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Specific Pathogen Detection Using Bioorthogonal Chemistry and Diagnostic Magnetic Resonance

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Abstract

The development of faster and more sensitive detection methods capable of identifying specific bacterial types and strains has remained a longstanding clinical challenge. Thus to date, the diagnosis of bacterial infections continues to rely on the performance of time-consuming cultures. Here, we demonstrate the use of bioorthogonal chemistry for magnetically labeling specific pathogens to enable their subsequent detection by nuclear magnetic resonance. Antibodies against a bacterial target of interest were first modified with *trans*-cyclooctene and then coupled to tetrazine-modified magnetic nanoprobes, directly on the bacteria. This labeling method was verified using surface plasmon resonance as well as by using a miniaturized diagnostic magnetic resonance device capable of highly specific detection of *Staphylococcus aureus*. Compared to other copper-free bioorthogonal chemistries, the cycloaddition reaction described displayed faster kinetics and yielded higher labeling efficiency. Considering the short assay times and the portability of the necessary instrumentation, it is feasible that this approach could be adapted for clinical use in resource-limited settings.

Faster assays combined with rapid diagnosis of bacterial types and strains would not only improve global health but would effectively lower healthcare costs.[1, 2] Some examples of these detection methods include fluorescence imaging using the sugar molecules maltohexaose and trehalose for highly specific labeling of targeted bacteria,[3, 4] and colorimetric assay based on enzyme-gold nanoparticle system.[5] In an effort to develop a simple diagnostic test that could be routinely used for the detection of bacterial pathogens in resource-limited clinical settings, we extended a bioorthogonal binding method to the identification of bacterial pathogens. The method is based on a two-step strategy in which antibodies are first modified to bind specific targets and are then coupled to multiple magnetic probes. Signals from these probes can subsequently be detected by nuclear magnetic resonance (NMR). Furthermore, in cases where target concentrations are low, this method has the added advantage of being able to amplify target signals.[6, 7] A major challenge to developing diagnostic tests for bacteria has been achieving accurate detection of target pathogens from complex specimens, which may contain multiple bacterial strains. We have now overcome this hurdle by employing the cycloaddition bioorthogonal reaction

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of *trans*-cyclooctene (TCO)-modified antibodies to robustly and efficiently label bacteria with tetrazine (Tz)-modified magnetic nanoprobes (MNPs).[8] The labeled pathogens were then detected using a newly developed diagnostic magnetic resonance (DMR) system. Here, the clinical potential of this method is demonstrated by the accurate and rapid (<30 minutes) detection of *Staphylococcus aureus* (*S. aureus*) in human sputa.

It is often the case that the use of large affinity ligands results in low conjugation efficiency to nanoprobes and thus ineffective binding of the conjugates to targeted cells.[6] As a result, each new preparation of immunoconjugates for cellular labeling requires an optimization process to maximize its binding to targeted samples. As an alternative strategy, the use of bioorthogonal covalent reactions have become of increasing interest as a novel platform for biological labeling. Such reactions are not only highly selective toward their binding partners but also have fast reactivity in biological solutions and at ambient temperatures.[9–11] The approach is also modular and generalizable. Namely, a vast array of affinity ligands for cellular targeting can be prepared using bioorthogonal components, and generic probes can be used for labeling.[12] The Tz/TCO cycloaddition in particular has demonstrated great potential: it is catalyst-free, shows good stability in biological media, displays much faster reaction kinetics compared to other bioorthogonal reactions, and is capable of significantly amplifying probe-loading onto targets.[13, 14] Indeed, recently, this reaction was successfully adapted to the molecular profiling of cancer cells[6, 7, 15] as well as to *in vivo* imaging.[16]

The cycloaddition reaction of Tz and TCO offers a modular platform for effectively coupling magnetic probes to the surface of a particular target using a generalized labeling strategy. Figure 1a describes the developed two-step approach for bacterial detection based on the Tz/TCO reaction. We initially coupled amine-reactive Tz to the amine groups present on the MNPs; MNPs were comprised of an iron oxide core and a dextran coating.[17] Likewise, affinity ligands were modified with amine-reactive TCO. Samples were first incubated with TCO-conjugated antibodies to specifically target the bacteria before coupling with Tz-modified MNPs (Tz-MNPs). This labeling method rendered the bacteria superparamagnetic, and thus enhanced the transverse relaxation of the samples, as determined by NMR. After loading the samples into disposable tubes, their relaxation rates (R_2) were measured using a miniaturized DMR system.[18]

The binding kinetics of the probes to TCO molecules were characterized using surface plasmon resonance (SPR).[19] To determine the association constant of the Tz-modified probes to TCO, sequentially increasing concentrations of Tz-MNPs were flowed over the SPR sensor surface, which was functionalized with TCO molecules. With a measured second-order rate constant of $k_2 \sim 7.4 \times 10^4\ M^{-1}\ sec^{-1}$ at room temperature, the reaction of Tz-MNPs with TCO proved fast and highly stable under the continuous flow stream of the SPR (Figure 1b). The capacity of the MNPs for multivalent binding and the fast kinetics of Tz/TCO thus indicated its suitability for rapid magnetic labeling of targeted bacteria.

S. aureus was selected as the specific target for pathogen detection, for which a highly selective antibody against the bacteria (anti-SA) was used as the affinity ligand. The antibodies were subsequently modified with TCO; an average of ~15 TCO molecules were conjugated to each antibody, as determined by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Figure S1). Each MNP was functionalized with approximately ~8 fluorescent moieties and ~56 Tz molecules, as measured by the SPDP assay.[20] The bacteria were first tagged with TCO-anti-SA before being sequentially coupled to Tz-MNPs. Fluorescence microscopy (Figure 2a) showed that bacterial labeling using the bioorthogonal approach was both efficient and consistent. In contrast, control samples showed only punctate traces of green fluorescence, thus confirming the minimal nonspecific binding of the Tz-probes (Figure 2b).

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The specificity of the Tz/TCO labeling method was further investigated by applying the assay to other relevant pathogens. ~10^5 CFU of the target bacteria (*S. aureus*), an acid-fast species (*Mycobacterium smegmatis*), as well as gram-negative (*Haemophilus influenzae*, *Escherichia coli*, *Pseudomonas aeruginosa*), and gram-positive strains (*Streptococcus pneumoniae*) were each mixed with TCO-anti-SA and then incubated with Tz-MNPs in a 300 μL volume. Each sample was then concentrated by centrifugation and transferred to the sample chamber of the DMR device for analysis (~10^4 CFU in 2 μL volume). The measured ΔR_2 value for each sample was then converted to cellular relaxivity Δr_2 (ΔR_2 divided by cell concentration), a value which is proportional to the number of MNPs per bacterium.[21] For *S. aureus*, the Δr_2 was ~9-fold higher than the background, whereas the Δr_2 for other pathogens showed small or no increase over the baseline (Figure 2c). Corresponding fluorescence measurements by flow cytometry also correlated well with the DMR results (Figure 2d), thus demonstrating the reliable dual-labeling capability of the method for both optical and magnetic detection. Since the DMR device requires only small sample volumes (~2 μL), it could be useful as a complementary diagnostic technique in cases where only scant samples are available.

We next evaluated the efficacy of this bioorthogonal labeling method using clinically relevant samples. Expectorated samples were prepared by spiking *S. aureus* (~10^5 CFU) into human sputa (1 mL volume). Reagents for three different bioorthogonal groups were synthesized to compare their labeling efficiency in clinical samples; namely Tz/TCO, Tz-norbornene (Tz/Norb) and dibenzylcyclooctyne-azide (DBCO/Azide). For the Tz/Norb method, antibodies were tagged with norbornene and Tz-MNPs were used as the binding probes;[22, 23] for the strain-promoted DBCO/Azide method, azide-modified antibodies and DBCO-MNPs were used (Figure S2).[24] Following sputum liquefaction, all samples were labeled with antibodies conjugated to the above-listed small molecules and their corresponding probes. The labeled samples were then divided for subsequent bacterial counts and DMR measurement. At the optimal probe concentration (50 μg/mL) and incubation time (15 minutes), the Tz/TCO labeling method yielded considerably higher DMR signals, outperforming Tz/Norb and DBCO/Azide methods even after longer overnight (O/N) incubation times (8 hours). This result is consistent with previous reports which have shown that the Tz/TCO system has kinetics that are several orders of magnitude faster than other bioorthogonal reactions (Figure 3a inset).[14, 22, 24]

The modular Tz/TCO approach provided a facile method for enhancing the specificity and sensitivity of bacterial detection. In addition, by enabling the attachment of multiple MNPs per target, the Tz/TCO system resulted in a far higher DMR signal (>350%) to that of direct antibody-MNP conjugates (Figure 3b). The detection threshold for *S. aureus*, using the Tz/TCO MNPs was determined to be ~200 CFU with cutoff ΔR_2 value of 5% (Figure S3). The detection sensitivity can be further enhanced by increasing the r_2 relaxivity of the magnetic particles, which is achieved by using materials with strong magnetization and by increasing the size of the magnetic nanocrystals. Indeed, by replacing the MNPs with doped iron oxide nanocrystals (NCs) with higher transverse relaxivity (r_2 = 380 mM^{-1} sec^{-1}, Figure S4),[25] the DMR sensitivity could be further improved (>300%). While the cross-linked iron oxide MNPs (transverse relaxivity r_2 = 70 mM^{-1} sec^{-1}) are sufficient for initial labeling and detection experiments, the highly magnetic NCs would be useful for detection of pathogens in scant samples. The Tz/TCO method could also be extended to antibodies against other target pathogens for labeling and detection (Figure S5). Since DMR detection requires small volumes of samples, a parent specimen can be divided into smaller aliquots, and each sample can be profiled for different pathogens via modular Tz/TCO approach. Such a capacity would be particularly useful in cases of infection where specific bacterial strains are suspected or need to be ruled out in complex biological samples. The recent outbreak of enterohemorrhagic *Escherichia coli* (EHEC) strain O104:H4 exemplifies such needs. We are

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currently investigating this approach for the detection of *Mycobacterium tuberculosis* in clinical sputum samples.

In summary, we have developed a magnetic labeling assay capable of rapid pathogen detection using bioorthogonal conjugation chemistry. The cycloaddition between TCO-labeled *S. aureus* and Tz-modified MNPs enables specific bacteria to be labeled and subsequently detected. The magnetic labeling approach is efficient, chemoselective for the targeted pathogen (determined by the antibody used), and applicable to sputum samples. By using this magnetic labeling technique together with the above-mentioned miniaturized detection device (DMR), the strategy could serve as a promising diagnostic platform for pathogen detection within a point-of-care clinical setting.

Supplementary Material

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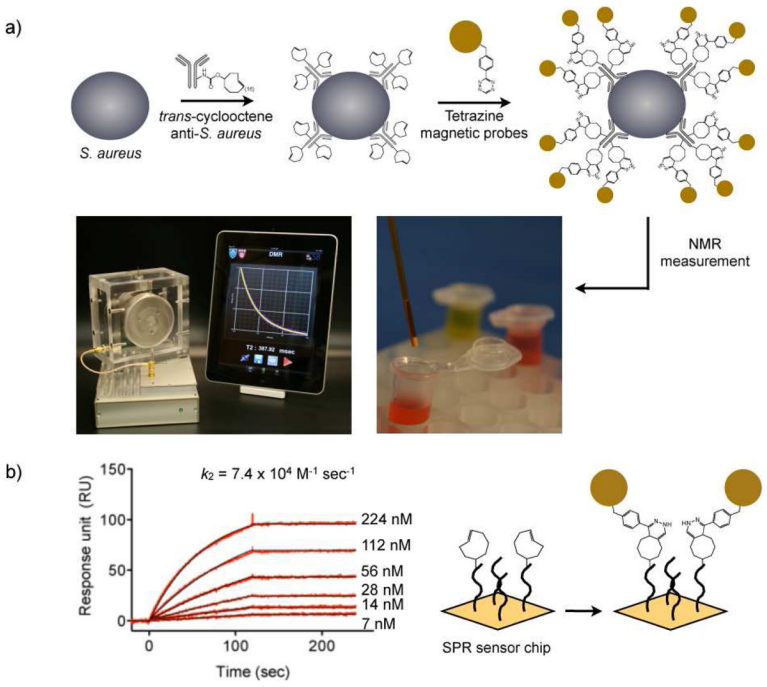

Figure 1.

(a) Schematic of the two-step magnetic labeling method involving the cycloaddition of *trans*-cyclooctene (TCO) to tetrazine (Tz). *Staphylococcus aureus* (*S. aureus*) were first targeted with TCO-modified antibodies and then coupled to Tz-modified magnetic nanoprobes. Given that each antibody is bound by ~15 TCO molecules, multiple probes can be attached to each marker. The labeled cells were subsequently loaded into a disposable tube for detection using a diagnostic magnetic resonance device. (b) Surface plasmon resonance (SPR) measurements (left) and a schematic of the binding kinetics between Tz-modified probes and surface-immobilized TCO (right). The fast second-order rate constant ($k_2 = 7.4 \times 10^4\ M^{-1}\ sec^{-1}$), determined by flowing the probes at different concentrations, is largely due to the multivalent binding of Tz-MNPs.

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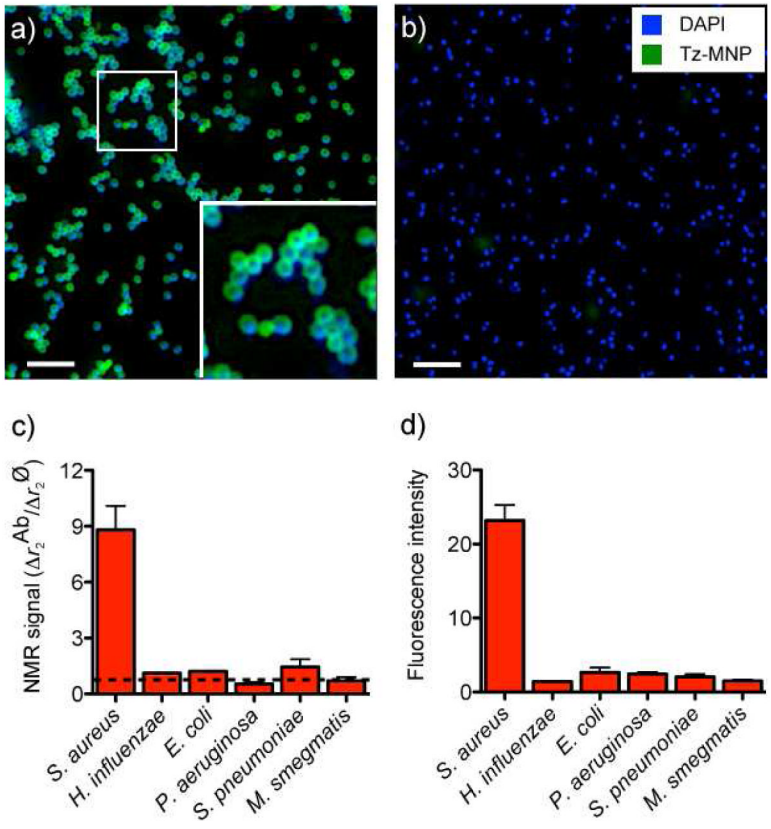

Figure 2.

Selective bacterial labeling using bioorthogonal chemistry. a) DAPI stained *S. aureus* (blue) were targeted with TCO-anti-SA and labeled with fluorescent Tz-modified probes (green). Labeling of the bacterial membrane can be clearly seen in the enlarged inset image (bottom right; higher magnification of boxed region). Scale bar, 5 μm. b) Minimal fluorescence from the probes was observed on the control non-targeted bacterial samples. c) Detection of *S. aureus* using the diagnostic magnetic resonance (DMR) device (~10^4 CFU in 2 μL volume). A panel of bacterial strains were incubated first with TCO-antibodies (specific for *S. aureus*) and then with Tz-MNPs, before undergoing measurement with DMR. The displayed nuclear magnetic resonance (NMR) signals were determined by calculating the ratio of sample Δr_2^{Ab} to non-targeted control $\Delta r_2^{\emptyset}$. Bioorthogonal labeling yielded accurate and elevated NMR signals for the targeted *S. aureus*, but not for other strains. d) Fluorescence measurements of the bacterial samples, as determined by flow cytometry. The mean fluorescence intensity was normalized by calculating the ratio between each bacterial sample and the non-targeted control. *S. aureus* showed considerably higher normalized fluorescence intensity than other bacterial strains. Overall, the fluorescence measurements obtained by flow cytometry showed an excellent correlation with measurements obtained by DMR.

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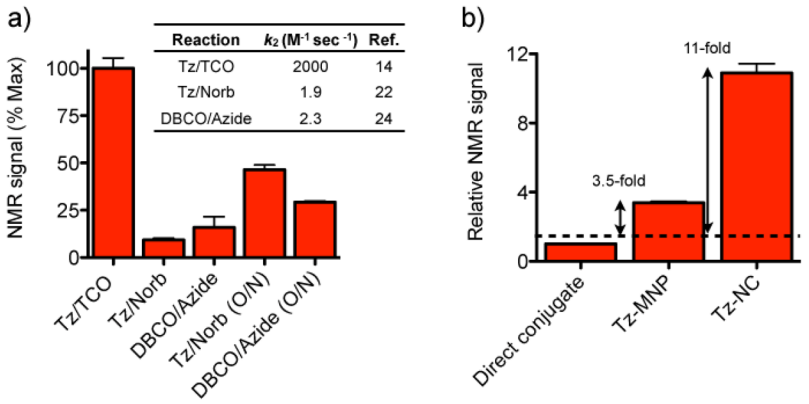

Figure 3.

Comparison of the effectiveness of different bioorthogonal labeling strategies. a) After liquefying human sputa containing *S. aureus* (~10^5 in 1 mL sputa), the bacteria were targeted and labeled using three different reactions. The inset table summarizes previously reported k_2 second-order rate constants for the different bioorthogonal chemistries. The Tz/TCO system was more effective in labeling bacteria than either of the alternative catalyst-free bioorthogonal chemistries, even with overnight (O/N) incubation (8 hours). b) The two-step labeling approach proved better at labeling bacteria than direct immunoconjugation. By using materials with higher magnetization (nanocrystals/NC *versus* magnetic nanoprobes/MNP), the NMR signal and detection sensitivity could be enhanced further.