Endotoxin Characterization – Effects of Metal Ions on Endotoxins Zeta Potential under Various Concentrations and pH Conditions

Elvina Clarie Dullah, Clarence M. Ongkudon#

Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA.
Corresponding author. E-Mail: clarence@ums.edu.my; Tel: +6088-320000; Fax: +6088-320993.

ABSTRACT Endotoxin has unique characteristics such as ability to form a stable interaction with other biomolecules as well as high temperature and pH tolerance. These characteristics make its removal difficult especially during the production of biotherapeutic drugs. Endotoxins contamination in biopharmaceutical products can result in sepsis, tissue damage, inflammation, fever and even lead to death. The choice of an efficient method in removing endotoxins from biopharmaceutical products is rather perplexing as the method could affect the biological properties of the products. Previous studies have found that divalent metal ions, such as zinc sulphate, calcium chloride and magnesium chloride have better aggregative effects on endotoxins compared to that on plasmid DNA, thus, these metal ions may be potentially useful in the selective removal of endotoxin from biopharmaceutical products. The main focus of this study was to investigate the effects of metal ions effects on endotoxins under various parameters such as pH and concentration. In the present study, zeta potential analysis was employed to measure the effects of metal ions on endotoxin. The observed experimental data showed significant changes in zeta potential of endotoxins when compared with the control (i.e., untreated endotoxin). Apparently, the magnitude of zeta potential of endotoxins changed after treatments with metal ions at different pH and concentrations. Therefore, it can be concluded that the apparent effect of metal ions on endotoxins zeta potential increases in the order of Zn^{2+}<Ca^{2+}< Mg^{2+}. This study serves as a basis for improved endotoxin monitoring in biomanufacturing.

KEYWORDS: Endotoxin; Lipopolysaccharide; Zeta potential; Divalent metal cations; Endotoxin characterization

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INTRODUCTION

Endotoxins are part of Gram-negative bacteria which are released during the cell death or cell growth (Petsch & Anspach, 2000). It is basically known as the defense tool for bacteria when released *in vivo* during cell growth. However, it could trigger an inflammatory response when introduced in human body at as low as 1 ng. kg^{-1}. h^{-1} (Buttenschoen *et al.*, 2010). Endotoxins, also known as lipopolysaccharide (LPS) consists of three major parts including lipid A, core and O-antigen. The toxicity of endotoxins originates from a conservative part of endotoxin which is Lipid A (Frecer *et al.*, 2000). The level of toxicity produced depends on its conformational structure where it will become more toxic when its spatial structure is more conical (Bui, 2012). Recombinant therapeutic products have been widely produced ever since the biotechnology industry was established. Gram negative bacteria, ie: *Eschericia coli* are the most extensively used bacteria for the production of recombinant therapeutics products as it is well characterized. However, endotoxin contamination becomes a major concern whenever these bacteria are used in biomanufacturing. Endotoxin removal is rather difficult due to its ability to resist high temperature and stability at any pH condition (Gorbet & Sefton, 2005). Researchers have developed a number of endotoxin removal methods which are mainly chromatography - based. Nevertheless, the most versatile and effective method has yet to be found. Previous study reported that metal ions have the potentials for removing endotoxins from recombinant products. This is attributed to the flexibility of endotoxin in interacting with most immobilized and free metal ions (Ongkudon *et al.*, 2012). Previous study showed that metal ions, specifically divalent cations have the ability to precipitate endotoxins (Mack

et al., 2014). Zinc sulphate (Zn^{2+}), calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+}) were believed to have the ability to selectively remove endotoxins from plasmid DNA molecules. Theoretically, the interaction between divalent cations and endotoxins is based on the electrostatic principle which follows the Schulze-Hardy rule (Cao *et al.*, 2015). Zeta potential analysis can be employed to measure this interaction based on the surface charge changes.

METHODOLOGY

Chemicals

Calcium chloride, magnesium chloride and zinc sulphate were dissolved in deionized water. Endotoxin was purchased from Sigma-Aldrich and dissolved using endotoxin-free water to obtain stock solutions of 80uM.

Zeta potential analysis

Cation concentrations of 0.1M, 0.5M, 1.0M, 1.5M and 2.0M were prepared using deionized water. Endotoxin working solution was made by dissolving the stock solution in sterile deionized water to obtain a final concentration of 8uM. For zeta potential analysis, 300ul of 8uM endotoxin solution was added into separate microcentrifuge tubes containing different concentrations of cation solutions. The control working solution contained only endotoxin in sterile deionized water. Each solution was analyzed using the NanoPlus Zeta Potential and Nano Particle Analyzer (Particulate Systems) for 10 accumulation points and three times repetition. The temperature was set at 25°C.

RESULT AND DISCUSSION

Effects of different cation concentrations on endotoxin zeta potential

At 8uM, the average mean zeta potential of the control endotoxin (without cation) was -43.53 mV **(Figure 1)**. The data suggested that the endotoxin was in anionic aggregated form based on the zeta potential value. The addition of 0.5M divalent cations, i.e; Mg^{2+}, Ca^{2+} and Zn^{2+} showed reduction of endotoxin zeta potential value to -0.52 mV, -11.85 mV and -14.30 mV respectively **(**Figure 1**)**. The reduction of zeta potential value indicated interaction of endotoxin with each of the divalent cations. The effect of Mg^{2+} on endotoxin was more prominent as the reduction of zeta potential was higher compared to zeta potential of endotoxin in the presence of Ca^{2+} and Zn^{2+}. As the concentration of cations was increased to 2.0M, the zeta potential of endotoxin was further reduced. The mean zeta potential of endotoxin in Mg^{2+} and Ca^{2+} solutions were 0.02 mV and 0.09 mV respectively, while in Zn^{2+} solution, the zeta potential of endotoxin was -1.24 mV. This observation suggested that endotoxin aggregates were more cationic in the presence of Mg^{2+} and Ca^{2+} compared to Zn^{2+}. Table 1 summarized the zeta potential value of endotoxin under different cation concentration.

Effects of different cations pH on endotoxin zeta potential

The initial pH of endotoxin without divalent metal cations was 6.8 with a zeta potential value of -43.53 mV. Incubation of endotoxin with Mg^{2+}, Ca^{2+}, Zn^{2+} ions at pH 5 reduced the initial zeta potential of endotoxin to -0.52 mV, -11.85 mV and -14.30 mV respectively **(Table 2)**. Charge neutralization occurred when endotoxin was treated at the lowest pH, i.e, pH 1 of Mg^{2+} (Figure 2) and Ca^{2+} (Figure 3). The mean zeta potential value was 0.59 mV in the presence of Mg^{2+} and 0.11 mV in the presence of Ca^{2+}. There was no charge neutralization on endotoxin zeta potential after incubation in pH 1 Zn^{2+} where the value was -0.69 mV (Figure 4). These results indicated that in the

presence of Mg^{2+} and Ca^{2+} at the lowest pH, endotoxins were subjected to cationic aggregation while in Zn^{2+} solution, the aggregation was anionic.

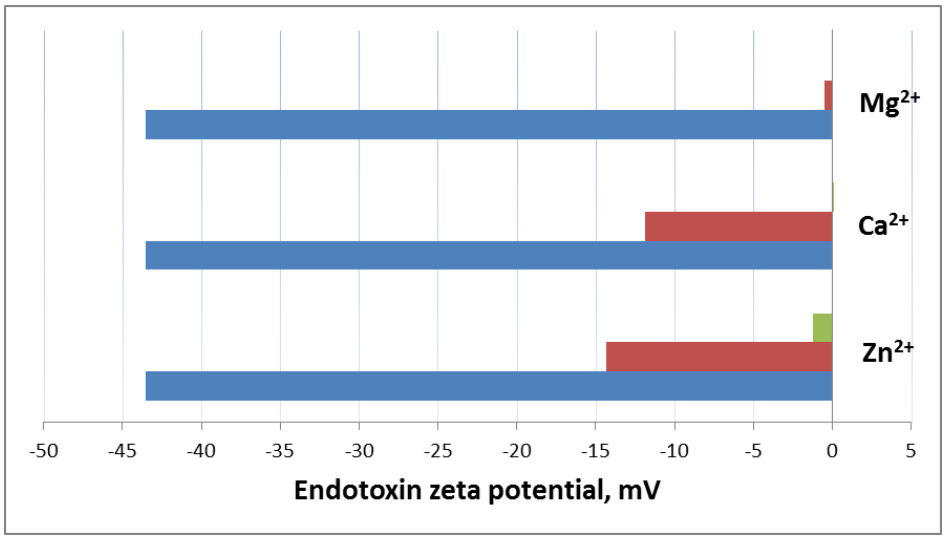

Figure 1. Effects of cation concentration on endotoxin zeta potential. (: 2.0M cation; : 0.5M cation; : 0M cation)

Table 1. Endotoxin zeta potential (mV) under different concentrations of cation

Cation concentration (M)	Zeta potential (mV)		
	Ca2+	Mg2+	Zn2+
0	-43.53	-43.53	-43.53
0.5	-11.85	-0.52	-14.30
2.0	0.09	0.02	-1.24

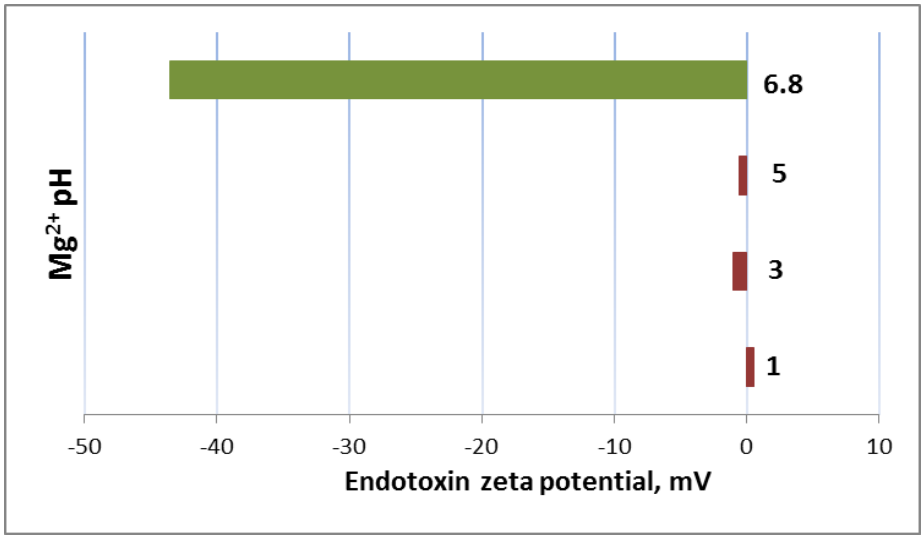

Figure 2. Effects of Mg^{2+} pH on endotoxin zeta potential (: Untreated endotoxin; : Treated endotoxin with Mg^{2+})

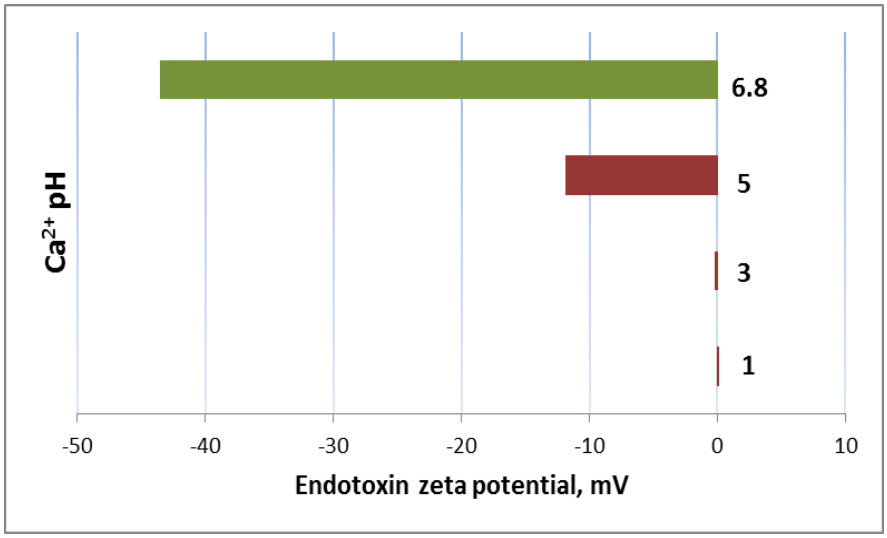

Figure 3. Effects of Ca^{2+} pH on endotoxin zeta potential (: Untreated endotoxin; : Treated endotoxin with Ca^{2+})

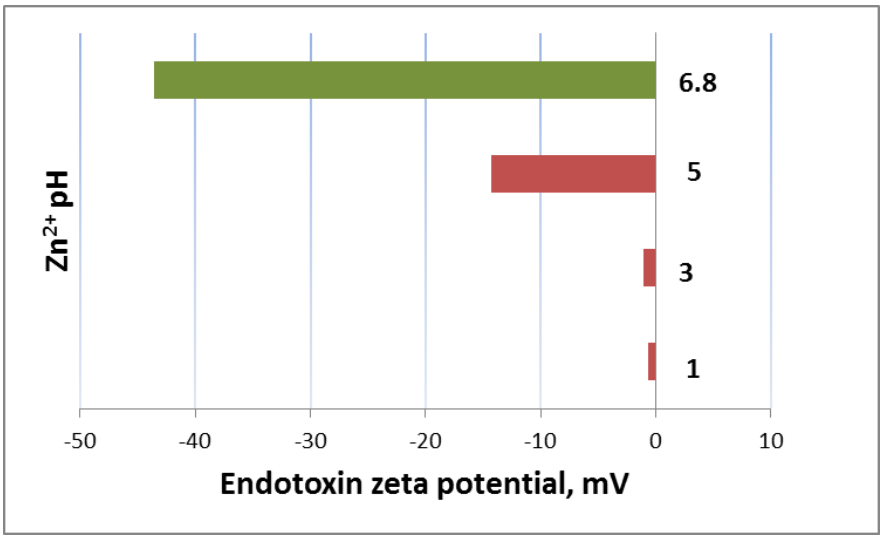

Figure 4. Effects of Zn^{2+} pH on endotoxin zeta potential (: Untreated endotoxin; : Treated endotoxin with Zn^{2+})

Table 2. Endotoxin zeta potential (mV) after being treated with cations under different pH

Cation pH	Zeta potential (mV)		
	Ca^{2+}	Mg^{2+}	Zn^{2+}
1	0.11	0.59	-0.69
3	-0.15	-1.02	-1.06
5	-11.85	-0.52	-14.30

CONCLUSION

This article highlighted the effects of divalent metal cations on endotoxins zeta potential which increased in the order of Zn^{2+}, Ca^{2+}, and $Mg2^{+}$ under different concentrations and pH conditions. A further understanding on how these divalent metal cations interact with endotoxin is important as it could form the basis for monitoring and controlling endotoxin contamination in biomanufacturing.

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