Assessment of Purification Methods for the Removal of Endotoxins from Polymers Generated by E. coli

Jason R. Pauldine

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Assessment of Purification Methods for the Removal of Endotoxins from Polymers Generated by E. coli

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Chemistry
With Honors

May 2016

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Date: 11/17/16
Abstract

In the medical field, specialty polymers have great potential to meet the demands for bioabsorbable materials as base materials for new technologies. Targeted drug delivery, scaffolds for stem cell growth and intraoperative hemostasis are just a few of the many potential applications for these polymers. Bacterially derived polymers, which are known to be biocompatible, often contain harmful endotoxins. Removal of these endotoxins is necessary to create a safe biocompatible product that meets the FDA requirements on endotoxin limits for implants. This study examines the effectiveness of methanol and sodium hydroxide purification of Clear Coli™ developed by Lucigen and on LSBJ and RSCO2 strains of E. coli. The two purification methods, a non-solvent precipitation and a 0.2 M sodium hydroxide treatment method, were evaluated for their efficacy in removing endotoxin. Poly(3-hydroxybutyrate) was produced in all three strains harboring the recombinant enzymes PhaC1(STQK), PhaA, and PhbB. Sodium hydroxide proved to be the most effective for method of removing endotoxins. After a single round of NaOH purification, 87% of endotoxins were removed from LSBJ and all detectable endotoxins were removed from RSCO2. It required three rounds of methanol purification to achieve a 77% reduction of endotoxin in LSBJ and removal of all detectable levels of endotoxin for RSCO2. Clear Coli was easiest to purify as one round of methanol purification removed 70% of LAL detected endotoxins from the polymer.
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Acknowledgements

I would like to thank Ryan Scheel for his tireless guidance and instruction, which made this project possible. I am especially grateful for his assistance with performing the LAL endpoint chromogenic assays. I would also like to thank Ata Pinto for being a resource to help troubleshoot cell culture issues. Finally, a sincere thanks you to Dr. Christopher Nomura for his guidance, and to Dr. William Shields and the Honors Program at ESF for making this project possible through funding.
Introduction

Specialty polymers are increasingly being utilized as biomedical materials for a wide variety of medical products. Some of these applications include targeted drug delivery, bioabsorbable scaffolds for stem cell growth of organs, and intraoperative hemostasis of osseous bleeding (Malafaya, Silva & Reis, 2007; Tiwari, Panthari, Katare & Kharkwal, 2014). Due to the incredible variety of applications for biopolymers, the physical properties must also be able to meet a variety of different requirements (Ige, Umoru & Aribio, 2012). In general these polymers must at a minimum be bioabsorbable and biocompatible. Many naturally derived polymers are known to be biocompatible, however these materials can contain elements that produce an unfavorable immune response, thereby limiting the amount of material that can be used in a medical setting.

Polyhydroxyalkanoates (PHAs) are polyesters that are generated from native and recombinant organisms (Lu, Tappe!, and Nomura, 2009). Non-native producers such as Escherichia coli are frequently chosen to produce such materials because of the relative ease with which this microorganism can be manipulated (Fidler, 1992). E. coli is a gram negative bacteria with an outer membrane that contains lipopolysaccharides (LPS). This LPS consists of three components; lipid A, a core oligosaccharide, and an O-antigen polysaccharide (Wu et al, 2014). Lipid A, the hydrophobic anchor of lipopolysaccharide (LPS), consists of six acyl chains that are responsible for eliciting a powerful immune response in humans (Raetz & Whitfield, 2002). Human response to endotoxins includes fever, disseminated intra-vascular coagulation, tissue necrosis, and septic shock. Human endotoxin studies have
shown that humoral response to endotoxins are approximately 250 times greater in humans than mice (Morrison & Ryan, 1987). An intravascular dose as little as 2-4 ng/ml was found to be sufficient enough to elicit a systemic inflammatory response in one human study. (Calvano & Coyle, 2012). The FDA has set upper limits in respect to endotoxin levels. For medical devices, the limit is 0.5 EU/ml or 20 EU per device for products that directly or indirectly contact the cardiovascular or lymphatic system (U.S. Department of Health & Human Services Food and Drug Administration, 2012).

Removal of endotoxins from polymers is not a novel idea. Considerable research has been performed to assess the most efficient way of removing endotoxins from polymers that, at the same time, does not significantly diminish yield, reduce purity, or compromise PHA integrity. Current methods for endotoxin removal from polymers include temperature-controlled extraction and precipitation, purification using activated charcoal, and sodium hydroxide digest (Furrer, Panke & Zinn, 2007; Wampfler et al, 2010; Lee et al, 1999).

The first goal of this study was to determine the endotoxin levels of crude PHAs procured from E. coli strains RSCO2 and LSBJ. The second goal of this study was to test the hypothesis that both methanol purification and sodium hydroxide purification are effective means of removing residual endotoxin from E. coli strains RSCO2 and LSBJ. The third goal of this study was to assess the endotoxin level of non-purified PHAs produced from Clear Coli™, transformed with the same plasmid as the aforementioned strains.
Materials and Methods

Two separate experiments were performed to assess distinct aspects of endotoxin levels. For Experiment 1, all reagents were made using endotoxin free water and in glassware that was endotoxin free. This was performed to assess relative endotoxin levels of bacterial strains post growth and to evaluate endotoxin levels after a single methanol purification. For Experiment 2, no endotoxin precautions were made with the exception of the materials and glassware associated with the steps directly prior to endotoxin assays. Water used for growth cultures in the production of PHAs was ultra-filtered water (NANOpure), which was also tested for residual endotoxins in this study. This second experiment was performed to simply evaluate the change in endotoxin levels after being subjected to repeated methanol purification as well as a sodium hydroxide purification. Three bacterial strains were chosen as the designated gram-negative species to be studied and are listed in Table 1. In all strains the plasmid pBBR-STQKAB was used to transform the bacteria (Table 1). Kanamycin was added at a concentration of 50 mg L\textsuperscript{-1} for plasmid selection and retention whenever appropriate.

Preparation of glassware

All glassware used in endotoxin free production of PHA's and in the preparation of the endotoxin assays were either endotoxin free from the manufacturer or made endotoxin free through heat destruction. Heat destruction of endotoxins was accomplished by baking glassware at 240°C for 1 hour. In order to allocate samples for testing, portions of the polymers were cut using surgical grade
Mayo scissors and surgical grade Kelly clamp hemostats. These instruments were flame sterilized by propane torch until red-hot and immediately cooled using endotoxin free water and allowed to dry. This practice was used to expedite the sampling process, and was performed for every individual sample prepared for endotoxin testing.

**PHA production in Clear Coli™**

Clear Coli™ purchased from Lucigen was used as a control strain for endotoxin. This genetically modified *E. coli* strain has a deletion of two of the six acyl chains in lipid IV<sub>A</sub> and is reported to not induce an endotoxin response in mammals. Electrocompetent Clear Coli™ cells were transformed with 3 µl of pBBRSTQKAB using electroporation (1500V, 5ms; BTX ECM 399). Transformed cells were then used to inoculate 500 µl of LB and recovered at 37°C for one hour with 200 rpm rotary shaking. A 250 µl aliquot of recovered cells were then plated onto endotoxin free LB Miller plates (10 g L<sup>-1</sup> tryptone, 5 g L<sup>-1</sup> yeast extract, 10 g L<sup>-1</sup> sodium chloride, 15 g L<sup>-1</sup> agar) with kanamycin (50 mg L<sup>-1</sup>) and grown for 24 hours at 37°C. Three colonies were chosen and used to inoculate a growth medium of 50ml LB Miller with kanamycin. This seed culture was then grown for 36 hours at 37°C incubation with 200rpm rotary shaking. A 2 ml aliquot of seed culture was used to inoculate a growth medium of 100 ml LB Miller with kanamycin in baffled shake flasks. Cell cultures were grown for 96 hours at 30°C with 250rpm rotary shaking.
PHA production in LSBJ and RSC02

Chemically competent LSBJ and RSC02 *E. Coli* cells were transformed with 3 µl of plasmid using a 1 minute of 42°C heat shock following standard procedures (Sambrook & Russell, 2001). Transformants were cooled for 2 minutes on ice and then used to inoculate 500 µl of LB and allowed to recover at 37°C for one hour with 200 rpm rotary shaking. A 250 µl aliquot of recovered cells were then plated onto endotoxin free LB Miller plates with kanamycin and grown for 16 hours at 37°C. Three colonies were chosen and used to inoculate a growth medium of 50 ml LB Miller with kanamycin. This seed culture was then grown for 16 hours at 37°C incubation with 200 rpm rotary shaking. A 2ml aliquot of seed culture was used to inoculate a growth medium of 100 ml LB Miller with kanamycin in baffled shake flasks. Cell cultures were grown for 48 hours at 30°C with 250 rpm rotary shaking.

Harvest of cells

Following the growth period of all strains, cells were harvested in preparation for polymer extraction. Combined cell culture was divided into two separate 400ml containers and centrifuged at 4000g for 45 minutes at room temperature to pellet the cells. Supernatant was poured off and the cells washed with 40ml water of approximately 35% ethanol mix with vortexing. Once cells were fully reconstituted, they were distributed equally among four 50 ml Falcon tubes and were pelleted again for 30 minutes with the same centrifuge parameters as above and then supernatant decanted. Cells were then washed with 20ml water with vortexing, and pelleted again for 20 minutes. The supernatant was decanted,
cells reconstituted with 4 ml of water, and then stored at -80°C. After the freezing overnight, the cells were lyophilized for a minimum of 24 hours in preparation for polymer extraction.

**Extraction and purification of PHAs**

Freeze dried cells were extracted by soxhlet using chloroform for a minimum of 5 hours. Following extraction, chloroform was concentrated using rotary evaporation until a solid polymer film was visualized. Polymer was then placed on a high vacuum unit for a minimum of 2 hours to remove any residual chloroform. A crude sample was then aseptically excised and set aside using endotoxin-free tools.

Once a crude sample had been set aside, remaining polymer was brought back into solution with 20 ml of chloroform with heating (55°C). The solution was then cooled and added dropwise by pipette into 150 ml of ice cold methanol solution (~4°C) with moderate stirring. The precipitated sample was then transferred to a scintillation vial and placed under high vacuum to remove all chloroform. A small portion was aseptically excised and retained as the first methanol purification sample. The remaining polymer was then subjected to two additional rounds of methanol purification as described above.

**Sodium hydroxide purification of PHAs**

To perform the sodium hydroxide digest at the conclusion of the final centrifuge step during cell harvest, the supernatant was decanted, and the pellet was then instead reconstituted with 20 ml of 0.2 M NaOH with vortexing. Centrifuge
tubes (50ml) were then placed on their side and incubated at 37°C with 100 rpm transverse shaking for 5 hours. The solution in each tube was then neutralized with 1 M HCl and centrifuged at 2500g for 20 minutes. Supernatant was decanted and the cells reconstituted with 4ml of endotoxin free water, then placed in a -80°C freezer for storage. After the freezing overnight, cells were placed on a lyophilizer for a minimum of 24 hours in preparation for polymer extraction.

**Endotoxin assay**

Assessment of endotoxin levels in polymer samples was accomplished using the Pierce™ Limulus Amebocyte Lysate Chromogenic Endotoxin Quantitation Kit. Polymer samples were prepared for testing following a previously described protocol, with adaptation (Furrer, Panke, & Zinn, 2007). Briefly, polymer samples were prepared by aseptically transferring approximately 200 mg of weighed sample into endotoxin free glass vials. A 4ml aliquot of endotoxin free water was added to samples. Sample vials were then incubated at 37°C with 200 rpm rotary shaking for 24 hours. For samples that were tested for endotoxin leeching over time, water within the sample vials was changed at the 24 hour mark and placed back into the rotary incubator for an additional 24 hours. Both 24 hour and 48 hour water samples were tested for endotoxin levels. Endotoxin assays were carried out following the manufacturer's recommendations. The spectrometer used for this study was a Biotek Synergy H4 microplate reader.
Results

Endotoxin levels in strains experiment 1

The Clear Coli™ strain showed the highest endotoxin value by LAL assay (Table 2, Figure 1). Reduction of endotoxin values was also the greatest for this strain post methanol purification. The strain RSCO2 showed 22% more endotoxin than did LSBJ, however it showed only a 41% reduction after a single round of methanol purification compared to a 50% endotoxin reduction for LSBJ.

Endotoxin reduction in strains experiment 2

Similar to experiment 1, one round of methanol purification removed 5% more endotoxin from LSBJ than RSCO2 (Table 3, Figure 2). A second round of methanol purification removed an even greater degree of endotoxin for LSBJ relative to RSCO2 on the order of 19%. Subsequent methanol purification however showed a greater degree of endotoxin removal for RSCO2 than LSBJ. A third purification removed all detectable levels of endotoxin in RSCO2, whereas the LSBJ strain endotoxin levels were reduced by only ~78% overall. The final purification step was largely ineffective at removing endotoxins from LSBJ with a reduction percentage of only 3.6%. The sodium hydroxide purification of RSCO2 reduced the endotoxin to undetectable levels by assay after one treatment (Table 4). The endotoxin was reduced by ~87% for the LSBJ strain.
Endotoxin leech over time

The water sample taken at the 48-hour mark for Clear Coli™ was shown to contain roughly 83% less endotoxin than the 24-hour mark (Table 5). The water samples for both the 24-hour and 48-hour marks for the RSC02 strain was shown to be identical by assay.

Discussion

The high endotoxin values for the crude Clear Coli™ showed that the LAL assay was unable to distinguish between the 4-acyl chain variant and the standard 6-acyl chain endotoxins. According to Lucigen, a false-positive endotoxin value is produced from the interaction of the 4'-monophosphoryldiglucosamine backbone of LPS with the LAL reagent. Standard practice to reduce false positives of this strain is to run samples through a nickel column purification step before LAL testing. Unfortunately, this practice cannot be applied as easily to polymers as it can with general recombinant proteins. Methanol purification was significantly more effective at removing LPS from the Clear Coli™ strain than either LSBJ or RSC02. This suggests that the genetically modified LPS molecules are much more loosely attached to the polymer than the unaltered LPS of LSBJ and RSC02.

Values for the crude samples for Experiment 2 were reported as “overflow” because the concentration of endotoxin was beyond the upper limit of the spectrometer unit’s ability to report them. A likely cause was that the additional endotoxin introduced as a result of not using endotoxin free water was enough to bring the concentration above the maximum threshold. Taking this into
consideration, endotoxin reduction values for Tables 3 and 4 were calculated using the least possible crude concentration. This represents the minimum endotoxin concentration at which the spectrometer reports an overflow value.

A single round of methanol purification produced similar endotoxin reduction percentages in both experiment 1 and 2, with LSBJ averaging more endotoxin reduction than RSC02. A subsequent methanol purification for both strains yielded an endotoxin reduction percentage slightly higher than the first purification. However, the strain RSC02 displayed the greatest reduction upon the third methanol purification, whereas the LSBJ strain was largely unaffected by the final purification. This resulted in virtually all of the endotoxin having been removed from RSC02 while approximately 12% of the original endotoxins remained for the strain LSBJ. A possible explanation for the large discrepancy in the effectiveness of the final methanol purification between the two strains may very well be attributed to the technique itself. It was critical during the purification that the dissolved polymer be added slowly and dropwise into stirring methanol. Any addition of polymer that was added faster than a dropwise rate could very well have escaped the full effectiveness of the purification. Nevertheless, it was apparent that a minimum of three rounds of purification was necessary to achieve elimination of nearly all endotoxin.

Sodium hydroxide purification proved to be the most effective way to remove endotoxins from both strains. After a single purification step, virtually all detectable levels of endotoxin was removed from the RSC02 strain, while LSBJ attained greater than a 87% reduction in endotoxin levels.
Assessment of endotoxin levels after a water exchange for Clear Coli™ showed a significant 83% concentration reduction, while the RSC02 levels remained identical. It is likely that the altered endotoxin in Clear Coli™ is not strongly retained in the polymer, and can be removed by water rinses. Endotoxins appear to remain strongly attached to the polymer produced by the RSC02 strain. The fact that the endotoxin levels remained identical after a water exchange suggests that there is an endotoxin equilibrium that is attained with incubation over a 24 hour period. Subsequent water exchanges would have little effect on endotoxin levels for the RSC02 strain.

**Conclusion**

Sodium hydroxide purification was found to be far more effective at removing endotoxins from RSC02 and LSBJ than methanol purification. A single round of sodium hydroxide was, at very minimum, equivalent to three rounds of methanol purification. Clear Coli was able to be further purified with a water rinse, whereas the RSC02 strain showed no endotoxin changes after a water exchange. This suggests that the normal 6-acyl chain LPS of gram-negative bacteria is strongly attached to the polymer produced by them. The altered 4-acyl chain lipopolysaccharides produced by Clear Coli were significantly reduced by the water rinse, suggesting that the altered LPS is only loosely attached to the polymer. Interestingly, a single water rinse was shown to remove more endotoxin from the polymer produced by Clear Coli than a round of methanol purification. Further studies need to be performed to assess whether water alone can purify Clear Coli.
produced polymer below the detection limit for the LAL endotoxin assay. Further studies also need to be performed to assess whether or not the sodium hydroxide caused any molecular changes to the polymer. While it is possible to either significantly reduce or remove all detectable endotoxin from LSBJ and RSCO2 strains using methanol purification, this study showed that sodium hydroxide is a far more efficient procedure that both reduces cost and waste material.
References


### Tables

**Table 1: Strains and Plasmid**

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear Coli™</td>
<td>ΔgutQ ΔkdsD ΔlpxL ΔlpxM ΔpagP ΔlpxP ΔeptA msbA148</td>
<td>1</td>
</tr>
<tr>
<td><em>E. coli</em> LSBJ</td>
<td><em>E. coli</em> LS5218 fadB::Cm, ΔfadJ</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em> RSC02</td>
<td><em>E. coli</em> LSBJ ΔarcA</td>
<td>3</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBBRSTQKAB</td>
<td>pBBR1MCS-2 derivative, phaC1 (STQK), phbAB</td>
<td>4</td>
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</table>

All strains and plasmid used during this study.


**Table 2: Endotoxin Levels of Strains**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Crude EU/mg</th>
<th>Methanol Purified EU/mg</th>
<th>Percent Change</th>
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<tbody>
<tr>
<td>Clear Coli™</td>
<td>0.160</td>
<td>0.049</td>
<td>69.5%</td>
</tr>
<tr>
<td>RSC02</td>
<td>0.136</td>
<td>0.080</td>
<td>41.2%</td>
</tr>
<tr>
<td>LSBJ</td>
<td>0.106</td>
<td>0.053</td>
<td>49.8%</td>
</tr>
</tbody>
</table>

Values were calculated as the average of quadruplicate experiments.
Table 3: Percent Reduction of Endotoxin From Methanol Purification

<table>
<thead>
<tr>
<th>Strains</th>
<th>First Purification</th>
<th>Second Purification</th>
<th>Third Purification</th>
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<tr>
<td>RSCO2</td>
<td>&gt;37.4%</td>
<td>40.9%</td>
<td>99.9%</td>
</tr>
<tr>
<td>LSBj</td>
<td>&gt;42.3%</td>
<td>59.8%</td>
<td>3.57%</td>
</tr>
</tbody>
</table>

Cumulative endotoxin level reduction percentage

<table>
<thead>
<tr>
<th>Strains</th>
<th>First Purification</th>
<th>Second Purification</th>
<th>Third Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSCO2</td>
<td>&gt;37.3%</td>
<td>&gt;62.9%</td>
<td>&gt;99.9%</td>
</tr>
<tr>
<td>LSBj</td>
<td>&gt;42.3%</td>
<td>&gt;76.8%</td>
<td>&gt;77.7%</td>
</tr>
</tbody>
</table>

Percent reduction was calculated using endotoxin values corresponding to the maximum detection limit of the spectrometer. Values were calculated as the average of quadruplicate experiments.

Table 4: Percent Reduction of Endotoxin From Sodium Hydroxide Purification

<table>
<thead>
<tr>
<th>Strains</th>
<th>Crude EU/mg</th>
<th>Purified EU/mg</th>
<th>Percent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSCO2</td>
<td>&gt;0.021</td>
<td>Below detection limit</td>
<td>&gt;99.9%</td>
</tr>
<tr>
<td>LSBj</td>
<td>&gt;0.021</td>
<td>0.0028</td>
<td>&gt;87.1%</td>
</tr>
</tbody>
</table>

Percent reduction was calculated using endotoxin values corresponding to the maximum detection limit of the spectrometer. Values were calculated as the average of quadruplicate experiments.

Table 5: Endotoxin Values Post Water Exchange

<table>
<thead>
<tr>
<th>Strains</th>
<th>24 Hour Endotoxin Value EU/mg</th>
<th>48 Hour Endotoxin Value EU/mg</th>
<th>Percent Change</th>
</tr>
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<tbody>
<tr>
<td>Clear Coli™</td>
<td>0.0139</td>
<td>0.0024</td>
<td>82.6%</td>
</tr>
<tr>
<td>RSCO2</td>
<td>0.0135</td>
<td>0.0135</td>
<td>0%</td>
</tr>
</tbody>
</table>

Values were calculated as the average of quadruplicate experiments.
Table 6: Endotoxin Values of Water Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Endotoxin EU/ml</th>
</tr>
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<tbody>
<tr>
<td>Endotoxin Free Water</td>
<td>0</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0</td>
</tr>
<tr>
<td>Nanopure Water</td>
<td>0.695</td>
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</tbody>
</table>

Values were calculated as the average of quadruplicate experiments.

Figures

Figure 1: Endotoxin Levels of PHBs Pre and Post Methanol Purification. Clear Coli showed the greatest percent reduction of endotoxin at 70%, while LSBJ endotoxin percent reduction was 41% and RSCO2 reduction was shown as 50% after one round of methanol purification. RSCO2 retained the greatest amount of endotoxin post methanol purification. Values were calculated as the average of quadruplicate experiments.
Figure 2: Endotoxin Values of methanol purified RSC02 and LSBJ at each purification step are shown above. The RSC02 strain showed no detectable endotoxin after the third purification while the LSBJ strain showed no further endotoxin removal after the second methanol purification step. Values were calculated as the average of quadruplicate experiments.