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Removal of Endotoxins and Nucleic Acids Using Submicron-sized Polymeric Particles

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Submicron-sized polymeric particles (SSPP) were used to remove nucleic acids and endotoxins from cell lysates. The positively charged SSPP selectively adsorb nucleic acids and endotoxins and form complexes with them. The complexes can be easily removed by sedimentation or centrifugation. The removal of nucleic acids and endotoxins using SSPP also can be accomplished in the presence of cell and cell debris. Therefore, nucleic acids and endotoxins can be removed in an initial step of the down-stream processes. In bakers yeast and *E. coli* lysate systems, the level of DNA could be reduced more than three orders of magnitudes and endotoxins more than seven orders of magnitudes concurrently with the cell debris removal process using SSPP.

Endotoxins are pyrogenic, producing fever in man or other mammals. It is essential that the endotoxins should be removed for the safe parenteral administration of products produced by natural sources or by recombinant DNA technology. The sensitivity of mammals to endotoxins is extraordinary (27). Contamination levels of less than 1 ng/ml elicit a strong fever response and can even result in death. Therefore, manufacturers of pharmacologicals and therapeutics face rigorous government mandates to assure endotoxin-free products. As biotechnology industries approach the mass production phase of biologicals for internal use, the elimination of endotoxins from sensitive biologicals becomes a formidable problem. This is of particular concern to those companies contemplating the use of cloned, gram-negative bacteria as vectors for the production of their biologicals.

Although extended high temperatures (250°C for an hour) or strong acid and/or alkali treatments can be used to inactivate endotoxins, these conditions are almost always prohibitively harsh for physiologically important biologicals. Conceptually, however, it is possible to reduce or eliminate endotoxins under more moderate, physiological conditions (pH, temperature, salt ect.) by exploiting the physical properties of endotoxins. These include ultrafiltration (7, 22), adsorption to activated charcoal (20) or Limulus amebocyte lysate (LAL) (5, 9), ion-exchange chromatography (25, 26), and affinity chromatography (8, 10, 18). All of these methods have been reported to be effective in endotoxin reduction; however, not all of the procedures are highly reproducible and may be accompanied by significant loss of the product being purified. In addition, the adsorption capacity of adsorbents is generally low.

In the production of biological products, especially pharmaceuticals, diagnostic enzymes, or polysaccharides, the removal of nucleic acids is also a serious problem (8, 28). Nucleic acids increase the viscosity of cell lysates and extracts, thus interfering with down-stream processes. Furthermore, some of the physical properties of nucleic acids are similar to those of proteins and polysaccharides, thus posing difficulties in separation. The clogging of chromatographic columns and impeding of centrifugation or membrane processes are some well known general problems caused by the presence of nucleic acids in cell lysates or extracts (11, 19, 21). This effect is amplified when scaled-up and leads to a reduced capacity for adsorbing the product and to a shorter column lifetime.

The polycationic polyethyleneimine has been used to remove nucleic acids from cell lysates or extracts (1, 2). Although effective nucleic acid removal can be attained using polyethyleneimine, one severe limitation on the use of polyethyleneimine is the fact that the residual monomer is a carcinogen (19, 23). Therefore, an effective removal process which meets all government-

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mandated requirements is sought.

Submicron-sized polymeric particles (SSPP, diameter 0.01-1 μm) have been used to facilitate separation processes for biological products (12, 15), removal of cell and cell debris (12, 16), liquid-liquid extraction (12, 15), and filtration (6, 12). The advantages in using SSPP are (1) the large surface area available for interaction or adsorption, (2) high colloidal stability to allow sufficient time for interaction, and (3) rapid adsorption equilibrium. In addition, the unit operations involved are simple and easy for scaling-up and continuous process. In this article we discuss current developments in the removal of nucleic acids and endotoxins using SSPP.

MATERIALS AND METHODS

Submicron-sized Polymeric Particles and Water Soluble Polymers

Acrylic SSPP with quaternary amino groups or tertiary amino groups (provided by Rohm and Haas, Spring House, PA, USA) were used for this study. The mean diameter of quaternary amino SSPP (SSPP I) was 0.1 μm and its charge density was 270 $\mu C/cm^2$. The mean diameter of tertiary amino SSPP (SSPP II) was 0.08 μm and its charge density was 340 $\mu C/cm^2$.

The methacrylic SSPP and water soluble polymers with quatenary amino groups to which one to eight carbon tailes are attached (provided by Rohm and Haas Co., Spring House, PA, USA) were used to study the effect of the carbon chain length on endotoxin binding.

Endotoxin Assay

One hundred μl of calf thymus DNA (50 μg, Sigma Chem. Co., St. Louis, MO, USA) was added to increasing concentrations of SSPP, vortexed, observed for visible precipitate, centrifuged at 15,000×g for 2 minutes and 100 μl of the supernatants were assayed for endotoxins by the Limulus amebocyte lysate (LAL) test (14).

The lyophilized lysate (Pyrotell, Associate of Cape Cod, Inc., Woods Hole, MA, USA) in a glass vial was reconstituted using 5 ml pyrogen-free water (Associates of Cape Cod, Inc., Woods Hole, MA, USA). Usually, all of the reconstituted lysate was consumed during a single experiment. Unused portions of the reconstituted lysate were frozen at -20°C. Frozen lysate was thawed and used in the next experiment, and any remaining at that time was discarded.

One hundred μl of the supernatants (or pyrogen-free saline or 0.03 EU control standard endotoxin/ml) was added into the sterilized pyrogen-free test tubes (12×75 mm, Elkay Product, Inc., Shrewsbury, MA, USA) containing 100 μl of reconstituted lysate (total volume=200 μl). The vortex-mixed suspensions were incubated for 60 minutes at 37°C. A solid clot (+) was arbitrarily assigned a value of 1.0, a semi-solid (i.e., loose, easily broken) clot (±) was assigned a value of 0.5 and no clot (-) was assigned a value of 0.0. The endotoxin test score is the sum of the values in the duplicate set.

To study the removal of endotoxin from the cell lysate, *E. coli* lysate was diluted 40 times with a sodium acetate buffer (35 mM, 1 mM EDTA, pH 5.0). To 1.5 ml of the diluted lysate 0.5 ml of various concentrations of SSPP or polymers were added. These samples were vortexed to mix then centrifuged at 1,000 rpm for 7 minutes. The addition of SSPP or water soluble polymers facilitated the sedimentation of *E. coli* cell debris leaving clear supernatants (12, 16). The endotoxin level and DNA concentration in the supernatant were determined.

The LAL test used in this study has been accepted by the U.S. Pharmacopeia Convention, Inc., and the Food & Drug Administration as a valid biological assay method to determine the presence and activity of endotoxins in parental drug preparation and their delivery systems (24). The procedure of the LAL test is described elsewhere (17, 24).

Residual DNA Test

The *E. coli* cell lysate was prepared as described in the endotoxin assay. In order to detect the concentration of DNA remaining in the cell extract after SSPP treatment, the radioactive *E. coli* DNA [thymidine-methyl-^{14}C] (2 μCi/ml; 12 μCi/mg) (New England Nuclear Corp., Boston, MA, USA) was added to the *E. coli* lysate prior to the addition of SSPP. The radioactivity remaining in the supernatant was measured after centrifugation.

Bakers yeast was lysed by the addition of 0.12 M NH_4OH containing 2 mM EDTA while stirring at room temperature. Lysis was completed in 5 h (pH 8.1). To 10.5 ml of the unfractionated lysate 70 μl of *E. coli* DNA [thymidine-mehtyl-^{14}C] (2 μCi/mL; 12 μCi/mg) was added and thoroughly mixed. The DNA that was added to the yeast lysate (12 μl; 0.14 μCi) was at a final concentration of 0.001 μl/ml (0.013 μCi/ml). This provided approximately 30,000 cpm/ml in a scintillation system that was approximately 90% efficient. Under these conditions, the weight contribution of *E. coli* DNA to yeast DNA in the crude lysate was negligible. One and a half ml of the unfractionated lysate containing tracer amounts of radioactive DNA was taken. SSPP I and acetate buffer were added to achieve a final volume of 2 ml such that the SSPP varied from 0 to 10 mg/ml in a constant amount of lysate (and therefore, DNA). Six duplicates were thoroughly mixed and centrifuged at 15,000×g for 2 minutes at room temperature. Aliquots (250 μl) of the the supernatant were mixed with 250 μl acetate buffer and 10 ml Aquasol scintillation cocktail.

RESULTS AND DISCUSSION

Removal of Endotoxins

Table 1 shows the removal of endotoxins from calf thymus DNA using a positively charged SSPP (C8QUAT,

Table 1. Removal of Endotoxins from Calf Thymus DNA.

SSPP added (μg)	Visible ppt (+ or -)	Endotoxin Test[1] (-)	(±)	(+)	Endotoxin Test Score[2]
0	-			xx	2
50	+			xx	2
100	+			xx	2
200	+			xx	2
300	+	xx			0
400	+	xx			0

[1] A solid clot (+) was arbitrarily assigned a value of 1.0, a semi-solid (i.e., loose, easily broken) clot (±) was assigned a value of 0.5 and no clot (-) was assigned a value of 0.0.
[2] The endotoxin tet score is the sum of the values in the duplicate test.

methacrylated resin with quaternary amino groups to which eight carbon tails are attached). The endotoxin level of calf thymus DNA solution was 6 endotoxin unit, (EU/ml DNA solution). When more than 300 μg SSPP was added into the DNA solution, the endotoxins were not detectable in the supernatant with the LAL test. Therefore, the endotoxin level was reduced to a level lower than the detection limit of the LAL assay, 0.03 EU/ml. This suggests that SSPP can be used to remove endotoxins.

Endotoxin removal from a complex system, *E. coli* lysate, was conducted using positively charged SSPP or water soluble polymers. The endotoxin level of the *E. coli* lysate diluted 40 times was higher than 3×10^5 EU/ml. With the addition of positively charged SSPP or water soluble polymers, the endotoxin level of the supernatant could be reduced to lower than 0.03 EU/ml (Table 2). This demonstrates that the addition of SSPP or water soluble polymers reduces the endotoxin level greater than 7 orders of magnitudes (from 3×10^5 EU/ml to below 0.03 EU/ml).

When more than 0.4 mg/ml of water soluble polymers was added, the endotoxin test results were all positive except the tests with 3 carbon chain water soluble polymer. This may be due to the stabilization of the water soluble polymer with overdosing (13, 14) or the fault positive nature with the interaction of the water soluble polymer of the Limulus amebocyte lysate. It is, however, not clear why the 3 carbon chain water soluble polymer shows negative test results.

As a functional group on SSPP, the tertiary amine was as efficient as the quaternary amine. The endotoxin level in the supernatant was also below 0.03 EU/ml when 0.3 mg/ml to 0.5 mg/ml of tertiary amino SSPP was added (Table 2). The effective concentration range of the tertiary amino SSPP, however, was narrower than that of the quaternary amino SSPP.

The length of the carbon chain as a linkage between the functional group and the backbone of SSPP or water soluble polymers did not affect the efficiency of the endotoxin removal as shown in Table 2. The same concentration of SSPP or water soluble polymers (0.2 mg/ml) was required to reduce the endotoxin level below 0.03 EU/ml within the length of carbon chains of 1 to 8 carbons.

Table 2. Removal of Endotoxins from *E. coli* Lysate.

Length of carbon chain attacted to; Type of SSPP or Polymer	Endotoxin Test Concentration of SSPP or Polymer (mg/ml) 0.05	0.1	0.2	0.3	0.4	0.5	0.75	1.0
Quaternary Amino								
SSPP	+	+	-	-	-	-	-	-
1 Polymer	+	+	-	-	+	+	+	+
SSPP	+	+	-	-	-	-	-	-
3 Polymer	+	+	-	-	-	-	-	-
SSPP	+	+	-	-	-	-	-	-
4 Polymer	+	+	-	-	+	+	+	+
SSPP	+	+	-	-	-	-	-	-
8 Polymer	+	+	-	-	+	+	+	+
Tertiary Amine								
1 SSPP	+	+	+	-	+	-	+	+

The original concentration of endotoxin was higher than 3×10^5 EU/ml.
+, indicates that the endotoxin level in the supernatant was higher than 0.03 EU/ml; -, indicates that the endotoxin level was lower than 0.03 EU/ml.

Therefore, the positively charged SSPP and water soluble polymers adsorb endotoxins and form complexes with them. It has been reported that the anion-exchange resins such as DEAE-Sephadex and DEAE-Sepharose CL-6B adsorb endotoxins (24-26). The electrostatic interaction between phospho or carboxyl groups of endotoxins and positively charged functional groups on the ion-exchange resins is the basis for the adsorption of endotoxins on ion-exchange resins. The adsorption capacity of SSPP is expected to be much higher than that of conventional ion-exchange resins with their high specific surface area (3×10^5 cm^2/g particle) (12). In addition, the adsorption can be accomplished in suspensions rather than chromatographic columns, thus significantly reducing the processing time. The removal of endotoxins may then be accomplished by simple sedimentation or centrifugation.

Removal of Nucleic Acids

Calf thymus DNA was adsorbed and removed completely from the solution by adsorption on the positively charged SSPP (SSPP I and II) as indicated by decreases in absorbance at 260 nm (Fig. 1). Ribonucleic acid (RNA) was also removed by the adsorption on the positively charged SSPP. Bakers yeast RNA (Sigma Chem. Co., St. Louis, MO, USA) was almost completely removed from the solution by adsorption on the quaternary amino SSPP (SSPP I, Fig. 2).

In the presence of proteins, the adsorption of nucleic acids on the positively charged SSPP decreased. At a given concentration of SSPP I, the adsorption yield of calf thymus DNA decreased with increases in the con-

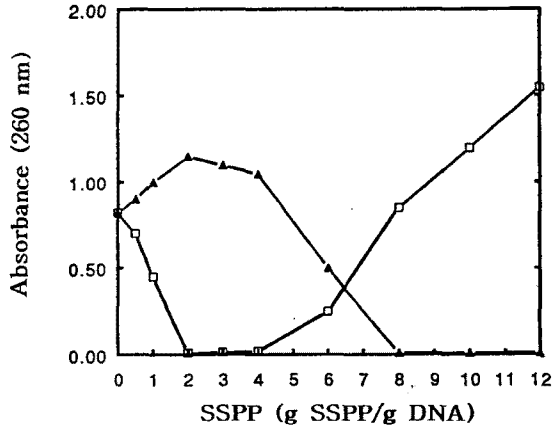

Fig. 1. Optimum concnetration of positively charged submicron-sized polymeric particles (SSPP) for removal of calf thymus DNA (10 mM potassium phosphate, pH 7.2).
—□—, SSPP I; —▲—, SSPP II.

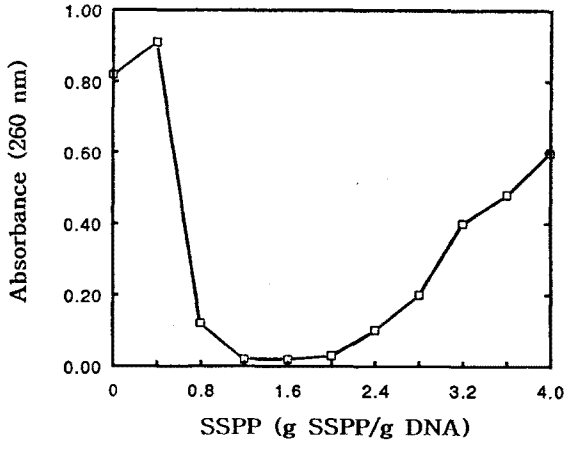

Fig. 2. Optimum concentrations of the quaternary amino submicron-sized polymeric particle (SSPP I) for the removal of bakers yeast RNA (10 mM potassium phosphate, pH 7.2).

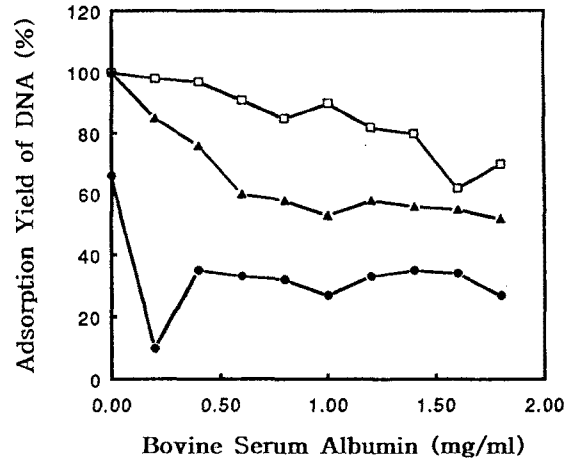

Fig. 3. Effects of the protein concentration on the adsorption of DNA on quaternary amino SSPP (SSPP I).
—□—, 0.2 mg SSPP I/ml; —▲—, 0.4 mg SSPP I/ml; —●—, 0.6 mg SSPP I/ml.

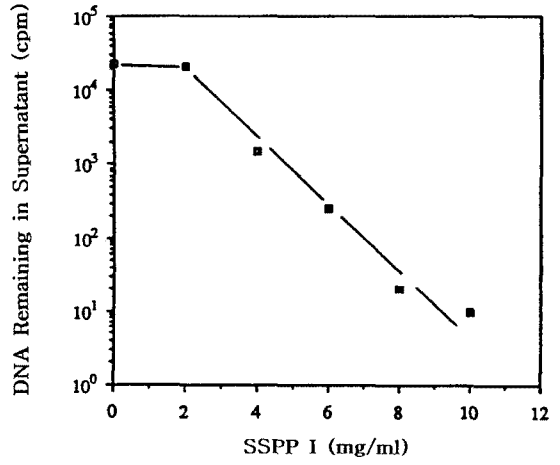

Fig. 4. Removal of DNA from bakers yeast lysate with SSPP I.

centration of bovine serum albumin (Fig. 3). The adsorption of proteins on the SSPP, however, can be controlled by manipulation of solution conditions such as the concentration and type of salt and pH (12, 15). In the control of the protein adsorption, the selection of salt is the most important factor because the adsorption of nucleic acids on SSPP is highly dependent on the type of salt. The adsorption of nucleic acids on SSPP decreases at high concentrations of potassium chloride or sodium chloride (higher than 0.6 M) as on hydroxyapatite (3, 4). The adsorption of nucleic acids on SSPP, however, is not affected by the concentration of potassium phosphate or sodium phosphate. The adsorption yield of nucleic acids remained the same regardless of the concentration of potassium phosphate or sodium phosphate up to the 1.2 M tested (13, 14).

In complex systems such as cell lysate, the addition of 0.2 mg/ml of a positively charged SSPP also removed almost completely the DNA present in the *E. coli* lysate. Therefore, the removal of endotoxins and DNA can be accomplished concurrently with the cell and cell debris removal process using SSPP.

Fig. 4 shows the removal of DNA in a bakers yeast cell lysate using the quaternary amino SSPP (SSPP I). The concentration of DNA in the yeast lysate was reduced by three to four orders of magnitude when 8 mg SSPP I/ml was added during the cell debris removal process. The same results were obtained in an *E. coli* cell lysate system. This indicates that nucleic acid removal can be accomplished concurrently with the cell debris re-

moval process in a single purification step using SSPP. In this experiment, radioactive *E. coli* DNA [thymidine-methyl-^{14}C] was added to the yeast lysate to detect the concentration of DNA remaining in the supernatant.

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