Published in final edited form as:

Chem Commun (Camb). 2015 November 14; 51(88): 15964–15966. doi:10.1039/c5cc05772h.

Aggregation-Based Detection of *M. smegmatis* Using D-Arabinose-Functionalized Fluorescent Silica Nanoparticles

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Abstract

Fluorescein-doped silica nanoparticles (FSNPs) functionalized with D-arabinose (Ara) showed strong interactions with *Mycobacterium smegmatis* (*M. smegmatis*) and caused bacteria to aggregate. This aggregate formation was used as a means to detect *M. smegmatis* at the concentration of 10^4 CFU/mL.

Tuberculosis (TB) is among the most widespread plagues that mankind faced for centuries. In 2013, there were estimated 9 million new cases of TB reported and 1.5 million deaths.¹ The resurgence of TB in recent years calls for new diagnostic and therapeutic strategies.² Rapid diagnosis is the first critical step to ensure effective treatment. A number of methods are currently in use for TB diagnosis.³ Culture has been the gold standard, and involves growing the bacteria from the patient's sample in growth medium or on an agar plate and looking for the microorganism.⁴ It has high sensitivity, but results can take several weeks to obtain due to the slow growing nature of TB.⁵ The nucleic acid test employs PCR (polymerase chain reaction) to identify nucleic acid sequence that is specific for *M. tuberculosis*.⁶ Results can be obtained quickly with reasonable accuracy, however, the infrastructure and material costs are relatively high.⁷ In resource-limited regions where TB is the most widely spread, smear microscopy remains the most popular. It is over a century old technique that relies on the waxy and highly impermeable nature of the TB cell wall.⁸ In smear microscopy, a thin film of the sputum sample is smeared on a glass slide. This is then followed by a series of treatment with dyes and reagents leaving only the acid-fast bacteria, i.e., TB, stained, which can be visualized under an optical or fluorescence microscope. Smear microscopy is inexpensive, but is tedious to perform and has limited sensitivity. It may require as many as 10^6 bacterial/g of tissue to detect by light microscopy.⁹ Fluorescence microscopy has shown to increase detection sensitivity by 10% or more than the light microscopy.¹⁰

In this article, we report an alternative strategy to detect mycobacteria by aggregation using D-arabinose (Ara)-functionalized nanoparticles. Mycobacteria cell wall contains

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[†]Electronic Supplementary Information (ESI) available: Experimental details on the synthesis of glyconanoparticles, TEM, DLS and TGA characterization of nanoparticles, carbohydrate density measurement, TEM sample preparation and additional TEM images, SEM images and cell viability data. See DOI: 10.1039/x0xx00000x

peptidoglycan, arabinogalactan and mycolic acids covalently linked together.^{11,12} Ara is a vital component in mycobacterial cell wall, critical to the survival of the bacteria. Exogenous Ara is transported to the mycobacterial cytoplasm,^{13–15} and is then used in the synthesis of cell wall associated polysaccharides such as arabinogalactan and arabinomannan.^{16, 17} Anton et.al. reported that the addition of Ara to mycobacterial aggregates de-clumped the bacteria,¹⁸ and the authors suggested a potential existence of an Ara-binding lectin on mycobacteria. Encouraged by this observation, we hypothesize that by conjugating Ara on nanoparticles, the multivalent Ara-presenting nanoparticles would interact with mycobacteria to form clusters, which can be visualized under microscopy.

Fluorescein (FITC)-doped silica nanoparticle (FSNP) was used in this study. FSNP was prepared by co-condensing tetraethyl orthosilicate with FITC-derivatized silane (Scheme 1a)^{19, 20} to give particles of 30.4 ± 1.9 nm (TEM) or 38.6 ± 1.7 nm (DLS) (Fig. S1). Ara was conjugated to nanoparticles using the photocoupling chemistry developed in our laboratory (Scheme 1b).^{21–24} FSNP was first functionalized with silane-derivatized perfluorophenyl azide (PFPA-silane) to give PFPA-FSNP (Fig. S2), which is evidenced by the appearance of asymmetric stretch of the azide ($-N_3$) at ~ 2119 cm^{-1} in the FTIR spectrum (Fig. S3). Ara was then covalently conjugated to PFPA-FSNP by irradiating the particles in the presence of an aqueous solution of Ara (Fig. S4).^{25, 26} The density of Ara conjugated on the nanoparticles was determined by thermal gravimetric analysis (TGA) to be 3.8×10^{-16} $\mu\text{g}/\text{nm}^2$ (Fig. S5, Table S1). D-Glucose (Glc), D-galactose (Gal) and β -cyclodextrin (CD)-conjugated nanoparticles were used as the control (Scheme S1). Glc was used as the positive control because Glc-NP also showed interacting with mycobacteria in a previous study.²⁷ Gal is also a carbohydrate substrate for mycobacterial transporters, and is used as a positive control. CD was used as a negative control. The densities of Glc, Gal, and CD on the nanoparticles were determined by TGA to be 2.6×10^{-16} , 2.1×10^{-16} and 2.0×10^{-16} $\mu\text{g}/\text{nm}^2$, respectively (Table S1).

M. smegmatis was used as the model mycobacterium due to its similarity to the pathogenic *M. tuberculosis*.^{28, 29} It is fast growing and is widely accepted as a mycobacterium model for the development of drugs against TB.^{30, 31} First, time study was performed to study the time needed to have maximal interactions between Ara-FSNP and *M. smegmatis*. In the experiment, Ara-FSNP nanoparticles were treated with *M. smegmatis* strain mc² 155 at 37 °C for 2, 3, 4, 5, 6 h. After excess nanoparticles were removed by centrifugation, the samples were examined under an optical microscope. Results showed that nanoparticles incubated for 5–6 h had significantly higher interactions and aggregate formation with *M. smegmatis* (Fig. S6). This protocol was then used for the subsequent studies. Thus *M. smegmatis* mc² 155 was incubated with the nanoparticles at 37 °C for 6 h, and the excess particles were removed by centrifugation. TEM images showed that nanoparticles conjugated with Ara had significantly higher interactions with *M. smegmatis* than nanoparticles modified with Glc, Gal or CD (Fig. 1). Furthermore, aggregates were formed upon treating *M. smegmatis* with Ara-FSNPs (Fig. 1a). No such aggregates were seen when the mycobacteria were treated with Glc-FSNPs, Gal-FSNPs or CD-FSNPs (Fig. 1b–d).

The interactions were next investigated by fluorescence microscopy. In this case, *M. smegmatis* was first treated with a red fluorescent nucleic acid staining dye, SYTO® 61.

Ara-FSNP, Gal-FSNP or CD-FSNP was then incubated with the mycobacteria (10^8 CFU/mL) at 37 °C for 6 h. After excess nanoparticles were removed, the sample was examined under laser scanning confocal microscopy (LSCM). When the sample was excited at 633 nm, red fluorescent aggregates of mycobacteria were seen (Fig. 2a). At 488 nm excitation, the green fluorescent Ara-FSNPs were visible in the areas where *M. smegmatis* aggregates were present (Fig. 2b). When *M. smegmatis* was incubated with Gal-FSNP (Fig. S6a) or CD-FSNP (Fig. S6c), very little aggregated bacteria were seen. More importantly, neither Gal-FSNP nor CD-FSNP was observed around the bacteria (Fig. S6b, S6d). These results demonstrate that the interaction of FSNP with *M. smegmatis* is highly carbohydrate-specific. Only Ara-FSNP interacted strongly with *M. smegmatis* and caused the bacteria to aggregate. To test that the Ara-nanoparticle induced aggregation was specific for mycobacteria, Ara-FSNP was treated with a Gram-negative bacterium, *E. coli* strain ORN 178, and a Gram-positive bacterium, *Staphylococcus epidermidis* (*S. epidermidis*) strain 35984, at 37 °C for 6 h, respectively. After excess nanoparticles were removed, the samples were examined under TEM. Neither *E. coli* or *S. epidermidis* formed aggregates (Fig. S7), indicating that Ara-FSNP induced bacteria aggregation is highly specific for mycobacteria.

The aggregation of *M. smegmatis* by Ara-FSNP was then applied for the detection of mycobacteria. Different concentrations of *M. smegmatis* were incubated with Ara-FSNP at 37 °C for 6 h. After excess nanoparticles were removed, samples were examined under a light microscope (Fig. 3). Obvious aggregates were observed for *M. smegmatis* ranging from 10^7 CFU/mL to 10^4 CFU/mL (Fig. 3a–d). The aggregates were also seen under fluorescence microscope where green-fluorescent Ara-FSNP were clearly visible (Fig. 3e–h). At below 10^4 CFU/mL, no obvious aggregates were detected. When *M. smegmatis* was incubated with Gal-FSNP or CD-FSNP, however, no aggregates were observed in these samples under either optical or fluorescence microscope (Fig. S8). These results demonstrate that the Ara-nanoparticle induced aggregation can be used for the detection of mycobacteria, and the sensitivity reached 10^4 CFU/mL.

In conclusion, we have demonstrated that the conjugation of Ara on nanoparticles resulted in strong interactions with *M. smegmatis*, causing the mycobacteria to aggregate. The interaction is specific for Ara, and no bacteria aggregation was observed when treated with particles functionalized with Glc, Gal or CD. Furthermore, Ara-functionalize nanoparticles did not induce bacteria aggregation for *E. coli* ORN 178 or *S. epidermidis* 35984. This general strategy of using Ara-functionalized nanoparticles to facilitate mycobacteria aggregation was employed to detect *M. smegmatis* at a concentration as low as 10^4 CFU/mL. The mechanism by which Ara-mediated bacterial aggregation is yet to be determined. Nevertheless, the work described here represents a straightforward method, and may potentially be developed into an alternative test for the detection and imaging of TB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH (R01GM080295 and R21AI109896), and University of Massachusetts Lowell.

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Figure 1.
TEM images of *M. smegmatis* strain mc²155 after incubating for 6 h with (a) Ara-FSNPs, (b) Glc-FSNPs, (c) Gal-FSNPs, (d) CD-FSNPs.

Figure 2.
LSCM images of SYTO® 61 dye-stained *M. smegmatis* after incubating with Ara-FSNPs. Bacteria concentration of the sample was 10^8 CFU/mL. (a) Images taken at 633 nm excitation showing SYTO® 61-stained red *M. smegmatis*. (b) Images taken at 488 nm excitation showing green FSNP.

Figure 3.
(a–d) Optical, and (e–h) LSCM images of *M. smegmatis* after incubating with Ara-FSNPs. Bacteria concentrations were (a, e) 10^7 CFU/mL, (b, f) 10^6 CFU/mL, (c, g) 10^5 CFU/mL, (d, h) 10^4 CFU/mL.

Scheme 1.

(a) Synthesis of FSNP. (b) Synthesis of PFPA-functionalized FSNPs and conjugation of Ara onto FSNP.