Multimodal Magneto-Fluorescent Nanosensor for Rapid and Specific Detection of Blood-Borne Pathogens

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Supporting Information

ABSTRACT: Detection of bacterial contaminants in blood and platelet concentrates (PCs) continues to be challenging in clinical settings despite available current testing methods. At the same time, it is important to detect the low bacterial contaminants present at the time of transfusion. Herein, we report the design and synthesis of a dual-modal magneto-fluorescent nanosensor (MFnS) by integrating magnetic relaxation and fluorescence modalities for the wide-range detection of blood-borne pathogens. In this study, functional MFnSs are designed to specifically detect *Staphylococcus epidermidis* and *Escherichia coli*, two of the predominant bacterial contaminants of PCs. Specific interaction between the target pathogen and functional MFnS resulted in the change of water proton’s magnetic relaxation time, indicative of sensitive detection of the target bacteria from low to high colony-forming units (CFUs). In addition, the acquired magnetic relaxation signal of the MFnS further facilitated quantitative assessment of the slow and fast growth kinetics of target pathogens. Moreover, the presence of fluorescence modality in MFnS allowed for the detection of multicontaminants. Bacterial detection was also performed in complex media including whole blood and PCs, which further demonstrated its robust detection sensitivity. Overall, our study indicated that the designer MFnS will have potential for the wide-range detection of blood-borne pathogens and features desirable qualities including timeliness, sensitivity, and specificity.

KEYWORDS: magneto-fluorescent nanosensors, blood-borne pathogen, magnetic relaxation, fluorescence, sepsis

INTRODUCTION

Bacterial contamination of blood and blood products, particularly platelet concentrates (PCs), continues to pose a major challenge in the world of transfusion medicine. This, in turn, often leads to various life-threatening medical conditions including sepsis. According to Centers for Disease Control and Prevention estimates, 1 in 1000 transfused units may be contaminated with bacteria at the time of transfusion. Additionally, the frequency of bacterial contamination in transfused PCs is 10 times higher than that in red blood cells. *Staphylococcus epidermidis* (S.e.), *Staphylococcus aureus* (S.a.), *Escherichia coli* (E.c.), and *Bacillus cereus* (B.c.) are the most frequent bacteria found in contaminated PCs. However, the storage temperatures and agitation necessary to maintain the temperatures ranging from 20 to 25 °C, contribute significantly to the bacterial growth within the storage temperatures ranging from 20 to 25 °C. Currently, several methods are available for the screening of PCs for bacterial contaminants. For example, culture-based testing, real-time polymerase chain reaction (PCR), and flow cytometry (FCM) are commonly used techniques. While these methods are sensitive, they carry their own limitations and may not completely meet the required standard for detection.

In particular, the culture-based testing method is one of the most commonly used techniques for bacterial detection. Although this method is inexpensive, it is extremely inefficient with regard to time and labor. The real-time PCR is often prone to false-positive results because of DNA contamination of the PCR reagents. FCM is also used for the detection of PC contaminations. While effective, several modifications including culturing of PCs in bacterial growth media are needed for low concentration levels of bacteria in platelets, which limits the detection capability. Other obstacles for FCM include slow-growing bacteria not reaching titers at high enough levels to be detected because of differential growth kinetics. Some of these methods are also used for the testing of blood cultures for bloodstream infection detection. Hence, new technologies are arguably needed that can detect pathogens in simple and complex media with minimal sample preparation, without the need for further sample amplification, and with low turnaround time.

In recent years, the unique combination of nanotechnology and magnetic relaxation (MR) achieved through the fabrication of magnetic relaxation nanosensors (MRnSs) has...
been extensively used for the rapid detection of bacterial targets with greater sensitivity. These MRNPs are synthesized by conjugating antibodies/affinity ligands on the surface of a superparamagnetic iron oxide nanoparticle (IONP). The underlying principle behind the detection lies in the switching of MRNPs between dispersed and clustered states due to interaction with the targeted bacterial contaminants, which results in a simultaneous change in the spin—spin relaxation time (T2 MR) of the water protons.

Moreover, the magnitude of ΔT2 MR can be directly correlated with the target concentration. In likewise fashion, the use of magnetic nanoparticles in conjunction with fluorescence technology leads to significant improvements in the detection of pathogens at high concentrations, hence allowing a wide detection range. In our previous findings, the integration of MR technology with fluorescence technology leads to significant improvements in the detection of pathogens at high concentrations, hence allowing a wide detection range. In our previous findings, the integration of MR technology with fluorescence technology allowed the rapid and sensitive detection of low and high concentrations of E. coli O157:H7 [1–100 colony-forming units (CFUs)/mL] in water and liquid food samples. These MRNPs may be directly correlated with the target concentration.

In this study, we have designed new MFNs for the rapid and specific detection of various blood-borne pathogens. These functional MFNs (Figure 1) are also suitable for the simultaneous detection of multicontaminants in PCs and in whole blood. S. epidermidis was chosen as one of the target pathogens for the present study because it accounts for 25% of bacterial contaminations in PCs. On the other hand, E. coli was selected because of its faster growth rate than S. epidermidis, in order to evaluate the differential growth kinetics in one step using our designer MFN. Overall, the present work represents novel multimodal MFNs nanoplatforms for the rapid, sensitive, and differential detection of multiple pathogens in simple and complex media.

**MATERIALS AND METHODS**

**Materials.** ACS reagent grade iron salts [ferric chloride (FeCl3·6H2O) and ferrous chloride (FeCl2·4H2O)], hydrochloric acid, and ammonium hydroxide were purchased from Fisher Scientific. Poly(acrylic acid) (PAA), 2-morpholinoethanesulfonic acid (MES), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide ( NHS) were purchased from Sigma-Aldrich and used as received. Bacterial strain S. epidermidis and E. coli were acquired from American Type Culture Collection (ATCC). S. epidermidis monoclonal antibody (MA1-35788) was purchased from Invitrogen, and E. coli monoclonal (ab25823) was obtained from Abcam. Nutrient broth and nutrient agar were purchased from Becton Dickinson. The fluid thioglycolate medium was procured from BBL. Human PC and whole human blood were purchased from Becton Dickinson.

**Synthesis and Purification of Functional MFNs.** Encapsulation of Fluorescent Dyes in PAA-Coated IONPs (IONP-COOHs). First, IONP-COOH was synthesized according to our previously reported method and briefly described in Scheme S1. Fluorescent Dil/DIR dyes were encapsulated within the PAA coatings of IONPs using a solvent diffusion method. Briefly, 2.0 μL of a Dil/DIR dye (3.0 mmol) reconstituted in 100 μL of dimethyl sulfoxide was added in a dropwise manner to 4.0 mL of IONP-COOH (4.0 mmol) with constant mixing at 1100 rpm. Finally, the resulting IONP-Dil-COOH and IONP-Dir-COOH solutions were purified using a magnetic column with phosphate-buffered saline (PBS; pH 7.4; final concentration [Fe] = 3.0 mmol) as the eluent and a QuadroMAX LS column from Milteniyibiotec. Dynamic light scattering (DLS; Figure S1) and UV–vis (Figure 2) studies further confirmed the successful encapsulation of the dyes within the PAA coatings of the IONPs.

**Formulation of Antibody-Conjugating MFNs.** Antibody-conjugating magnetic nanoparticles were synthesized following our previously reported protocol. Briefly, the following solutions were prepared: (1) 4.0 mL of IONP-Dil/Dir-COOH (3.0 mmol) added to 1 mL of PBS (pH 7.4); (2) 5.0 mg of EDC in 250 μL of a MES buffer (0.1 M, pH 6.0); (3) 3.0 mg of NHS in 250 μL of a MES buffer (0.1 M, pH 6.0); (4) 5.0 μg of the corresponding antibodies IgG, the S. epidermidis mAb, or E. coli mAb in 225 μL of PBS (pH 7.4). After the preparation of solution 2, it was immediately added to solution 1, followed by the addition of solution 3, with brief mixing. This reaction mixture was incubated for an additional 3 min, and the final addition of solution 4 was done in a dropwise manner. The antibody-conjugated nanoparticles (MFNs-S.e., IONP-Dil-S.e-mAb;
MFNs-E.c., IONP-DiR-E.c.-mAb) were kept in a table mixer at room temperature for 3 h and then incubated at 4 °C overnight. After conjugation, further purification was performed using a QuadroMACS magnetic column (LS) to separate the unconjugated antibodies and other free reagents. The final concentration of the antibody-conjugating MFNs was adjusted to [\text{Fe}] = 2.0 mmol.

**Characterizations of the Functional MFNs.** Size, ζ Potential, Fluorescence, and MR Measurements. Using Malvern’s Nano-ZS90 Zetasizer, we measured the average size and overall surface charge (ζ potential) of the IONP-COOH and MFNs encapsulated with two different fluorescent dyes. For average size measurements, three consecutive measurements were taken from a sample, whereas one measurement was taken per sample for collecting the ζ potential. The average size and ζ potential of IONP-COOH were found to be 82 ± 1 nm and −24 mV, respectively (Figure 1). The average size and surface charge of IONP-DiR-mAb (MFNs-S.e.) were 87 ± 2 nm and −27 mV, respectively. For IONP-DiR-mAb (MFNs-E.c.), the average size and surface ζ potential were found to be 90 ± 1 nm and −29 mV, respectively (Figure S2). Using TECAN’s infinite M200 PRO high-throughput plate reader, the UV–vis and fluorescence of MFNs-S.e. and MFNs-E.c. were measured. Fluorescence experiments showed emission at 575 and 796 nm for MFnS-S.e. and MFnS-E.c., respectively, which confirmed the effective encapsulation of both dyes (Figure 2). Bruker’s magnetic relaxometer mq20 (0.47T, B = 20 MHz) was used for spin−spin T₂ MR experiments and showed T₂ MR at 152 and 165 ms for MFnS-S.e. and MFnS-E.c., respectively (Figure S3), treated as baselines for all detection experiments.

**Bacterial Culture.** The lyophilized pellet of bacteria obtained from ATCC was resuspended in 1.0 mL of nutrient broth. Ten to 1.0 mL of the bacterial suspension was added 5.0 mL of fresh media. A bacterial growth medium (BBL Bacterial Culture) was used for the cultivation of both bacteria in 200 μL, [Fe] = 2.0 mmol). A similar protocol was repeated when the lyophilized pellet of bacteria obtained from ATCC was resuspended in 1.0 mL of nutrient broth. To the 1.0 mL of this solution was added 200 μL of antibody-conjugating MFNs (MFNs-S.e. and MFNs-E.c., respectively) (Figure S3), treated as baselines for all detection experiments.

**Detection of Bacterial Contamination in Complex Media Using MR Technology.** All T₂ MR measurements were performed at 37 °C using Bruker’s magnetic relaxometer. A constant amount of MFNs (200 μL, [Fe] = 2.0 mmol) was added to PBS solutions (500 μL, 1X, pH 7.4) spiked with increasing concentrations (ranging from 1 to 200 CFUs/mL) but also in 10% whole blood and in serum (Figure S4), as determined by DLS measurements over a period of 2 months. Spectrophotometric studies showed an absorption band at λ₉₀₀ = 554 nm and an emission band at λₙ₅₅ = 575 nm for IONP-DiS-S.e.-mAb and an absorption band at λ₉₀₀ = 759 nm and an emission band at λₙ₅₅ = 796 nm for IONP-DiR-E.c.-mAb (Figure 2). The emission spectra were compared with those of the corresponding free optical dyes, indicating that the synthesized MFnSs were not contaminated with any free optical dyes after purification using a magnetic column. These results were further confirmed for the successful encapsulation of fluorescent dyes within the PAA coatings of MFnSs.

Next, for the dual-modal (T₂ MR and fluorescence) detection of pathogens, the following steps were followed. The purified antibody functional MFnSs (200 μL, [Fe] = 2 mmol) was incubated for 15 min with bacterial solutions (500 μL) from low to high concentrations (1–200 CFUs/mL), and the sensitive T₂ MR measurements were collected in each case. Following the collection of T₂ MR readings, the solutions were centrifuged at 2880g for 10 min to remove any unbound MFnSs. The remaining MFnS-bound bacterial cell pellets were

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**RESULTS AND DISCUSSION**

**Bacterial Pathogen Detection Using a Dual-Modal Functional MFnS.** To achieve sensitive and multiplex detection of pathogens, functional MFnSs were designed and synthesized as shown in Figure 1. In this study, surface modification on MFnS allowed us to specifically detect blood-borne bacterial pathogens. Moreover, the addition of fluorescence modality enabled wide-range detection and discrimination between different bacteria.

Towards this goal, IONP-COOHs were formulated according to the previously reported solvent precipitation method (Scheme S1). Following the synthesis, fluorescent dyes were encapsulated within the PAA coatings of IONPs. The resuspended IONP-DiR-COOH ([Fe] = 3.0 mmol) showed a diameter of 84 ± 2 nm and a surface charge of −25 mV. The DiR-encapsulating IONPs (IONP-DiR-COOH; [Fe] = 3.0 mmol) reported a diameter of 85 ± 1 nm and contained a charge of −25 mV (Figure S1). These fluorescent-labeled IONPs were found to be stable for an extended period of time (Table S1). Next, monoclonal IgG1 antibodies specific for S. epidermidis (anti-S.e.-mAb) and E. coli (anti-E.c.-mAb) were conjugated on the IONP’s surface using water-based carbodiimide (EDC/NHS) chemistry. Detailed characterization studies including the size and ζ potential were carried out and are shown in Figure S2. The resuspended IONP-DiS-S.e.-mAb ([Fe] = 2.0 mmol) showed a diameter of 87 ± 2 nm and a surface charge of −27 mV. IONP-DiR-E.c.-mAb ([Fe] = 2.0 mmol) reported a diameter of 90 ± 1 nm and contained a charge of −29 mV. As shown, dispersed MFnSs were synthesized with an increase in the overall size after conjugation with antibodies. These functional MFnSs were found to be stable for a longer period of time not only in PBS (1X, pH 7.4) but also in 10% whole blood and in serum (Figure S4), as determined by DLS measurements over a period of 2 months.
resuspended in PBS, and the corresponding fluorescence measurements were recorded. According to our hypothesis, when Ab-MFnSs are placed in a solution with bacterial colonies, they migrate around the bacteria’s outer membrane via specific interactions between the surface functional IgG1 Ab and bacterial epitope. Because of this binding and clustering of the nanosensors around the bacterial colonies, interaction between the magnetic nanosensors and their surrounding water protons is inhibited, which results in a change in the spin–spin MR time ($T_2$ ms). In the presence of a small amount of bacteria (low concentration), binding of MFnS results in a higher $\Delta T_2$ value. As the concentration is raised, the nanosensors disperse within a given voxel because of the presence of additional bacterial CFUs. This phenomenon results in a reduced $\Delta T_2$ value, demonstrating that detection via MR is highly sensitive for low counts of target pathogens.

In our initial set of experiments, MFnS-based detections were evaluated using *S. epidermidis* and *E. coli*, which were cultured in nutrient broth (Figure S3A,B) and serially diluted in PBS (1X, pH 7.4) with increasing concentrations (CFU counts confirmed by plate counting). Each diluted solution (500 μL) was incubated for 15 min with the corresponding Ab-MFnS (200 μL, [Fe] = 2 mmol) at 37 °C and then transferred to a magnetic relaxometer ($B = 0.47T$) for collection of the $\Delta T_2$ values. Baseline $T_2$ MR values for both Ab-MFnSs were collected using PBS with no bacterial colonies (Figure S3) for the calculation of $\Delta T_2$. As shown in Figure 3A, this, the bacterial solutions were removed from the relaxometer and then centrifuged at 2880g for 10 min to remove any unbound nanosensors. The remaining bacterial cell pellet was then resuspended in 100 μL of PBS (1X, pH 7.4). Each of these samples (80 μL) was added to a 96-well plate, and the fluorescence intensities from the samples were recorded. The fluorescence results (Figure 4A,B) showed a linear increase in the emission intensity (linearity plot of fluorescence; Figure S6) at higher concentrations, where more MFnSs are available to bind with increased bacterial colonies, leading to amplified fluorescence signals from the encapsulating optical dye molecules in MFnS. This dual-modal detection modality would increase the sensitivity and accuracy for both high- and low-concentration solutions. Separate sets of experiments were carried out to assess the specificity in pathogen detection using our customized Ab-MFnS. Anti-S.e.-mAb-conjugated IONPs (MFnS-S.e.) were mixed with 500 μL of 1X PBS (pH 7.4) containing 10 CFUs of *E. coli*. This resulted in little to no change in the $\Delta T_2$ MR signal (●, Figure 3A). However, when this experiment was carried out in the presence of 10 CFUs of *S. epidermidis*, a sharp change in $\Delta T_2$ MR = 55 ms was recorded. Similar specificity experiments were performed for *S. epidermidis* detection using anti-E.c.-mAb-conjugated IONPs (●, Figure 3B). As expected, the fluorescence experiments showed minimum binding in these control experiments (●, Figure 4A,B). These results indicated the specific pathogen detection capabilities of our functional Ab-MFnS. Taken together, the above experiments indicated that specific detection by MR is more efficient for low bacterial concentrations, whereas fluorescence-based detection is more accurate for higher CFUs.

### Rapid and Sensitive Detection of Pathogens Using MFnSs

In order to demonstrate the faster detection capability and sensitivity of the formulated MFnS, time-dependent assays were performed for *S. epidermidis* and *E. coli*, separately. Samples were prepared by mixing 500 μL of 1X PBS spiked with 2 CFUs/mL of *S. epidermidis/E. coli* and 200 μL of specific Ab-MFnS ([Fe] = 2 mmol) at 37 °C. The $T_2$ MR data were collected at different time points over a period of 15 min (Figure 5). The results showed our MFnS was able to detect specific pathogen within 2 min of incubation. Minimal variation between the $\Delta T_2$ values over 15 min for either
transferred to a relaxometer tube for solutions were incubated for 15 min at 37 °C. Each experiment was performed in triplicate, and the data are represented as mean ± standard deviation errors.

Figure 5. Time-dependent MR assays for (A) *S. epidermidis* and (B) *E. coli* performed to demonstrate the sensitivity of magnetic nanosensor and pathogen interactions. Each experiment was performed in triplicate, and the data are represented as mean ± standard deviation errors.

Rapid Pathogen Detection in Blood and PC Using Functional MFnS. After validation of the specificity and sensitivity of the Ab-MFnS nanoplatform in a simple buffer (1X PBS), we proceeded to evaluate the analytical performance of Ab-MFnS in more complex media, including whole blood and PC. These media were selected to mimic a real-world application for our nanosensors, both being shown to be suitable for bacterial growth. Initially, MFnS-S.e. (200 μL, [Fe] = 2 mmol) was added to two serially diluted parallel samples of nutrient broth-cultured *S. epidermidis* (500 μL, 1–200 CFUs/mL), each containing 200 μL of blood and PC. These solutions were incubated for 15 min at 37 °C and then transferred to a relaxometer tube for *T₂* measurements. The MR results from Figure 6A,C demonstrated that lower concentrations (1–50 CFUs/mL) of bacteria yielded dose-dependent changes in the *ΔT₂* values, and as expected, the *ΔT₂* values showed minimal changes at higher concentrations.

Figure 6. (A and C) *S. epidermidis* and (B and D) *E. coli* cultured in nutrient broth. Specific Ab-MFnS (200 μL, [Fe] = 2 mmol) was added to serially diluted samples (500 μL) of *S. epidermidis* or *E. coli*, each containing 200 μL of blood (A and B) and PC (C and D). After 15 min of incubation at 37 °C, the samples were transferred to a relaxometer tube, and *T₂* MR measurements were performed. Each experiment was performed in triplicate, and the data are represented as mean ± standard deviation errors.

Similar results obtained for *E. coli* detection in whole blood and PC using the functional MFnS-E.c., and the results are shown in Figure 6B-D. Overall, the MFnS sensitivity in complex media followed a trend similar to that in the simple buffer. Different parameters are important in the evaluation of effective bacterial detection, including the sample volume, where a larger volume is known to yield more accurate results. However, larger blood sample volumes result in a reduced number of PCs available for transfusion. Therefore, the need for a low level of detection is significant, which can be achieved by the MR mechanism of the proposed MFnS. Our study showed that the binding of MFnS at low pathogen concentrations yielded very sensitive MR signals, as shown in Figure 6.

Simultaneous Detection of Two Target Bacteria Using MFnS. Next, the specificity of our nanosensor was further tested using a MR technique and fluorescence detection in a PC featuring an equal mixture of two pathogens, *S. epidermidis* and *E. coli*. The goal of this experiment was to determine whether our Ab-MFnS could differentiate between two pathogens present in a clinical/environmental sample. To evaluate the multiplex detection capability of MFnS, MFnS-S.e. and MFnS-E.c. were mixed in equal proportions for the simultaneous detection of two bacteria present in a PC. After incubation for 15 min at 37 °C, the solution was transferred to a relaxometer tube, and *T₂* measurement was performed. As seen from Figure 7A, the presence of a bacterial mixture leads to an average *T₂* signal for both the low and higher bacterial counts and, hence, cannot be precisely used to discriminate between two pathogens. Interestingly, fluorescence spectra were measured from suspension of the bacterial cell pellet (procedure as previously described), and two distinct fluorescence emission spectra were obtained for *S. epidermidis* (DiI: λ_{max} = 575 nm) and *E. coli* (DiR: λ_{max} = 796 nm). This is demonstrated for the detection of both contaminants in one step (Figure 7B). As seen, two different concentrations of bacterial solutions were also tested and can be precisely used to discriminate between two pathogens. Control experiments were also performed with no spiked bacteria and media only.
Rapid Assessment of Growth Kinetics of Bacteria Using MFnS. Finally, the assessment of growth kinetics of *S. epidermidis* and *E. coli* was carried out as a validation test for our functional MFnS. In order to reduce and prevent platelet-associated bacterial sepsis, it is important to detect both fast and slow growth kinetics of bacteria. To accomplish this, specific Ab-MFnS (200 μL, [Fe] = 2 mmol) were mixed with 400 μL of a bacterial growth medium (BBL fluid thioglycollate medium) containing 400 μL of PC, with the final concentration of spiked bacteria adjusted to 10 CFUs/mL. This was then incubated at 37 °C for 48 h. At every 4 h of incubation, ΔT2 MR data were collected. As shown in Figure 8, PCs contaminated with *S. epidermidis* showed slower growth than those with *E. coli*, and significant bacterial proliferation was observed for *E. coli* between 10 and 12 h. These results demonstrated that our MFnS is also able to distinguish and define *S. epidermidis* as a slow-growing pathogen, while showing *E. coli* proliferating at a much faster rate. This is an important tool because platelet storage usually does not exceed 96 h, limiting both the culture times and potentially bacterial counts to levels below 10 CFUs/mL, which could be even lower especially for slow-growing bacteria such as *S. epidermidis*.

**CONCLUSIONS**

In summary, we have generated a multimodal nanosensor platform that can simultaneously screen and identify multiple pathogenic bacteria in a simple buffer system and complex media. The paired techniques of MR and fluorescence complement one another, providing a powerful tool for the rapid detection of bacterial contamination as low as 1 CFU. This nanosensor system also demonstrated the capability of discriminating between two pathogenic bacteria including *E. coli* and *S. epidermidis*. Importantly, cross-reactivity tests established the specificity of MFnS-based detection. Furthermore, a comparison of the analytical performance of MFnS in complex samples including whole blood and PCs with that of a simple buffer revealed that the sensitivity was not compromised, and similar detection limits were achieved in each case. Our multifunctional MFnS has demonstrated the potential for the rapid and sensitive detection of blood-borne pathogens. Additionally, time-dependent assays performed for low concentrations of *E. coli* and *S. epidermidis* further confirmed the sensitivity of MFnS detection. Likewise, our nanosensor system was able to distinguish and identify the characteristically fast and slow growth kinetics of each bacteria, an important aid in the accurate detection and treatment of bacteremia. Taken together, our multimodal MFnS can greatly enhance the ability to detect contaminants in blood products in the early stages, which would markedly improve patient mortality rates. Moreover, this nanosensor technology would significantly aid physicians and care providers for the prompt identification of pathogens, improving therapy and reducing factors responsible for antibiotic-resistant bacteria.

**ASSOCIATED CONTENT**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsanm.9b01158.

Synthesis and characterizations of IONPs, functional MFnS, and supporting characterization data including hydrodynamic diameter, ζ-potential, stability, fluorescence, and MR experiments, as described in the text (PDF).

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**Author Contributions**

The project was designed by S.S. and T.B. The experiments were performed by T.T., T.B., W.B., L.H., V.J., and R.E., and the manuscript was written by S.S., T.B., and R.E. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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