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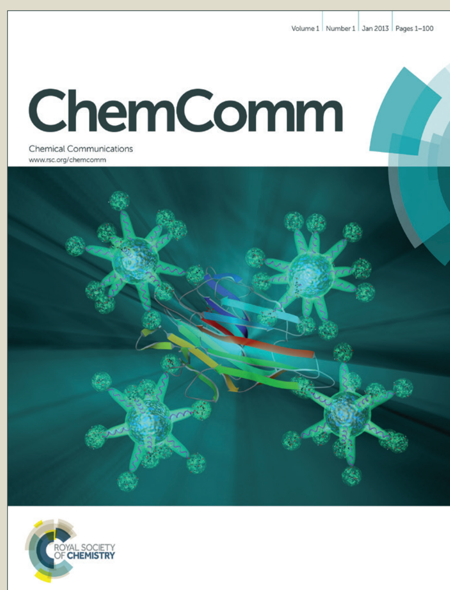

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COMMUNICATION

Peptide-Perylene Diimide Functionalized Magnetic Nano-platforms for Fluorescence Turn-on Detection and Clearance of Bacterial Lipopolysaccharide†

Fang Liu,[a] Jing Mu,[a] Xiangyang Wu,[a] Surajit Bhattacharjya,*[b] Edwin Kok Lee Yeow,*[a] Bengang Xing*[a]

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A simple and unique strategy has been successfully designed for sensitive detection and rapid clearance of bacterial lipopolysaccharide (LPS) by integration of core-shell $Fe_3O_4@SiO_2$ magnetic nanoparticles with a perylene-diimide (PDI) conjugated LPS-recognition peptide.

Bacterial lipopolysaccharide (LPS), a key component in the outer membrane of Gram-negative bacteria, is well known as bacterial endotoxin that may cause serious septic shock or many other health problems in the intensive care.[1] The extreme toxicity of bacterial LPS has thus urgently initiated extensive research efforts toward the development of novel and specific strategies to sensitively identify, and more importantly, to rapidly remove such highly toxic endotoxin from biological and pharmaceutical products. One commonly used FDA-approved technique for LPS detection is the enzymatic limulus amoebocyte lysate (LAL) assay. However this assay requires a rather long and complicated testing procedure, and moreover, the results from LAL assay are also susceptible to environmental variations, especially in pH, and temperature.[2] Very recently, several fluorescent, colorimetric or electrochemical sensors based on small molecules,[3] CD14-derivatived peptides,[4] liposomes,[5] or polymers[6] have been reported for sensitive determination of bacterial LPS in aqueous solutions. Although all of these attempts have shown great promise with their individual detection limit range (from micromolar to picomolar level),[5a-6] none of these assays indicated the capacity for the effective separation of LPS from the detection mixtures. Given the clinical importance of LPS, a simple and specific sensing strategy with more promising functions to clear bacterial endotoxin remains a big challenge in current research. Although several conventional techniques such as ultrafiltration, two-phase extraction, chromatography and functionalized nanoparticles have been utilized to remove LPS contaminants from certain biological preparations,[7] these techniques may not report the specific and sensitive determination of LPS directly, and their LPS clearance could be potentially affected by environmental changes (e.g. pH).[7c] Therefore, the development of a stable and easily controlled method with a combination of sensitive detection and efficient clearance of LPS in complex biological samples such as bacterial cell lysates and human serums will still be of great importance in pharmaceutical industry and such highly desirable studies have not been fully explored yet.

Here we first present a simple and novel dual-functional magnetic nanoparticle (MNPs) for effective detection and removal of bacterial LPS by introduction of a specific perylene diimide (PDI) conjugated LPS-binding peptide onto nanoparticles surface. PDI derivatives were chosen mainly because of their high thermal and photo stability, high quantum yield, and more importantly, their favorable self-assembly properties in aqueous solutions.[8] In addition, along with our continuous efforts in selective bacterial imaging and photo-inactivation of pathogens,[9] a set of designed LPS recognition peptides have been screened that exhibited promising binding affinity to the bacterial surfaces. In this rational design, a simple and high-affinity LPS binding peptide with the sequence of YVLWKRKRKFCFI-NH_2 has been selected as the targeting ligand to conjugate with magnetic $Fe_3O_4@SiO_2$ core-shell structure through a short linker containing PDI as a fluorescent reporting group. In the absence of LPS, the fluorescence of the PDI molecules may undergo self-quenching due to its strong $\pi \cdots \pi$ stacking interactions.[10] However, in the presence of LPS, the specific LPS binding would trigger conformational changes of the peptide[9] that may lead to the disruption of the hydrophobic stacking between adjacent PDI molecules and induce a significant fluorescence recovery (Fig. 1). Furthermore, the LPS captured by the recognition peptide on MNPs can be easily removed by a simple magnet, therefore

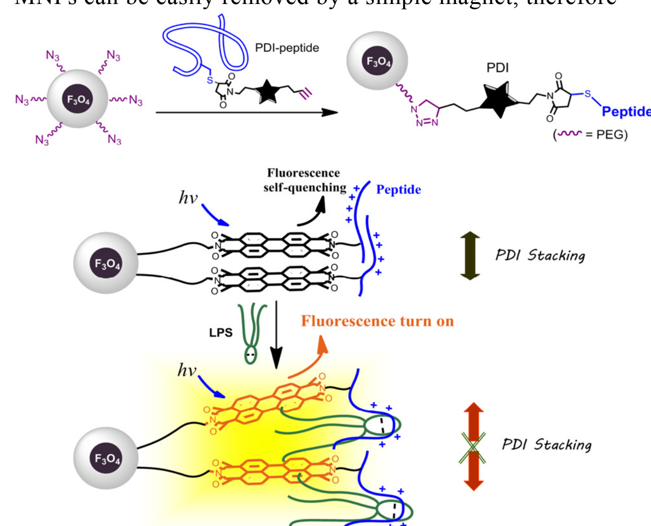

Fig. 1 LPS recognition peptide functionalized $Fe_3O_4@SiO_2$ core-shell MNPs for fluorescence turn on detection and clearance of LPS.

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separating the toxic bacterial LPS from the contaminated biological mixture, and thus lowering the risk of LPS toxicity to both the environment and human healthcare units.

In this study, the affinity peptide engineered MNPs were chosen as solid platforms because they have demonstrated great promise in biomedical applications including cancer imaging and therapy,[11] bio-separation,[12] targeted drug or gene delivery,[13] and the sorting of living cells and bio-molecules.[14] The $Fe_3O_4@SiO_2$ core-shell MNPs were prepared according to the method reported previously.[11,15] To achieve effective particle functionalization, an azido-dPEG$_4$ linker was first coupled to the $Fe_3O_4@SiO_2$ surface and then followed by conjugation of MNPs with the alkyne modified PDI peptide moieties through "Click-chemistry" (Fig. 1). The formation of the PDI-peptide functionalized MNPs ($MNP_{PDI\text{-}peptide}$) with the size distribution of about 25 nm was finally confirmed by spectroscopic and TEM analysis. The optimal amount of peptide conjugate on $MNP_{PDI\text{-}peptide}$ was determined to be 10 nmol/mg (Fig. S1-S4, ESI†).

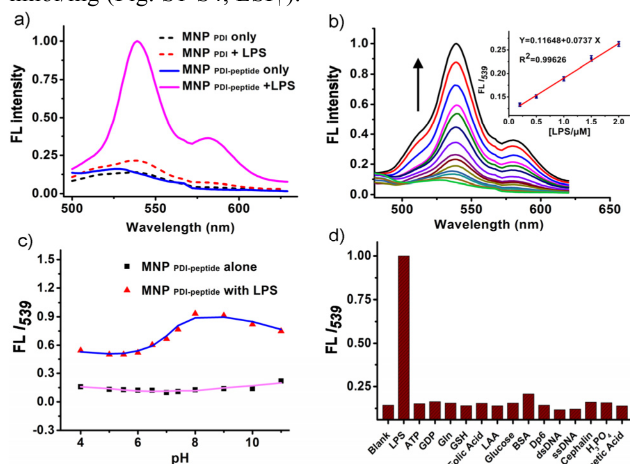

Fig. 2 a) Fluorescence spectra (excited at 450 nm) of $MNP_{PDI\text{-}peptide}$ and MNP_{PDI} with or without LPS (10 μM) in TBS buffer; b) Fluorescence of $MNP_{PDI\text{-}peptide}$ (100 μg/mL) after incubation with different concentrations (from 0, 0.1 to 25 μM) of LPS in TBS buffer at 37 °C. The inset shows the plot and linear fitting of normalized fluorescence at 539 nm vs the concentration of LPS (0-2.0 μM); c) The fluorescence of 100 μg/mL $MNP_{PDI\text{-}peptide}$ in the absence or presence of LPS (10 μM) under different pH conditions; d) The selective fluorescence increase upon addition of LPS against various biologically important species (at a concentration of 10.0 μM).

We further examined the possibility of $MNP_{PDI\text{-}peptide}$ responding to bacterial LPS and the induced fluorescence change in buffer solutions. Typically, 100 μg/mL of $MNP_{PDI\text{-}peptide}$ were incubated with 10 μM LPS in Tris-buffered saline solution (TBS, 50 mM, pH 7.4). As expected, there was very weak fluorescence detected before addition of LPS in $MNP_{PDI\text{-}peptide}$ solutions (Fig. 2a), mainly due to the self-quenching caused by the π···π stacking of PDI on particle surfaces.[10] However, upon the addition of LPS for 15 min, there was over 7-fold fluorescence enhancement at the emission wavelength of 539 nm and no obvious further change was observed as the incubation time increases (Fig. 2a, Fig. S5, ESI†). As control, the MNP_{PDI} conjugate but no LPS recognition peptide was also used to respond to the addition of LPS (Fig. 2a). No obvious fluorescence change was observed, clearly confirming that the LPS binding peptide functioned as an indispensable moiety to specifically target bacterial LPS, hence changing the conformation of stacked PDI molecules and eventually restoring the fluorescence from $MNP_{PDI\text{-}peptide}$. In addition, the analysis also showed that the fluorescence enhancement was LPS concentration dependent. The plot of fluorescence intensity at 539 nm versus the LPS concentration displayed a good linear relationship ($R^2 = 0.996$) with a LPS concentration range up to 2 μM (Fig. 2b) and the detection limit of LPS in TBS buffer was determined to be 28 nM (Fig. S6, ESI†), which was comparable to most of the LPS sensing systems reported previously.[3a,4-5] Furthermore, the fluorescent intensity of $MNP_{PDI\text{-}peptide}$ itself at 539 nm did not change dramatically in a wide pH range from 4 to 11. Upon addition of LPS (10 μM), the fluorescence intensity was increased about 4~7 times at all tested pH conditions (Fig. 2c), clearly suggesting that the as-prepared $MNP_{PDI\text{-}peptide}$ could serve as an environmentally stable and reliable platform for sensitive LPS detection.

Moreover, a further selectivity study was also performed by incubation of $MNP_{PDI\text{-}peptide}$ with various small or macro-biomolecules (Fig. 2d, Fig. S7-S8, ESI†). Compared to the results from $MNP_{PDI\text{-}peptide}$ with LPS, no significant fluorescence response was detected against all the tested analytes with concentration up to 10 μM including the highly negatively charged BSA or DNA, which has been reported to potentially interfere with LPS detection[5a], confirming that the specific LPS-peptide interaction could easily replace the nonspecific electrostatic attraction between the negatively charged bio-molecules and LPS affinity peptides. The activated peptide conformation change[9] will greatly induce the recovery of the quenched fluorescence by disrupting π···π stacking of PDI on the particle surface. These results indicated that the $MNP_{PDI\text{-}peptide}$ could work as a highly selective fluorescent turn-on sensor for toxic LPS determination.

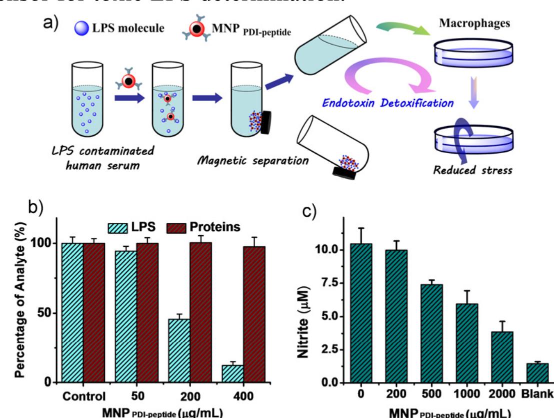

Fig. 3 a) Scheme of LPS removal based on $MNP_{PDI\text{-}peptide}$, b) Quantified magnetic LPS removal and protein recovery from *E. coli* cell lysates, c) NO generated from RAW 264.7 cells pre-treated with serum before and after LPS detoxification. Blank: serum treated cells without LPS.

More importantly, we also exploited the feasibility of using $MNP_{PDI\text{-}peptide}$ to remove this toxic bacterial component. Typically, the procedure to remove LPS consisted of two simple steps: 1) mixing of $MNP_{PDI\text{-}peptide}$ with suspension of lysed bacterial cells (e.g. *E. coli.* DH5α etc) or LPS contaminated human serum for 15 minutes; 2) applying a small magnet to attract $MNP_{PDI\text{-}peptide}$ to the wall of the vials to

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capture LPS (Fig. 3a). The optimum concentration of LPS and protein components from mixtures were determined by LAL assay or standard Braford method, which were used to evaluate the LPS clearance efficiency.[1,2] As shown in Fig. 3b, the removal of LPS was strongly dependent on the concentration of $MNP_{PDI-peptide}$ and an effective LPS removal (e.g. over 87% from lysate of 10^9 cfu/mL *E. coli.* DH5α cells) could be easily achieved when 400 μg/mL of $MNP_{PDI-peptide}$ was used, while no obvious loss of the total proteins was observed during the process of LPS removal. Moreover, after a simple multi-cycle removal (e.g. 3 or 4 cycles) with a fixed amount of $MNP_{PDI-peptide}$ (e.g. 200 μg/mL) each time, the LPS could be completely cleared from about 6800 endotoxin units (EU) per mL and there was even less than 1 EU/mL left in the bacterial lysates (Fig. S9a-S10, ESI†). As contrast, when similar clearance was conducted with Gram-positive *B. subtilis* lysates, only non-specific background signal was observed before and after treatment, suggesting that $MNP_{PDI-peptide}$ could selectively remove the LPS components from Gram-negative bacteria. Moreover, we also examined the feasibility of $MNP_{PDI-peptide}$ to detoxify LPS contamination by efficient removal of LPS endotoxin from human serum sample. Similarly, LPS removal strongly depends on the concentrations of $MNP_{PDI-peptide}$ and more efficient LPS clearance (e.g. > 89%) could be easily achieved under higher concentration of $MNP_{PDI-peptide}$ (e.g. 2.0 mg/mL) even only upon a single cycle particle separation (Fig. S9b, ESI†). Finally, the detoxification effect after removal of LPS was also evaluated in macrophages cultures by measuring the concentration of nitrite,[16] one stable metabolite of NO that has been well established as a pre-inflammatory stimulus to reflect the cell stress upon the simulation of bacterial LPS (Fig. 3c). Compared to the control study without any LPS in serum, the macrophage RAW 264.7 cells incubated with LPS contaminated serum for 24 hours would obviously induce the generation of high level of nitrite, indicating the occurrence of cell stress stimulated by LPS. As contrast, similar serum incubation but with efficient LPS removal resulted in low level of nitrite generation. The decreasing nitrite concentration observed in macrophage culture medium was consistent with the lower level of remaining LPS after effective MNPs separation (Fig. 3). Importantly, the $MNP_{PDI-peptide}$ itself did not demonstrate obvious cytotoxicity (e.g. cell viability of 87%) even under the concentration of nanoparticle conjugate up to 400 μg/mL (Fig. S11, ESI†), suggesting that the trace amount of residue $MNP_{PDI-peptide}$ in whole cell environment during the magnetic LPS removal would not be sufficient to induce obvious cellular toxicity.

In summary, by combining $Fe_3O_4@SiO_2$ core-shell MNPs with specific affinity ligand based on PDI conjugated LPS recognition peptide, we first developed a simple and novel strategy for effective fluorescent detection of LPS and rapid clearance of such toxic bacterial endotoxin. Based on the specific interactions between LPS and affinity peptides on MNPs surface, self-stacking of adjacent PDI molecules would be disrupted, which broke self-fluorescence quenching and thus restored the fluorescence. This peptide functionalized MNPs could sensitively identify LPS with the promising detection limit down to nano-molar level and it is highly selective for bacterial LPS when compared to other interfering bio-analytes. More importantly, such simple and effective LPS detection nanoparticle also indicated low risk of LPS toxicity to the environment and human healthcare by rapidly clearing bacterial LPS from complicated bacterial lysates and LPS contaminated serum samples. We envision that such simple and novel development would hold great potentials for the future treatment of bacteria associated sepsis with minimum complicated sample pre-treatment in extensive clinical practice.

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Notes and references

[a] *Division of Chemistry and Biological Chemistry; School of Physical & Mathematical Sciences; Nanyang Technological University; Singapore. 637371, E-mail: Bengang@ntu.edu.sg; Edwinyeow@ntu.edu.sg*
[b] *Division of Structural Biology & Biochemistry, School of Biological Sciences, Nanyang Technological University, Singapore, 637371. E-mail: Surajit@ntu.edu.sg*

† Electronic Supplementary Information (ESI) available: Synthesis and characterization of magnetic nanoparticles and PDI-peptide conjugates, additional experimental details and figures. See DOI: 10.1039/b000000x/

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Peptide-Perylene Diimide Functionalized Magnetic Nano-platforms for Fluorescence Turn-on Detection and Clearance of Bacterial Lipopolysaccharide †

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A fluorescence dye perylene diimide (PDI) functionalized LPS recognition peptide was conjugated to $Fe_3O_4@SiO_2$ core-shell nanoparticle to form a dual-functional magnetic nanoplatform, which offers the great possibility for their promising applications in fluorescence sensing and rapid clearance of bacterial endotoxin.

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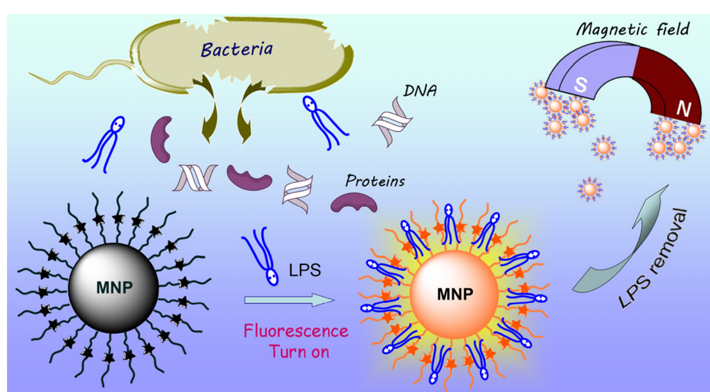

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