endotoxins, it is critical for the pharmaceutical industry and healthcare community to have an early and sensitive detection of endotoxin.

[0003] The Limulus Amebocyte Lysate (LAL) test was commercially introduced in the 1970s as a way to detect endotoxins. LAL is derived from the blood cells, or amebocytes, of the horseshoe crab, Limulus polyphemus. The original LAL test constituted a cascade of serine proteases which are triggered by trace levels of endotoxin, culminating in a gel clot at the end of the reaction. Factor C, which normally exists as a zymogen, is the primer of this coagulation cascade. In vivo, Factor C is the perfect biosensor, which alerts the horseshoe crab of the presence of a Gram-negative invader. The hemostatic end-point entraps the invader, killing it and limiting further infection.

[0004] The LAL test can be modified to use different methods to measure the response of the amebocytes against the endotoxins. These methods include the so-called Gel-Clot method, turbidimetric and chromogenic methods. These LAL tests are recommended in international pharmacopoeias as the method for detecting bacterial toxins both in the raw materials used for the production of medicines and for the final products. These tests are also useful for the cosmetics industry and in food production as it is the method recommended by the FDA (Food and Drug Administration) for the detection of pyrogens.

BRIEF SUMMARY OF THE INVENTION

[0005] Provided herein are methods of detecting an endotoxin in a sample using a chromogenic assay comprising a limulus amebocyte lysate (LAL) substantially free of coagulogen.

[0006] Embodiments herein are directed to a method of detecting an endotoxin in a sample using a chromogenic assay, the method comprising: (a) contacting the sample with a reagent comprising limulus amebocyte lysate (LAL) and a chromogenic substrate; and (b) measuring a chromogenic effect resulting from a change in the chromogenic substrate in the presence of endotoxin in the sample, wherein the LAL is substantially free of coagulogen.

[0007] In some embodiments, the chromogenic substrate is a p-nitroaniline covalently bonded to a greater than three amino acids. In some embodiments, the chromogenic substrate is Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1). In some embodiments, the change in the chromogenic substrate occurs due

to an enzymatic reaction. In some embodiments, the enzymatic reaction is cleavage of a chromophore from a polypeptide. In some embodiments, the chromogenic effect is measured by detecting at absorbance at 380 nm – 420 nm. In some embodiments, chromogenic effect is measured by detecting absorbance at 405 nm.

[0008] In some embodiments, the reagent is a liquid. In some embodiments, the reagent is an aqueous liquid. In some embodiments, the reagent is lyophilized and then reconstituted in an aqueous liquid prior to contacting with the sample.

[0009] In some embodiments, the LAL is lyophilized, and then reconstituted prior to contacting with the sample. In some embodiments, the chromogenic substrate is lyophilized, and then reconstituted prior to contacting with the sample.

[0010] In some embodiments, the sample is a biological sample. In some embodiments, the sample is selected from the group consisting of a parenteral dosage form, vaccine, antibiotic, therapeutic protein, therapeutic nucleic acid, therapeutic antibody, and biological product. In some embodiments, the LAL substantially free of coagulogen has less than 5% (wt/wt) of coagulogen relative to total protein in the LAL as measured by SDS-PAGE and confirmed by Western blot. In some embodiments, the LAL substantially free of coagulogen has less than 2% (wt/wt) of coagulogen relative to total protein in the LAL. In some embodiments, the LAL substantially free of coagulogen has less than 0.5% (wt/wt) of coagulogen relative to total protein in the LAL. In some embodiments, the chromogenic assay is conducted using single cuvette spectroscopy, multiple cuvette spectroscopy, or a microplate reader.

[0011] In some embodiments, the method further comprises comparing the chromogenic effect to a standard to determine the quantity of endotoxin in the sample.

[0012] In some embodiments, the disclosure is directed to a method of detecting an endotoxin in a biological sample using a chromogenic assay, the method comprising: (a) contacting the biological sample with an aqueous reagent comprising limulus amebocyte lysate (LAL) and Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1); (b) measuring the change in absorbance at 405nm resulting from the enzymatic cleavage of pNA from Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1) in the presence of endotoxin in the sample; wherein the LAL is substantially free of coagulogen.

[0013] In some embodiments, the method of the present disclosure has increased sensitivity. In some embodiments, the method has a sensitivity of >0.001 EU/mL endotoxin.

[0014] In some embodiments, the method of the present disclosure is directed to a composition comprising limulus amebocyte lysate (LAL) and a chromogenic substrate, wherein the LAL is substantially free of coagulogen. In some embodiments, the chromogenic substrate is Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1). In some embodiments, the LAL and chromogenic substrate are lyophilized. In some embodiments, the LAL and chromogenic substrate are in an aqueous solution.

[0015] In some embodiments, the method of the present disclosure is directed to a kit comprising: (a) limulus amebocyte lysate (LAL), wherein the LAL is substantially free of coagulogen; (b) a chromogenic substrate; and (c) instructions for detecting an endotoxin using the LAL and chromogenic substrate.

[0016] In some embodiments, the LAL is lyophilized. In some embodiments, the LAL is in an aqueous solution. In some embodiments, the LAL and the chromogenic substrate are in a single container. In some embodiments, the kit further comprises a sterile container comprising the LAL. In some embodiments, the sterile container is a sterile vial. In some embodiments, the kit further comprises a control standard endotoxin.

[0017] In some embodiments, the disclosure is directed to a method of making limulus amebocyte lysate (LAL) substantially free of coagulogen, the method comprising: (a) providing a composition comprising LAL, wherein the LAL comprises coagulogen; (b) adjusting the buffer; and (c) subjecting the composition of (b) to tangential flow filtration using a 20 kDa to 50 kDa filter, thereby isolating LAL substantially free of coagulogen.

[0018] In some embodiments, the TFF is performed at a flow rate of 350 ml/min to 500 ml/min. In some embodiments, the buffer is a Tris buffer or MES buffer. In some embodiments, the buffer has a pH of about 7.0 to 8.0. In some embodiments, the filter is tangential flow filtration (TFF). In some embodiments, the TFF filter is a modified polyethersulfone (mPES) membrane filter.

"-" for no gel clot and "-/+" for soft gel clot. (B) Western blot analysis of tangential flow filtration (30 kDa) products run under reducing conditions for three runs. Antibody dilutions were as follows: α-coagulogen 1:1,000, α-proclotting enzyme 1:10,000, α-sushi 3 1:100.

[0027] FIG. 8: (A) Quantification of coagulogen using Western blot analysis using α-coagulogen. (B) The data of Fig. 8(A) plotted to a standard curve. The data suggests about 33% of total LAL protein is coagulogen.

[0028] FIG. 9: SDS-PAGE of coagulogen. When run33 under reducing conditions, three bands of coagulogen are present. Protein sequencing confirmed that the three bands are coagulogen fragments. A = coagulogen1: pos 21 GDPNVPTCLC (SEQ ID NO: 2); B = coagulogen2: pos 39 KVIVSQEKTD (SEQ ID NO: 3); C = coagulogen3: pos 67 GFSIFGGHPA (SEQ ID NO: 4).

[0029] FIG. 10: Stability of two separate lots of L-co was determined over ten weeks at 50 mOD. The data suggests that the L-co was stable over the 10 week period.

[0030] FIG. 11: The presence of coagulogen in commercially available LAL from Lonza, Charles River Laboratories International, and Associates of Cape Cod, Inc. (ACC) was tested using SDS-PAGE with a protein stain and with Western Blot analysis. This confirmed that LAL from all three suppliers contained coagulogen.

DETAILED DESCRIPTION OF THE INVENTION

[0031] It should be appreciated that the particular implementations shown and described herein are examples and are not intended to otherwise limit the scope of the application in any way.

[0032] The published patents, patent applications, websites, company names, and scientific literature referred to herein are hereby incorporated by reference in their entirety to the same extent as if each was specifically and individually indicated to be incorporated by reference. Any conflict between any reference cited herein and the specific teachings of this specification shall be resolved in favor of the latter. Likewise, any conflict between an art-understood definition of a word or phrase and a definition of the word or phrase as specifically taught in this specification shall be resolved in favor of the latter.

[0050] In some embodiments, the change in chromogenic substrate occurs due to an enzymatic reaction. In some embodiments, the enzymatic reaction results in cleavage of a peptide bond, thereby cleaving a chromophore substituent (e.g., p-NA) from a polypeptide. For example, the activated enzyme can catalyze the release of pNA from a colorless peptide substrate, e.g., Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1). In some embodiments, the peptide substrate is a p-nitroaniline covalently bonded to greater than three amino acids. In some embodiments, embodiments, the chromogenic substrate is Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1). In some embodiments, the chromogenic assay measures free pNA. In some embodiments, the chromogenic assay measures free pNA photometrically at an absorbance of 380 nm to 410 nm, e.g., 400 nm to 410 nm, or 405 nm. Methods of measuring absorbance are well known to those in the art. In some embodiments, the chromogenic assay is conducted using single cuvette spectroscopy, multiple cuvette spectroscopy, or a microplate reader to measure absorbance.

[0051] The free pNA can be measured photometrically at 380 nm to 410 nm, e.g., 405 nm, after the reaction is stopped with stop reagent. The concentration of endotoxin in a sample is calculated from a standard curve of absorbance values of solutions containing known amounts of an endotoxin standard.

[0052] One standard chromogenic assay for detecting endotoxin comprises contacting a sample with a reagent, wherein the reagent comprises limulus amebocyte lysate (LAL). In some embodiments, the reagent is a liquid, e.g., and aqueous liquid. Alternatively, the reagent can be lyophilized, and then reconstituted in an aqueous liquid, e.g., sterile water or buffer solution, prior to being contacted with the sample. In some embodiments, the reagent is a liquid. In some embodiments, the reagent is an aqueous liquid. In some embodiments, the reagent is lyophilized and then reconstituted in an aqueous liquid prior to contacting with the sample. In some embodiments, the LAL is lyophilized, and then reconstituted in the aqueous liquid prior to contacting with the sample. In some embodiments, the chromogenic substrate is lyophilized, and then reconstituted in an aqueous liquid prior to contacting with the sample. In some embodiments, lyophilization allows for a longer and/or more robust storage of the reagent, LAL, and or chromogenic substrate in the chromogenic assay. For example, in some embodiments, the lyophilized reagent, LAL and/or chromogenic substrate allows for greater than 20%, greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than

of endotoxin in a sample. In some embodiments, the method further comprises comparing the chromogenic effect of the endotoxin in a sample to a known endotoxin standard to determine the quantity of endotoxin in the sample.

[0061] In some embodiments, the disclosure is directed to a method of detecting an endotoxin in a biological sample using a chromogenic assay, the method comprising: (a) contacting the biological sample with an aqueous reagent comprising limulus amebocyte lysate (LAL) and Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1); (b) measuring the change in absorbance at 405nm resulting from the enzymatic cleavage of pNA from Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1) in the presence of endotoxin in the sample; wherein the LAL is substantially free of coagulogen.

[0062] By removing the coagulogen from the LAL, the chromogenic assay can have increased sensitivity. In some embodiments, the method of the present disclosure has increased sensitivity. In some embodiments, the method has a sensitivity of less than 0.05 EU/mL, less than 0.03 EU/mL, less than 0.01 EU/mL, less than 0.008 EU/mL, less than 0.006 EU/mL, less than 0.005 EU/mL, less than 0.004 EU/mL, less than 0.003 EU/mL, less than 0.002 EU/mL, or less than 0.001 EU/mL.

[0063] The disclosure additionally is directed to compositions comprising (1) LAL substantially free of coagulogen, and (2) a chromogenic substrate. In some embodiments, the chromogenic substrate in the composition comprises pNA. In some embodiments, the chromogenic substrate in the composition is Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1). In some embodiments, the composition comprises 60% to 80% LAL substantially free of coagulogen preparation, and 20% to 40% chromogenic substrate (wt/wt). In some embodiments, the composition comprises about 6% to about 25% LAL substantially free of coagulogen. In some embodiments, the composition comprises 70% LAL substantially free of coagulogen preparation, and 30% chromogenic substrate (wt/wt). In some embodiments, the composition comprises 70% LAL substantially free of coagulogen preparation, and 30% Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1)(wt/wt). In some embodiments, the compositions as described herein are in a single container, e.g., a single vial. In some embodiments, the compositions described herein are lyophilized. For example, the disclosure specifically describes a lyophilized composition comprising 70% LAL substantially free of coagulogen preparation, and 30% Ac-Ile-Glu-Ala-Arg-pNA (SEQ ID NO: 1)(wt/wt).