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Fluorescence Spectroscopy in Study of Human-like Collagen and Endotoxin Interactions

PEI MA[1,2,†], YUAN-YUAN YU[1,2,3,†] and DAI-DI FAN[1,2,*]

[1]Shaanxi Key Laboratory of Degradable Biomedical Materials, School of Chemical Engineering, Northwest University, Taibai North Road 229, Xi'an 710069, Shaanxi Province, P.R. China
[2]Shaanxi R & D Center of Biomaterials and Fermentation Engineering, School of Chemical Engineering, Northwest University, Xi'an 710069, Shaanxi Province, P.R. China
[3]Shaanxi Xueqian Normal University, Xi'an 710100, Shaanxi Province, P.R. China

*Corresponding author: Fax: +86 29 88302348; Tel.: +86 29 88305118; E-mail: fandaidi@nwu.edu.cn
†These authors contributed equally to this work.

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Removal of endotoxins from recombinant proteins is a critical and challenging step in the preparation of intravenous therapeutics, because of their potent biological activities causing pyrogenic and shock reactions. However, the interaction between protein and endotoxin makes it more complicated to remove endotoxin from target protein. In this paper, the binding of endotoxin with human-like collagen was investigated by fluorescence spectroscopy and the effect of surfactants on the human-like collagen-endotoxin complex was studied. The fluorescence titration results revealed that endotoxin has a strong ability to quench the intrinsic fluorescence of human-like collagen through static quenching mechanism according to the Stern-Volmer equation. The binding constants and the corresponding thermodynamic parameters at different temperatures were calculated, which showed that the hydrophobic force played a major role in the interaction of endotoxin with human-like collagen. In addition, Triton X-100 was described to remove endotoxin from human-like collagen. All these results and theoretical data clarified that endotoxin could interact with human-like collagen to form the complex and Triton X-100 has proven to be effective in the process of endotoxin removal, which could be a useful guideline for further endotoxin removal from recombinant protein.

Keywords: Human-like collagen, Endotoxin, Fluorescence, Surfactant, Triton X-100.

INTRODUCTION

Endotoxin is an integral part of the outer cellular membrane of Gram-negative bacteria and they are generally considered lipopolysaccharides (LPS). It has been recognized as a major cause of the pyrogenic and shock reactions at very low concentrations in human beings and many animals during the administration of biotherapeutics, *e.g. via* intravenous application of a contaminated medicament[1-4]. As Gram-negative bacteria, *Escherichia coli* is still a production host of choice to produce recombinant DNA products such as peptides and proteins in the biotechnology industry. Therefore, the removal of these physiologically active agents from final bioproducts is essential before their therapeutic application, especially in situations where endotoxins bind product proteins.

Endotoxins consist of three biologically, chemically, genetically and serologically different parts. These are a non-polar lipid component, called lipid A, a core oligosaccharide and a long heteropolysaccharide chain representing the surface antigen (O-antigen)[5]. The most conservative part of endotoxins is lipid A, showing very narrow structural relationship in different bacterial genera. It consists of a β-1, 6 linked disaccharide of glucosamine, covalently linked to 3-hydroxy-acyl substituents with 12-16 carbon atoms *via* amide and ester bonds (Fig. 1). For the removal of endotoxins it is important to point out that the core region close to lipid A and lipid A itself are partially phosphorylated ($pK_1 = 1.3$, $pK_2 = 8.2$ of phosphate groups at lipid A), thus endotoxin molecules exhibit a net negative charge in common protein solutions[6]. Therefore, endotoxin is to be expected to interact with proteins, especially the net-positively charged protein as well as generally with hydrophobic moieties.

Fluorescence quenching is an important technique to study the interaction between small molecule and bio-macromolecule with its exceptional sensitivity, selectivity, convenience and abundant theoretical foundation[7,8]. It is a process, in which fluorescence intensity of the solute decreases by variety of molecular interactions such as excited state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional quenching[9,10].

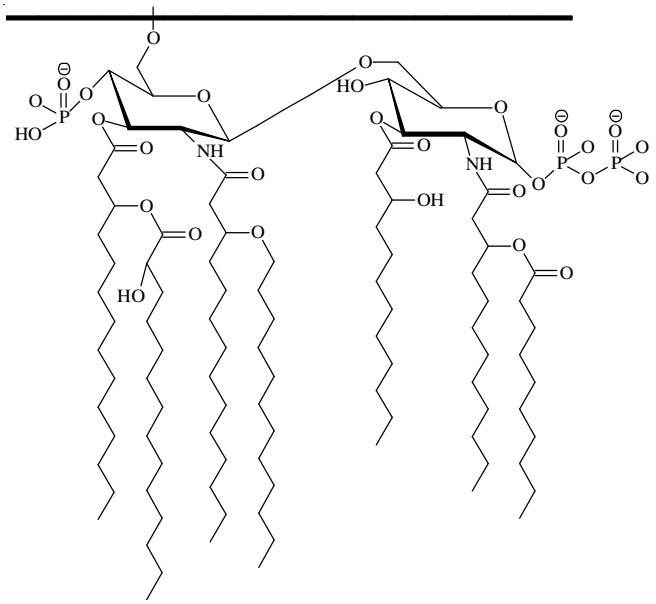

Fig. 1. Schematic view of the chemical structure of lipid-A

Human-like collagen (HLC) is a recombinant protein expressed by recombinant *Escherichia coli* BL 21 containing human collagen's cDNA transcribed reversely from mRNA[11,12]. Furthermore, HLC has been used for the construction of artificial bone, skin, hepatic and vascular tissue engineering for its excellent biocompatibility[13-16].

In the paper the existence of the HLC-endotoxin complex was determined and fluorescence spectrometry was applied to study the interaction between HLC and endotoxin. Besides, the role of surfactants to the HLC-endotoxin complex was also studied. The investigation of protein-endotoxin complex could have great significance and provide a guide for the endotoxin removal from recombinant protein.

EXPERIMENTAL

Human-like collagen (HLC) was produced in our lab (China patent number: ZL01106757.8). Control standard endotoxin (CSE) was purchased from Xiamen Houshiji Co. Ltd. *Tris* and Triton X-100 were obtained from the company of Amresco and Genview, respectively. All other reagents were of analytical grade. Glassware was heated in the oven at 210 ºC for 3 h. All solutions were prepared with endotoxin-free water.

Endotoxin assay: To determine the concentration of endotoxin a LAL (*Limulus* amebocyte lysate) test kit Pyrosate (Associate of Cape Cod, Inc., Woods Hole, MA, USA) was used with the sensitivity of 0.25 EU mL^{-1}. A positive reaction was indicated by the formation of a solid gel that did not collapse upon inversion of the tube. A negative result was characterized by the absence of such a gel.

Assay of human-like collagen: The concentration of HLC was measured by immunodetection method according to the description in "QIA express Detection and Assay Handbook" (Qiagen).

Interaction between human-like collagen and endotoxin: 1 mL Q resin was installed in 5 mL eppendorf tube and 3 mL 1 M NaOH was added. After standing for 0.5 h and centrifugation for 5 min at 7000 g, the supernatant was aspirated carefully from Q resin. Next the resin was washed with endotoxin-free water to neutral pH and then centrifuged and separated from the supernatant. The resin was conditioned with endotoxin-free *Tris*-HCl buffer (pH 7.5, 0.05 M) and sample a, b and c (Table-1) was then added, respectively. The mixture in the tubes were gently shaken for 1 h at 4 ºC and then centrifuged. Finally the supernatant was assayed by LAL test kit. All measurements were performed in triplicate.

TABLE-1
HLC AND ENDOTOXIN CONCENTRATION OF SAMPLE a, b AND c

Sample	HLC conc. (mg mL^{-1})	Endotoxin conc. (EU mL^{-1})
a	1	0
b	0	5
c	1	5

Fluorescence spectrometry: The solution of human-like collagen was prepared with endotoxin-free water to concentration of 1 mg mL^{-1}. Five aliquots of 5 mL protein solutions with 0, 5, 10, 15 and 20 EU mL^{-1} CSE were prepared and agitated vigorously for 15 min.

The excitation wavelength of 279 nm and the emission wavelength of 305 nm were chosen and the emission spectra in the range of 280-400 nm were recorded with the slit width of 1.5 nm.

Surfactant effects on the HLC-endotoxin complex: 1 mg mL^{-1} HLC solution was prepared by endotoxin-free *Tris*-HCl buffer (pH 7.5, 0.05 M). 1 % (V/V) SDS, TWEEN-80, TWEEN-20, Triton X-114, Triton X-100 and STAB were added to six tubes with 2 mL protein solution contained 5 EU mL^{-1} CSE, respectively, then agitated vigorously for 15 min and incubated at 4 ºC for 0.5 h. After static adsorption equilibrium of Q resin, the remaining procedure was conducted according to part 2.4.

RESULTS AND DISCUSSION

Interaction between human-like collagen and endotoxin: The LAL assay is the gold standard for endotoxin detection and quantification. As seen in Table-2, there was no potential endotoxin contamination in the sample a, and 5 EU endotoxin could be absorbed by the 1 mL Q resin in the sample b. However, when 5 EU endotoxin was mixed with HLC solution, it could not be effectively absorbed by 1 mL Q resin (sample c). The results indicated that human-like collagen could interact with endotoxin and the HLC-endotoxin complex forms, which may result in the masking of endotoxin molecules and consequently to a partial escape from removal procedures.

TABLE-2
LAL RESULTS OF HLC-ENDOTOXIN INTERACTION

Sample	LAL result
a	-
b	-
c	+

It has been reported that the electrostatic force may be the main mechanism of the interaction between endotoxin and protein[17]. However, the isoelectric points of HLC and endotoxin are about 6 and 2, respectively[18] and both of them

are negatively charged under the experimental condition (pH 7.5). Therefore, the electrostatic force could not be the predominant factor in this research.

Fluorescence spectrometry: There are three types of fluorophores in protein, namely, tryptophan residue, tyrosine residue and phenylalanine residue[19]. Because of very low quantum yield of phenylalanine, normally fluorescence of tryptophan and tyrosine residue is investigated in researches. Previous studies reveal that there is no tryptophan residue in the molecule of human-like collagen. Generally, when 280 nm excitation wavelength is used, the intrinsic fluorescence of protein comes from both tryptophan and tyrosine residues, whereas 293 nm wavelength only excites tryptophan residues[19,20]. According to the wavelength at which the maximum fluorescence intensity decreases we can find which residue is located in or near the binding position. In this study, 280 nm was chosen as the excitation wavelength throughout the experiment. Tyrosine is the dominant intrinsic fluorophore in human-like collagen. In fact emission of HLC is dominated by tyrosine which absorbs at longer wavelength and displays the larger extinction coefficient.

As shown in Fig. 2, HLC had a strong fluorescence emission band by fixing the excitation wavelength at 280 nm and the fluorescence emission intensity of HLC decreased regularly as the concentration of endotoxin increased. These results indicated that endotoxin could quench the intrinsic fluorescence of HLC.

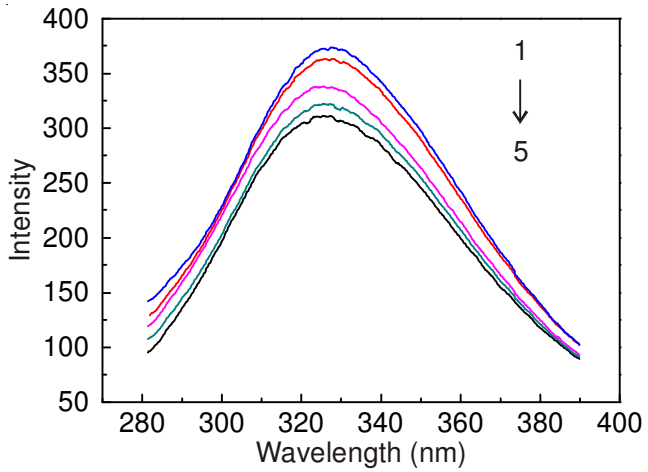

Fig. 2. Fluorescence quenching spectra of HLC with various concentrations of endotoxin. (C_{HLC} = 1 mg mL^{-1}; curves 1 → 5 (C_{et} × EU mL^{-1}): 0, 5, 10, 15 and 20, respectively)

Fluorescence quenching is usually classified into static quenching and dynamic quenching. The fluorescence quenching data are usually analyzed by the Stern-Volmer equation[19], which allows for calculating of quenching constants.

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. K_{sv} is the Stern-Volmer quenching constant. In this paper, [Q] is the concentration of endotoxin.

In the range of endotoxin concentration from 5 to 20 EU mL^{-1}, the curve in Fig. 3 exhibits good linear relationships with correlation coefficient R^2 = 0.982. According to eqn. (1), the quenching rate constant K_{sv} (L mol^{-1} S^{-1}) was determined by linear regression of a plot of F_0/F *vs*. [Q]. Generally, the maximum scatter collision quenching constant K_{sv} of various kinds of quenchers with biopolymer is 100 L mol^{-1} S^{-1}. However, K_{sv} of HLC quenching procedure initiated by endotoxin are greater than 100 L mol^{-1} S^{-1}, which means that the process is static.

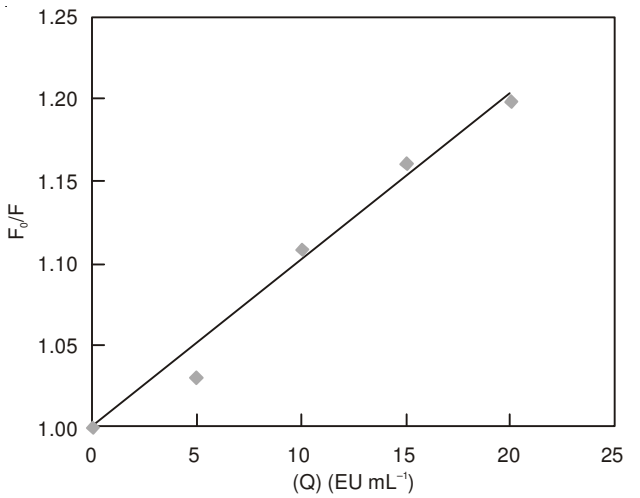

Fig. 3. Stern-Volmer curves of HLC quenched by endotoxin. (C_{HLC} = 1 mg mL^{-1})

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the following equation[21,22]:

$$\log\frac{(F_0 - F)}{F} = \log K_A + n\log[Q] \quad (2)$$

where, K_A is the binding constant to a site, n is the number of binding sites per HLC molecule and [Q] is the concentration of endotoxin. According to eqn (2), the binding parameters can be obtained by the plot of log[(F_0-F)/F] against log[Q].

The fitted plots of log[(F_0-F)/F] *versus* log[Q] were obtained by the measured fluorescence data at different temperatures. The values of K and n were calculated from the values of intercept and slope of the plots (Fig. 4), respectively and the corresponding results are listed in Table-3. The value of n is about 1.5, which exhibits that HLC and endotoxin formed the complex of mol ratio 2:3.

The binding forces between protein and small molecule may include hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, *etc*.[23]. In order to clarify the interaction between HLC and endotoxin, the thermodynamic parameters were calculated based on the binding constant at different temperatures. Because the temperature effect is pretty small, the enthalpy change (ΔH) can be regarded as constant if the temperature range studied is not too wide. Therefore, the values of ΔH, ΔS (entropy change) and ΔG (Gibbs free energy change) can be determined from the following equations:

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

$$\Delta G = -RT\ln K \quad (4)$$

$$\Delta H = \frac{RT_1T_2\ln(K_2/K_1)}{T_2 - T_1} \quad (5)$$

TABLE-3
BINDING CONSTANT AND THERMODYNAMIC PARAMETERS FOR THE SYSTEM OF HLC AND ENDOTOXIN

t/°C	K (× 10^4 L mol^{-1} s^{-1})	n	ΔH (KJ mol^{-1})	ΔS (JK^{-1} mol^{-1})	ΔG (KJ mol^{-1})
25	2.4 × 10^{10}	1.36	7.23 × 10^5	2.42 × 10^3	-23.9
30	2.95 × 10^{12}	1.65		2.39 × 10^3	-28.7

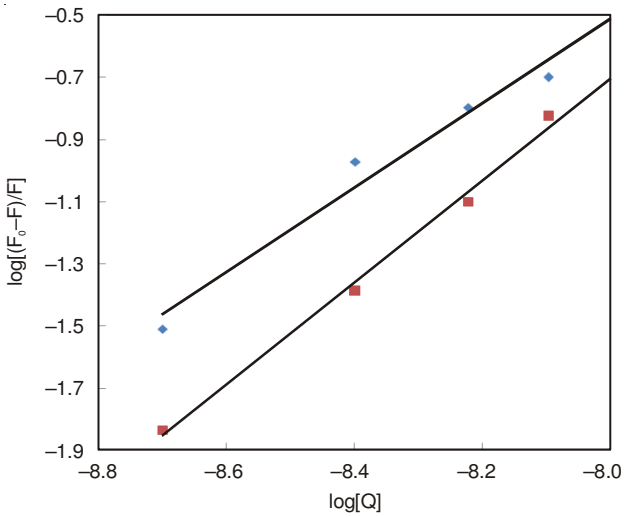

Fig. 4. Relationship of log[(F_0-F)/F] *versus* log[Q] (a: 25 °C, b: 30 °C). [Q]: C_{et} × g L^{-1}, C_{HLC} = 1 mg mL^{-1}

where K is the binding constant at the corresponding temperature[24], R is the gas constant and T is the absolute temperature.

The values of ΔH, ΔS and ΔG are listed in Table-3. For the system of HLC and endotoxin, ΔH and ΔS are positive and ΔG is negative. The negative values of ΔG and positive ΔH mean that the binding process was spontaneous and the formation of the HLC-endotoxin complex was an endothermic reaction. According to the rules on relationship between the sign and magnitude of the thermodynamic parameters and various kinds of interaction in the binding process[25], the positive ΔH and ΔS indicate that the reaction is entropy-driven and the acting force for the binding reaction between HLC and endotoxin is mainly hydrophobic forces.

The major hydrophobic part of the endotoxin is lipid A and proline and hydroxyproline residues in HLC are both hydrophobic amino acids. These hydrophobic groups might interact with each other and form the HLC-endotoxin complex.

Surfactant effects on the HLC-endotoxin complex: As seen in Table-4, Triton X-100, Triton X-114 and STAB could effectively dissociate the HLC-endotoxin complex. The three surfactants have similar structure (Fig. 5) and they are mainly composed of hydrophilic head and hydrophobic long-chain. It could be assumed that endotoxin interacted with the hydrophobic region of the surfactant and alleviated the hydrophobic interaction between endotoxin and HLC, thus released the HLC molecule simultaneously. The mechanism could be represented in Fig. 6. However, SDS with the similar structure could not dissociate the HLC-endotoxin complex. The reason might be that the electrostatic repulsion might be the dominant factor when endotoxin encountered SDS, which contained the negative charged sulfate.

As a non-ionic surfactant, Triton X-100 has less impact on the protein conformation and lower cytotoxicity than ionic surfactant. Besides, it is low cost compared with Triton X-114 and has been widely used in the cell-mediated immunity. Therefore, Triton X-100 was chosen for the endotoxin removal during the follow-up experiment.

TABLE-4
RESULTS OF LAL ASSAY FOR DIFFERENT SURFACTANTS

Surfactants (type)	Endotoxin conc. (EU mL^{-1})	LAL results
SDS (anionic)	5	+
Tween-80 (nonionic)	5	+
Tween-20 (nonionic)	5	+
TritonX-114 (nonionic)	5	-
TritonX-100 (nonionic)	5	-
STAB (cationic)	5	-

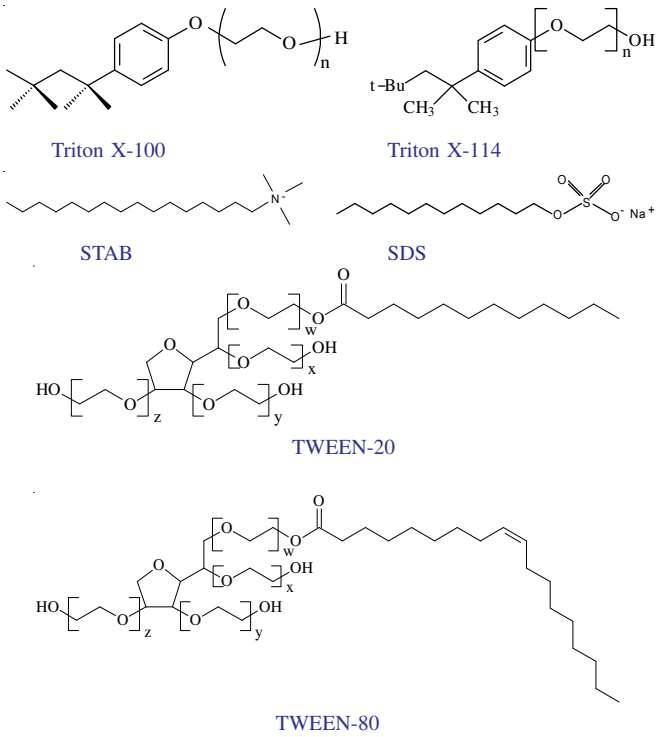

Fig. 5. Chemical structure of the surfactants used in this study

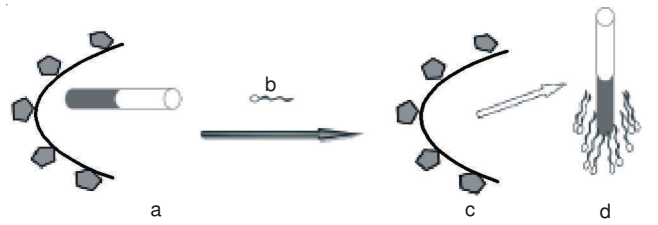

Fig. 6. Mechanism of the interaction between surfactant and the HLC-endotoxin complex. (a: HLC-endotoxin complex; b: surfactant; c: HLC molecule; d: hydrophobic interaction between surfactant and endotoxin. The hydrophobic group is represented by grey and the hydrophilic group as white)

Conclusion

This study presents the interaction of endotoxin with human-like collagen using fluorescence emission spectrum and the effect of surfactant on the endotoxin-HLC complex. It was shown that the fluorescence of HLC has been quenched for reacting with endotoxin. Static quenching mechanism was involved in the binding procedure. Human-like collagen and endotoxin formed the complex of mole ratio 2:3 and the binding process is spontaneous and is predominantly owing to hydrophobic forces. Besides, Triton X-100 was observed to have the ability of dissociating the endotoxin-HLC complex. These results may contribute to some useful evidence for further understanding the photodynamic process and mechanism of HLC-endotoxin complex and provide great importance for endotoxin removal from recombinant protein.

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