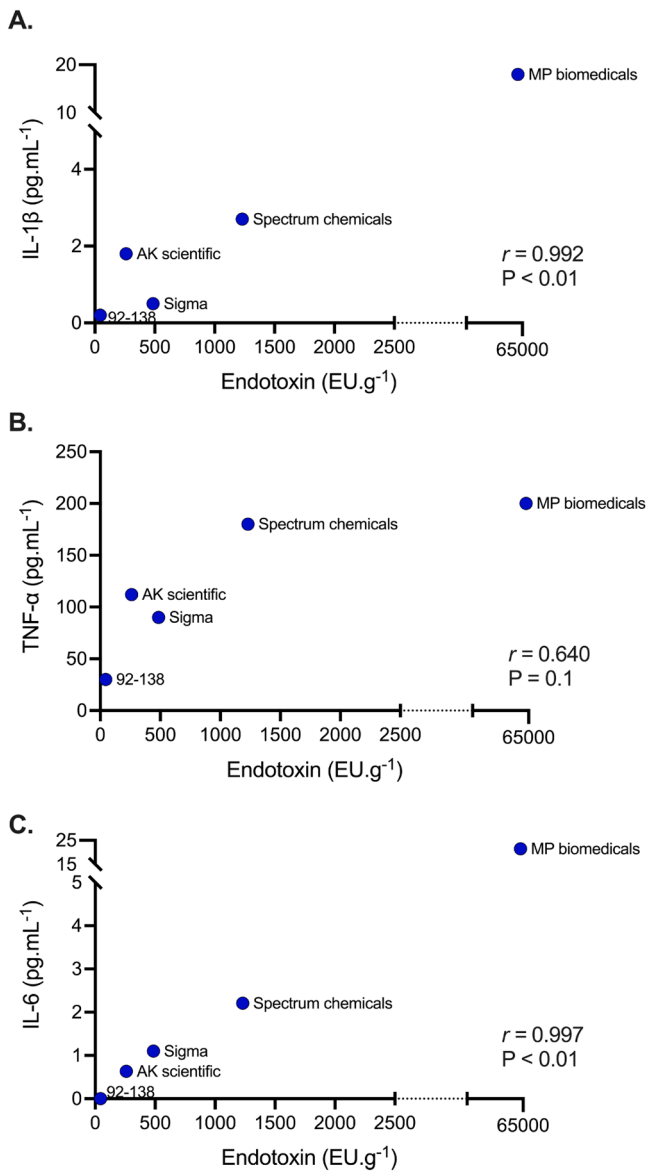

Fig. 7. Correlation between endotoxin levels in chitosan and proinflammatory cytokine secretion in J774A.1 macrophages. Scatter plot illustrating the correlation between endotoxin levels in non-purified chitosan and the secretion of (A) IL-1β, (B) TNF-α and (C) IL-6 after 24 h of in vitro stimulation of macrophages with 0.1 mg/mL chitosan from different suppliers. Pearson's *r* was used to determine correlation coefficients. α was set at 0.05.

followed by post hoc Tukey test, were utilized. $P < 0.05$ was considered statistically significant. For correlation of groups the Mann–Whitney test was applied. Pearson's *r* was used to determine correlation coefficients. α was set at 0.05.

3. Results

3.1. Characterization of commercial chitosan reveals discrepancies in supplier specifications and moderate to high endotoxin contamination

First, the DDA and molar mass distribution of chitosans obtained from various suppliers were assessed using nuclear magnetic resonance (NMR) spectroscopy and size exclusion chromatography multi-angle light scattering (SEC-MALS), respectively. Results are shown in Table I below.

Among the four sources tested, only one supplier provided a specific DDA value, while the remaining sources provided a range. Measured DDA values fell generally within the ranges or provided by the suppliers, with the following exception: The Sigma MMW chitosan had a DDA of 72.4 %, slightly below the supplier's specified minimum of 75 %. The Spectrum Chemicals chitosan exhibited a DDA of 91.6 %, deviating from the 99 % value indicated in its certificate of analysis. The DDA of the in-house produced chitosan was 92 %, aligning with the targeted value (Table I).

MW data were provided as a range by only one out of the four suppliers. SEC-MALS analysis determined the Mn of Sigma MMW chitosan to be 184 kDa, slightly below the specified 190–310 kDa range. The Mn of the in-house produced chitosan was 138 kDa. Polydispersity index (PDI) values were not reported by any manufacturer. Our measurements indicate that commercial chitosan samples exhibited a PDI ranging from 2.00 to 2.37, whereas in-house produced chitosan showed lower polydispersity, with a value of 1.30. MW data are presented in Table I.

Second, endotoxin levels in the commercial chitosans were evaluated using a chromogenic Limulus Amebocyte Lysate (LAL) assay (Table II). None of the four suppliers provided endotoxin quantification data. MP Biomedicals' chitosan exhibited the highest level of contamination (62,160 EU/g), while AK Scientific's chitosan had the lowest (259 EU/g). The in-house produced chitosan exhibited the lowest overall endotoxin level, at 20 EU/g.

3.2. Optimizing chitosan purification: 1 N NaOH for 48 h achieves optimal endotoxin reduction while preserving chitosan's physicochemical properties

We selected the most contaminated chitosan sample to test and optimize the purification process. Chitosan from MP Biomedicals was purified using three NaOH concentrations (0.3 N, 1 N, and 3 N) over two time periods (4 and 48 h). While all concentrations were effective in reducing endotoxin levels, the 1 N and 3 N NaOH for 48 h condition proved to be the most efficient, lowering endotoxin content from 62,160 EU/g to approximately 600 EU/g (Fig. 2).

To assess whether the endotoxin removal procedure affected the physicochemical properties of chitosan, the DDA and Mn were measured after purification and compared to their initial values (Fig. 3**A**). At 3 N NaOH for both 4 and 48 h, a moderate reduction in Mn was observed compared to the untreated chitosan. DDA remained stable across all conditions.

3.3. Optimized NaOH purification applied to all chitosans effectively removes endotoxins while preserving chitosans' structural integrity

Based on the findings above, the purification process was subsequently applied to all chitosan samples using 1 N NaOH for 48 h. Depyrogenated chitosans displayed a significant decrease in endotoxin content (Fig. 4). The most substantial reduction was observed for MP Biomedicals' and AK Scientific's chitosan, with a decrease of approximately 100-fold to 590 EU/g and 2.5 EU/g, respectively. Sigma MMW and Spectrum chitosans exhibited ~50-fold reductions (Fig. 4). These results show that the purification process was effective in significantly reducing endotoxin levels across all chitosan samples.

NMR analysis (Fig. 5**A**) showed no change in the DDA before and after the purification for any of the chitosans. Additionally, the SEC-MALS results revealed a slight reduction in the Mn (Fig. 5**B - left Y axis**) following purification, while the polydispersity index remained stable after purification (Fig. 5**B - right Y axis**).

3.4. Endotoxin contamination in chitosan increases proinflammatory cytokine release from macrophages in vitro

Following chitosan purification, we conducted a comparative in vitro study to evaluate the impact of endotoxin contamination on J774A.1 macrophage cytokine profiles, focusing on potential excessive

immunoactivation. Significant variations were observed in the secretion of pro-inflammatory cytokines interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) across chitosans from different suppliers (Fig. 6). Cell viability was not significantly influenced by any of the treatments (data not shown).

Chitosan from MP Biomedicals, which had the highest endotoxin levels before purification, induced the most pronounced IL-1β secretion (18 pg/mL); followed by chitosan from Spectrum Chemicals (3.2 pg/mL) (Fig. 6**A**). This observation aligns with a strong correlation between endotoxin content and IL-1β levels (Pearson's $r = 0.992$, $p < 0.01$) (Fig. 7**A**). After purification, IL-1β levels decreased significantly to 1 pg/mL for MP Biomedicals and to 0.1 pg/mL for Spectrum Chemicals.

A similar pattern was observed for TNF-α. MP Biomedicals' chitosan elicited the highest levels (210 pg/mL), followed by Spectrum Chemicals (170 pg/mL) (Fig. 6**B**). After purification, these concentrations decreased significantly to 56 pg/mL and 15 pg/mL, respectively. A moderate correlation was found between TNF-α levels and endotoxin content ($r = 0.64$, $p = 0.1$) (Fig. 7**B**).

The strongest correlation was observed for IL-6, a key cytokine induced by endotoxins. MP Biomedicals' chitosan again elicited the highest levels (22 pg/mL), followed by Spectrum Chemicals (2 pg/mL). Purification significantly reduced these concentrations to (2 pg/mL) and (1 pg/mL) for MP Biomedicals and Spectrum chitosans, respectively (Fig. 6**C**).

4. Discussion

The analysis of commercial chitosans highlighted the limited characterization typically provided by manufacturers. While some products reported DDA ranges, only one included an estimated MW. The measurements using NMR and SEC-MALS revealed occasional discrepancies, highlighting the variability and incomplete specifications often associated with commercial chitosans. These discrepancies may stem from differences in analytical methods, sample preparation, or assay limitations (Lavertu et al., 2003; Weißpflog et al., 2021).

In addition, their endotoxin content was assessed using the LAL assay. Varying levels of endotoxin contamination were detected, ranging from 259 EU/g to 62,000 EU/g, consistent with previous findings (Ravindranathan et al., 2016). We hypothesise that chitosan may become contaminated with LPS due to either 1) initial contamination from raw material (e.g., crustacean shells) (Prester et al., 2010) which may not be fully removed during production or purification processes that vary between manufacturers, or 2) contamination introduced during manufacturing if clean/aseptic conditions are not properly maintained (Health Canada, 2023).

Next, we developed an endotoxin removal treatment, leveraging NaOH's ability to hydrolyze ester and amide bonds in the lipid A component of LPS (Sandle, 2013). Various NaOH concentrations and incubation times were tested. Although all conditions reduced endotoxin content, the most effective were 1 N and 3 N NaOH for 48 h, without altering chitosan's DDA. However, exposure to 3 N NaOH led to a more pronounced decrease in chitosan's Mn, without further reduction of the endotoxin content. Therefore, treatment with 1 N NaOH for 48 h was identified as optimal, efficiently removing endotoxins while preserving chitosan's physicochemical integrity, with only a slight reduction of its molar mass.

Chitosan is generally stable in basic solutions. Its backbone, composed of glucosamine/N-actetyl glucosamine monomers, connected by ether (glycosidic) bonds, is resistant to cleavage under alkaline conditions unless subjected to extreme conditions, such as highly alkaline conditions and elevated temperatures for prolonged periods (Novikov et al., 2023). For instance, during alkaline deacetylation of chitosan, significant depolymerization can occur (e.g. around 80 % decrease in MW after deacetylation with 50 % NaOH solution at 145–150 °C) (Chebotok et al., 2006). Thus, the slight reduction in molar mass observed under the most aggressive conditions tested here is not unexpected.

Conversely, LPS are much more susceptible to degradation in alkaline environments and can be hydrolyzed by low to moderate NaOH concentrations even at room temperature (Caroff & Karibian, 2003; Lee Sang et al., 1999). Their lipid component (lipid A) contains multiple base labile ester and amide groups that are cleaved under these conditions (Lu et al., 2008; Sandle, 2013). The proposed purification method, which involved incubating chitosan powder with 1 N NaOH for 48 h, resulted in up to a 100-fold reduction in endotoxin contamination while preserving the structural properties of chitosan.

The in vitro study revealed that endotoxin contamination in commercially available chitosans influenced the cytokine profile of macrophages. We observed notable variations in the secretion of key pro-inflammatory cytokines including IL-1β, TNF-α, and IL-6 with strong correlations between endotoxin levels and the production of IL-1β and IL-6 and a moderate correlation for TNF-α. Importantly, all chitosans exhibited similar cytokine responses following purification, indicating that the structural properties of chitosan do not appear to influence the in vitro immune response in J774A.1 macrophages. The observed variations between chitosans are thus primarily attributed to contamination.

Taken together, these findings support the starting hypothesis suggesting a potential influence of endotoxin contamination on chitosan-induced immune response. These findings also provide valuable insights into the ongoing debate regarding whether chitosan's immunostimulatory effects are dependent on the TLR-4 signaling pathway (Carroll et al., 2016; Ghattas et al., 2025; Villiers et al., 2009; Zhang et al., 2014). We propose that discrepancies in the literature may stem from inadequate assessment of chitosan purity, particularly regarding endotoxin contamination, which could lead to skewed interpretations of data. Endotoxins, as potent TLR-4 agonists (Paramo et al., 2015), could be responsible for the immune activation observed in some studies, suggesting that the effects attributed to chitosan may, in fact, be influenced by these contaminants.

Finally, to highlight the importance of endotoxin level control and the relevance of the proposed purification method in enhancing safety, consider the following real-world clinical scenario. The U.S. Food and Drug Administration (FDA) sets the endotoxin limit for non-intrathecal, non-spinal parenteral drug administration at 5.0 EU per kilogram of body weight (U.S. Food & Drug Administration, 1985). In a clinical study for rotator cuff repair using chitosan (ClinicalTrials.gov ID: NCT05333211), the treatment involves a 100 mg dose of chitosan. If chitosan from MP Biomedicals were used as the source material in that clinical study, its initial endotoxin level of approximately 60,000 EU/g would result in an endotoxin content of approximately 6000 EU per dose. For a 70 kg individual, this corresponds to around 90 EU/kg, far exceeding the FDA's safety threshold. However, applying the purification method significantly reduced the endotoxin level to ~600 EU/g, without altering the chitosan's physicochemical properties. Under these conditions, the same 100 mg dose would result in an endotoxin exposure of approximately 1 EU/kg, which is well within the FDA's regulatory limit.

A potential limitation of the study is that, while the purification method was shown to be effective at small scale, its performance at larger scales has not yet been assessed. Further optimization may be necessary to ensure the same level of efficiency and consistency upon scale up. For large-scale applications, techniques such as tangential flow filtration (TFF) may be worth exploring, as they offer a more standardized alternative to multiple centrifugation steps, which are often labor-intensive and difficult to scale.

In summary, we found discrepancies between the measured and reported physicochemical properties of commercial chitosans and showed that they contain moderate to high levels of endotoxins. We also developed a simple and effective method for endotoxin removal that preserves chitosan's structural properties, offering a practical strategy to improve its safety profile for biomedical applications.

CRediT authorship contribution statement

Majed Ghattas: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Anik Chevrier:** Methodology, Writing – review & editing. **Dong Wang:** Methodology, Writing – review & editing. **Mohamad-Gabriel Alameh:** Supervision, Funding acquisition. **Marc Lavertu:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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