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An elastography analytical method for the rapid detection of endotoxin†

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We present a flexible analytical method for the study of coagulation systems by monitoring elastography (EG). The rapid detection of endotoxin is achieved by the EG analysis of endotoxin-induced limulus amebocyte lysate coagulation. This method is superior to other methods using the same reagents in not only sensitivity but also detecting time.

Endotoxin is a type of lipopolysaccharide, which is released from the outer membrane of gram-negative bacteria during their growth or by the action of antibiotics.[1,2] It can lead to the secretion of proinflammatory cytokines and cause lethal symptoms such as pyrogenic or septic shock reactions.[3,4] Thus, the rapid and sensitive monitoring of endotoxin levels is essential in potentially contaminated medical devices or pharmaceuticals. Conventional endotoxin assays are based on the reactions between endotoxin and limulus amebocyte lysate (LAL), an aqueous extract of blood cells from the horseshoe crab.[5] This is also the gold standard for endotoxin determination as approved and validated by international Pharmacopoeias. Three main methodologies of LAL assay are now commercially available, including gel-clot, turbidimetric and chromogenic methods, which are all based on photometrical detections.[6–8] These methods are highly sensitive. However, many drawbacks exist. For example, they require skillful operators and are always time consuming. Recently, many ingenious biosensors for the detection of endotoxin have been developed[9] and various signals such as fluorescent,[10,11] colorimetric,[12,13] surface plasmon resonance,[14] luminescent[15] and electrochemical signals[16–19] have been obtained to reveal endotoxin levels. These methods have many merits such as high sensitivity and portability. Nevertheless, fluorescence methods rely on the use of high-cost instruments;[11] furthermore, some colorful or electro-active interferences may lead to the failure of colorimetric and electrochemical methods. Therefore, to avoid these limitations, improved methodologies or techniques are still highly required.

In the presence of endotoxin, LAL reagents undergo a coagulation process, which can be quantitatively detected by monitoring the electrochemical signals[20] or energy storing features of a piezoelectric resonator.[21,22] Inspired by the Thrombelastograph® (TEG®) Hemostasis System 5000 that measures blood states, we have established an analytical system to measure the elasticity during the endotoxin-induced coagulation process. A physical model was constructed and used to interpret the relationship between the coagulation events and the obtained electrical signals, which indicated the elastography (EG). Compared with other methods used to monitor the coagulation process, the circuit design of this EG method was considerably simpler without any amplifiers or filters. Nevertheless, the sensitivity did not lag behind. A limit of detection (LOD) as low as 0.002 EU mL^{-1} was achieved for the detection of endotoxin. This EG analytical method was also compared with other techniques for the endotoxin assay. It was demonstrated that the proposed EG assay is among the most sensitive and time-effective methods. In addition, this analytical method can also be applied for monitoring other coagulation systems and could be extended for more generalized purposes, which may have great potential utility in the future.

Fig. 1a shows the image of the proposed EG analytical system, which consists of a printed circuit board, a cam driving mechanism, a thermostatic bath and an elasticity detecting unit. The elasticity detecting unit is further divided into torsion wire, an angular displacement sensor and the probe (Fig. 1b). The probe suspended beneath the torsion wire is immersed in the sample solution held in a special stationary cylindrical cup, which is oscillated through an angle of 4°45′ driven by the cam driving mechanism at constant temperature. Each rotation cycle lasts 10 seconds. During the coagulation process of the sample, the cup is coupled with the probe to a certain degree. The shear stress generated by the cup rotation

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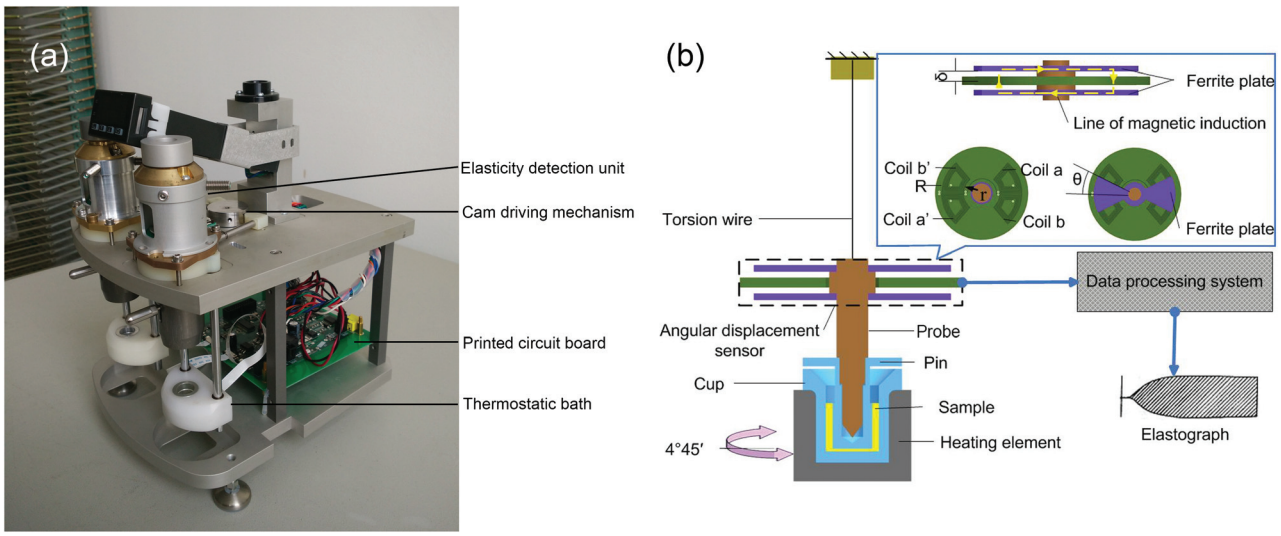

Fig. 1 (a) Image of the developed EG system. (b) Schematic of the elasticity detecting unit.

is transmitted to the probe and the oscillation amplitude of the probe has a direct relationship with the intensity of the generated clot in the cup, which is transformed into displacement information represented by electrical signals using an angular displacement sensor. The signals are then analyzed by the data processing system. When the clot is retracted or dissolved, the connection between the cup and the probe is relieved and the movement of the sample cup is not transmitted to the probe, which may also be reflected by the electrical signals.

The angular displacement sensor consists of coils (including coils a, a′, b, and b′) and two ferrite plates. From electromagnetic theory, the self-inductance of coil (L) can be calculated by

$$L = \frac{W^2}{R_m} \tag{1}$$

$$R_m = \frac{l}{\mu A_0} + \frac{4\delta}{\mu_0 A} \tag{2}$$

in which W stands for the number of coils, R_m is the reluctance of the magnetic circuit (H^{-1}), l is the length of the magnetic core, θ is the angle of the ferrite plate and coil, A_0 is the cross-sectional area of the magnetic core, A is the cross-sectional area of the air magnetizer $\left(A = \frac{\theta}{2}(R^2 - r^2)\right)$, μ is the permeability of the magnetic core, μ_0 is the permeability of air, and δ is the air gap length. Because the magnetic resistance of the magnetic core is considerably smaller than that of the air gap, which is negligible, L has the following approximate value:

$$L = \frac{W^2\mu_0 A}{4\delta} = \frac{W^2\mu_0(R^2 - r^2)}{8\delta}\theta \tag{3}$$

If the ferrite plate rotates in a counterclockwise angle (φ) compared with the initial position, the self-inductances of the two coils are given by:

$$L_a = \frac{W^2\mu_0(R^2 - r^2)}{8\delta}(\theta + \varphi) \tag{4}$$

$$L_b = \frac{W^2\mu_0(R^2 - r^2)}{8\delta}(\theta - \varphi) \tag{5}$$

$$\Delta L = L_a - L_b = \frac{W^2\mu_0(R^2 - r^2)}{4\delta}\varphi \tag{6}$$

The probe oscillating angle φ is detected by a highly accurate rotating stage (RSA100) with the displacement resolution of 0.00125°. It was observed that ΔL is proportional to φ. The two coils are connected with the adjacent bridge arm of the bridge. The output electrical signal (y) stands for the variation of the self-inductance of the coil (ΔL). The relationship between y and φ is obtained from the experimental data:

$$y = 11.847\varphi \tag{7}$$

φ can be calculated from the classic formula:

$$\varphi = \frac{Tl}{GI_p} = \frac{32Tl}{G\pi D^4} = 11.609T \tag{8}$$

in which T is the torque, I_p is the polar moment of inertia, l is the length of the tungsten wire (21.7 mm), D is the diameter of the tungsten wire (0.175 mm), and G is the shear modulus (tungsten: 409 GPa).

The output signal (y) is also equal to the value below:

$$y = 137.079T \tag{9}$$

In addition, the relationship between T and the shear stress of the pin (τ) is as follows:

$$T = 2\pi RhR\tau + \int_0^R 2\pi r^2\tau dr = \left(2\pi R^2 h + \frac{2}{3}\pi R^3\right) \quad \tau = 0.004641\tau \tag{10}$$

in which R is the pin radius (3 mm) and h is the depth of the pin immersed in the sample (8 mm).

The output signal (y) can be rewritten as:

$$y = 0.636\tau \quad (11)$$

From eqn (7), (9) and (11), we can conclude that the electrical signal y is proportional to the altered angle, torque and shear stress of the pin. Therefore, the oscillation amplitude of the probe can be well represented by the electrical signal y. In addition, the elastography constituted by the recorded y during the detection period may contain abundant information on the coagulation process.

We choose endotoxin-induced LAL coagulation to check the utility of this EG analytical method. LAL coagulation involves cascade enzyme reactions (Fig. S1†).[23] In brief, endotoxin activates factor C, which in turn activates factor B. The proclotting enzyme can thus be transformed to the clotting enzyme, which produces coagulin. Finally, the elasticity is changed, which can be detected by this proposed EG analytical method. From a typical elastography, the oscillation amplitude increases rapidly and then decreases slowly, which is the sign of clotting and fibrolysis action (Fig. S2†). Such biological events are always undetectable by other methods for endotoxin assay, which only achieve the qualitative or quantitative detection of endotoxin. On the contrary, our EG analytical method provides comprehensive information about endotoxin-induced LAL coagulation and is superior to the other methods because it provides simple fluorescent, colorimetric or electrochemical signals.

In order to obtain the detection range and LOD of the analytical method, different amounts of endotoxin were employed to induce coagulation in the reaction cup. Fig. 2a shows that more endotoxin leads to a faster coagulation. A linear relationship is established between the logarithms of the time to reach the oscillation amplitude of 0.1 and the endotoxin concentration (Fig. 2b). The regression equation is $y = 2.660 - 0.234x$, in which y is the logarithm of time and x is the logarithm of the endotoxin concentration; $n = 3$, $R^2 = 0.990$. The linear range obtained was from 0.01 to 10 EU mL^{-1} and LOD was down to 0.002 EU mL^{-1}. Note that EU is a unit expressing endotoxin activity. We then compared the analytical performance of this method with that of some recently reported endotoxin assays (Table 1). It was observed that the sensitivity of this method is among the highest ones.

The proposed EG assay for the detection of endotoxin was also challenged with real samples to confirm its practical utility. Table 2 lists the detected results of physiological saline (PS) and cell culture medium samples spiked with different amounts of endotoxin. Satisfactory recoveries were obtained. The relative errors were also rather small, which is promising for the accuracy and fine practicality of this method. However, when the samples contained some clot or gelatinous contents, the EG signal could not completely reflect the endotoxin-induced coagulation, which is one limitation of this method,

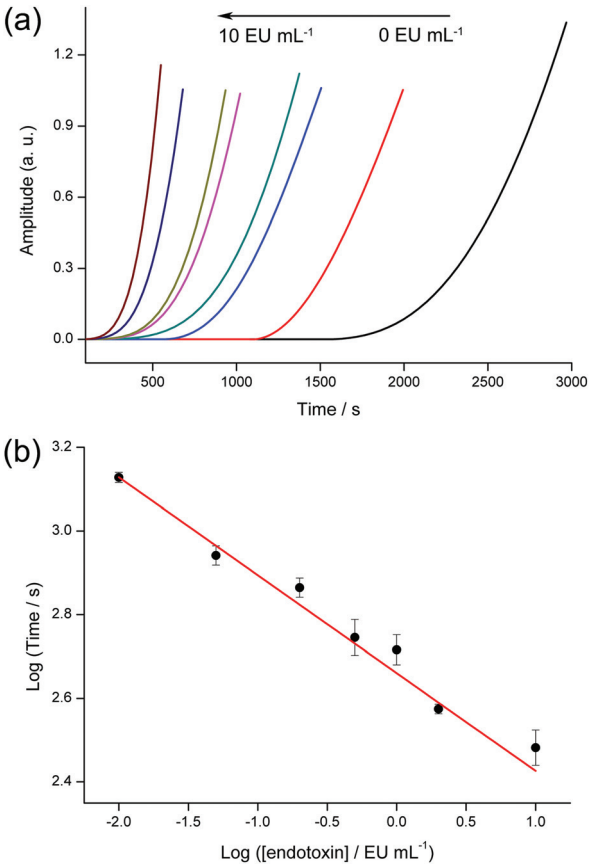

Fig. 2 (a) EG curves of the cases with different amounts of endotoxin: 0, 0.01, 0.05, 0.2, 0.5, 1.0, 2.0, 10.0 EU mL^{-1} (from right to left). (b) The linear relationship between the EG signal *versus* the logarithmic value of the endotoxin concentration.

and other endotoxin assay methods should be used to complement the detection.

To evaluate the convenience of this EG method for the endotoxin assay, the detection time durations of the EG method, an electrochemical method, and a turbidimetric method were compared. The electrochemical detection procedure was carried out according to our previously reported work.[20] The turbidimetric method was performed using the kinetic turbidimetric LAL reagent kit from Chinese Horseshoe Crab Reagent Manufactory Co., Ltd (Xiamen, China). The three methods measured variations of the turbidity, the electrochemical signals of potassium ferricyanide, and the electric signals representing elasticity during the endotoxin-induced coagulation. We prepared five standard endotoxin samples with the same reagents and protocols to check the identity of the three methods. As shown in Fig. 3, the results of the three methods are consistent with each other. Moreover, the EG assay is considerably more time-effective compared with the other two methods. The detecting time could be reduced by 12%–55%.

Coagulations are common physiological phenomena and reflect abundant information about biological systems. For example, the deficiency, inhibition or activation of clotting

Table 1 Recent endotoxin assay methods

Technique	Mechanism	LOD	Ref.
Pyrogen	Rabbit pyrogen test	0.5 EU mL^{-1}	24
Differential pulse voltammetry	Recombinant factor C zymogen	1 EU mL^{-1}	25
Differential pulse voltammetry	Cascade LAL reaction to generate *p*-nitroaniline	0.01 EU mL^{-1}	2
Amperometry	Electrochemical detection of gel-clot	0.03 EU mL^{-1}	20
Amperometry	Competitive assay	0.07 EU mL^{-1}	26
Electrochemical impedance spectroscopy	Endotoxin neutralizing protein	0.03 EU mL^{-1}	27
Flow cytometry	Magnetic aptasensor	3.6 ng mL^{-1}	11
Surface plasmon resonance	Molecularly imprinted polymers	15.6 ng mL^{-1}	14
Colorimetry	Gold nanoparticles aggregation	33 nM	28
Elastography	Endotoxin-induced elastography variation	0.002 EU mL^{-1}	This work

Table 2 Determination of endotoxin levels spiked in PS samples and in cell culture medium

Samples	Added (EU mL^{-1})	Detected (EU mL^{-1})	Recovery (%)	Relative error (%)
PS	0.1	0.108	108	6.214
	0.5	0.527	105.4	3.751
	1.0	1.045	104.5	5.419
	2.0	2.038	101.9	7.844
Cell culture medium	0	0.422	—	2.381
	0.1	0.516	94.0	4.359
	0.5	0.935	102.6	6.321
	1.0	1.409	98.7	5.636

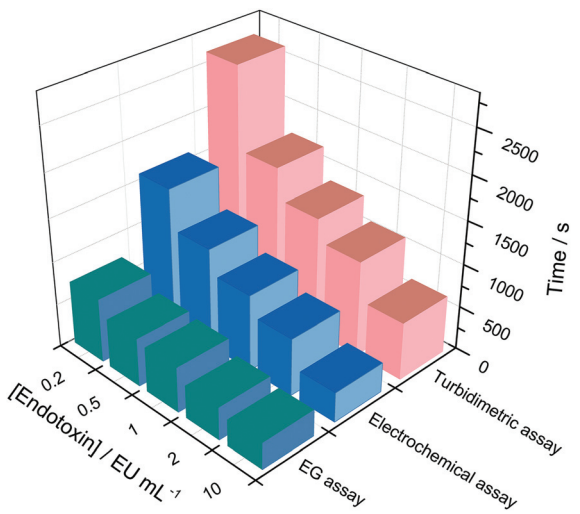

Fig. 3 Comparison of the proposed EG assay, an electrochemical method and a turbidimetric method.

factors can be concluded from abnormal coagulations such as prolongation of the coagulation time. Herein, we proposed a general analytical method for the study of coagulation systems by monitoring elastography. As an application of this method, we realized the measurement of endotoxin-induced LAL coagulation. This biosensor for endotoxin has many advantages such as high sensitivity, fast response and fine reproducibility. In view of these advantages of the EG assay for endotoxin, we anticipate that this analytical method could be expanded to detect a wide range of coagulation systems, which may have great potential utility in both fundamental research and biomedical diagnostics in the future.

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