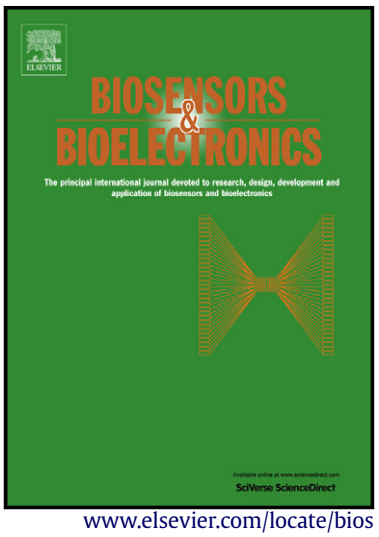

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Recent Advances in Biosensor Based Endotoxin Detection

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Abstract

Endotoxin also referred to as pyrogens are chemically lipopolysaccharides habitually found in food, environment and clinical products of bacterial origin are unavoidable ubiquitous microbiological contaminants. Pernicious issues of its contamination results in high mortality and severe morbidities. Standard traditional techniques are slow and cumbersome, highlighting the pressing need for evoking agile endotoxin detection system. The early and prompt detection of endotoxin assumes prime importance in health care, pharmacological and biomedical sectors. The unparalleled recognition abilities of LAL biosensors perched with remarkable sensitivity, high stability and reproducibility have bestowed it with persistent reliability and their possible fabrication for commercial applicability. This review paper entails an overview of various trends in current techniques available and other possible alternatives in biosensor based endotoxin detection together with its classification, epidemiological aspects, thrust areas demanding endotoxin control, commercially available detection sensors and a revolutionary unprecedented approach narrating the influence of omics for endotoxin detection.

Keywords: Endotoxin, Sepsis, Biosensor, Omics, LAL assay, Recombinant Factor C, Lab-on-a-chip

1. Introduction

Endotoxin are complex lipopolysaccharides (LPS) which form an inherent fraction of the outer cell wall of all gram negative bacteria and are responsible for the organisation and stability of the cell wall (Kim et al., 2012). These are composed of three distinct regions: O-specific antigen forming the surface antigen, core polysaccharide and a non-polar lipid A. Endotoxin are responsible for toxic effects causing fever (Su et al., 2012), multi organ failure (Lawrence et al., 2011), septic shock (Ding et al., 2007), sepsis (Gutsmann et al., 2010) meningococcemia and severe morbidities like neurologic disability, hearing loss and loss of a limb (Kofyman et al., 2011). Figure 1 corresponds to flow diagram representing the lethal effects of endotoxin.

Severe endotoxin infections coupled with septic shock transmit an elevated mortality rate in spite of proper antibiotic treatment and intensive care (Goscinski et al., 2004). Detection of endotoxin is essential for quality control in biological products, medical devices, serological products, parenteral drugs, recombinant therapeutic products and food & water security (Yeo et al., 2011; Lazcka et al., 2007). These necessities the need for a rapid and sensitive biosensor for detection and monitoring of endotoxin levels even in extremely small concentrations (Su et al., 2012). Biosensors are compact analytical detection devices that use living organisms or biologically derived molecules such as antibodies, nucleic acids, or enzymes, to recognize specific target analyte or a group of closely related analytes in the sample matrix through discrete or continuous electrical signal, colorimetric or fluorescent indicators (Turner 2013). Despite of the large arena of biosensor applications, the field of biosensor has been virtually classified into two broad categories (i) high throughput expensive instruments delivering high sensitivity and selectivity with capable of measuring complex limitless biological interactions and components (ii) inexpensive, portable and easy to use by layman and even capable of home analysis (Turner 2013). Recently focus has also been geared up for development of diverse strategies for endotoxin detection for example biosensors based on bioluminescence using luciferase enzyme (Noda et al., 2010), fluorescent dyes (Voss et al., 2007), mass change (Ooe et al., 2007), frequency-amplitude responses (Ong et al., 2006) and endotoxin recognition systems that use endotoxin-specific substances such as endotoxin neutralizing proteins (Kaconis et al., 2011),

endotoxin binding proteins (Davies et al., 2011), cationic antibacterial proteins (Ding et al., 2007), peptides (Voss et al., 2007), artificial molecules like synthetic receptor (i.e. amino acid-functionalized polydiacetylene liposome) (Rangin and Basu et al., 2004) and pyrene derivatives (Zeng et al., 2010). A comprehensive literature survey has been reviewed over the past ten years with an emphasis on biosensor based endotoxin detection (figure 2).

List of potential gram negative bacteria (Table 1) corresponding with their toxin produced, causal diseases, and its sources are mentioned. With an annual worldwide incidence of approximately 18 million, it is the largest cause of mortality in intensive care units (Daniels 2009). It represents a growing concern and significant problem in high-risk patients with an associated 30-40% mortality rate (Blanco 2008, Karlsson 2007). About 250,000 deaths occur annually in US alone and an estimate of nearly 750,000 patients are annually diagnosed with sepsis. Details of which are summarized in Figure 3.

Centre for Disease Control and Prevention (CDCP) ranked sepsis has one of the top ten leading causes of death worldwide. In case of children and elderly aged above 85 years, sepsis infection ranged from 0.2 cases per 1000 and 26.2 per 1000 admissions respectively. The mortality rate was 28.6% and ranged from 10% in children to 38.4% in elderly people. It leads to a total annual cost of $16.7 billion annually and an average of $2200 per case (Angus et al., 2001). Sepsis is both the leading cause of admission and death in ICUs (Blanco et al., 2008). Levy et al found that there were 12% more people dying from sepsis-related causes in hospitals across Europe (Levy et al, 2012). Mortality and morbidity rates due to endotoxin remain paramount high and have not improved significantly over the past 25 years. The outcome of failure to detect and monitor endotoxin levels can result in serious sequelae in survivors and large scale deaths. Conventional techniques are extremely slow, laborious, invasive and cumbersome. This present scenario immediately demands an endotoxin detecting biosensor to monitor endotoxin levels in human biological products and medical devices. Efforts are being made to enhance the specificity and sensitivity of biosensors. Development of biosensors has always been confronted with new challenges and still holds promising scope for novel sensor based futuristic world.

2. Thrust areas demanding endotoxin control:

Endotoxin control is of paramount importance primarily for health and safety issues. There are many sectors demanding mandatory monitoring of endotoxin levels such as food industry, pharmaceutical, health care, agriculture, sewage treatment plants, transportation warehousing and utilities, defence, environment, textile industry, cell culture and recombinant therapeutic products (Turner et al., 2013, Garcia et al., 2013, Pankhurst et al., 2011, Thorne et al., 2010). List of detail sectors demanding mandatory monitoring are listed in figure 4.

Although lipopolysacharrides are firmly anchored within the bacterial cell wall, they are continuously liberated into the surrounding medium. Its release mainly happens during cell death, antibiotic use and even during growth and division. Since bacteria can grow in nutrient poor media such as water, saline and buffers, endotoxins can be found almost everywhere. High concentrations are found where bacteria accumulate or are to be used for technical purposes, such as bioprocessing. Endotoxin concentration also head to high levels during natural calamities like cyclones (GL et al., 2006). Exposure to bacterial endotoxin in the occupational and residential environment has been found to mediate a variety of adverse health effects (Rylander, 2008). Among agriculture, transportation and cotton textile prolonged exposure of workers in occupational environment has been associated with intermittent respiratory symptoms (e.g., chest tightness). Long-term occupational exposure to organic (e.g cotton dust) that contains endotoxin is associated with chronic respiratory symptoms and excessive decline in forced expiratory volume in 1 sec. Bacteria growing in the cotton dust itself can produce endotoxin during storage and is responsible for producing the bronchospastic response associated with byssinosis (Wang et al., 2003). Current conventional techniques lacklustre performance urges to focus on newer techniques with more reliability and increased performance with a range of different samples and sample volumes. Our review emphasizes on biosensor for easy and rapid detection and monitoring of endotoxin in human biological products and medical devices.

3. Detection of endotoxin:

Endotoxin detection has assumed prime importance and concern in various sectors. Detecting, monitoring and quantifying have always been serious issues. Failure of detection often results in a plethora of hazardous symptoms, if left untreated even results in death. Hence, a range of diverse methods using different techniques are chalked out and tested for sleuthing endotoxin. Table 2 summarize various approaches engaged to detect endotoxin. First conventional methods are briefly described followed by endotoxin based sensors and finally by Limulus Amebocyte Lysate assay (LAL).

3.1 Conventional and traditional methods for endotoxin detection:

Conventional methods are standard established techniques such as rabbit pyrogen test (Schindler et al., 2007), culturing and plating (Jacobs et al, 2008), polymerase chain reaction (Zarain et al., 2012) and immunology methods (Jechorek et al., 2008). These have extensively served for bacterial endotoxin detection and till now are continuously being employed.

3.1.1 Culturing and plating method:

Culturing and plating method are the traditional and oldest known conventional indirect assay that served as the standard technique for bacterial endotoxin detection (Rafel and Pat 2000). The serious disadvantages include, each microorganism demands its own culture media for its optimum growth and needs its own supplements. Hence, for each microbe a new fresh culture media has to be designed. Adding more trouble, each microbe requires its own time to yield either positive or negative result. In case of *Campylobacter*, 4-9 days are required to yield a negative result and 14-16 days are required to yield a confirmatory positive result (Brooks et al., 2004). Correlation between endotoxin concentraion and bacterial count along with spore concentration has been successfully established. (Vu et al., 2012, Nys et al., 2000).

3.1.2 Rabbit pyrogen test:

The Rabbit pyrogen test is an in vivo qualitative test for detecting endotoxin (Schindler et al., 2007). Rabbits are considered as ideal choice because they have similar endotoxin tolerance as humans. The 3Rs concept introducing refine, reduce and replace animal testing has significantly led to reduction of rabbit pyrogen testing (Hoffmann et al., 2005). The major drawback being it is costly, time consuming, subjective, prompted protests from animal's rights activists and finally its inability to quantify the endotoxin level (Dobrovolskaia et al., 2010). The limit of raise in rectal temperature is assigned to a maximum of 0.55^0C for 180 min after injection. The sensitivity was found to be 0.5 EU/ml (Hoffmann et al., 2005).

3.1.3 Polymerase chain reaction:

PCR is a common and indispensable technique used widely to amplify a single or a few copies of DNA to millions of copies with a very high degree of precision (Joshi et al., 2011). A number of PCR variants have been introduced to detect pathogenic bacterial endotoxin such as Real-time PCR (Mafu et al., 2009), Reverse Transcriptase PCR (RT-PCR), Nested PCR (Zarain et al., 2012) and Multiplex PCR (Josefson et al., 2011). It has also got an added advantage of getting coupled to other techniques like Surface Acoustic Wave sensor (SAW), Fluorescence in situ hybridization (FISH), Most Probable Number (MPN-PCR), Lightcycler real-time PCR (LC-PCR), PCR-Enzyme linked immunosorbent assay (PCR-ELISA) and sandwich hybridization assay. Among the conventional techniques available, PCR is much faster and less time-consuming. In addition to this PCR is fully automated, doesn't require pre-enrichment steps, can be coupled to other techniques and provide sensitive results. Amidst all available PCR variants, multiplex PCR has got solemn advantage of simultaneous detection of several organisms by using different primers to amplify DNA regions (Poritz et al., 2011, Lazcka et al., 2007). The key limitations with most of PCR variants include that it can't distinguish between living viable cells and non-living dead cells. However, this lacuna can be overcome by using RT-PCR and EMA-LAMP (Lu et al., 2009). Inspite of all the advantages, the other associated limitations

include it is labor intensive, time consuming and requires post- amplification processing steps like gel electrophoresis. A correlation has been successfully established between quantitative PCR analysis and endotoxin concentration (Johansson et al., 2011).

3.1.4 Immunological methods:

It is the solemn technology toted of successfully detecting all microbes and its various products including bacterial cells, spores, viruses, exotoxins and endotoxin. It has been successfully employed in detection of *Escherichia coli, Salmonella, L.monocytogenes, Staphylococcal and Camplylobacter* (Velusamy et al., 2010, Jay et al 2005). Serological typing remains as one of the most widely used immunological based techniques and an important tool in identifying bacteria, even between different strains (Van Belkum et al. 2007). Latex agglutination test has been employed for detecting bacterial cells and reverse passive agglutination for toxins that are soluble (Feng 2001, Cunha et al., 2007). Fluorescence based method is another subtyping technique which is also based on specific interactions between surface associated antigens and antibodies labelled with fluorescein or fluorescein labelled conjugate which is added for visualization of the antigen-antibody binding (Mohan et al., 2008). Apart from traditional antigen-antibody based immunological assays, it can also be performed for detection of metabolites, such as toxins e.g. *Clostridium difficile* (Musher et al., 2007). Another option is to look for antibody variants namely monoclonal, polyclonal and recombinant antibodies. Polyclonal antibodies have been successfully used to detect *L.monocytogenes* (Feldsine et al., 1997a), *Campylobacter* species (Hochel et al., 2007), *Fusarium* sp. (Meirelles et al., 2006). However, polyclonal antibodies are limited both in terms of their specificity and abundance (Leonard et al., 2003). Because these methods are highly time-consuming and labor intensive, there is a need for more rapid and reliable techniques.

3.2 Biosensor based endotoxin detection

Biosensors are miniature sized, fully automated promising analytical tools delivering high selectivity and sensitivity. They use specific biocomponents such as enzymes, antibody, nucleic acid, lectin, cells

or tissues and their subsequent transducing systems include optical, electrochemical, piezoelectric, thermometric, magnetic or acoustic. Although biosensors are generally classified according to various transduction modes and recognition elements. Here we classified endotoxin detecting biosensors only on the basis of transduction system, which are basic devices giving an output quantity in the form of a measurable energy having a correlation with the input quantity. According to the form of transduction adopted, these can be categorized in one of three main classes. These classes are: 1) optical detection methods, 2) electrochemical detection methods and 3) mass detection methods (Figure 5). Along with it, a comparison chart eluciting general advantages and disadvantages of individual biosensor has been presented (Table 3).

3.2.1 Optical biosensors

Optical transducers are particularly attractive for direct or indirect detection of pathogenic bacteria and its toxin. Availability of a number of variants due to different spectrochemical properties has made it instant popular. These sensors can even detect diminutive changes in spectophotometric parameters such as refractive index or thickness which occur when target analyte binds to receptors immobilized on the transducer surface. They are further categorised into fluorescence and surface plasmon resonance.

3.2.1.1 Fluorescence detection

Fluorescence is an emission phenomenon in which a fluorophore absorbs light or electromagnetic radiation and emits light at visible range. Sometimes, the emitted radiation may be at shorter wavelength or at same wavelength than the excited radiation. The later is called resonance fluorescence. Since it is accompanied by loss of radiative energy, the fluorescent light is always at a longer wavelength than the absorbed light called stokes shift (Velusamy et al., 2010, Lazcka et al., 2007) (Figure 6). Detection of endotoxin from *Listeria* species using fluoroscent sandwich assay has achieved a detection limit of 10 ng/ml (Leung et al., 2007, James et al., 1996). It has also been blended to simultaneously detect *Escherichia coli* and *Staphylococcus aureus* with lower detection limit to 10^2 cells (Xue et al., 2009). Fluorescence coupled to fresnel reflection spectroscopy has been reported to detect as low as 200 cells/ml of *Legionella pneumophila* (Li et al., 2013). Further simultaneous

detection been possible using capture antibody targeted detection (CAT-FISH) was developed to detect both *Escherichia coli* O157:H7 and *Staphylococcus aureus* (Stroot et al., 2012). A chemi-luminiscence based endotoxin detection was reported as early as in 1998 by Romaschin et al., with a detection limit of 0.017-1.6 EU/ml (Romaschin et al., 1998). A luminiscence based biosensor for endotoxin detection has been reported for *Hafnia alvei* PCM 1186 strain (Hreniak et al., 2004). Reports of using Enhanced green fluorescent protein (EGFP) as a signalling scaffold protein for a fluorescent bacterial endotoxin detecting biosensor has surfaced (Goh et al., 2002). A new endotoxin detection sensor using bioluminiscence from mutant luciferase enzyme to a detection limit of 0.0005 EU/ml has been reported (Noda et al., 2010). An entirely new colorimetric and fluorometric sensor capable of visual determination of endotoxin with a detection limit of 270 pM has been accomplished (Lan et al., 2012).

3.2.1.2 Surface plasmon resonance

SPR based transduction are surface sensitive method for detecting measurable changes in refractive index caused by structural alterations in the vicinity of a thin film metal surface. At prism-metal interface, Total Internal Reflection (TIR) occurs when a light from high refractive index meets interface at lower refractive index at an angle larger than the critical angle. TIR of an incident light elicits a propagating plasmon wave by leaking an evanescent field wave, which decays at an exponential rate and possess one wavelength (Lotierzo et al., 2004) (Figure 7). SPR based biosensors has been employed in detecting *salmonella* (Barlen et al., 2007). A gold nanorod-based optical DNA biosensor for the diagnosis of pathogens like *Chlamydia trachomatis* pathogen has been reported (Parab et al., 2010). SPR using bacteriophage as bioreceptors has been successfully used to diagnose *Escherichia coli* O157:H7 and methicillin-resistant *Staphylococcus aureus* (MRSA) (Tawil et al., 2012). A modification of regular SPR called long-range surface plasmons (LRSPs) coupled to magnetic nanoparticle assay (MNP) can detect *Escherichia coli* O157:H7 at concentrations as low as 50 CFU/ml (Wang et al., 2012). A real time monitoring of *E coli* K-12 and vaious bacterial products including spores, proteins and viruses are successfully reported (Zibaii et al., 2010, Bhatta et al., 2010)

SPR based sensors have been exclusively used in screening for δ-endotoxin of *Bacillus thuringiensis* (Okumura et al., 2001).

3.2.1.2 Electrochemiluminescence

Electrochemiluminescence (ECL) is a kind of chemiluminescence which is immediately preceded by an electrochemical reaction. From stable precursors, highly reactive species are generated which upon reacting with each other produces light. The main advantage being controlled oxidation/reduction reaction at electrode surface which subsequently reins the time and position of the light emitting reaction (Hu et al., 2010). Electrochemiluminescence has been successfully employed for detecting both gram negative and positive bacteria, with a detection range of 10^5–10^8 CFU/ml (Chaney et al., 1999). Immunomagnetic electrochemiluminiscence has been successfully engaged to detect E. coli O157 with a detection limit of 25 cells/100 ml (Shelton et al., 2001). Leach et al has blended the detection of E. Coli O157:H7 with a detection limit of 10^2 cells/ml and 10^3 cells/ml in buffer and spinach rinsed enriched samples respectively (Leach et al., 2010). Reports of binding of LPS to CD14 has surfaced which can later be augmented to fabricate a working biosensor (Burkhardt et al., 2007).

3.2.2 Electrochemical biosensors

Electrochemical based transducers are an additional alternative strategy been successfully applied for identification, monitoring and quantification of gram negative bacteria and its related endotoxin (Lu et al., 2013). The principle lying behind is that the chemical reactions produce change in measure of electrons or ions, which affects electrical parameters of solutions (Sarkar et al., 2010). Based on parameters like current, potential and impedance, they have been classified into amperometric, potentiometric and impedance.

3.2.2.1 Amperometric methods

Amperometric based transducers are the most commonly used electrochemical sensors which are been regularly used for pathogen detection and possess superior sensitivity than other used methods. The

principle working behind is a simple linear relationship between analyte concentration and electron transfer i.e. current (Velusamy et al., 2010). A disposable amperometric immunosensing strip was prepared to rapidly detect *E Coli* O157:H7 with lower detection limit of 6 CFU/strip in PBS buffer and 50 CFU/strip in milk (Lin et al., 2008). A new amendment to the regular amperometric sensor using bienzyme sensor developed for detection of *Escherichia coli*. The lower detection limit was established to be 9.7×10^2 cells/ml (Tang et al., 2006). Adding a fresh amendment, disposable amperometric magnetoimmunosensors was developed to detect *Streptococcus pneumoniae*. The lower detection limit was found to be 1.5×10^4 CFU/ml for serotype 37 and 6.3×10^5 CFU/ml for R6 strain respectively (Campuzano et al., 2010). An assay using amperometric based sensors to detect endotoxin from *salmonella minnesota* at concentrations as low as 0.1 ng/ml has been accomplished (Priano et al., 2007). Recently, a new amperometric based biosensor detecting 0.03 EU/ml has been reported using endotoxin induced limulus amebocyte lysate gel-clot process (Miao et al., 2013).

3.2.2.2 Potentiometric methods

Potentiometric based sensors represent attractive tools for pathogen detection. The first biosensor that is able to detect *Staphylococcus aureus* in real-time was designed to detect in two different formats. A non-covalent adsorption of aptamers showed lower detection limit to be 8×10^2 CFU/ml. With the non-covalent approach, the sensitivity was 10^7 CFU/ml (Zelada-Guillen et al., 2012). Reports of using potentiometric sensors for testing pork freshness has been reviewed (Kaneki et al., 2004). These have been successfully integrated to quantitatively detect endotoxin with two varying incubation period of 1h and 3h with a lower detection limit of 5000 EU/l and 1000 EU/l. (Inoue et al., 2010).

3.2.2.3 Impedance spectroscopy

Electrochemical Impedance spectroscopy has been successfully fabricated to detect *E coli* with detection range of 10^3-10^8 CFU/ml (Lu et al., 2013). Similar results for detection of *Salmonella typhimurium* was obtained with lower detection limit of 500 CFU/ml (Nandakumar et al., 2008). An altered version with simultaneous detection of both *E coli* O157:H7 and *Staphylococcus aureus* with a

detection limit of 10^2 CFU/ml has been reported (Tan et al., 2011). Additionally, endotoxin detection through impedance spectroscopy has been recently reported with a detection range of 0.001-1 ng/ml (Su et al., 2012). Going a step ahead, similar result was reported with a detection limit of 0.005 ng/ml (Su et al., 2012). Electronic tongue using impedance spectroscopy for endotoxin detection has been reported (Heras et al., 2010). Endotoxin detection using impedance spectroscopy has been successfully harnessed to detect as low as 0.01 ng/ml (Kim et al., 2012). Using metal complex immobilized gold electrode, a real-time sensitive impedance spectroscopy has been designed with a lower detection limit of 0.0001 ng/ml (Cho et al., 2012). A boosting sensor with a capability of multiplex endotoxin detection from various sources has been fabricated with a detection limit of 200 μg/ml (Oliveira et al., 2011). Reports of an advanced real-time endotoxin detection using antibiotic peptide polymixin B to detect endotoxins with a sensitivity as low as 0.2 – 0.8 ng/ml concentrations has been accomplished. (Ding et al., 2007). New methods using planar interdigital sensors with polymixin B as bioreceptors are reined with a detection limit of 0.05 EU/ml (Rahman et al., 2013).

3.2.3. Mass based sensors

Mass based transducers record the minuscule changes on the probe or transducer surface. Principally, small crystals are used such as piezoelectric whose resonating frequency depends on the mass of crystal as well as electrical signal applied. Any change in mass due to binding of chemicals to surface of crystals can be detected by change in resonance frequencies of crystals.

3.2.3.1 Piezoelectric method

A newly designed Piezoelectric-Excited Millimeter sized Cantilever (PEMC) detects *Escherichia coli* O157:H7 at 50-100 cells/ml (Campbell et al., 2007). Real time monitoring of viable *E coli* O157:H7 has been made feasible by piezoelectric biosensor-quartz crystal microbalance (Guo et al., 2012). An electric nose working on piezoelectric Quartz crystal microbalance (QCM) sensors successfully detected six different pathogens (Shih et al., 2010). An electric tuning fork is designed to monitor *Pseudomonas aeruginosa* biofilm dynamics (Waszczuk et al., 2012). Ren et al successfully detected

resistant *Mycobacterium tuberculosis* using piezoelectric system (Ren et al., 2013). Even it has been successfully used to detect potential biological warfare agents surrogates *Escherichia coli MRE 162, Bacillus atrophaeus, Cydia pomonella granulosis virus (CpGV) and ovalbumin* (Alava et al., 2009). Reports of reusable QCM based sensors for detection of staphylococcal enterotoxin A has been established (Salmain et al., 2012). Endotoxin detection using QCM with lower detection limit of 0.01 pg/ml has been accomplished (Xiong et al., 2005). Similar reports of endotoxin detection have been surfaced with lower detection limit of 0.1 pg/ml has been reported (Xiong et al., 2012).

3.2.3.2 Magnetoelastic method

Magnetoelastic based sensors (Figure 8) monitors the viscosity changes of LAL assay in response to endotoxin. These are basically amorphous ferromagnetic ribbons or wires featuring high mechanical tensile strength. The working articulation link being, resonant frequency is inversely proportional to length of the sensor (Shinde et al., 2012, Velusamy et al., 2010). Detection of *Salmonella typhimurium* using magnetoelastic sensors has been reported with a detection limit of 5×10^1 CFU/ml (Lakshmanan et al., 2007). Magnetoelastic sensors can detect *Staphylococcus aureus* with a concentration as low as 10^3 for culture media and 10^4 for milk has been reported (Huang et al., 2010). Investigation has also been carried out using *Staphylococcus aureus* in different sample media, to estimate the effects of varying media on detection. The varying detection limits ranged for culture media and milk was observed to be within 10^3 and 10^4 cells/ml (Si-Jing et al., 2010). Even wireless in situ sensors detecting biofilm formation of *Pseudomonas aeruginosa* has been reported (Pang et al., 2008). Reports of *Neisseria meningitidis* detection has surfaced at a concentration as low as 10 - 60 ng/µl (Patel et al., 2010). Successful detection of endotoxin using stress responsive magnetoelastic sensors monitoring viscosity changes in gel formation has accomplished a detection limit of 0.0105 EU/ml (Ong et al., 2006).

4. LAL based method

W. H. Howell for the first time reported the clotting of horseshoe crab blood and successively F. Bang detected the causative agent to be endotoxin produced from gram negative bacteria. LAL provides a magnanimous way to detect endotoxin without having to sacrifice animals. There are three principal LAL test methods- (Figure 9) gel clot method, turbidimetric method and chromogenic method.

Gel clot assay is the simplest and most widely used qualitative test. It yields a binary result, either in the form of positive or negative. The reactions occurring in the test tube are fundamentally same, as that in nature when a horseshoe crab is injured. After proper incubation at 37ºC, if the gel withstands 180 º inversions, it is rated positive otherwise rated as negative for endotoxin (Akbar et al., 2012).In turbidimetric methods, the concentration of insoluble coagulin increases which results in increase in turbidity. Rate of turbidity increase has been correlated to increase in endotoxin concentration. In chromogenic method chromogenic substrates are used to yield colour and this intensity of colour is taken as parameter towards endotoxin concentration in test sample. These synthetic chromogenic substrates are co-lyophilized along with the LAL reagent. This substrate has an amino acid sequence that is homologous to one of the points of cleavage of the natural substrate, coagulogen. The intact substrate is colourless, whereas free *p*NA is yellow with an absorption peak at 405 nm.

5. Endotoxin induced defence mechanism in horseshoe crab

Granular amebocytes found in circulating hemolymph forms the major defence system of horseshoe crabs. These form the major chunk of circulating amebocytes (approx. 99%) and a small fraction of non-granular amebocytes (1%). Each amebocyte is composed of electron dense large granules (L-granule) and less electron dense small granules (S-granule). L-graules are selectively rich in clotting factors whereas S-granules are rich in anti-microbial peptides. The defence system of amebocyte is initiated by zymogen factor C, upon exposure to endotoxin, which eventually leads to a cascade of events leading to gelation and later immbolization and engulfment of gram negative bacteria (Figure 10).

Factor C plays a major key role as an activator to immune system by binding to endotoxin. It is a single chain glycoprotein with molecular mass 123kDa. It is composed of two chains i.e. H- Chain and L-Chain with molecular mass 80kDa and 43kDa respectively (Mutu et al., 1991). On binding of endotoxin, an autocatalytic activity triggers with the cleavage of Phe-Ile bond in resulting disulfide linked H, A and B-Chain as activated factor C. In activated factor C, B-Chain is a serine protease domain and H-Chain composed of several domains for endotoxin recognition and adhesion. H-Chain is composed of signal region, cys-rich region, EFG domain, sushi domain and lectin region (Iwanaga, 1998, Mutu et al., 1991). In various researches it is found out that these Cys-rich region Pro-rich region act as connecting region (Park SY, 2008). The EFG-domain derives from epidermal growth factor and Sushi domain also known as short consensus repeats (SCR) are evolutionary conserved protein domains (Park SY, 2008). EFG domain, sushi domain and lectin are combined as selectin and involved in recognition of source of infection and diversion of defensive action towards it (Figure 10). Factor B and proclotting enzyme are structurally similar with molecular weight 64kDa and 54kDa respectively (Figure 10). Activated factor C interact with factor B to make it functional by cleaving at two site combining L-chain and H-chain. In both Factor B and proclotting enzyme a clip domain is present. Clip-domain plays role in immune system as antimicrobial activity, cell adhesion and recognition etc (Chun-Yu Lin et al. 2006). Activated factor B cleave at initiation position of serine protease in proclotting enzyme converting it into clotting enzyme. Coagulogen is the gel forming unit of the entire cascade system with molecular mass 20kDa. It contains 3 unit i.e. A Chain, Peptide C and B Chain out that peptide C act as separator to A and B Chain. Clotting enzyme cleaves the coagulogen at two terminal of peptide C at the Arg-Lys and Arg-Gly linkage making Chain A and B joining to form insoluble coagulin gel. This proteolytic activity feature of the activated clotting enzyme can be used on synthetic chromogenic i.e. Gly-Arg-p-nitroaniline substrates instead of coagulogen to detect the endotoxin as it separate *p*-Nitroaniline. The substrate *p*-Nitroaniline is yellow and can be used as a marker to detect endotoxin with high selectivity and specificity. This method is efficiently advantageous.

6. Commercially available endotoxin detecting sensors

Endotoxin detection has become an absolute prerequisite, since the largescale commercial production of various injectables. Regulation of endotoxin levels has been made mandatory mainly because it can trigger several severe physiological reactions (Pennamareddy et al., 2009). Hence, it assumes high relevance in pharmaceutical production, life science and medical research (Ding et al., 2010). The commercial flooding of various endotoxin detection kits in market clearly signifies the demand and necessity of early detection, regulation and monitoring of endotoxin levels in various sectors. When compared to the official United States Pharmacopeia (USP) rabbit pyrogen test, the LAL test was found to be not only more sensitive to endotoxin, but also simpler, more rapid and less expensive to perform. The commercially available kits are based on various principles, which are listed in details in table 4. Each strategy offering an unique advantage over the other. All are sensitive, reliable, efficient, and usually recommended over the older, slower pyrogen test using rabbits. Each method has its own lower endotoxin detection limit and incubation time, depending on the kit used.

As the limelight focused towards the Limulus Amebocyte Lysate (LAL), it emerged as the preferred endotoxin detection method. With time, variants of LAL in the form of Gel Clot LAL, Kinetic turbidimetric, End point chromogenic LAL, Kinetic Chromogenic LAL and Recombinant factor C (rFC) coupled to fluorescence are introduced. Each method has its own pros and cons. For eg- Gel clot assay is a qualitative assay providing a simple positive/negative result and is often cited in most pharmacopeial monographs as the official referee test. Kinetic turbidimetric offers an economical quantitative choice for water and parenteral fluids. Endpoint chromogenic LAL offers less product interference and more importantly is a quantitative test. Kinetic chromogenic LAL provides greater sensitivity together with low product interference from proteins, vaccines and other biologicals. Going further step ahead, to reduce the dependence on horseshoe crabs, recombinant factor C is coupled to fluorescence to reduce false positive results with improved sensitivity.

In 1970, the first commercial LAL kit was introduced and later in 1977 the Food and Drug Administration (FDA) described conditions for the use of LAL as an end product test for endotoxin in

human biological products and medical devices. The FDA noted, it is widely recognized that the LAL test is faster, more economical and requires a smaller volume of test sample than does the rabbit pyrogen test. In addition, the procedure is less labor intensive than the rabbit test, making it possible to perform many tests in a single day. The endotoxin detection kit assures safety from endotoxin contamination of pharmaceutical products, parenteral drugs, biological products, medical devices and recombinant protein products for consumer or patient use.

7. Omics approach for endotoxin detection:

Omics tools with its novel strategies have led to development and refinement of new research fields allowing comprehensive analysis in various biological systems. They are being developed like never before at an ever increasing pace. Based on omics profile, a number of novel strategies and approaches are redefined and transformed from a simple reductionist approach to a holistic perspective of whole elements interacting together in complex biological systems. Recent advances in omics tools and sequencing techniques have provided new insights into the biological world of pathogens along with its toxins and host limitless interactions. The ability to examine fully sequenced and annotated genomes has greatly accelerated the application of genetic approaches to elucidate various mechanisms. There is a hope that with omic tools, the early detection of toxic effects and their molecular mechanisms of action of various toxins including endotoxin can be easily studied. Current omics technologies provide a range of high-throughput methods which are extremely useful for unravelling the concealed details and indepth insight, allowing a rational outlook in every fragment of human welfare.

Genetic engineering accompanied with omics technologies has led to discovery of novel microenzymatic assay system using recombinant factor C (rFC), opening a new era in endotoxin testing. Cloning of biologically functional rFC using vectors like *Escherichia coli*, yeast, mammalian cells and baculoviral system has been successfully reported. In the later case, remarkable sensitivity of 0.001 EU/ml has been accomplished. As a zymogen, rFC is armed with multiple binding sites for endotoxin binding and get catalytically activated, by trace levels of endotoxin. The resulted acitivated

rFC cleaves a synthetic chromogenic substrate such as Boc-Val-Pro-Arg-pNA producing a yellow colour or fluorogenic substrate such as Boc-Val-Pro-Arg-MCA yielding fluorescence to form a quantifiable product, which can be correlated to endotoxin concentration (figure 11). Recombinant factor C forms a rapid, convenient, reliable, perpetual, economical, environmentally friendly and standardized source of recombinant LAL. It does not cross talk with contaminants like β-glucan, thus eliminating all false positive and elevated results, ease-of-us, excellent lot-to-lot consistency and does not depend on animal source, horseshoe crabs. Hence, redeeming diminishing horseshoe crabs from extinction. Omics with its revolutionary techniques has left a profound effect on the fields of bioinformatics, systems biology, proteomics, genomics, pharmacogenomics, metabolomics, metabonomics, phenomics, transcriptomics, fluxomics, metagenomics, toxicogenomics, lipidomics and various other fields.

8. New trends:

A Lab-on-a-Chip (LOC) is a microfabricated analytical device designed to couple microfluidics system with semiconductor processing on the same chip. It s a such a technology integrating one or several laboratory functions on to a single chip of only millimeters to a few square centimeters in size. It is purposefully built to analyze small volume samples even less than pico litres with high throughput biochemical analysis for end users including for home analysis. They consist of etched microchannels made of silicon, glass or polymer. The test fluids (blood, saliva, urine) in the channels can then interact with different elements such as electrodes, photodetectors, chemical sensors, pumps and valves and can then be quantified. LOC are extensively in use for endotoxin detection by NASA. It is termed as Lab-on-a-Chip Application Development-Portable Test System (LOCAD-PTS). It is a handheld device for rapid detection of biological and chemical substances on surfaces aboard the space station. Operaror has to swab surfaces within the cabin, mix swabbed material in liquid form to the LOCAD-PTS, and obtain results within 15 minutes on a display screen. The study's purpose is to effectively provide a rapid indication of biological cleanliness to help crew monitor microorganisms in the cabin environment. LOCAD-PTS is available with three different sensitivities - 0.01, 0.05 and 0.005 EU/ml.

The next remarkable up thrust requires an all printed biosensors, but till now limited success has been achieved in the form of printed biosensors. It has been aptly achieved to some extent by using curable polymer inks and the rest being produced using a combination of vapour deposition of thin layers of metal such as palladium, followed by laser extirpation to pattern these into individual electrodes. The alternatives choices left for successful fabrication includes use of all printed Mn-ZnO batteries (1–10 mA h), monochrome emissive (110 V) (Turner, A.P.F., 2013).

9. Summary and future prospects:

Although conventional standard endotoxin detecting methods are sensitive but are too slow to be completely reliable on them for diagnostic needs. Their slow pace makes them highly undesirable. Therefore, novel techniques with new approaches have to be designed. Although some initial success has been achieved but there is still a long run ahead. Optical sensors accompanied with their range of diverse spectrochemical properties are no doubt strong contenders but their high cost and certain other complexity issues bring them to back foot. Electrochemical techniques are sensitive enough and a lot easier to use but is pushed to corner because of lower selectivity. Likewise, Mass based sensors too has been exploited but till now limited success has been achieved. Although biosensors for detecting endotoxin are currently lacks in increased sensitivity and selectivity. Owing to its miniaturization, low cost, portability, possibility of multiple use, reuse, easy disposable, time saving, early diagnostics and numerous commercial applications their release into market is obvious success.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest and each author share similar contribution to this article.

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Table 1: List of potential gram negative bacteria corresponding with their toxin produced, causal diseases, and its sources.

Pathogenic organism	Toxin	Disease	Source
Escherichia coli,	Endotoxin	Sepsis, Cholecystitis, cholangitis, neonatal meningitis	Contaminated food and water
Salmonella paratyphi		*Campylobacter* enteritis, bacteremia,Food borne illness, typhoid, bacteremia, Endovascular infections	
Shigella dysenteriae	Endotoxin	Bacillary dysentery or shigellosis	Fecal contamination
Vibrio cholera		Gastroenteritis, wound infection, septicemia	
Staphylococcus aureus	Endotoxin	Septic arthritis, Pneumonia, Toxic shock syndrome	Dermal contacts
Brucella melitensis	Endotoxin	Brucellosis , Infective endocarditis	Contaminated fluids of animal origin and their products
Coxiella burnetti	Endotoxin	Q fever, pneumonia	Aerosolization of contaminated fluids
Mycobacterium tuberculosis		Tuberculosis	
Rickettsia rickettsi	Endotoxin	Rocky Mountain spotted fever, Rickettsialpox	Infected tick bite
Treponema pallidum	Endotoxin	Syphilis	Sexual contact
Neisseria gonorrhoeae		Ophthalmia neonatorum systemic neonatal infection, endocervicitis urethritis	

Pasteurella multocida	Endotoxin	Osteomyelitis, and septic arthritis	Animal bite, scratch or lick
Klebsiella pneumoniae, *Pseudomonas aeruginosa*	Endotoxin	Sepsis, pneumonia, bacteremia, thrombophlebitis, urinary tract infection Sepsis, pneumonia, urinary tract infections, and bacteremia	Nosocomial Infections

Table 2: **Depicts technique, sample used and lower detection limit of individual biosensor**

Technique	Organism	Lower detection limit (as reported)	Incubation time (as reported)	Reference
Rabbit pyrogen test	*Escherichia coli*	0.5 EU/ml	180 min	Hoffman et al., 2005
ELISA	-	10 pg/ml	-	Mohanan et al., 2011
Fluoroscence	*Escherichia coli*	10 ng/ml	30 s	Leung et al., 2007
Bio-luminiscence	*Escherichia coli* UKT-B	0.0005 EU/ml	15 min	Noda et al., 2010
Fluorescence	*Escherichia coli 055:B5*	270 pM	5 min	Lan et al., 2012
Surface plasmon resonance	*Bacillus thuringiensis* HD-73	-	-	Okumura et al., 2001
Amperometric	*salmonella minnesota*	0.1 ng/ml	-	Priano et al., 2007
Potentiometric		1000 EU/l	3 h	Inoue et al., 2010
Potentiometric	*Escherichia coli* O55:B5	0.0002 EU/ml	30 min	Yeo et al., 2011
Impedance		0.005 ng/ml	10 min	Su et al., 2012
Impedance	*Salmonella enterica sv.*	10 pg/ml	15 min	Heras et al.,2010
Impedance	*Escherichia coli* O111:B4	0.1 µg/ml	-	Rahman et al., 2013

Impedance	*S. enterica, S. marcescens, K. pneumonie, Escherichia coli*	200 µg/ml	20 min	Oliveira et al., 2011
Impedance	*Escherichia coli 055:B5*	0.01 ng/ml	15 min	Kim et al., 2012
Piezoelectric	-	0.01 pg/ml	-	Xiong et al., 2005
Piezoelectric	-	0.1pg/ml	-	Xiong et al., 2012
Magnetoelastic	-	0.0105 EU/ml	20 min	Ong et al., 2006
Magnetoelastic	*Neisseria meningitidis*	10 ng/µl	5 min	Patel et al., 2010
Magnetoelastic	*Staphylococcus aureus*	10^3 and 10^4 cells/ml		Si-Jing et al., 2010

Table 3: Summarizes a comparison in terms of general advantages and disadvantages associated with individual biosensor.

Sensor	Classification	Advantages	Disadvantages	References
Optical	Surface Plasmon Resonance	• Numerous spectroscopic properties can be exploited • Multiplex capability • Free from electromagnetic interferences • No reference electrode is needed • Less chance of electrical safety hazards	• Subjected to light interference, • Long response times if mass transfer to the reagent • phase is needed • Path length instability due to matrix swelling • Reagent photolability and photoleaching	Wang et al., 2012, Parab et al., 2010, Okumura et al., 2001,
	Fluoroscence	• Ability to fluoresce without the No need for an exogenous enzyme substrates • Spontaneous	• Fluorophore toxicity • IiInterference with normal biological processes	Li et al., 2013, Bateman et al., 2007, Goh et al., 2002

		process Fluorescnce is acquired spontaneously •		
		• Multi-photon excitation		
	Electrochemi-luminescence	• High signal to noise ratio • Absence of background optical signal • Controlled oxidation/reduction reaction • Enhanced selectivity and sensitivity	• Tris(bipyridine)ruthenium(II) ($[Ru(bpy)_3]Cl_2$) dichloride is volatile and toxic. • ($[Ru(bpy)_3]Cl_2$) needs to be in high concentraton ($\simeq$ 100 mM) for higher sensitivity	Hu et al., 2010 Leach et al., 2010
Electrochemical	Amperometric	• Applied potential can be adjustedadjusted to maximize the response for the analyte of interest	• Dependent on analyte flow rate • Free-diffusing redox mediators hinders continuous monitoring of the analyte of interest • Consumption of analyte	Miao et al., 2013, Velusamy et al., 2010, Rabeah et al 2009

	while minimizing the response for interfering analytes •		
	• hHigh signal/noise ratio • Llow consumption of analyte		
Potentiometric	• Linear response • Non-destructive (no consumption of analyte) • Short response time • Immune to color or turbidity changes	• Stable and accurate Rreference electrode is required • Possible Ddeviation from Nernst's equation • Electrodes can be fouled by proteins or other organic solutes • Iinterference by other ions • Electrodes are fragile and have limited shelf life	Zelada-Guillen et al., 2012, Inoue et al., 2010

	Impedance	• Exploits both current and potential parameters • Low noise • High sensitivity • Multiplex • High sensitivity and selectivity	• Complex data analysis for quantification • Expensive • Detection limits are poor	Rahman et al., 2013, Kim et al., 2012, Oliveira et al., 2011,
Mass	Piezoelectric	• High modulus of elasticity • Iinsensitive to electromagneti c fields and radiation • Rapid response time • Reduced influence of interferences	• Lack of an exact correlation between mass addition and frequency change Elevated temperatures causes an additional drop in internal resistance and sensitivity Cannot be used for truly static measurements • Lack of an exact correlation between mass • addition and frequency change for solution phase sensing • Sensitivity to environmental	Ren et al., 2013, Xiong et al., 2012, Guo et al., 2012

		conditions	
Magnetoelastic	• Wireless, *in situ* and *in vivo* monitoring • High sensitivity and dynamic range • Physical robustness	• Difficult to quantify, ifUsually the amplitude of the senor's vibration is very small • In this case it is very hard to get the exact value • Costly and difficult • Achieving miniaturization is difficult	Shinde et al, 2012 Patel et al., 2010, Ong et al., 2006

Table 4: Commercially available sensors

Kit	Model No.	Principle	Time	Lower detection limit
Lonza BioScience's	QCL-1000	Endpoint chromogenic assay	16 minute	0.1 EU /ml
	Pyrogent 5000	Kinetic turbidimetric assay	1 hour	0.01 EU/ ml
	Kinetic-QCL	Kinetic chromogenic assay	1 hour	0.005 EU/ml
	PyroGene	Endpoint fluorogenic assay using rFC	1 hour	0.01 EU/ ml
InvivoGen	HEK-Blue LPS Detection Kit	Specific interaction of TLR4 with LPS	< 1 hour (needs overnight incubation)	0.03 ng/ml
Thermo Scientific	Pierce LAL Chromogenic Endotoxin Quantitation Kit	Endpoint chromogenic LAL assay	30 minutes	0.1 EU/ml
GenScript	ToxinSensor Chromogenic LAL	Endpoint chromogenic	45 minutes	0.005 EU/ml

	Endotoxin Assay Kit	LAL assay		
Sigma	E-TOXATE Kits	Endpoint chromogenic LAL assay	-	0.05 EU/ml
EndoLISA	EndoLISA Endotoxin Detection	ELISA	-	0.05 EU/ml
	EndoZyme rFC Assay	Fluoroscence	-	0.005 EU/ml
Associates of Cape Cod	Pyrosate Kit	Gel clot assay	30 minutes	0.25 EU/ml 1.0 EU/ml

Highlights

- The sensing efficiency of various endotoxin based biosensors has been compared.
- Graphical illustrations of working mechanism for possible biosensor are provided.
- A detailed study on the commercially available biosensor kits has been reviewed.
- Special focus has been given on OMICS (rFC) approach for endotoxin detection.
- Lab-on-a-chip, printed biosensors and LOCAD-PTS has been briefly discussed.

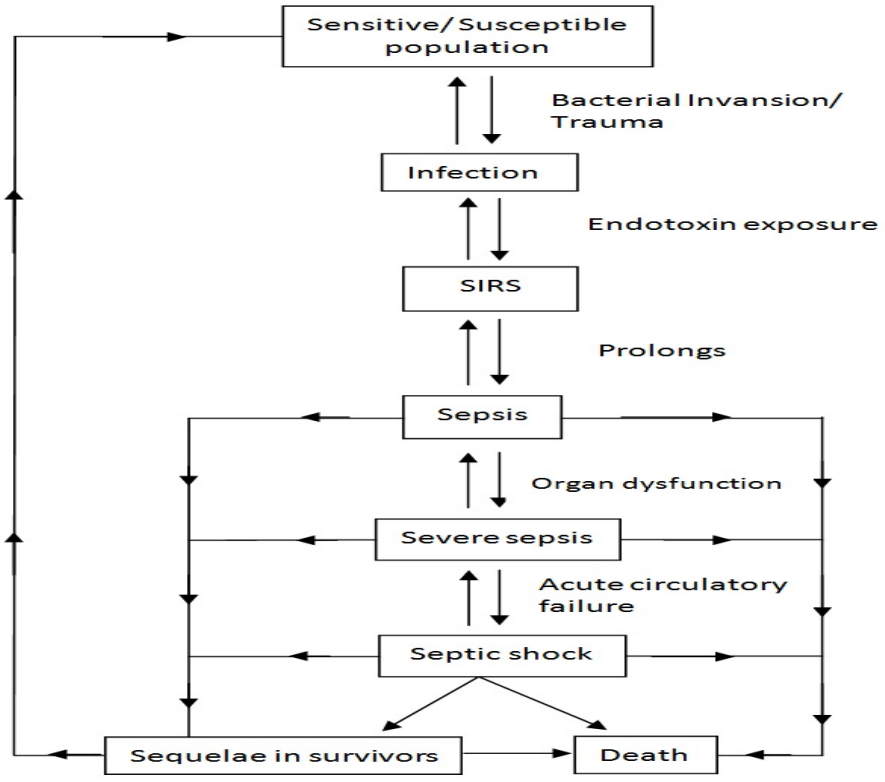

Figure 1: Flow diagram representing the lethal effects of endotoxin. Whenever a sensitive or susceptible population due to bacterial infection or trauma get infected and finally get exposed to endotoxin, they develop SIRS. If left untreated or unchecked it prolongs to sepsis. Sepsis coupled with organ dysfunction lead to severe sepsis, which coalesces with acute circulatory failure to finally give rise to septic shock followed by irreversible sequelae in survivors and death.

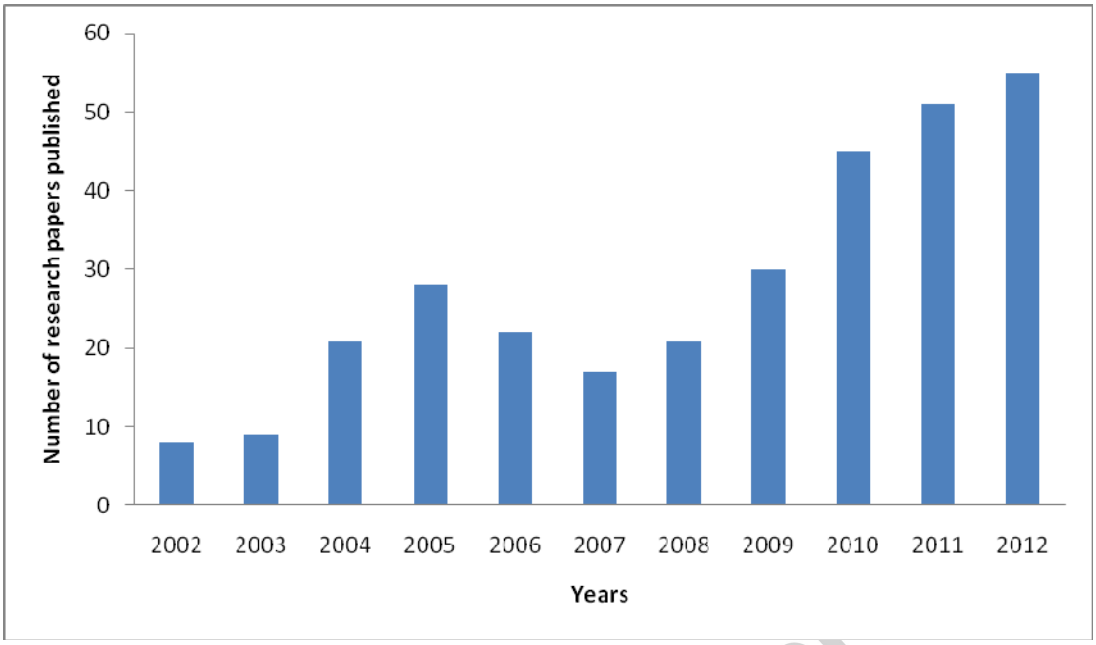

Figure 2: Graph of a search on the term "biosensor based endotoxin detection" during the period 2002 to 2013, using the SCI database. This graph clearly depicts a linear increase in manuscripts published on biosensor based endotoxin detection with the highest number of papers published in the year 2012.

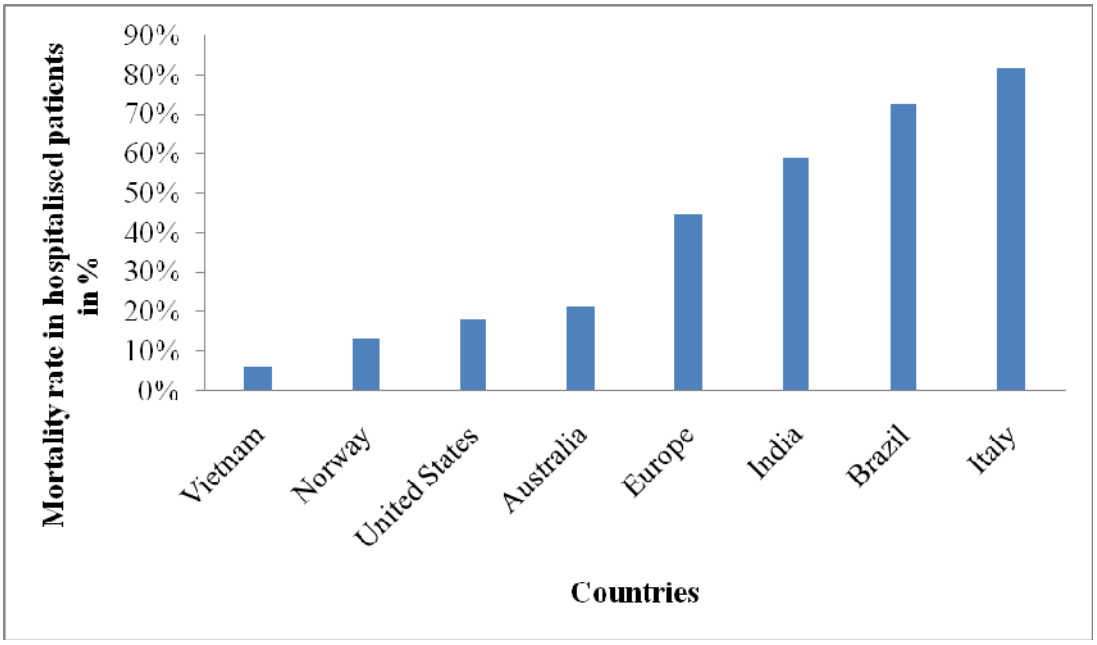

Figure 3: Depicts percentage of mortality in hospitalized patients with respect to different countries.

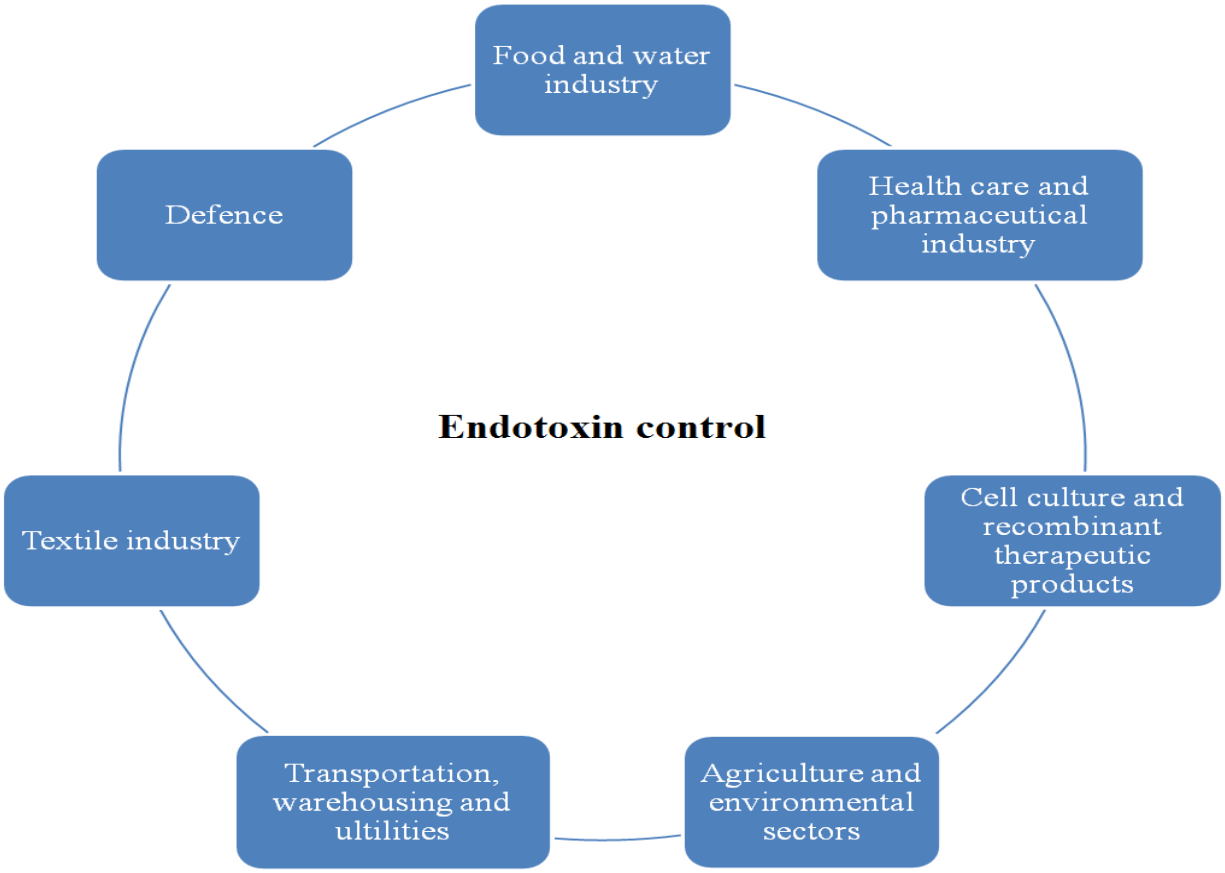

Figure 4: Sectors demanding mandatory endotoxin monitoring

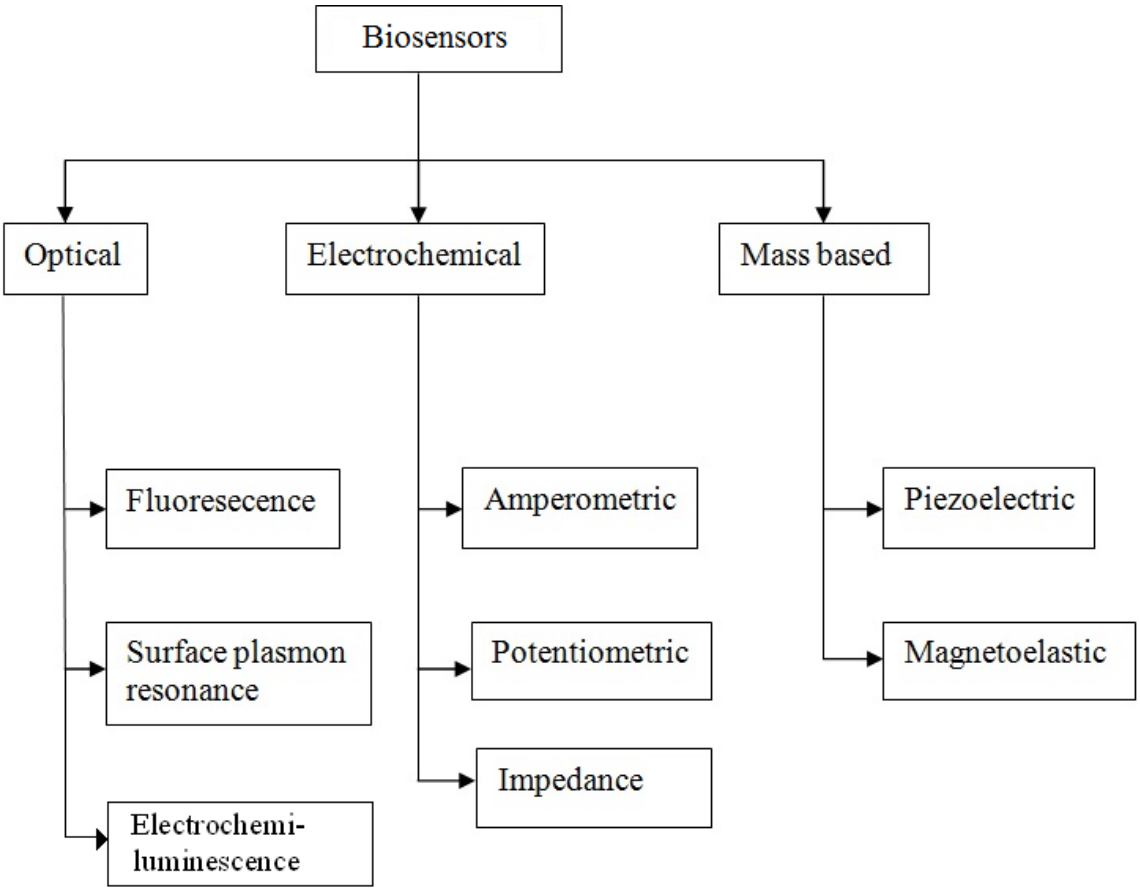

Figure 5: Biosensor Classification based on transduction methods

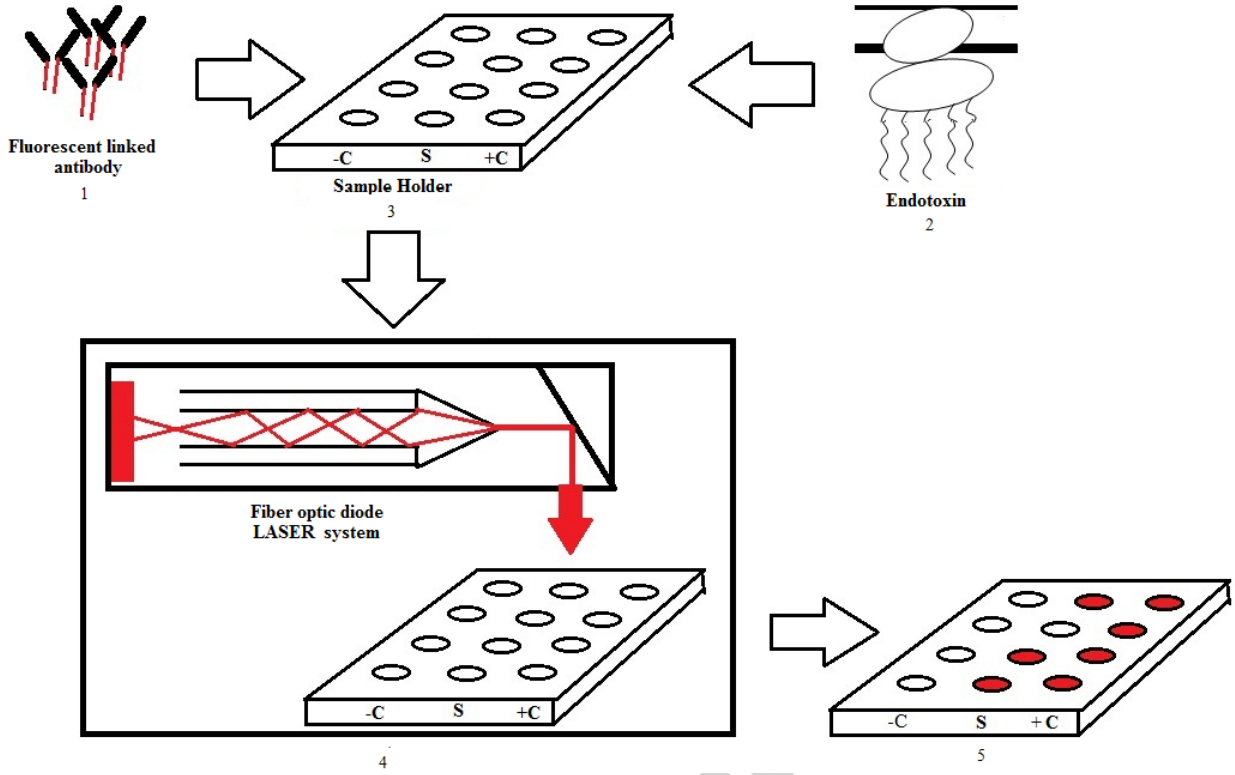

Figure 6: Schematic Representation of Fluorescence detection

(1)Antibodies are tagged with suitable fluorophores. (2) Samples contaminated with endotoxin is added to microtitre plates. (3) Sample holder with corresponding wells for negative control, sample and positive control. After proper incubation, wells are washed with suitable wash buffer to remove any unbound antibodies. (4) Fibre optic diode laser system runs through each well exciting the bound fluoroscent linked antibody. (5) Finally, the amount of fluoroscence exhibited in each well can be correlated to the amount of endotoxin.

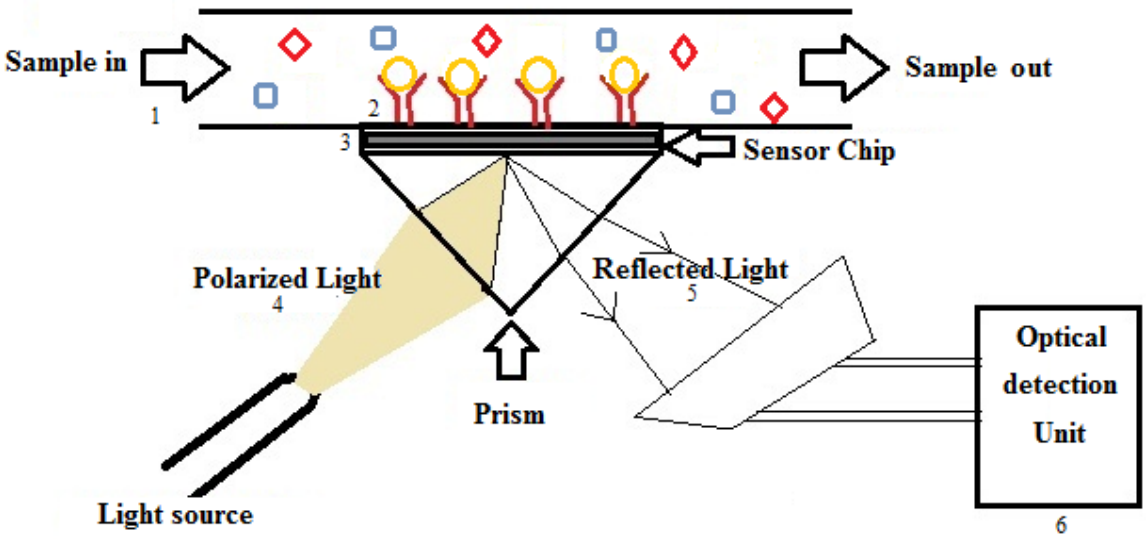

Figure 7: Diagrammatic representation of Surface plasmon resonance based biosensor.

(1) Test analyte is drawn in through the column containing bioreceptors. (2) Bioreceptors (endotoxin binding capture antibodies) are immobilized on the sensor chip. (3) Sensor-glass with high refractive index and an external medium with low refractive index. (4) P-Polarized light with fixed wavelength and in precise angle is incident on sensor-prism interface emanating a highly specific signature of surface plamon resonance. (5) Binding of endotoxin to antibodies results in shift of surface plasmon resonance pattern. (6) Optical detection unit measures any change in the emitted energy pattern of reflected light.

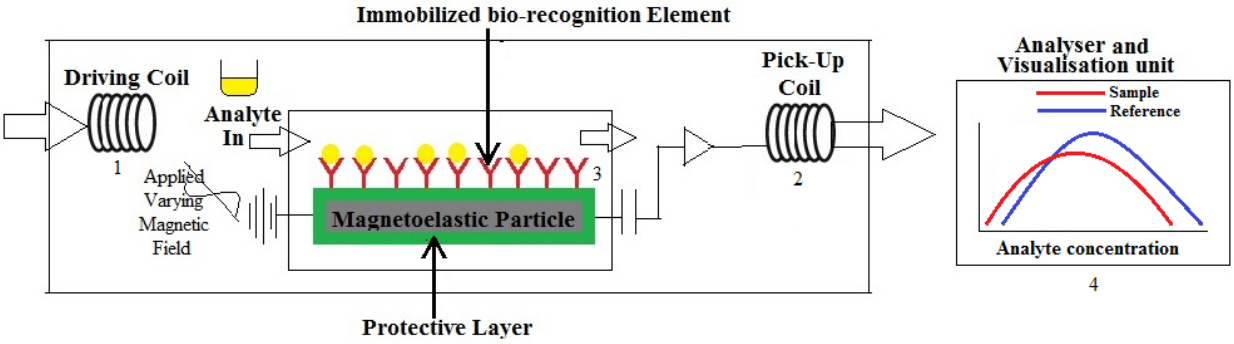

Figure 8: **Systematic representation of magnetoelastic biosensor**

1. Through driving coil varying magnitude of current is passed producing corresponding magnetic fields. (2) Pick up coil receives the resultant signal which is later tranferred to display unit. (3) The native resonance frequency of magnetoelastic biosensor alters on binding of the test analyte to the immobilized bioreceptors. (4) The change in resonant frequency can be viewed as a dip in amplitude, confirming the presence of endotoxin in test analyte.

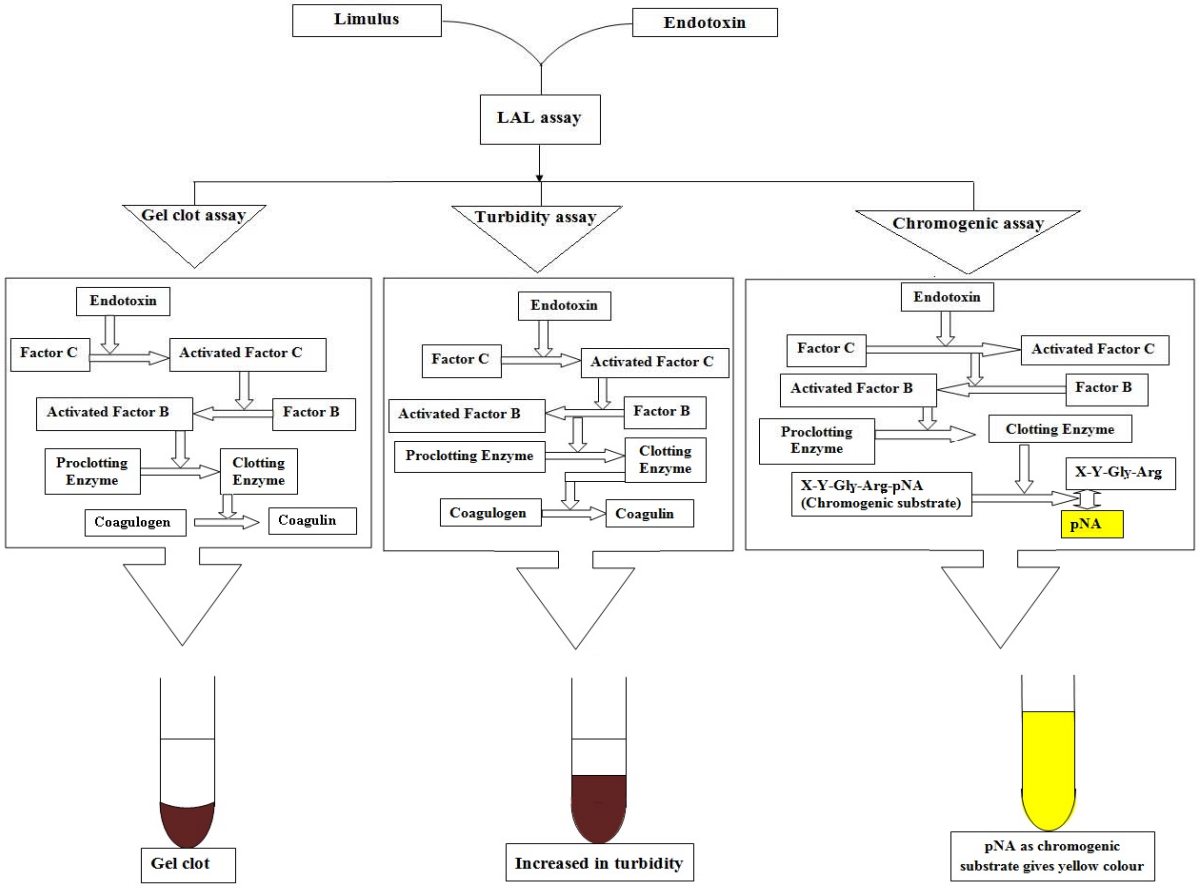

Figure 9: LAL provides a magnanimous way to detect endotoxin without having to sacrifice animals. There are three principal LAL test methods. Flow sheet representing LAL based assay of Gel clot method, Turbidimetric method and Chromogenic methods.

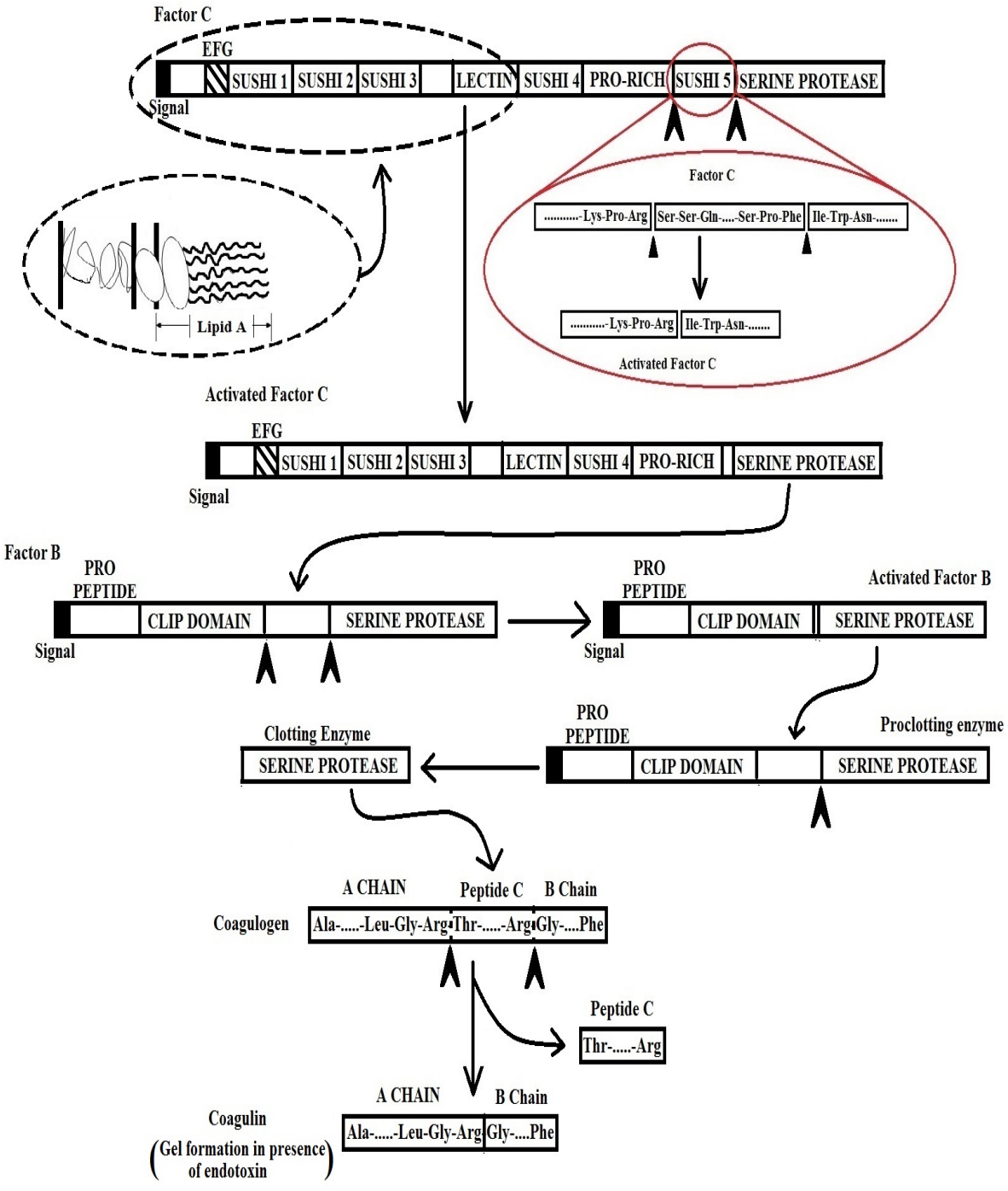

Figure 10: Diagrammatic illustration of enzymatic cascade in hemolymph of horseshoe crab, upon exposure to endotoxin.

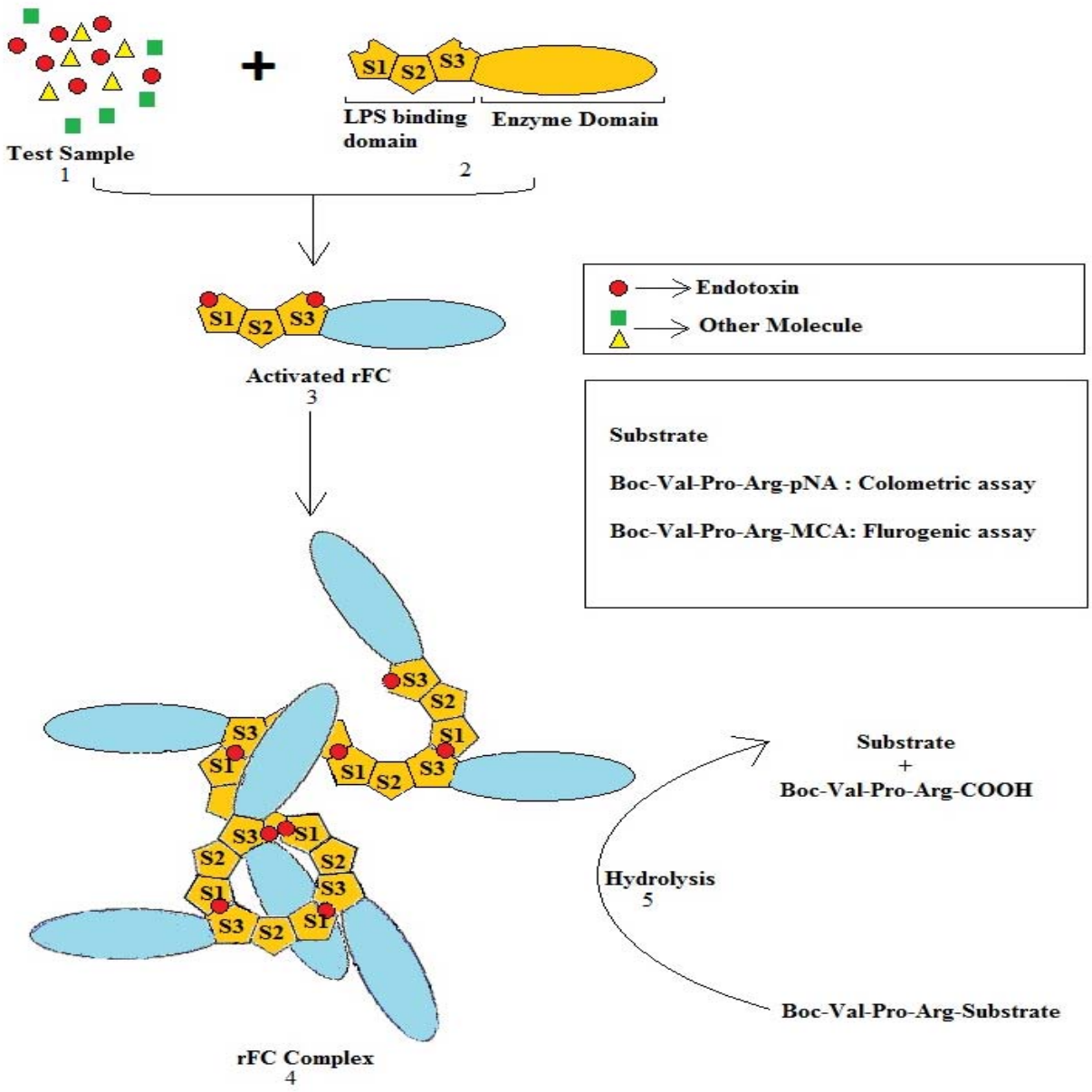

Figure 11: Depicts biologically functional rFC yielding quantifiable results.

1. Test sample with endotoxin and other concomitant molecules. 2. rFC with LPS binding domain and enzyme domain capable of cleaving colorimetric or florogenic substrate. 3. LPS binds in specific multiple binding sites of rFC.4. Binding of rFC to each other forming a complex. 5. Both complex and non-complexed form can hydrolyse fluorogenic and colorimetric substrates.