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Ubiquitous Detection of Gram-Positive Bacteria with Bioorthogonal Magnetofluorescent Nanoparticles

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Abstract

The ability to rapidly diagnose gram-positive pathogenic bacteria would have far reaching biomedical and technological applications. Here we describe the bioorthogonal modification of small molecule antibiotics (vancomycin and daptomycin), which bind to the cell wall of gram-positive bacteria. The bound antibiotics conjugates can be reacted orthogonally with tetrazine-modified nanoparticles, *via* an almost instantaneous cycloaddition, which subsequently renders the bacteria detectable by optical or magnetic sensing. We show that this approach is specific, selective, fast and biocompatible. Furthermore, it can be adapted to the detection of intracellular pathogens. Importantly, this strategy enables detection of entire classes of bacteria, a feat that is difficult to achieve using current antibody approaches. Compared to covalent nanoparticle conjugates, our bioorthogonal method demonstrated 1-2 orders of magnitude greater sensitivity. This bioorthogonal labeling method could ultimately be applied to a variety of other small molecules with specificity for infectious pathogens, enabling their detection and diagnosis.

Keywords

Nanoparticles; bacteria; infection

Infectious bacterial diseases continue to be a leading cause of death, disability, and socioeconomic disruption worldwide.¹ Whilst the majority of such infections are most prevalent in developing countries, co-infections and hospital-acquired infections are also major problems in developed countries. Infectious diseases are responsible for approximately 17 million deaths each year, and represent one-third of global mortality. In particular, infections resulting from gram-positive bacteria remain a leading cause of morbidity and mortality in humans.^{2, 3} The ability to rapidly diagnose gram-positive pathogens in a point-of-care setting would thus have a significant impact on improving the treatment of such diseases.

Gram-positive bacteria are differentiated from gram-negative bacteria based on the structure of their cell walls. The walls of gram-positive bacteria are composed of a thick peptidoglycan layer and thus stain positively by Gram stain.⁴ The most common gram-positive pathogenic bacteria in humans are cocci (sphere-shaped bacteria) which include

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Supporting Information Available. Additional data for microscopy, FACS, and fluorescence measurements. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

staphylococci and streptococci. A number of antibiotics have been developed to treat gram-positive infections, many of which work either by inhibiting cell wall synthesis or by blocking transcription/translation processes. Vancomycin is a commonly used glycopeptide antibiotic, whose action primarily results in inhibition of cell wall synthesis. Specifically, vancomycin exerts its antibacterial activity by forming hydrogen bonds with the terminal D-alanyl-D-alanine (D-Ala-D-Ala) moieties of the *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) peptide subunits.^{5, 6} This binding prevents incorporation of the NAM/NAG-peptide subunits into the major structural component of gram-positive cell walls, the peptidoglycan matrix, and thus results in inhibition of cell wall synthesis and ultimately bacterial cell death. The increasing prevalence of vancomycin-resistant organisms, however, have now led to the development of newer generation antibiotics including daptomycin, linezolid and pristinamycin. Daptomycin binds to the cell wall of gram-positive bacteria *via* its hydrophobic tail, resulting in perturbation and depolarization of the cell membrane.⁷

In view of the unique mechanism by which both vancomycin and daptomycin bind the bacterial cell wall of gram-positive bacteria, we reasoned that bioorthogonal derivatives of these antibiotics could be useful for the rapid detection and capture of gram-positive bacteria. Here, we describe the synthesis of *trans*-cyclooctene (TCO) derivatives of these antibiotics, which *via* the complementary application of tetrazine (Tz) decorated magnetofluorescent nanoparticles, are used to detect gram-positive bacteria. Furthermore, we also show that this two-step labeling procedure is superior to labeling *via* direct antibiotic-nanoparticle conjugates. The described labeling method was investigated on multiple bacterial strains for subsequent optical and magnetic detection, as well as for bacterial profiling.

RESULTS

Vancomycin-TCO binds to gram-positive bacteria

Figure 1A illustrates the labeling mechanism using vancomycin-TCO (vanc-TCO) as the targeting ligand.⁵ The binding of vanc-TCO to gram-positive bacteria was achieved *via* a five-point hydrogen bond interaction between the drug and the D-Ala-D-Ala moieties of the NAM/NAG peptides. Tetrazine-modified magnetofluorescent nanoparticles (MFNP-Tz) were subsequently applied, which resulted in magnetic and fluorescent labeling of the microbes through a bioorthogonal cycloaddition reaction. Vanc-TCO was synthesized by modifying the primary amine group on the glycopeptide with *trans*-cyclooctene *N*-hydroxy-succinimidyl (NHS) ester (TCO-NHS).⁸ The purity and identity of the synthesized product were confirmed *via* HPLC, ESI-MS and HRMS (Figure 1B). When tested on *Staphylococcus aureus* (*S. aureus*), incubation with vanc-TCO and MFNP-Tz resulted in highly effective bacterial targeting. Subsequent confocal microscopy showed bright fluorescence on the surface and outer layer of the bacterial cells targeted by the bioorthogonal labeling approach (Figure 2A). Without vanc-TCO incubation, however, the signal intensity was negligible (Figure S1A). Transmission electron microscopy was likewise able to show the presence of nanoparticles, evenly coated across the surface of bacterial cells (Figure 2B). In contrast, non-targeted bacteria showed a smooth surface devoid of nanoparticles (Figure S1B).

Bacterial detection *via* vancomycin cycloaddition to nanoparticles

We next examined how effectively different bacterial strains could be detected. The following panel of gram-positive bacteria was tested: *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Enterococcus faecalis* (*E. faecalis*). The following gram-negative species were used as

negative controls: *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Klebsiella pneumoniae* (*K. pneumoniae*). Using these bacteria, we initially determined the optimal vanc-TCO dose for bacterial labeling. All bacterial samples were incubated with varying concentrations of vanc-TCO, before subsequent labeling with MFNP-Tz (Figure 3A). For all gram-positive bacteria, fluorescence intensities increased proportionally with vanc-TCO concentration until saturation was attained. Treating higher concentrations of vanc-TCO (50, 100 μM) resulted in lower labeling efficiencies than at 20 μM due to the bactericidal effect of the drug conjugates. Therefore, the optimal dose of vanc-TCO for targeted labeling was $\sim 20 \mu\text{M}$. The equilibrium binding constant K_d ($= 5.7 \mu\text{M}$) of vanc-TCO was statistically similar ($p > 0.28$) across all gram-positive species. The observed variations in fluorescence intensities were likely due to different D-Ala-D-Ala content across species. In agreement with our results, previous studies based on surface plasmon resonance have shown that the K_d for vancomycin binding to self-assembled monolayers of D-Ala-D-Ala is $\sim 1.5 \mu\text{M}$.^{9, 10} The gram-negative species (controls) showed negligible fluorescent signals at all vanc-TCO concentrations tested. Flow cytometry likewise confirmed that only the gram-positive bacteria were labeled efficiently and evenly throughout the cell population (Figure S2).

We subsequently compared the labeling efficacy of the bioorthogonal two-step approach to that of direct covalent conjugates of vancomycin-nanoparticles (MFNP-vanc, one-step). Bacterial samples were magnetically targeted using both labeling methods, and the transverse relaxation time (T_2) of all samples was measured using a miniature (micro) nuclear magnetic resonance (μNMR) system.^{11, 12} The T_2 values were then converted to cellular relaxivity values ($1/T_2$ per bacterial concentration), which are proportional to the amount of MFNPs loaded onto each bacterium.¹¹ From this comparison, we found that the bioorthogonal approach offered far more efficient labeling of gram-positive targets. Indeed, the cellular relaxivities of bioorthogonally targeted bacteria were up to 6-fold higher than those of direct conjugates (Figure 3B). Such high MFNP loading ultimately enabled the performance of highly sensitive and rapid bacterial detection. For example, the detection limits of *S. aureus* and *S. epidermidis* using the bioorthogonal labeling method were 1,300 CFU and 35,100 CFU, respectively (Figure 3C), and each assay took less than 30 minutes. It should be noted that this detection sensitivity would be further enhanced by optimizing the labeling parameters and/or by reducing the size of the μNMR detection coil. The μNMR assay reported here also showed a good correlation with fluorescence measurements (Figure 3D), which thus verified the reliability of the developed labeling method for multimodal detection.

Functional assays

To further characterize the binding properties of vanc-TCO, we performed competitive inhibition studies in which unmodified vancomycin was introduced together with vanc-TCO, before reacting with MFNP-Tz. As the concentration of unmodified vancomycin increased, the labeling efficacy of gram-positive bacteria gradually decreased (Figure 4A, B). The inhibition binding constant (K_i) was $\sim 6 \mu\text{M}$ for all gram-positive bacteria, a value which was similar to that of vanc-TCO. Up to the established optimal dose of vanc-TCO (20 μM), all gram-positive bacteria (*e.g.*, *S. aureus*) showed high viability (over 80% for the first 30 minutes); as such this now opens up avenues for further diagnostic applications (Figure 4C). The bactericidal effect of vanc-TCO, however, became more evident as incubation times increased, demonstrating that vancomycin still retained its bactericidal activity even after the chemical modification with TCO. It is thus possible that vanc-TCO could be used for both pathogen detection as well as for treatment of infection. Control bacteria (*E. coli*) did not show any bactericidal effect with vanc-TCO during either short or long treatment periods.

Detection of intracellular bacteria

In vivo, bacteria are often present within phagocytic cells and thereby escape detection; this is especially true in chronic inflammation. We therefore tested whether the bioorthogonal labeling method could be used for intracellular detection of such pathogens. Macrophages in culture were incubated with *S. aureus* and then washed to remove extracellular pathogens. The cells were subsequently treated with vanc-TCO and MFNP-Tz, as described above but with some modifications. First, when targeting cells with MFNPs, we added a small amount of cell permeabilization solution containing saponin. The agent was previously found to be better at maintaining the viability and integrity of live cells than other permeabilizing agents.¹³ The semipermeabilization allowed facile entry of MFNPs by penetration through the cell membranes and not by phagocytosis. Second, we incubated the particles for a longer time (40 min) to ensure the delivery of MFNPs into the macrophages and their subsequent binding to intracellular bacteria. Figure 4D shows that *S. aureus* could indeed be labeled intracellularly within live macrophages. Since the treatment of MFNPs for intracellular labeling was done in presence of the permeabilizing agent and not after permeabilization, the MFNPs, after entering the macrophages by penetrating through cell membranes, would have more chance to bind specifically to the pathogens. To eliminate the possibility that our observations were simply the result of normal phagocytosis, we performed the identical experiments on *E. coli* as well as in the absence of bacteria; neither experiments showed the presence of intracellular MFNPs (Figure S4). It is therefore conceivable that the intracellular detection of gram-positive bacteria could be entirely based on small molecule reactants (vanc-TCO and fluorescent dye-Tz). This would avoid cell permeabilization steps, and would perhaps allow the performance of *in vivo* labeling following systemic administration of the reaction partners.

Bacterial nanoparticle detection via daptomycin cycloaddition

The above-described bioorthogonal strategy could be extended to the development of affinity ligands using other antibiotics. As an example, we used daptomycin, which is another potent drug that targets gram-positive bacteria. As for vanc-TCO, the primary amine group in daptomycin was modified to attach a TCO molecule. Figure 5A depicts the binding mechanism of daptomycin-TCO (dapt-TCO) to the bacterial cell wall. In the presence of Ca^{2+} , dapt-TCO oligomerizes and binds to the bacterial cell wall, which then causes the formation of membrane pores and consequent depolarization.^{14, 15} Dapt-TCO was synthesized from TCO-NHS ester and daptomycin. Figure 5B shows HPLC and ESI-MS traces that confirm the identity and purity of the bioorthogonal reactive probe. Complementary application of dapt-TCO and MFNP-Tz consequently resulted in highly specific labeling of gram-positive bacteria, with increased relative fluorescence intensities of up to 220% (Figure 5C) and cellular relaxivity of $1.32 \text{ s}^{-1}[\text{CFU}/\mu\text{l}]^{-1}$ (Figure 5D). Although daptomycin has been reported to have higher bactericidal effects than vancomycin,¹⁶ dapt-TCO proved less effective than vanc-TCO in bacterial binding, presumably because its binding *via* oligomerization sterically hinders the access of MFNPs to the reactive TCO groups. The fact that daptomycin penetrates into the cell membrane could also cause a portion of dapt-TCO to be present within the bacterial cell, limiting the access of MFNPs. Note that the one-step method yielded even lower bacterial labeling (close to noise level), which made it difficult to compare labeling efficiencies between these two methods.

DISCUSSION

Our results show that TCO-modified vancomycin analogs bind to the bacterial wall of gram-positive bacteria (*S. aureus*, *S. pneumoniae*, *S. epidermidis*, and *E. faecalis*) in a dose depended fashion. Bioorthogonal nanoparticles can thus be used to label gram-positive bacteria for subsequent detection by optical and magnetic sensing devices. The extent of

particle-binding varied across bacterial species; it was also dose dependent and inhibitable with unmodified antibiotics. Interestingly, however, the K_d was similar for all gram positive bacteria tested ($\sim 5.7 \mu\text{M}$) suggesting that while all bacterial strains might employ the same binding mechanism, they likely differ in the amounts of D-Ala-D-Ala moieties available. Furthermore, we show that the bioorthogonal vancomycin derivatives have antibacterial activity at much longer incubation periods (24 hours); this is because, like the parent compound, they interfere with the incorporation of the NAM/NAG-peptide subunits into the peptidoglycan matrix. Moreover, because vanc-TCO is permeable to mammalian cells, it could potentially be used to detect intracellular bacteria, such as those present in macrophages.

Direct vancomycin-nanoparticle conjugates have been described before and have been used for bacterial scavenging,¹⁷⁻¹⁹ therapy,^{20, 21} and detection.²² In the majority of these investigations, the NH_2 terminus modification of vancomycin is used to directly attach vancomycin to the surface of different nanomaterials. When bacterial binding using direct conjugates was compared to the two-step bioorthogonal procedure in the present study, significant differences were observed. Using optical detection as the read-out, binding efficacy using the bioorthogonal method was typically one to two orders of magnitude higher than that of direct conjugates (Figure S3). We believe that this is due to the TCO-antibiotics having easier access to the bacterial cell wall, resulting in their subsequent reaction with the high-valency nanoparticles, thus leading the nanomaterials to pack more densely on the bacterial surface. Similar behavior was observed with daptomycin, which employs a different cell-binding mechanism.

Beyond that of higher affinity and sensitivity for detection, the described bioorthogonal method has several advantages over direct conjugates. Direct antibiotic-nanoparticle conjugates are typically difficult to characterize (due to the small number of drug molecules on each nanoparticle), involve multi-step conjugation procedures, and have varying conjugation yields depending on the batch or type of molecules. The bioorthogonal approach described here, however, provides a useful platform technology enabling: 1) the preparation of highly homogenous TCO-antibiotic conjugates, 2) easy adaptation of the method to other antibiotics, and 3) the use of a simplified labeling process through the use of generic Tz-derivatized probes.

Further investigation is currently underway to extend this bioorthogonal labeling method to the detection of gram-negative pathogens. A prevailing challenge has been the limited availability of universal small molecule binders. At present, many of the compounds available unfortunately act only on intracellular compartments, and are also highly cationic. As an alternative strategy, certain gram-negative strains (*e.g.*, enterohemorrhagic *E. coli* O157:H7) could be selectively detected using the same antibody-based bioorthogonal approach, recently used for the detection of protein biomarkers in mammalian cells.²³

CONCLUSION

We envision a number of important applications for the two-step bioorthogonal chemistry described here. The sensitive detection of gram-positive bacteria will certainly be one key application. By using the appropriate Tz-modified nanoprobe, detection could be performed *via* optical, magnetic, or any other sensing modality.²⁴ To demonstrate rapid and facile bacterial detection, we used a μNMR device, which does not require sample purification and which can detect nanoprobe in fluid phase.^{25, 26} It is possible, however, that the bioorthogonal strategy could be used to perform efficient purification and extraction of bacteria, since the TCO-Tz reaction is one of the fastest and most selective known bioorthogonal reactions.^{8, 23} Other applications that could likewise benefit by the

incorporation of the described method include intracellular detection of pathogens and point-of-care bacterial sensitivity testing.

MATERIALS AND METHODS

Synthesis of antibiotic-TCO conjugates

Unless otherwise noted, all reagents for syntheses of the antibiotic-TCO conjugates were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. (*E*)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate (*trans*-cyclooctene *N*-hydroxy-succinimidyl ester; TCO-NHS) was synthesized according to published procedures.⁸ For the synthesis of vanc-TCO and dapt-TCO, 8.4 mg (5.8 μ mol) of vancomycin or 9.4 mg (5.8 μ mol) of daptomycin (Cubicin; Cubist Pharmaceuticals, Lexington, MA) in dimethylformamide (DMF; 1 mL) or acetonitrile (2 mL), respectively, was added to a solution of TCO-NHS (400 μ L, 10 mg/mL in DMF) and triethylamine (8.1 μ L, 58 μ mol). The reaction mixture was stirred for 6 hours before being analyzed with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS) and high performance liquid chromatography (HPLC) using a Waters (Milford, MA) LC-MS system. For LC-ESI-MS analyses, a Waters XTerra® C18 5 μ m column was used. For preparative runs, an Atlantis® Prep T3 OBD™ 5 μ m or an XTerra® Prep MS C18 OBD™ 5 μ m column was used. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) was performed using a Bruker Daltonics APEXIV 4.7 Tesla Fourier Transform mass spectrometer (FT-ICR-MS) in the Department of Chemistry Instrumentation Facility at Massachusetts Institute of Technology. The yields were 23% (2.1 mg, 1.3 μ mol) for vancomycin and 29% (3.0 mg, 1.7 μ mol) for daptomycin. Characterization by LC-ESI-MS and HRMS-ESI resulted in the following values: vanc-TCO: LC-ESI-MS(−) m/z = 1599.7 $[M-H]^+$ −; LC-ESI-MS(+) m/z = 1600.7 $[M+H]^+$; HRMS-ESI $[M+H]^+$ m/z calculated for $[C_{75}H_{87}Cl_2N_9O_{26}]^+$ 1600.5218, found 1600.5230. Dapt-TCO: LC-ESI-MS(−) m/z = 1770.8 $[M-H]^+$ −; LC-ESI-MS(+) m/z = 1773.0 $[M+H]^+$; HRMS-ESI $[M+H]^+$ m/z calculated for $[C_{81}H_{113}N_{17}O_{28}]^+$ 1772.8019, found 1772.7970.

Preparation of nanoparticles

Magnetofluorescent nanoparticles (MFNPs) were synthesized as described previously.²⁷ The nanoparticles had a 3 nm core of $(Fe_2O_3)_m(Fe_3O_4)_n$ coated with a layer of crosslinked dextran. The average hydrodynamic diameter was 21 nm, and each particle had 22 free amine groups and 8.4 molecules of fluorescein conjugated on the surface. The measured r_1 and r_2 relaxivity values were 23 $s^{-1} mM^{-1}$ $[Fe]$ and 51 $s^{-1} mM^{-1}$ $[Fe]$, respectively. Amine-reactive tetrazine (Tz-NHS) was synthesized as reported previously.²² In brief, 3-(4-benzylamino)-1,2,4,5-tetrazine was used to produce 2,5-dioxopyrrolidin-1-yl 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoate. Tz-conjugated magnetofluorescent nanoparticles (MFNP-Tz) were prepared by reacting Tz-NHS with amine-functionalized MFNPs. Namely, MFNPs were added to a solution containing 500 times molar excess of Tz-NHS, and allowed to react in a 1:9 dimethylsulfoxide (DMSO)/phosphate buffered saline (PBS) solution containing 10 mM sodium bicarbonate at room temperature for 4 hours. Unreacted Tz-NHS was removed using Sephadex G-50 (GE Healthcare). To directly conjugate MFNPs with vancomycin, the amine groups of MFNPs were first converted to carboxyl groups by reacting them with 1000 times molar excess of succinic anhydride in PBS (containing 10 mM sodium bicarbonate), at room temperature for 4 hours. After purification of the nanoparticles with Sephadex G-50, the carboxylated MFNPs were reacted with *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma-Aldrich) and *N*-hydroxysulfosuccinimide (sulfo-NHS; Pierce) in PBS at room temperature for 1 hour (molar ratio of MFNP:EDC:sulfo-NHS = 1:1000:1000). NHS-activated MFNPs were then purified

with Sephadex G-50, reacted with vancomycin (1000-fold molar excess) in PBS for 2 hours at room temperature, and finally purified using Sephadex G-50.

Bacterial culture

Bacterial strains *Staphylococcus aureus* (*S. aureus*; #25923), *Streptococcus pneumoniae* (*S. pneumoniae*; #6318), *Staphylococcus epidermidis* (*S. epidermidis*; #29886), *Enterococcus faecalis* (*E. faecalis*; #29212), *Escherichia coli* (*E. coli*; #25922), *Pseudomonas aeruginosa* (*P. aeruginosa*; #142), and *Klebsiella pneumoniae* (*K. pneumoniae*; #43816) were purchased from ATCC (Manassas, VA). *S. aureus* and *S. epidermidis* were plated in mannitol salt agar (BD Biosciences, Sparks, MD) and colonies were cultured in Staphylococcus broth (BD Biosciences) for growth overnight. *S. pneumoniae* was plated onto selective streptococcus agar and the colony was seeded into Tryptic Soy Broth containing 5% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA) for growth. *P. aeruginosa* was plated on Pseudomonas isolation agar, and *E. faecalis*, *E. coli*, and *K. pneumoniae* were plated onto standard agar plates. For growth, *P. aeruginosa* and *K. pneumoniae* were cultured in Tryptic Soy Broth, *E. faecalis* was cultured in Tryptic Soy Broth containing 5% defibrinated sheep blood, and *E. coli* was cultured in Luria-Bertani (LB) media (BD Biosciences). Bacterial cell numbers were determined by plating onto standard agar plates and by counting the number of colony forming units (CFU).

Bacterial labeling and detection

Bacterial cells were first washed with PBS solution containing 2% fetal bovine serum (FBS) and 1 mg/ml bovine serum albumin (BSA; PBS-F). For two-step labeling, the washed cells were incubated with 1-20 μ M vanc-TCO in PBS-F at room temperature for 30 minutes. For competition studies, 1-200 μ M unmodified vancomycin was added to 20 μ M vanc-TCO. For two-step labeling using dapt-TCO, the drug conjugates were incubated in buffer solution containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM CaCl_2 , 2% FBS, and 1 mg/ml BSA at room temperature for 30 minutes. After washing twice with PBS-F, bacterial cells were incubated with 50 μ g/ml MFNP-Tz in PBS-F for 20 minutes at room temperature. For one-step labeling using directly conjugated MFNPs, bacteria were incubated with 50 μ g/ml MFNP-vancomycin in PBS-F for 30 minutes at room temperature. Unbound MFNPs were removed by washing the cells twice in PBS-F. For fluorescence spectrometry, μ NMR measurements, and flow cytometry, the cells were fixed in 10% paraformaldehyde (PFA) for 30 minutes, and then exchanged into PBS solution. Fluorescence measurements were taken using the Safire2 microplate reader (TECAN, Männedorf, Switzerland). Increased relative fluorescence intensity (%RFU) values were calculated as $100 \times (I_{\text{target}} - I_{\text{non}}) / I_{\text{non}}$, where I_{target} and I_{non} are intensity values of targeted and nontargeted bacteria, respectively. Flow cytometry was performed using LSRII (BD Biosciences). All labeling experiments and measurements were performed at least 3 times to confirm the reproducibility and robustness of the method.

Intracellular detection

RAW 264.7 mouse macrophages attached to culture slides were treated for 1 hour with either *S. aureus* or *E. coli* (200 CFUs per single macrophage) in serum-free Dulbecco's modified Eagle medium (DMEM) containing 100 μ M chloroquine (to prevent degradation of phagocytosed bacteria). Macrophages were then washed thoroughly with DMEM, and treated with 20 μ M vanc-TCO in DMEM containing 2% FBS (DMEM-F) for 1 hour. After washing away any unbound vanc-TCO with DMEM-F, 50 μ g/ml MFNP-Tz in DMEM-F containing 10% permeabilizing buffer (BD Phosflow) was added, before incubating the solution for 40 minutes. All treatments were performed in a 37 °C CO_2 incubator. Cells were then washed extensively with DMEM and stained with CellTracker Red (Invitrogen).

Microscopy

For confocal microscopy, bacterial cells were fixed in 10% PFA for 30 minutes, mounted onto poly(L-lysine) coated microscopic slides using Vectashield with propidium iodide (Vector Laboratories, Burlingame, CA), and observed under a laser scanning confocal microscope (LSM 5 Pascal, Carl Zeiss, Jena, Germany). For observing macrophages, cells were fixed in 10% PFA for 30 min and mounted using Vectashield including 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). For electron microscopy, bacterial cells were fixed in 2.5% glutaraldehyde for 30 minutes, dehydrated with a series of graded ethanol solutions, and mounted onto carbon-mesh coated copper grids (Ted Pella, Redding, CA) for subsequent observation under a transmission electron microscope (JEM 2011, Jeol Ltd., Tokyo, Japan).

Micro nuclear magnetic resonance (μ NMR)

All μ NMR measurements were performed using the portable NMR system recently developed for point-of-care operations.¹² The polarizing magnetic field was ~ 0.5 T. Transverse relaxation times were measured on 1–2 μ L sample volumes, using Carr–Purcell–Meiboom–Gill pulse sequences with the following parameters: echo time, 3 ms; repetition time, 4 s; number of 180° pulses per scan, 900; number of scans, 7. All measurements were done in triplicate, and data are displayed as mean $\pm$ standard error of mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chemistry underlying bioorthogonal magnetofluorescent nanoparticle (MFNP) labeling

(A) Vancomycin-*trans*-cyclooctene (vanc-TCO) targets gram-positive bacteria by binding onto its membrane subunits. Following incubation with MFNP-Tz, bacteria are labeled and can be detected via fluorescent or magnetic sensors. (B) HPLC (top) and ESI-MS (bottom) traces of vanc-TCO verifying its identity and purity.

Figure 2. Bacterial labeling

(A) Confocal microscopy and (B) transmission electron microscopy of *S. aureus* labeled with vanc-TCO and MFNP-Tz. Inset in top right of (B) shows labeling at a higher magnification.

Figure 3. Bacterial detection using vanc-TCO

(A) Fluorescence detection of MFNP-labeled bacteria as a function of vanc-TCO concentration. Relative fluorescence intensities were determined as described in the Methods. Data are expressed as mean $\pm$ standard deviation. (B) Detection (magnetic resonance) of bacteria following the two-step cycloaddition labeling method compared to that following the one-step labeling method (direct MFNP conjugates). Data are expressed as mean $\pm$ standard error. (C) Detection sensitivity for bacterial strains were determined by measuring serially diluted suspensions of labeled bacteria. (D) Correlation of magnetic resonance and fluorescence-based detection of the labeled bacteria.

Figure 4. Functional assays and intracellular detection

Competition studies of vanc-TCO using (A) fluorescence and (B) magnetic resonance measurements. (C) Viability of *S. aureus* was measured after treatment with vanc-TCO for either 30 minutes or 24 hours; measurements were made using the BacLight viability kit (Invitrogen). (D) Three-dimensional images (left: top view, right: perspective view) showing the intracellular detection of *S. aureus* (SA) in semipermeabilized live macrophages (MØ). Green: MFNPs; red: cytoplasm of macrophages.

Figure 5. Daptomycin-TCO for bacterial detection

(A) Binding mechanism of dapt-TCO to gram-positive bacteria. In the presence of Ca^{2+} , oligomerization occurs, and the multimeric complexes bind to the cell surface and form pores on the peptidoglycan layer. (B) HPLC and ESI-MS traces of dapt-TCO. (C) Detection of MFNP-labeled bacteria by fluorescence spectrometry at various concentrations of dapt-TCO (data expressed as mean $\pm$ standard deviation). (D) Detection of MFNP-labeled bacteria by magnetic resonance (data expressed as mean $\pm$ standard error).