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Facile labeling of lipoglycans with quantum dots

Carlos Morales Betanzos [a], Maria Gonzalez-Moa [a], Stephen Albert Johnston [a,b], Sergei A. Svarovsky [a,*]

[a] Center for Innovations in Medicine, The Biodesign Institute, Arizona State University, 1001 S. McAllister Av, Tempe, AZ 85287, United States
[b] School of Life Sciences, Arizona State University 1711 S. Rural Road, Tempe, AZ 85287, United States

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ABSTRACT

Bacterial endotoxins or lipopolysaccharides (LPS) are among the most potent activators of the innate immune system, yet mechanisms of their action and in particular the role of glycans remain elusive. Efficient non-invasive labeling strategies are necessary for studying interactions of LPS glycans with biological systems. Here we report a new method for labeling LPS and other lipoglycans with luminescent quantum dots. The labeling is achieved by partitioning of hydrophobic quantum dots into the core of various LPS aggregates without disturbing the native LPS structure. The biofunctionality of the LPS–Qdot conjugates is demonstrated by the labeling of mouse monocytes. This simple method should find broad applicability in studies concerned with visualization of LPS biodistribution and identification of LPS binding agents.

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Bacterial lipopolysaccharides (LPS), also known as endotoxins, are the major constituents of the outer surface of Gram-negative bacteria [1]. They occupy up to 90% of the bacterial cell surface and are responsible for septic shock that kills nearly 200,000 of critically ill patients in the US alone [2]. Not surprisingly, there is a great deal of interest in understanding mechanisms of LPS action for the developing of antisepsis drugs. The development of such agents depends on the availability of efficient labeling strategies for LPS molecules [3]. Ideally such labeling should be the least disruptive to the LPS functionality.

LPSs are complex, negatively charged lipoglycans composed of three distinct regions: (a) a fatty acid region called Lipid A; (b) a core region oligosaccharide composed of approximately 10 monosaccharides; and (c) a highly variable O-antigenic polysaccharide responsible for much of the bacterial pathogenicity and immunospecificity. Most labeling strategies rely on chemical modification of LPS molecules with organic dyes and normally require complex manipulations and purification steps [4,5] due to the aggregative tendencies of LPS molecules [6]. The chemical modification is not site-specific and depends on the availability of reactive groups that are not always accessible or available in LPS [1]. If such groups are not present, they are chemically introduced by oxidation of the O-antigenic glycans [5,7]. By introducing additional moieties to the LPS molecule these methods perturb its physical properties and biomolecular recognition events [8], making such probes unlikely candidates for elucidating the roles of glycan interactions.

Nanometer-sized crystals of semiconductors known as quantum dots (Qdots) have recently emerged as useful luminescent labeling agents [9]. These nanoprobes have significant benefits over organic dyes including long-term photostability, high luminescent intensity, and multiple colors with single-wavelength excitation that open up possibilities for multiplex detection. Coating of hydrophobic quantum dots with phospholipids [10] and synthetic amphiphilic polymers have been described [11]. Both methods rely on phase transfer of hydrophobic quantum dots from an organic solvent to an aqueous solution of amphiphilic molecules. Here, we report an application of hydrophobic quantum dots to non-covalent labeling of LPS and its derivatives. We show that this method may be broadly applicable to other lipoglycans as well. This method takes advantage of the universal amphiphilic nature of lipoglycans and does not introduce any chemical modalities to the LPS structure, making it ideally suitable for studying glycan interactions.

Materials and methods

Unless otherwise noted, all chemicals were purchased from Sigma, Inc. (Milwaukee, WI) and used without further purification. Smooth type LPS from *Escherichia coli* (serotypes O111:B4 and O55:B5) and *Pseudomonas aeruginosa* (type 10) were supplied by Sigma, Inc. (Milwaukee, WI). Lipid A was purchased from Avanti Polar Lipids (Alabaster, AL). FITC-labeled LPS from *E. coli* O111:B4 was from Sigma (cat# F3665). (Caution! LPS and Lipid A are pyrogens that may cause fever. It may be harmful if inhaled, ingested, or absorbed through skin. Good laboratory practice should be employed. Wear a lab coat, gloves, safety glasses and a respirator

* Corresponding author. Fax: +1 480 727 0756.
E-mail address: Sergei.Svarovsky@asu.edu (S.A. Svarovsky).

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mask). Deionized water was obtained from a Millipore ultrapure water filtration unit. Organic Qdots were purchased from Invitrogen (Carlsbad, CA). Sepharcyl HiPrep 16/60 (S-200 HR) was from GE Healthcare. Transmission electron microscopy (TEM) was done on a Philips CM12S electron microscope operated at an accelerating voltage of 80 kV. Samples were deposited onto carbon-formvar mesh grids and images were recorded using a Gatan model 791 digital camera. In solution nanosizing and zeta potential measurements were done on Zetasizer® Nano-ZS instrument (Malvern Instruments, UK). Spectrophotometric measurements were carried out on NanoDrop® ND-1000 instrument.

Labeling LPS with Qdots. The supplied solution of organic Qdots (QDot® 605 ITK™, catalog #Q21701MP, Invitrogen, Inc.) in decane (1 μM) was evaporated to dryness on SpeedVac at room temperature and re-dissolved in equal amount of chloroform. A 100 μL aliquot of the chloroform solution was diluted to 500 μL with chloroform and mixed with 100 μL of 10 mg/mL aqueous solution of corresponding lipoglycan (*E. coli* O111:B4, *E. coli* O55:B5, *P. aeruginosa* 10, or Lipid A). Methanol was added dropwise with occasional vortexing until complete mixing of both phases was achieved (400 μL of MeOH). This homogeneous mixture of 5/4/1 chloroform/methanol/water was then evaporated to dryness on a SpeedVac and the solid residue was re-suspended in 100 μL of ddH_2O. A saturated solution of tetramethylammonium hydroxide pentahydrate ($Me_4N^+OH^-5H_2O$) was added until pH 11 (ca. 25 μL). The solution was sonicated for 30 min and then passed through two consecutive Zeba columns (2 mL, Pierce) to remove salts and excess of free LPS. We further purified the LPS-coated Qdots by size-exclusion chromatography on Sepharcyl HiPrep 16/60 (S-200 HR) column (50 × 1 cm). The LPS–Qdots eluted in a narrow color band and were stored in the dark at 4 °C. Under these conditions, the LPS–Qdots are stable for at least one month without any visible signs of flocculation or deterioration in fluorescent intensity. In a control experiment, the above procedure was repeated without a lipoglycan; no solubilization of organic Qdots was observed in this case.

Preparation of control PEG20K-QDots. 17.2 μL of 8 μM solution of amino QDots (QDot® 605 ITK™ amino(PEG) Quantum Dots, catalog #Q21501MP, Invitrogen, Inc.) were diluted with 200 μL of 100 mM sodium borate buffer pH = 8.5. To this solution, 27.5 μg of Traut's reagent were added and the mixture was shaken at 750 rpm for 2 h at rt. Then, the buffer was exchanged with 300 μL of 1 × PBS by using a Zeba column (2 ml, Pierce). To the solution obtained, 2 mg of mPEG-maleimide 20 K (NEKTAR) was added and the mixture was reacted overnight at room temperature. Excess of mPEG-maleimide was eliminated by filtering the mixture through an Amicon Ultra-4 100 K centrifugal filter and washing two times with 1 × PBS. The solution obtained was used immediately as a control to label monocytes.

Monocytes culture. Mouse monocytes from the cell line RAW 264.7 (American Type Culture Collection, ATCC) were provided by Dr. Yung Chang from the Center of Infectious Diseases and Vaccinology at The Biodesign Institute at ASU. Monocytes were cultured in DMEM (Dulbecco Modified Eagle medium; GIBCO, Grand Island, NY, USA) with 10% (v/v) Fetal Calf Serum (FCS) at 37 °C in a 5% CO_2 atmosphere and 95% humidity until confluence.

Monocytes labeling. Monocytes were detached using non-enzymatic cell dissociation agent, (Cellstripper™ Cat # 25-056-CI, Mediatech Inc.) and washed twice with 1 × PBS. Elutriated monocytes (1×10^6) were incubated in polypropylene tubes with 100 μg/mL of FITC conjugated *E. coli* O111:B4 LPS or the LPS equivalent of Biomimetic probe or QDots-PEG20K control, suspended in a final volume of 300 μL of HBSS (Hank's Balanced Salt Solution, GIBCO, Grand Island, NY, USA). Incubation time was 1 h at 37 °C [12]. After incubation cells were washed twice, fixed with 1% paraformaldehyde solution and analyzed by flow cytometry (FACS Caliber, BD Biosciences Inc.). The same cell suspension was poured on to a poly(L-lysine)-coated glass slide, incubated 30 min at room temperature and analyzed using a fluorescence microscope (BX51, Olympus America Inc.).

Results and discussion

Labeling of smooth-type LPS

Due to its amphipathic nature, LPS has strong tendency to form aggregates in solution [13]. The aggregation behavior depends on the concentration and the nature of the LPS molecule. Smooth-type LPS is believed to self-assemble into micellar structures of over 1 MDa [6]. This self-aggregation behavior is a function of the lipid A component of LPS molecule that also confers its ability to bind to hydrophobic surfaces. We exploited this ability for labeling LPS with luminescent quantum dots (Qdots) as shown schematically in Fig. 1.

Aqueous solution of LPS was mixed with a solution of organic Qdots in chloroform-methanol-water, evaporated, and re-suspended in basic aqueous solution. These steps resulted in the homogenous mixing of the hydrophobic constituents and incorporation of the Qdots into the LPS micelles. We followed the formation of Qdot–LPS conjugates by the Dynamic Light Scattering (DLS). As an example, Fig. 2 shows the size distribution during the labeling process of smooth-type LPS from *E. coli* O55:B5. The size of LPS micelles prior to labeling is represented by a broad peak with a hydrodynamic diameter of 100 nm, which agrees with previously reported values [13]. After mixing with Qdots and adding the base, the size of the newly formed Qdots–LPS aggregates is reduced to 50 nm. The basification process makes the LPS monomeric [4] favoring the access of the Qdots to the lipidic part of the LPS. Under the UV light, the Qdot particles can be seen quickly transitioning into the aqueous solution, which is only possible if the particles are taken up by the amphiphilic LPS. No luminescence is observed in solution in the absence of LPS even after sonication. The sonication of the mixture of Qdot–LPS for 30 min makes the

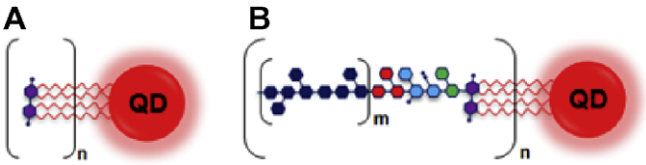

Fig. 1. (A) Lipid A-Qdots; (B) LPS–Qdots. *m* designates number of O-antigen repeating units; *n*, number of LPS molecules encapsulating the Qdot.

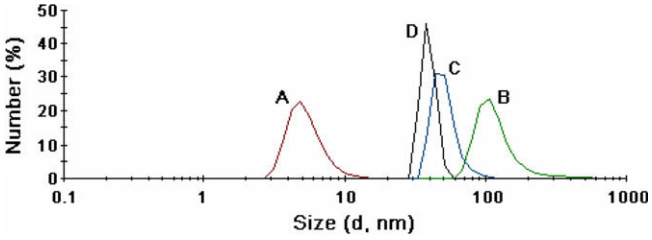

Fig. 2. Size distribution by number of particles obtained from the DLS analysis of the LPS *E. coli* O55:B5 serotype labeling process. Three key steps in the labeling process are included in the DLS study: (A) Qdots alone; (B) LPS alone; (C) after basification step, and (D) after sonication step. *X*-axis shows the diameter in nanometer and the *Y*-axis shows the percentage of particles of each specific diameter.

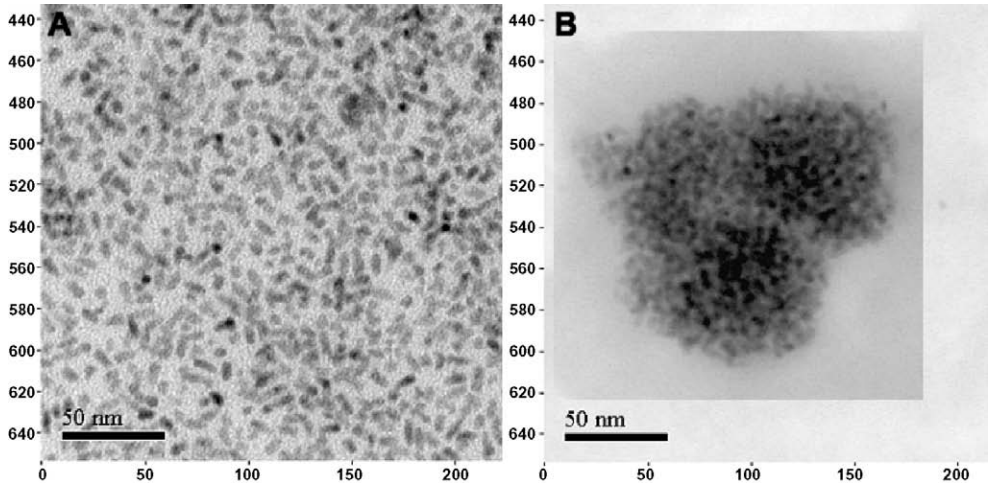

Fig. 3. Transmission Electron Microscopy image of (A) the organic Qdots from $CHCl_3$ solution; and (B) the purified Qdot-LPS *E. coli* O55:B5 conjugates in water.

aggregate more compact as the diameter is reduced to 38 nm and the size distribution becomes narrower, indicating increasing homogeneity of the Qdot–LPS conjugates.

The Qdot–LPS conjugates were purified by size-exclusion chromatography and studied by Transmission Electron Microscopy (TEM). Fig. 3 shows the sizes of the Qdots (ca. 5 nm) and the purified Qdot–LPS conjugates (ca. 50 nm) obtained in the TEM experiment. No free Qdots were observed in the aqueous solution of Qdot–LPS conjugate by TEM. These results agree with the data obtained by DLS shown in Fig. 2.

Labeling of other lipoglycans

Similarly, we labeled Lipid A, LPS from *P. aeruginosa*, *E. coli* O111:B4, and lipoteichoic acid (LTA)—a LPS equivalent in gram-positive bacteria. Interestingly, despite differences in molecular sizes, all of these conjugates had similar hydrodynamic diameters (centered around 30 nm) after conjugation to the Qdots (Supplementary Fig. S1). Even the complex formed by the smallest Lipid A molecule presents a size comparable to the one formed by the much larger smooth-type LPS. Since similar responses to basification and sonication were observed with the LPS alone, we conclude that presence of Qdots in the lipophilic core of the lipoglycans causes minimal disturbance to their native micellar structures. This highlights an important advantage of our labeling strategy over conventional labeling of individual LPS molecules in the developing assays for the binding analysis of endotoxin-receptor interactions. While in the case of individually labeled LPS molecules a possibility for false-positive binding, e.g. via membrane incorporation [14], is high, this is not the case with the Qdot-labeled LPS micelles since any molecule detached from the micelles is photosilent. Hence, only relevant saccharidic interactions can be detected with the Qdot–LPS probes.

Biological functionality of Qdot–LPS probes

To demonstrate that the Qdot labeled LPS keeps the integrity and biofunctionality of the bacterial LPS, and that it can bind as efficiently as the conventional FITC labeled LPS, we studied the recognition of LPS from *E. coli* O111:B4 by its natural cellular membrane receptors. A number of mammalian cells respond to LPS stimulation, with mononuclear phagocytes (monocytes) being the primary targets of the LPS action [15]. Although the exact mechanism of this interaction is not fully understood, it is evident

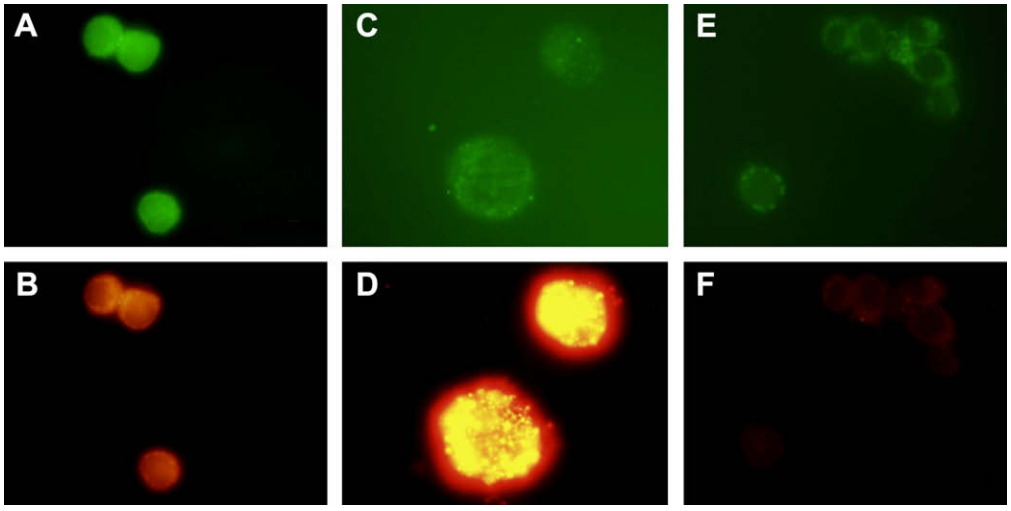

Fig. 4. Fluorescent microscope images of the labeling of mouse monocytes with (A, B) FITC-LPS; (C, D) Qdot-LPS; (E, F) Qdot-PEG20K. The top panel shows images taken using a filter with a wavelength range of 480–600 nm. The bottom panel shows images taken using a filter with a wavelength range of 530–650 nm.

that in order to elicit a response, LPS must first bind to the surface of the cells. We used monocytes as a model to study the interaction of Qdot-labeled LPS and compare it to the commercially available FITC-labeled LPS [8]. Also, to determine if that the LPS part of the complex is responsible for monocytes binding, we used Qdot-PEG20K as a control, where the Qdots were conjugated to a linear 20 kDa polyethyleneglycol (PEG) molecule. Fig. 4 shows fluorescent microscope images of the monocytes treated with the different probes, using two different filter sets for each. Figs. 4A and B correspond to FITC–LPS labeling, Figs. 4C and D depict the Qdot–LPS labeling, and Figs. 4E and F relate to the Qdot-PEG20K labeling. The LPS labeled with FITC and Qdots show binding to the surface of the monocytes, whereas no binding is observed with PEG20K QDots. The monocytes labeling was also followed by flow cytometry to get a more complete picture of the binding process (Supplementary Fig. S2). Both FITC- and Qdot-labeled LPS efficiently stained the monocytes, while the control Qdot-PEG20K did not.

In summary, we developed a new unobtrusive method for labeling lipoglycans with luminescent quantum dots. The labeling takes place by incorporation of lipophilic quantum dots into the native LPS aggregates. These highly luminescent LPS–Qdot complexes were formed and found to be stable in aqueous solution. Since the lipid functionality is concealed, LPS–Qdot constructs are ideally suited for studying interactions of the polysaccharide moiety of LPS in micellar presentation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.167.

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