Selective recognition of bacterial membranes by zinc(II)-coordination complexes†

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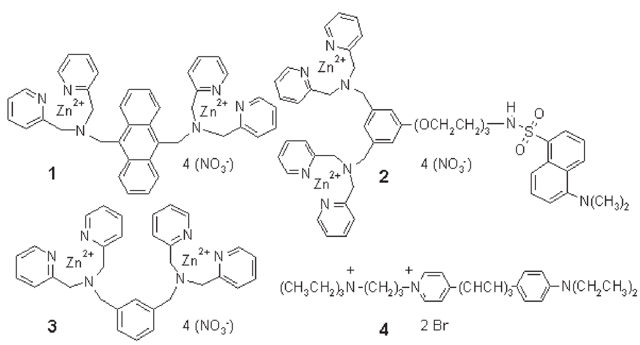
Two fluorophore–dipicolylamine–Zn^{2+} conjugates are shown by epifluorescence microscopy to stain the membranes of bacterial cells in preference to mammalian cells.

The selective recognition of bacterial *versus* mammalian membranes is an important function for both the immune system and antimicrobial drug candidates. This recognition is typically mediated by one of three cell surface components. First, the presence of a peptidoglycan cell wall is unique to bacteria and yeast, and thus is a common target of antibiotics like vancomycin[1] and proteins such as wheat germ agglutinin.[2] This approach is most successful with Gram-positive bacteria, which possess a cytoplasmic membrane surrounded by a thick cell wall that is exposed to the external environment. Gram-negative bacteria possess a second, outer membrane, which is composed of lipopolysaccharide (LPS) and covers the cell wall. In this case, a chain of sugar molecules, known as the O-antigen unit of LPS,[3] protrudes from the outer membrane into the surrounding environment providing a major target for antibodies.[4] Finally, the membranes of most bacteria contain significant amounts of anionic phospholipids, such as phosphatidylglycerol.[5] Thus, bacterial membranes are negatively charged and can be selectively targeted by cationic toxins like antimicrobial peptides.[6] The detection and imaging of bacteria has been achieved using bioconjugated polymers with fluorescent[7,8] or radioactive labels.[9] The versatility of these macromolecular probes has yet to be fully evaluated and some are likely to suffer from limitations such as poor biostability and undesired pharmacokinetics.[10]



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Here, we report, for the first time, that low-molecular-weight, fluorescent metal coordination complexes with anion binding capabilities can be employed as selective stains for bacterial membranes. Recently, we discovered that zinc dipicolylamine (Zn^{2+}–DPA) complexes have a strong affinity for bilayer membranes that are enriched with anionic phospholipids.[11] For example, the anthracene-derived bis(Zn^{2+}–DPA) conjugate, **1**, which was originally developed as a sensor for phosphate derivatives,[12] can also be used as a stain for mammalian cells undergoing apoptosis (programmed cell death).[13] During cell apoptosis the surface charge on the plasma membrane becomes increasingly negative due to the appearance of anionic phosphatidylserine.[14] Compound **1** selectively binds to these anionic membranes and thus identifies the cells as apoptotic.[15] Additional studies have demonstrated that a range of related Zn^{2+}–DPA conjugates can effectively discriminate between healthy and apoptotic mammalian cells.[16] These results suggested to us that Zn^{2+}–DPA conjugates may exhibit a similar binding preference for the negatively charged surfaces of bacterial cells.[17] Here we reveal that compound **1**, and new bis(Zn^{2+}–DPA) complex, **2**, can efficiently stain the membranes of Gram-negative *Escherichia coli* (K12) and Gram-positive *Staphylococcus aureus* (NRS 11) cells. Furthermore, these compounds are selective for the membrane *versus* the bacterial DNA or other intracellular phosphates. Finally, we show that these compounds preferentially bind bacteria over mammalian cells in the complex biological medium of saliva.

An attractive feature of compound **1** is that its fluorescence emission increases by almost an order of magnitude upon binding to a bilayer membrane.[18] This strong signal enhancement effectively eliminates the need to wash the bacterial cells after addition of the fluorophore. In the case of the *E. coli*, approximately 5×10^7 cells were centrifuged (3500X, 4 min), re-suspended in buffer (5 mM TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), 145 mM NaCl, pH = 7.4), and then treated with three compounds. First, the antimicrobial peptide KSL (50 μg/ml) was added to permeabilize the membrane.[19] Next, compound **1** (10 μM) and the DNA intercalator, 7-amino-actinomycin (7AAD, 500 ng/ml) were added and the mixture allowed to incubate for 15 minutes. Fig. 1A shows the *E. coli* cells as viewed in the "blue" filter set with a Nikon Eclipse TE2000-U epifluorescence microscope (see supporting information for additional details). The blue fluorescence of compound **1** (ex. 350 nm, em. 440 nm) is localized to the periphery of the *E. coli* cells. The staining is stable, and cells that were subsequently washed two times appear identical to those given in Fig. 1A (data

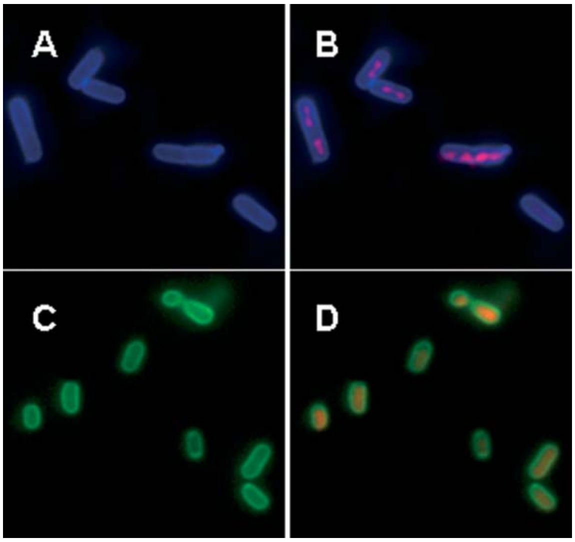

Fig. 1 *E. coli* cells were co-stained with either **1** or **2**, and 7AAD. Frames A and C show the blue and green emission due to fluorescence of **1** or **2**, respectively. Frames B and D overlay the co-staining by 7AAD, thus identifying the relative location of membrane and DNA (1500X).

not shown). Fig. 1B is an image overlay of the blue and red filter sets which capture emission from **1** and 7AAD (ex. 543 nm, em. 655 nm), respectively. The cells are not transfected with plasmids, therefore, the red staining by 7AAD is attributed to genomic DNA in the cell cytoplasm. In the absence of permeabilizing peptide, the membrane impermeant 7AAD does not enter the cytoplasm; whereas, the membrane is still stained by compound **1**.

The structure of bis(Zn^{2+}–DPA) **2** includes a dansyl fluorophore (ex. 335 nm, em. 560 nm) that is known to fluoresce more intensely when placed in a more hydrophobic environment.[20] This membrane-enhancement effect allowed bacterial imaging with **2** to be achieved without any washing steps; however, the background fluorescence was higher than in the case of **1**. Figs. 1C and 1D show that compound **2** behaves like **1** and stains the membrane of *E. coli* but not the intracellular DNA.

The results of additional studies of **1** and **2** with another Gram-negative bacterium, *Pseudomonas aeruginosa*, were identical to those with *E. coli* (data not shown). While these are only two strains out of thousands of Gram-negative bacteria, we believe that the same images will be obtained with most other strains given the ubiquitous presence of anionic membranes in bacteria. In Fig. 2 is the staining observed with the Gram-positive *S. aureus*. The images show that compounds **1** and **2** can effectively delineate the membrane of this smaller bacterium.

Having established that compounds **1** and **2** can effectively target and stain the membranes of Gram-negative and Gram-positive bacteria, we then determined the selectivity for bacteria in the presence of mammalian cells. It is well-known that the oral cavity is an area in which bacteria and mammalian cells co-exist. Therefore, selective staining studies were performed using the readily available medium of saliva.

Human saliva is known to contain a myriad of bacteria,[21] and primarily three types of mammalian cells; leukocytes (immune system), erythrocytes (blood), and detached buccal epithelial (oral lining) cells. These cell types can be counted and sorted from saliva samples using flow cytometry,[22] or they can be discerned by morphology under a microscope. Bacteria are known to adhere to the human oral epithelium,[23] and thus we attempted to image the bacteria on the surface of these cells. Human saliva (500 μL) was collected approximately 1 hour after the lunch meal from a healthy subject. Compound **1** (10 μM final concentration) was added to the sample which was then used for imaging without further manipulation. Fig. 3 shows an epithelial cell and its membrane-associated bacteria in the bright and fluorescence fields. The bacteria are clearly stained in preference to the membrane surface of the much larger host cell. Both rod-like bacteria and cocci can be observed in the fluorescence field. Unfortunately, these cells are too small to discern membrane *versus* intracellular staining. Similar experiments were performed using compound **2**; however, a brighter fluorescence background was obtained which prohibited the acquisition of images with the same high quality as **1**.

A unique feature of compounds **1** and **2** is they only associate with the membrane surface and they do not penetrate into the lipophilic interior of the bilayer. Thus, they distinguish between membranes on the basis of surface charge. This is in contrast to the poor membrane selectivity that is observed with lipophilic dyes. For example, cationic styryl dyes such as **4** (also known as FM 4-64) are used often as fluorescent probes for optical imaging of eukaryotic and bacterial cell membranes.[24] The strong driving force for membrane penetration and lipid mixing overwhelms any selectivity due to differences in membrane surface charge. The contrast in membrane selectivity is illustrated by the fluorescence images in Fig. 4. Cell staining experiments were performed using saliva samples treated with **1** (10 μM) and **4** (2 μg/ml). Fig. 4A shows an epithelial cell with an associated "clump" of bacteria in the red fluorescence field. Both the mammalian cell and associated

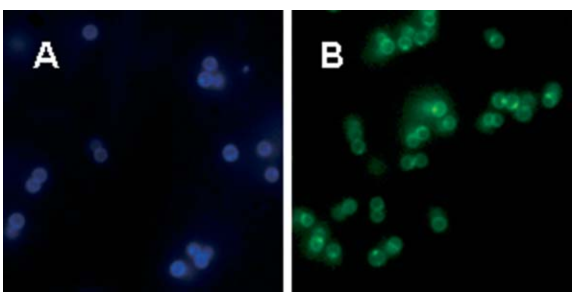

Fig. 2 *S. aureus* cells stained with **1** (frame A) and **2** (frame B).

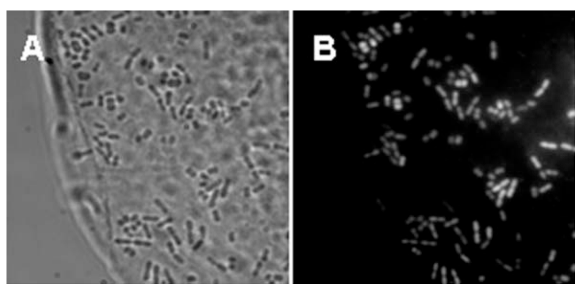

Fig. 3 Human saliva sample stained with **1**. The two frames show an epithelial cell with associated bacteria in the bright field (frame A) and blue fluorescence field (frame B). Image is uncoloured to emphasize contrast.

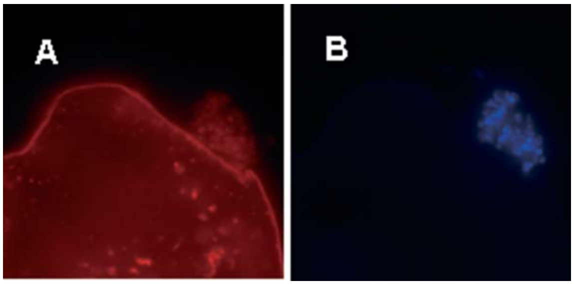

Fig. 4 Human epithelial cell with an associated "clump" of bacteria in the red field (frame A, **4**) and blue field (frame B, **1**).

bacteria emit strong fluorescence due to similar amounts of staining by **4** (ex. 558 nm, em. 734 nm). Fig. 4B shows the same group of cells in the blue field. Here the selectivity is dramatic, only the bacteria are stained by **1**.

The present study demonstrates the ability of fluorescently labeled bis(Zn^{2+}–DPA) coordination complexes to stain the surfaces of *E. coli*, *P. aeruginosa* and *S. aureus* cells. The fluorescent probes preferentially bind to the cell membrane over the intracellular DNA. Furthermore, these compounds can selectively stain bacteria over mammalian cells, even in the complex biological medium of saliva. The structures of these membrane-binding molecules are straightforward to modify and should provide a new platform for researchers to image and target bacteria with numerous reporter constructs and biological agents.‡

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Notes and references

‡ The toxicity of **1** and **2** to *E. coli* and *S. aureus* was investigated using the minimum inhibitory concentration (MIC) method outlined by the NCCLS.[25] In short, compounds **1–3** were serially diluted two-fold in Luria Bertani (LB) Miller broth, inoculated with 5×10^5 colony forming units (CFU) of bacteria per milliliter of media, and grown at 37 °C for 24 h. The MIC was determined as the lowest concentration of compound that inhibited bacterial growth as judged by the visual turbidity of the solution. Compound **1** was inactive to *E. coli* at concentrations up to 100 μM, while **2** inhibited growth at 25 μM. *S. aureus* growth was inhibited by **1** at a concentration of 12.5 μM, while **2** was toxic at 54 μM. Compound **3** was inactive with both microbes at concentrations up to 100 μM.

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