A Magnetic Gram Stain for Bacterial Detection**

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Bacterial cell walls are made up of peptidoglycans (polysaccharides crosslinked by unusual peptides) in addition to other components.[3] Bacteria are often classified into Gram-positive and Gram-negative strains by their visual staining properties using crystal violet (CV), a triarylmethane dye.[6] Herein we show that bioorthogonal modification of crystal violet with trans-cyclooctene can be used to render Gram-positive bacteria magnetic. This modification allows for class-specific automated magnetic detection, magnetic separation, or other magnetic manipulations.

The Gram stain is one of the most commonly used tools for detecting and differentiating bacteria. The method is routinely used for clinical diagnostic purposes, as well as detecting bacteria in environmental samples. The procedure involves staining bacterial samples with crystal violet, which binds to the peptidoglycan layer of Gram-positive and Gram-negative bacteria (Figure 1). Subsequent treatment with iodine solution results in crystal violet to form an insoluble complex. Gram-positive bacteria have a thick peptidoglycan layer, whereas Gram-negative bacteria only have a thin peptidoglycan layer covered by lipopolysaccharides and lipoproteins. Upon decolorization with alcohol or acetone, only Gram-positive bacteria remain purple, while Gram-negative bacteria loose the purple color.[3-5] Despite the simplicity and robustness of the staining procedure, the final detection still relies on optical microscopy, which is often susceptible to user-dependent sampling error. Strategies for quantitative and automated detection are highly desirable, especially for the diagnosis of infectious pathogens.

Magnetic, rather than optical, labeling and detection are advantageous because of their high sensitivity and ability to diagnose crude specimens without major purification.[6] For example, one could envision rapid and sensitive detection of bacterial samples in point-of-care settings by using a miniaturized micro magnetic resonance (μNMR) device.[7,8] Direct bacterial detection by μNMR is a sensitive diagnostic method[9] and potentially allows the exclusion of culturing steps and thus minimizes the time required for diagnosis. Alternative magnetic detection devices include giant magnetoresistance,[10] or Hall sensors.[31] Furthermore, rendering bacteria magnetic also has implications for magnetic separation,[11,12] cell sorting,[13] magnetic force microscopy,[14,15] or micromanipulation and force measurements using magnetic tweezers.[15]

We hypothesized that orthogonal triarylmethane-dye derivatives could be used as affinity ligands to bioorthogonally couple magnetic nanomaterials onto Gram-positive bacteria. We thus developed a crystal violet modified with trans-cyclooctene (CV-TCO). We show that this reagent can finally couple magnetic nanomaterials onto Gram-positive bacteria similar to the native crystal violet. Importantly, the CV-TCO can also serve as an anchor to attach tetrazine (Tz)-modified magnetic nanoparticles (or other Tz-derivatized reporters). The developed magnetic Gram stain method was then used to enable highly sensitive detection of Gram-positive pathogens by μNMR.

Crystal violet (CV; 4,4′,4″-dimethylaminotriphenylmethane) is a deep purple dye. We sought to develop a chromophore derivative where one of the anilino moieties is modified with a trans-cyclooctene (TCO) orthogonal group. We started the synthesis by the condensation of two equivalents of dimethylaniline with para-nitrobenzaldehyde under microwave (MW) irradiation at 90°C for four minutes in the presence of a catalytic amount of aniline (Scheme 1).[16] The aromatic nitro group was then reduced quantitatively by hydrogenolysis in presence of activated palladium affording the free amine 2 (Scheme 1). However, the formed adduct instantaneously oxidizes in presence of air, thus rendering purification and further conjugation difficult. The oxidation...
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staining and the cellular relaxivity in Gram-positive species, thereby confirming that CV-TCO on the bacterial surface was accessible for reaction with MFNP-Tz.

The labeling strategy was further applied to a panel of different bacterial species (Figure 3). Results showed that all Gram-positive species tested showed significantly higher cellular relaxivity values when compared to Gram-negative bacteria. Such magnetic labeling enabled the performance of highly sensitive and rapid detection of Gram-positive bacteria. Titration measurements with serially diluted bacterial samples established that the detection limit with the current experimental setup was approximately 4000 bacteria (Figure S5 in the Supporting Information). This detection method is significantly better than standard UV absorption detection, which has a detection limit of approximately $10^5$ bacteria (Figure S6 in the Supporting Information). It is likely that the sensitivity of the magnetic detector could be improved to the level of single cells by 1) further miniaturizing the µNMR detection coils, 2) implementing fluidic systems for bacterial enrichment (e.g., membrane filters, magnetic separation steps), and 3) employing different types of magnetic readers (e.g., Hall-effect sensors, giant magnetoresistive sensors).

Bioorthogonally labeled bacteria were also analyzed by confocal microscopy using MFNP-Tz (Figure 4A). Bacteria stained with CV-TCO showed uniform and high fluorescence signals in the bacterial cell wall, while the control experiments without CV-TCO showed no signal (Figure S7 in the Supporting Information). Similarly, transmission electron microscopy was performed in bacteria treated with CV-TCO but which were incubated with tetrazine-modified gold nanoparticles (AuNP-Tz). Gold nanoparticles were used instead of magnetic nanoparticles to obtain higher contrast. Gold nanoparticles were found distributed throughout the bacterial surface treated with CV-TCO, while bacteria without CV-TCO labeling showed a smooth surface devoid of nanoparticles (Figure 4B).

By modifying the above procedure, the detection strategy can be applied to detect both Gram-positive and Gram-negative bacteria. Performing the staining without the decolorization process would result in labeling both Gram-positive and negative species, since the Gram-negative species would also retain the CV-TCO (Figure S8A in the Supporting Information). This coloring of both species in the first step is in analogy to the conventional Gram stain where the first staining step “colors” all bacteria and the second decolorization step allows differentiation between the two Gram classes.

The staining and the cellular relaxivity in Gram-positive species, thereby confirming that CV-TCO on the bacterial surface was accessible for reaction with MFNP-Tz.
Gram-positive species retained their signals (Figure S8B in the Supporting Information). Through these sequential measurements, it is thus possible to obtain total bacterial counts (i.e. detection before decolorization) as well as their Gram-negative and Gram-positive composition (i.e. detection after decolorization).

In summary, we show that an orthogonal CV can be used to detect and broadly classify bacteria in biological samples. Staining bacteria with CV-TCO using the standard Gram stain procedure, followed by labeling with MFNP-Tz allows the detection and characterization of bacteria both by μNMR as well as by optical imaging. The “magnetic Gram stain” could be potentially implemented into automated point-of-care diagnostics, bacterial enrichment for subsequent analysis, as well as into therapeutic applications that utilize the antibacterial, antifungal, and antihelminthic properties of CV. The method could also be used to label bacteria in vivo for various imaging applications. Moreover, the staining strategy presented could be further extended to other small molecule affinity ligands (e.g., bioorthogonal carbol fuchsin or trehalose for mycobacterial species) to enable either universal or specific detection of other bacterial targets. This ability will not only facilitate the clinical diagnosis of a range of bacterial infections but will also promote advances in basic microbiological research.

Received: April 18, 2012
Published online: ■■ ■■. ■■■■■

Keywords: bacteria · biosensors · cycloaddition · imaging agents · nanoparticles

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