Quantum dot probes for bacteria distinguish *Escherichia coli* mutants and permit *in vivo* imaging†

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Fluorescent quantum dots coated with zinc(II)-dipicolylamine coordination complexes can selectively stain a rough *Escherichia coli* mutant that lacks an O-antigen element and permit optical detection in a living mouse leg infection model.

Recently, we discovered that small, fluorescent probes with zinc(II) dipicolylamine (Zn-DPA) units as targeting ligands act as universal stains for most, if not all, strains of bacteria.[1,2] The Zn-DPA ligands have a strong affinity for the anionic phospholipids and related phosphorylated amphiphiles that are ubiquitous on the bacterial cell surface.[3] As part of a program to create extremely bright fluorescent probes for detection of bacterial contamination in the environment and *in vivo* imaging of living animals, we are currently evaluating nanoparticle scaffolds. The fluorescent CdSe/ZnS core/shell nanoparticles known as quantum dots (QDs) are promising imaging agents with several attractive features such as broad absorption, narrow emission bands, extreme brightness, and high photostability.[4] The commercial availability of streptavidin-coated QDs (up to one hundred times brighter than streptavidin-FITC)[5] and the technical simplicity of mixing these nanoparticles with biotinylated targeting ligands make this an attractive way to generate imaging probes.[6] It appears to be a straightforward strategy with large targeting ligands, such as biotinylated antibodies (MW ~ 150 kDa), whose highly specific recognition abilities hardly change upon immobilization to the QD surface. Indeed, antibody–QD probes have recently been shown to selectively target the surfaces of *Escherichia coli*,[7] *Salmonella typhimurium*,[8] *Mycobacterium bovis*,[9] and oral bacteria.[10] However, the cell binding outcome when small biotinylated targeting ligands are used is not likely to be so predictable. The ligand recognition properties may change substantially depending on structural variables, *e.g.*, the steric size and polarity of the polymeric material that coats the QD and supports the immobilized streptavidin, the streptavidin loading level, accessibility of the cell surface target, multivalent complementarity between target and ligand, biotin–ligand linker length, *etc.*
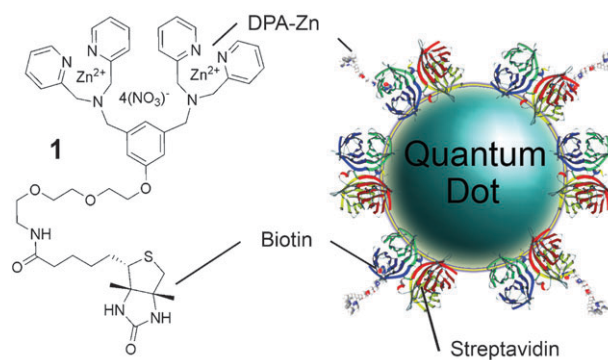

Fig. 1 Association of Zn-DPA–biotin conjugate **1** with a streptavidin-coated QD.

Here, we report that a relatively small Zn-DPA targeting ligand exhibits altered bacterial cell surface recognition properties when it is attached to a QD. Specifically, we have treated the biotinylated Zn-DPA probe, **1** (MW 0.7 kDa),[11] with separate samples of streptavidin-coated QDs and created a suite of extremely bright fluorescent imaging probes (Fig. 1) whose bacterial affinity is determined by the cell surface topology. The probes can distinguish different mutants of the same bacterial species. This topological information is complementary to that gained from smaller molecular probes, like the Gram stain and labeled antibiotics, which target different binding locations on the bacterial cell.[12]

A two-step procedure was followed to achieve bacterial staining. First, the Zn-DPA biotin conjugate **1** (4 μM) and streptavidin-coated red quantum dots (**RQD**, em. 655 nm, 1 μM) were mixed to give the **1**–**RQD** nanoparticle complex. Next, the complex was added to separate samples of three *E. coli* strains: JM83, UTI89, and AO16. After washing with buffer, the cells were examined using fluorescence microscopy. Intense surface staining was observed with the *E. coli* JM83
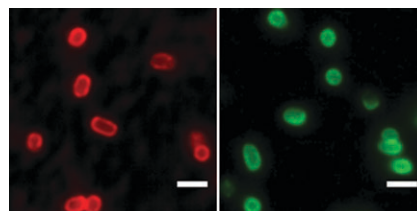

Fig. 2 Fluorescence micrographs of rough *E. coli* JM83 cells stained with **1**–**RQD** (left) and **1**–**GQD** (right). Scale bar is 2 μM.

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(Fig. 2) which is a rough strain (derivative of *E. coli* K12) that lacks the branched, O-antigen polysaccharide component extending from the lipopolysaccharide (LPS) in the exterior monolayer of the cell's outer membrane.[13] No cell staining was obtained with the smooth *E. coli* UTI89 and AO16 strains that have wild type LPS and extended O-antigen polysaccharides composed of ∼200 sugar units.[14] Repeating these staining experiments with streptavidin-coated green quantum dots (*i.e.*, **1–GQD**, em. 565 nm) produced the same micoscopy results (Fig. 2).‡ It appears that the UTI89 and AO16 cell surfaces are protected by a 'lawn' of O-antigen polysaccharides that prevent access of the relatively large nanoparticles to the phosphorylated 'lipid A' portion of LPS buried in the outer membrane. Thus, for Gram-negative *E. coli*, the nanoscale probes, **1–RGD** and **1–GQD**, are staining indicators of O-antigen length on the cell surface.

It is worth noting that cell staining does not occur if the order of reagent addition is reversed. That is, no bacterial staining is observed if the *E. coli* strains are treated first with the Zn-DPA–biotin conjugate **1** and then the streptavidin-coated **RQD**. It appears that the streptavidin-coated **RQD** cannot reach the biotin group on **1** after it binds to the bacteria. It is possible that the bacteria remove the biotinylated **1** from the surface *via* promiscuous biotin transport systems,[15] which would explain why there is no staining even with the *E. coli* JM83 cells that lack sterically protecting O-antigen polysaccharides.

The preformed **1–RQD** and **1–GQD** complexes were also tested for staining of Gram-positive *Staphylococcus aureus* NRS11 and *Enterococcus facaelis* cells. No cell binding was observed, which contrasts with the intense staining obtained previously using small fluorescent Zn-DPA probes.[1] These results suggest that anionic phospholipids in the Gram-positive bacterial membrane are crucial binding targets. The membrane is protected by a thick, surrounding cell wall which contains pores that are too small (maximum diameter of around 10 nm)[16] to allow passage of the functionalized quantum dots (hydrodynamic diameter 15–20 nm).[17]

The bacterial staining results indicate that tethering Zn-DPA affinity ligands to relatively large QDs produces fluorescent probes that can detect differences in cell surface topology. It should be possible to employ these extremely bright probes in highly sensitive multicolored staining schemes for rapid identification of bacterial species and mutant strains in contaminated samples. An added feature with streptavidin-coated QDs is the ability to quickly create a suite of multiplexed QD probes with different biotinylated ligands. These probes can be incorporated into staining arrays and analysed by pattern recognition methods.[18] This staining array technology is much faster than classical plating and culturing methods, and is well suited for 'point-of-care' medical applications.

A concurrent goal of the study was to determine the feasibility of Zn-DPA coated QD probes for *in vivo* imaging of bacterial infection in living mice. Optical imaging of bacteria is emerging as an effective method to study preclinical models of infection.[19] Bacteria may be genetically encoded with luminescent proteins that signal their presence, but when these reporters are unavailable, the bacteria must be labeled with a synthetic molecular probe. To achieve
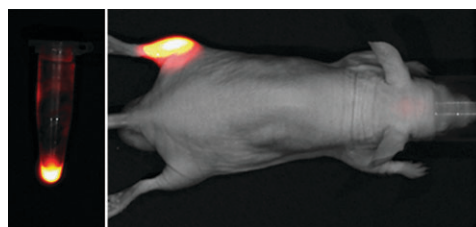

Fig. 3 Left: Fluorescence image of an Eppendorf tube containing *E. coli* JM83 cells labeled with **1–NIRQD**. Right: Nude mouse injected in the left rear calf muscle with the **1–NIRQD** labeled *E. coli* sample from the left panel.

maximum tissue penetration, the excitation and emission light should be in the window of 650–900 nm.[20] While a streptavidin-coated quantum dot tuned to emit at 800 nm (**NIRQD**) would seem to be ideal, its optimal excitation wavelength is below 500 nm,[6] which is problematic for *in vivo* imaging because penetration depth is diminished and there is substantial autofluorescence. Moving to longer excitation wavelengths substantially decreases the fluorescence brightness. So although Near-IR QDs have been reported by others as fluorescent probes for imaging of lymph nodes[21] and tumors,[22] it was not clear to us that *in vivo* brightness would be greater than that observed previously with a Near-IR Cy-7 fluorophore.[2a] Therefore, we conducted the following bacterial labeling and *in vivo* imaging experiment. *E. coli* JM83 cells (∼10[8] cells) were treated with the preformed probe **1–NIRQD** and the sample centrifuged and washed twice. The labeled bacterial cells were imaged using an IVIS Lumina imaging station and a Near-IR imaging filter set (Ex: 635 ± 20 nm, Em: 840 ± 30 nm, Low Binning, Fstop 1, acquisition time 10 s). The left panel in Fig. 3 shows a false colored fluorescent image of the labeled JM83 cells in an Eppendorf tube (as expected, **1–NIRQD** does not stain UTI89 and AO16 strains). Further analysis of these cells by fluorescence microscopy showed that the fluorescence was localized on the bacterial cell surface (essentially the same staining micrographs as Fig. 2). These labeled bacterial cells were injected into the rear left leg of a living nude mouse and the entire animal was imaged after five minutes. The right panel in Fig. 3 shows a photographic image of the mouse with the Near-IR fluorescence overlaid. Region of interest (ROI) analysis of the mouse fluorescence image indicated that the Near-IR signal from the site of bacterial infection is approximately 10-fold greater than the background autofluorescence from the mouse's back. While this level of contrast is potentially very useful, it is only 1.5 times higher than that obtained when the bacteria are labeled with a Zn-DPA probe containing a Near-IR Cy-7 fluorophore.[2a] Compared to organic dyes, the brightness advantage of visible wavelength QD probes is undisputed,[5] however, the photophysical advantages of **1–NIRQD** for *in vivo* imaging are less apparent.§ In any case, non-covalent labeling of bacteria with Near-IR Zn-DPA probes is technically straightforward and produces very bright *in vivo* images that should enable studies of bacteria motility and antibiotic efficacy in a mouse leg infection model.[19,23]

This study employed a mouse imaging protocol that was approved by the Notre Dame IACUC. The work was

Notes and references

‡ Control experiments demonstrated that the streptavidin-coated QDs alone do not stain any of the *E. coli* strains, but all three strains were intensely stained with small probes composed of Zn-DPA ligands attached to organic fluorophores.

§ This preliminary study does not address the question of whether **1**–**NIRQD** can recognize appropriate bacteria *in vivo*. As probes for *in vivo* imaging, QDs have likely advantages such as multivalency but they also have potential drawbacks, such as QD toxicity, slow rates of tissue diffusion, and slow rates of renal clearance. For further discussion, see ref. 17.

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