Monitoring and Optimizing the Lipopolysaccharides-plasmid DNA interaction by FLIM-FRET

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ABSTRACT In this work, *in vitro* FLIM-FRET experiments were performed to measure the interaction of divalent metal cations such as Zn2+ and Mg2+ with lipopolysaccharides (LPS) and plasmid DNA (pDNA). For Zn2+ induced interaction with LPS-DNA, the fluorescence lifetime of donor (Alexa Fluor 488 conjugate LPS) was 3.97 ns. Once acceptor (Alexa Fluor 594-labeled pDNA) was added, a sharp decrease in lifetime of 3.16 ns was observed. FRET efficiency for the interaction was calculated based on the change in fluorescence lifetime. The 20 % FRET efficiency calculated suggesting that significant interaction occurred. While for the interaction with Mg2+, donor lifetime alone was 3.72 ns. After the addition of acceptor, a slight decrease in lifetime was detected, 3.66 ns, corresponding to a low FRET efficiency of 1.6 % was recorded reflecting a very weak interaction. Data from FLIM images also showed that Zn2+ induced higher degree of aggregation compared to Mg2+. As part of the ongoing research project, the selectivity of Zn2+ over pDNA/ LPS and its ability to enhance aggregation are yet to be investigated.

KEYWORDS: *In vitro*; lipopolysaccharides; Forster Resonance Energy Transfer; Fluorescence Lifetime Imaging Microscopy; divalent metal cations

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INTRODUCTION

Lipopolysaccharides (LPS) located at the outer membrane of Gram-negative bacteria such as Escherichia coli are known to be responsible for their pathogenicity factor. Once released from the Gram-negative bacteria either due to cell division, cell destruction or cell death, it may trigger septic shock syndrome which may lead to death. LPS composed of three distinct regions: O antigen (forming the surface antigen), core oligosaccharide and non-polar lipid A.

Clinical products with bacterial origin exclusively vaccine demand effective LPS removal from plasmid DNA (pDNA) extraction. Past study by Ongkudon *et al.,* (2012) explored the use of divalent cation such as Zn^{2+}, Ca^{2+} and Mg^{2+} in inducing aggregation of LPS over pDNA. In the study, in the presence of Zn^{2+} was determined to be the highest particle size followed by Mg^{2+}. However better understanding on the structural changes and the role of Zn^{2+} in inducing LPS precipitation compared to other metal ions are still not well understood (Ongkudon *et al.,* 2014). In this study, we have devised new method in monitoring and analyzing the aggregation interaction of divalent metal ions with LPS and pDNA. For the first time, *in vitro* FLIM-FRET experiments were implemented to measure the interaction of divalent metal cations such as Zn^{2+} and Mg^{2+} with LPS and pDNA.

BACKGROUND THEORY

Basics of Fluorescence Phenomenon

The general aspects and basics of fluorescence and fluorescence microscopy are introduced here before going into detail to the FRET imaging technique. The phenomena of fluorescence can be explained by Jablonski diagram (Figure 1) below. When a fluorophore molecule absorbs specific quantum of light (energy), a valence electron from the ground state So is excited and moves to a higher energy level, generating the excited state Sn.. This transition process occurs very rapidly in femtoseconds and requires at least the energy $\Delta E = E_{ESn} - E_{0S0}$ to bridge the gap between excited and ground states in order for the excitation to occur. The excited fluorophore very quickly relaxes from the higher excited state (S_1 or S_2) by internal conversion and vibrational relaxation to lower excited state (S_0) in picoseconds. Upon returning to the lowest excited state it relaxes to ground state by emitting a photon with longer wavelength in the nanosecond time scale. This process is known as fluorescence emission. The Jablonski diagram below help in denoting each electronic state schematically for better understanding of fluorescence phenomenon.

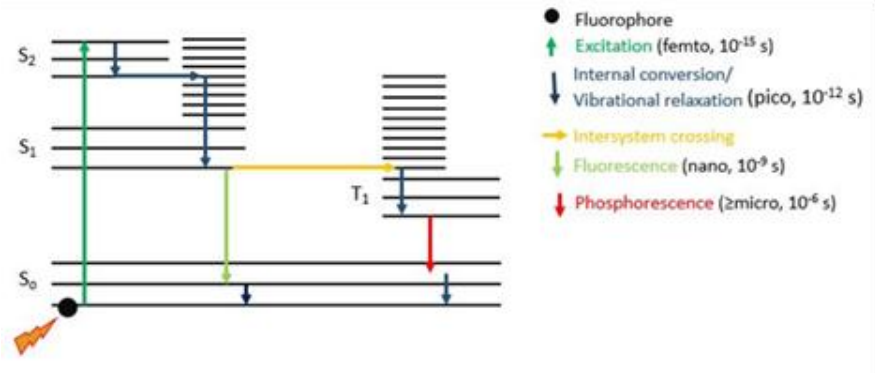

Figure 1. Jablonski diagram. When a quantum of light (a photon) is absorbed, the fluorophore leaves its ground state (S_0) and moves to higher excited state and quickly relax to lower vibrational excited state (blue arrow) and loose energy. Once falling to the ground state, the remaining energy is emitted via photon with a longer wavelength which emits a fluorescence (light green arrow).

Forster Resonance Energy Transfer (FRET)

FRET was first noted by Theodor Forster in 1948 as the radiationless transfer of energy occurs from a fluorophore molecules called 'donor' (D) in the excited state to a molecule in close proximity (< 10 nm) accepting the energy called 'acceptor' (A). Few factors need to be considered in order to increase the likelihood of FRET: a donor with a high quantum yield, an acceptor with a larger extinction coefficient, and the FRET pair with a larger spectral overlap (Periasamy, 2001). In addition with orientation of donor-acceptor pair also needs to be satisfied. Hence it is important to choose appropriate fluorophores ranging from exogenous and endogenous fluorophores, organic dyes, fluorescent proteins and quantum dots. The schematics of FRET is explained below (Figure 2) assisted with Jablonski diagram. A few factors may complicate the FRET studies: (i) the light used to activate the donor molecule may directly excite the acceptor as well (ii) the donor may leak into the channel used to detect the acceptor emission (iii) the FRET efficiency is affected by the expression levels of the donor and acceptor molecules (Becker, 2015).

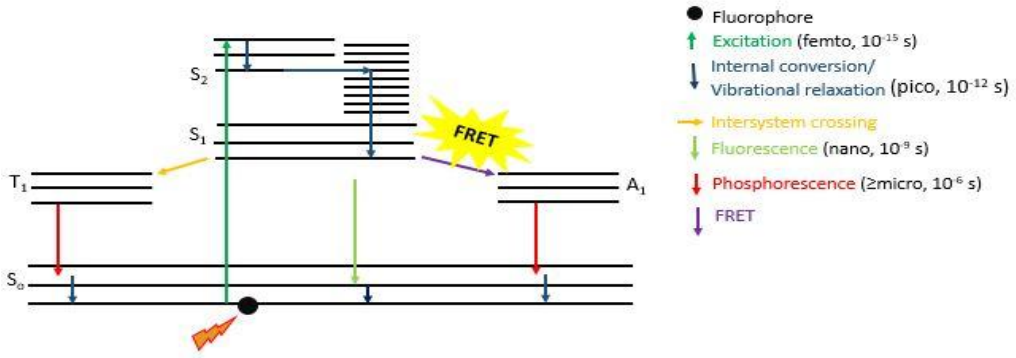

Figure 2. Jablonski diagram of FRET schematics. When a quantum of light (a photon) is absorbed, the fluorophore leaves its ground state (S0) and moves to higher excited state and quickly relax to lower vibrational excited state (blue arrow) and loose energy. If there is a coupling acceptor, a nonradiative decay channel is created and FRET will occur.

Fluorescence Lifetime Imaging Microscopy (FLIM)

Even though FRET is known as high resolution powerful tool to access molecular interactions down to 10 nm scale, the evolving technique of FRET-FLIM can be applied to accurately quantify interaction between two molecules by measuring the changes in fluorescence lifetime, which could overcome the complications observed during FRET. Fluorescence lifetime is the measure of average time a fluorophore spends in the excited state. FRET is calculated by measurings, the decrease in lifetime of donor in presence of acceptor. Unlike other FRET analysis, FLIM have the advantages of being independent of fluorophore concentration, excitation intensity fluctuation, photobleaching and sample thickness (Fortunati *et al.*, 2015).

Time correlated single photon counting (TCSPC) is the most common method to measure change in fluorescence lifetime. TCSPC-FLIM uses high-frequency pulsed laser beam to scan the sample which subsequently emits photons which is recorded in a time-correlated manner using sensitive detectors. . The TCSPC-FLIM offers quantifiable and highly informative approach to study interactions between two molecules. FRET-FLIM measures the fluorescence decay time of the FRET donor by calculating the average fluorescence lifetime at each pixel of the FLIM image (Wahl, 2009).

METHODOLOGY

Plasmid labelling with fluorescent dyes

Alexa Fluor 594 Ulysis Nucleic Acid Labeling Kit (Molecular Probes, Eugene, OR) was used following the manufacturer's instruction with minor modification.

Lipopolysaccharides from E. coli O55:B5

Alexa Fluor 488 conjugate LPS (Molecular Probes, Eugene, OR) was diluted according to the manufacturer's protocol with slight adjustment using ethanol-chloroform mixtures.

Metal cations preparation

ZnSO4.7H2O and MgCl2.6H2O were purchased from Systerm (ChemAR, Malaysia). Dulbecco's Phosphate Buffered Saline (DPBS 1x) was purchased Gibco (Life Technologies, New York, USA).

Fluorescence Lifetime Imaging

Time-Correlated Single Photon Counting (TCSPC) system (PicoQuant, Germany) attached to the Olympus FV-1000 confocal microscope (Olympus, USA) using the 60 x 1.2 W objective, at Institute of Medical Biology Microscopy Unit (IMU) The excitation light source was 485 nm pulsed diode laser with dichroic mirror of 405/488 and emission filter of 520/30 nm. Single-Photon Avalanche Diode (SPAD) detector was used to detect the photons arrivals and recorded by the PicoHarp 300 TCSPC module. Symphotime (PicoQuant) software was used to analyse the lifetime recorded. Mono and bi--exponential fittings were obtained for Alexa Fluor 488 conjugate LPS alone and in the presence of Alexa Fluor 594 tagged pDNA, respectively.

RESULT AND DISCUSSION

FLIM-FRET Techniques

The FLIM-FRET image result obtained by SymPhoTime software (PicoQuant) is shown in Figure 2. The lifetime was color-coded from 2 ns (blue) to 10 ns (red). In the reaction with Zinc, the lifetime of Alexa Fluor 488 conjugate (donor) LPS was 3.97 ns, upon addition of Alexa Fluor 594 (acceptor) labeled pDNA, a sharp decrease in lifetime 3.16 ns was observed. The FRET efficiency was calculated from the decrease in lifetime using the equation:

$$E = 1 - \frac{\tau_{DA}}{\tau_{D}}$$

where τ_{DA} is the lifetime of the donor in the presence of the acceptor while τ_{D} is the lifetime of the donor in the absence of the acceptor. The calculated, 20 % FRET efficiency is suggesting that interaction is significant. While for the interaction with Magnesium (b), the donor lifetime alone was 3.72 ns, upon the addition of acceptor only minimal change in lifetime 3.66 ns was observed, reflecting a FRET efficiency of 1.6 % showing a very weak interaction (Table 1). As mentioned previously by Ongkudon *et al.,* (2012) the presence of Zn2+ causing highest LPS aggregation size compared to Mg2+ and the live imaging of FLIM-FRET is shown in Figure 3.

Table 1. The measured lifetime and FRET efficiency are shown.

Divalent ion	τ_D (ns)	τ_{DA} (ns)	FRET Efficiency (%)
Zn^{2+}	3.97	3.16	20.4
Mg^{2+}	3.72	3.66	1.6

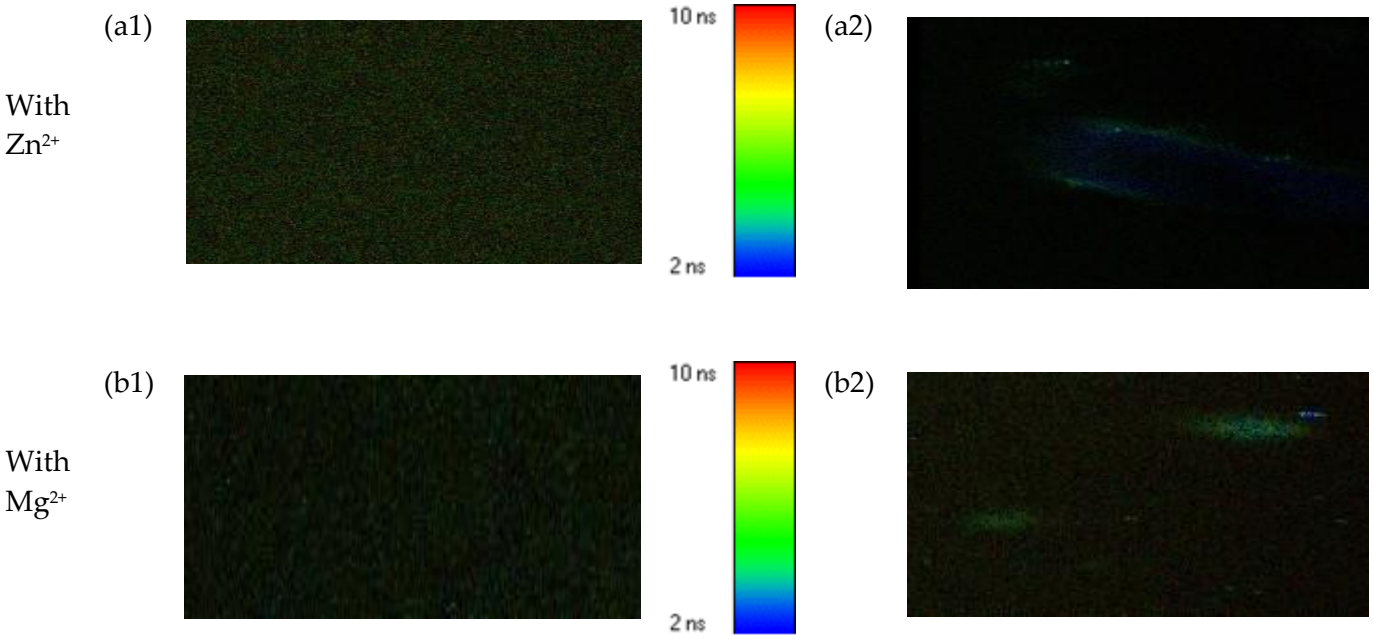

Figure 3. FLIM-FRET- image of LPS-pDNA interaction. The biomolecules interaction was done in vitro in Dulbecco's Phosphate Buffered Saline (DPBS 1X) pH 7.0 with panel (a) containing of metal ion of Zinc and (b) Magnesium. The lifetime (in ns) are represented using arbitrary color scales ranging from blue to red. Panel (a1) show the FLIM image of Alexa Fluor 488 conjugates LPS alone; Panel (a2) show Alexa Fluor 488 conjugates LPS interacting with Alexa Fluor 594 tagged pDNA. Panel (b1) show FLIM image of Alexa Fluor 488 conjugates LPS along; Panel (b2) is Alexa Fluor 488 conjugates LPS interacting with Alexa Fluor 594 tagged pDNA.

In this study, we applied in vitro FLIM-FRET as to overcome the complex microenvironment that may be present in the in vivo environment such as immune response factors. We reconstructed the in-vitro system mimicking the in-vivo system by the addition of salts and maintaining appropriate pH. Thus, the images with fluorescence lifetime information produced may correspond to the desired microenvironment. The TCSPC-FLIM images are shown in Figure 4 the lifetime scale is 2 ns to 10 ns indicated by the color-coding.

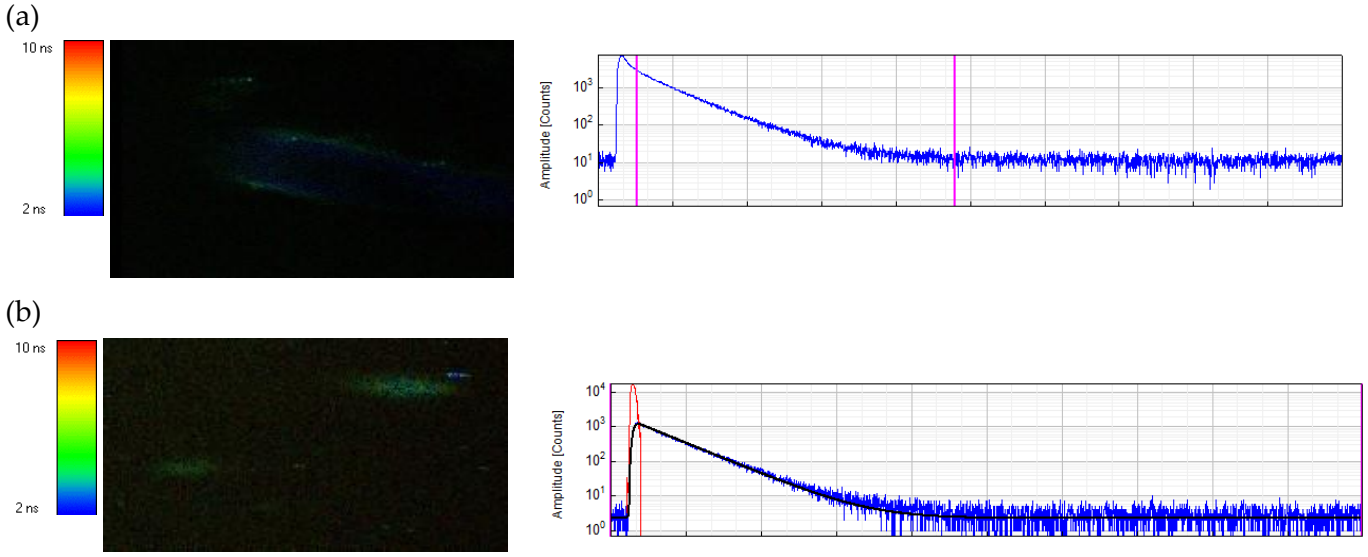

Figure 4. Time correlated single photon counting (TCSPC) decay plot is shown. (a) Fluorescence decay curve of Alexa Fluor 488 conjugate LPS interacting with Alexa Fluor 594 tagged pDNA with

the presence of Zn^{2+}. (b) Showing the decay curve of Alexa Fluor 488 conjugate LPS interacting with Alexa Fluor 594 tagged pDNA with the presence of Mg^{2+}.

CONCLUSION

In summary, we have presented FLIM-FRET method to monitor the LPS-pDNA physiological responses and conformational changes in the presence of metal cations such as Zn^{2+} and Mg^{2+}. The conformational changes also known as aggregation kinetics of LPS is supported by decreasing of donor lifetime decay as indicated by the FRET efficiency (*E*) calculated. Spatial distribution offered by FLIM is able to record changes of sample in real-time. However since the samples in this study was randomly distributed, a model system used to study lipid behavior, Giant unilamellar vesicles (GUVs) is considered to be implemented in the future study as to imitate the outer layer of gram-negative bacteria.

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