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Bioconjugated Magnetic Nanoparticles for Rapid Capture of Gram-positive Bacteria

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Abstract

In this paper, bioconjugated Magnetic Nanoparticles (MNPs) are developed for rapid capture gram-positive bacterium *Staphyloccocus aureus* (*S. aureus*). The MNPs was synthesized through a two-step sol-gel process, followed a bioconjugation of gentamicin (Gm), an aminoglycoside antibiotic, via the linker, glutaraldehyde. The average diameter of the magnetic core is 18 ± 3 nm and the thickness of shell is around 5 ± 3 nm. The XRD results indicate that core-shell MNPs consist of magnetic core, Fe_3O_4, and silica (SiO_2) shell. In addition, the core-shell MNPs show the ferromagnetic properties, whereas the monodipersed Iron Oxide Magnetic Nanoparticles (IONPs), which were produced in the first step, show the typical superparamagnetic properties with a blocking temperature (T_B) at 115 K. The interactions between *S. aureus* and core-shell MNPs with and without Gm have been further investigated by using a Transmission Electron Microscopy (TEM). Our results demonstrate that the diluted *S. aureus* with the concentration as low as 0.5 ×10^3 CFU/mL can be separated from the solution by the core-shell MNPs in one minute.

Keywords: Magnetic nanoparticles; Hysteresis loop; Core-shell structures; Bacteria capture; *Staphyloccocus aureus*

Introduction

Bacteria can lead to serious diseases and environmental contamination, and bring a huge public health burden [1]. *Staphylococcus aureus* (*S. aureus*) is a spherical gram-positive bacterium. Infections of *S. aureus* are often found in the skin, soft-tissue, bone, joint, and endovascular disorders [2,3]. Furthermore, *S. aureus* is one of the leading causes in foodborne diseases in the world [4]. Unfortunately, the unique cell-wall made of highly cross-linked peptidoglycan offer a rigid shell to protect the gram-positive bacteria from osmotic pressure, external hazard macromolecules permeability and antibacterial enzyme digesting [5], which make the treatment of their infections with much difficulty. Immunoassays [6,7] or PCR-combined immunoassay [8-11], have been used in detection and identification of *S. aureus*, while it normally takes a couple of days with several steps including pre-separation and incubation, and so on. To date, very few immunoassay could detect bacteria at concentrations of <10^3 cfu/mL without pre-enriching bacteria via a culture process. Thus, new approaches with the strong capability to rapid capture *S. aureus* are in high demand.

Bio-molecules, such as antibody [12], antibiotics [13-16], carbohydrate [17], and small organic molecules [18], have been conjugated nanoparticles (NPs) for bacterial labelling and detection. Recently, the conjugated antibiotic on the magnetic nanoparticles are able to bind on the receptor located on the cell-wall of the bacteria, and, therefore, capture and separate the bacteria under external magnetic fields. Previous research work reported by Xu et al. [13,14], antibiotic vancomycin functionalized FePt nanoparticles have shown the capability to capture the gram-positive bacteria by conjugating with vancomycin through the peptide bond.

It is well-known that most of the bacteria can double their population less than 20 min. Rapid capture of bacteria to avoid/minimize the contamination of environment, food, and infections caused by bacteria are strongly demanded. Very few research reports are related to capture low concentration of *S. aureus* in a short period, e.g. <20min.

Here, we report a different conjugation on magnetic nanoparticles with core-shell structures (MNPs). The MNPs consist of the magnetic core, Fe_3O_4, and silica (SiO_2) shell. SiO_2 shell have shown an excellent alternative candidate for coating on magnetic NPs due to its good thermo-mechanical properties, functional surface via silylation reaction, tunable nanoscale pores, and biocompatibility [19]. Furthermore, the antibiotic Gentamicin (Gm) is used to conjugate to the MNPs as shown in figure 1. Due to the thermal-resistant of Gm, it has been well-

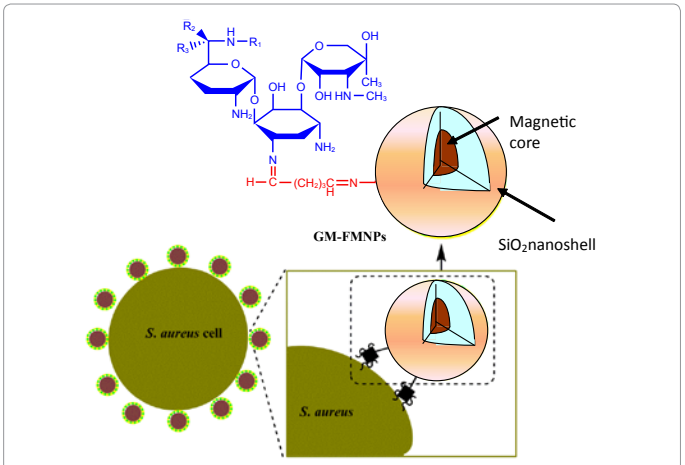

Figure 1: Schematic illustration of the Gm conjugating MNPs used for *S. aureus* capture in solution.

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accepted in orthopaedic surgery [20]. The amino acids of Gm shows the positive charges through protonation in physiologic solutions, which may contribute to the interaction of Gm with the Lipopolysaccharides (LPS) on the surface of bacteria. The strong interaction may allow the Gm-conjugating MNPs to capture *S. aureus* in a short period under an external magnetic field.

Materials and Methods

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich.

Synthesis of the iron oxide nanoparticles (IONPs)

The monodispersed Iron Oxide Nanoparticles (IONPs) were synthesized by thermal decomposition method [21]. Briefly, 1.32 g of iron (III) chloride (98%) and 7.4 g of sodium oleate (TCI, 95%) were dissolved in a mixture of 16.3 mL of absolute ethanol, 13.08 mL of water and 28.5 mL of hexane (95%). The solution was refluxed at 60°C for 4 hrs, followed by washing with a solution of ethanol and water (1:1, v%:v%) five times. The resultant iron-oleate precursor was then dried under vacuum overnight at 70°C. Afterwards, 1 g of wax-like precursor was re-dissolved in a solution of 177.3 µL oleic acid (99%) and 7.1 mL Triethalamine (98%). The solution was stirred vigorously and heated to 360°C rapidly under argon atmosphere, and then aged for 1 hr. After that the solution was cooled down, washed with hexane and ethanol (1:3) mixture and purified by centrifugation for three times. The NPs were finally dissolved in chloroform and kept at room temperature.

Preparation of core-shell MNPs

The MNPs consisting of iron oxide NPs core and silica shell were prepared through a modified sol-gel method [22]. In brief, 50 mg of iron oxide NPs were mixed in 20 ml of an aqueous solution of 2% Cetyl Trimethylammonium Bromide (CTAB). The mixture was stirred vigorously and heated upto 70°C to boil off chloroform. The NPs mixture was further filtered through a 0.45 µm syringe filter to remove large aggregates. Next, 5 mL of the filtered mixture was added into a solution of 43 mL water and 350 µL NaOH (2 M) and heated to 70°C. After the temperature stabilized, the mixture of 0.5 mL of Tetraethyl Orthosilicate (TEOS, 98%) and 10 µL of 3-Aminopropyltrimethoxysilane (APTMS) was slowly added to the CTAB aqueous solution. After 15 min, 127 µL of Trihydroxysilylpropylmethylphosphonate (THPMP) (42%) was added to the solution and stirred for another 2 hrs under dark. Then, the synthesized Fluorescent magnetic nanoparticles (FMNPs) were precipitated by adding excess methanol and collected by centrifugation. To remove CTAB, the FMNPs were further re-dispersed in a solution of 160 mg NH_4NO_3 and 60 mL 95% ethanol and heated at 60°C for 15 min, followed by repeated centrifuging and washing with ethanol. Finally, the FMNPs were dried under vacuum overnight and kept under dark ready for use.

Bio-conjugation of Gm to core-shell MNPs

40 mg of FMNPs was further dissolved in 7 ml water, followed by adding 1 ml of 25% glutaraldehyde solution. The mixture was then sealed and stirred at room temperature for 6 hrs. The glutaraldehyde modified nanoparticles were then washed three times with water. Next, 20 mg of the modified NPs were re-dispersed with 7 mL water, followed by adding 1 mL gentamicin solution (10 mg/mL). Meantime, 12 µL of Ethanolamine (EA) was mixed with another 20 mg of modified NPs in 7 mL water to prepare EA-MNPs, which is set as negative control nanoprobes. After overnight mixing, all the NPs were washed three times with water. Then, the gentamicin conjugated MNPs were redispersed in PBS (1% BSA) and incubated by shaking for 1 hr to block free formyl group on the surface of nanoparticles. After that the GM-FMNPs were washed with PBS and stored at 4°C under dark.

Characterization

Transmission Electron Microscopy (TEM) was performed to analyze the size and structure of nanoparticles, as well as their interaction with bacteria. The TEM images were obtained using a Philips CM-10 microscopy operating at 80 kv. The magnetic properties of both IONPs and MNPs were measured by Vibrating Sample Magnetometer 7404 (VSM, Lakeshore Inc.). The hysteresis loop was measured at room temperature under 1 kOe (1 T). The low temperature measures were carried out to determine the blocking temperature (TB).

Capturing *S. aureus* by Gm-MNPs

In a typical experiment, bacterium *S. aureus* (ATCC 33807) was cultured in LB broth for 24 hrs to reach a concentration of 5×10^7cfu/mL. After that, 1 mL of bacteria was collected by centrifugation. The bacteria were then dispersed in 900 µL PBS and mixed with 100 µL, 1 mg/mL GM-MNPs solution for 1 hr. The mixture was then applied in an external magnetic field (0.2 T) for 1 min. After washing twice, the magnetic confined complex was resuspended with 100 µL PBS. Meanwhile, Ethanolamine (EA) was used to conjugate to MNPs as a negative control to evaluate the Gm-bioconjugation of MNPs. EA has the same functional group as Gm to conjugate FMNPs. But, it is inert towards the E. coli.

Results and Discussion

The core-shell MNPs consist of the SiO_2 shell and a Fe_3O_4 core, through thermal decomposition of iron oleate complex. Figure 2a shows the monodispersed Iron Oxide Nanoparticles (IONPs) with the

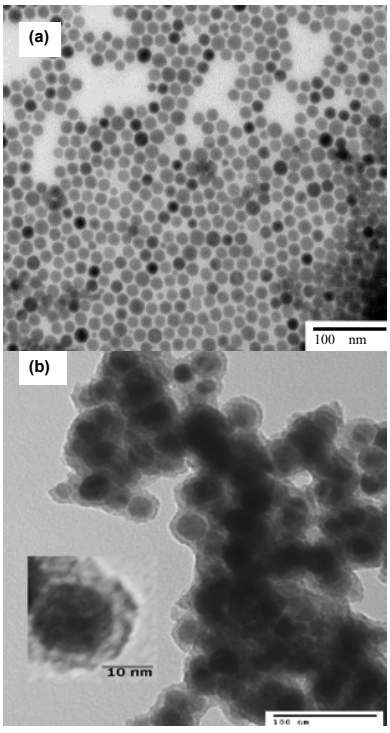

Figure 2: TEM micrographs of (a) iron oxide nanoparticles, and (b) core-shell structured MNPs

average diameter of 18 ± 2 nm. The core-shell structure of MNP can be identified in figure 2b. The average diameter of the core-shell MNPs is about 30 ± 5 nm, where, the core is about 23 ± 5 nm, and the shell is estimated at 5 ± 2 nm. The core-shell structured MNPs was investigated through the XRD. Two major phases were identified as shown in figure 3. The typical peak of semi-crystalline SiO_2 is broad and can be found at 23°C. Considering the result of TEM, the core is attributed to magnetite, Fe_3O_4, which has fcc structure with orientation of (311). Fe_3O_4 core is polycrystalline and have higher electronic density, and, therefore, show darker color, whereas the semi-crystalline SiO_2 has lighter color in TEM micrograph.

The produced IONPs exhibit the typical super paramagnetic properties with the blocking temperature of 115K, while the core-shell structured MNPs have the ferromagnetic properties as shown in Figure 4. The change of magnetic properties could be caused by the increased particles size of magnetic core which increases the barrier energy of the rotation of the magnetic spin.

The silica shell coating on the magnetic core can prevent the IONPs from further oxidation, and enable to the bioconjugation via silylation reaction. Glutaraldehyde (Glu), which has two carbonyl (–C=O) groups at both of the ends, links silica shell and Gm through

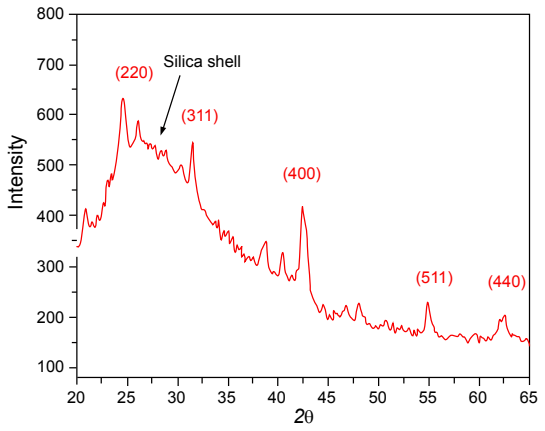

Figure 3: XRD profile of the core-shell structured MNPs; the small inset is the magnetization of IONPs as a function of temperature in the applied field of 50 Oe at a temperature range of 5 to 300 K using field 15 cooling (FC) and Zero-Field Cooling (ZFC) procedures.

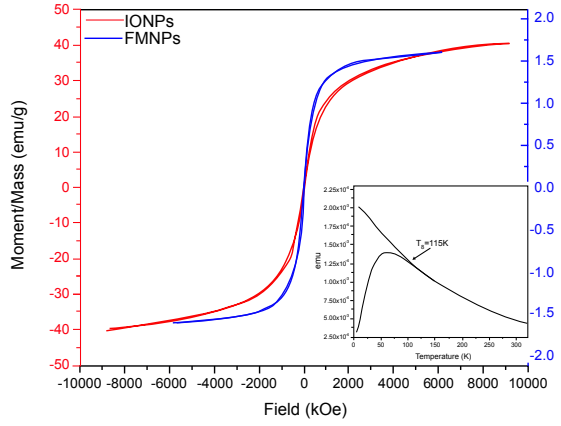

Figure 4: Hysteresis loops of iron oxide nanoparticles and the core-shell structure.

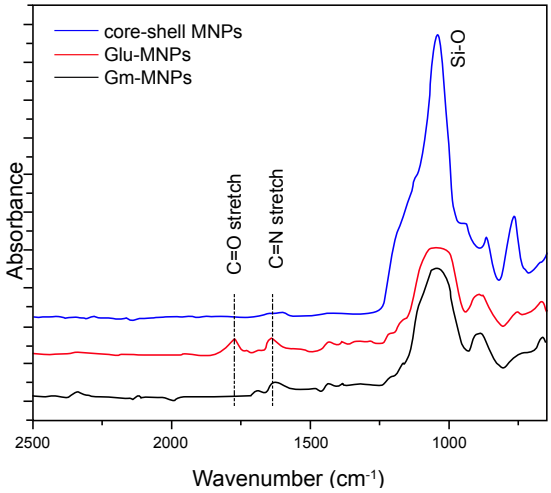

Figure 5: FTIR spectra of core-shell structured MNPs, Glu-MNPs, and Gm-MNPs.

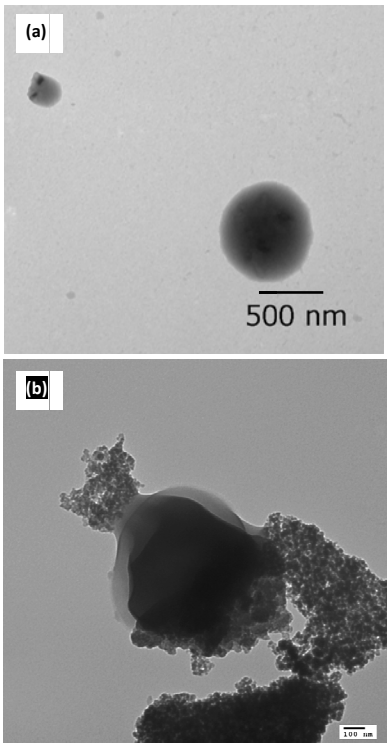

Figure 6: TEM micrographs of *S. aureus* interacting with core-shell structured MNPs without (a) and with (b) the Gm conjugation.

the carbon-nitrogen double bonds (C=N), i.e. Schiff base, due to the reaction of the amine group and the carbonyl group. Fourier Transform Infrared Spectroscopy (FTIR) was further carried out to confirm the conjugation. The Si-O-Si stretch is found at 1070 cm^{-1} in figure 5. There is -C=N- stretch of the imine group, -C=N-R, at 1640 cm^{-1} in the spectra of Gm-FMNPs. In addition, no C=O stretch at 1760 cm^{-1} is found in the spectrum of Glu-FMNPs.

We further investigate the interaction between *S. aureus* and MNPs with/without Gm through TEM. In figure 6a, there is no interaction between MNPs without Gm conjugation and *S. aureus*. Whereas, GM-MNPs are found to aggregate on the surface of the gram positive

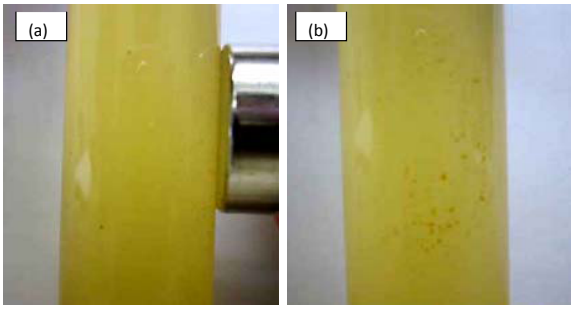

Figure 7: (a) Diluted *S. aureus* under external magnetic field (0.2 T). (b) Magnetic capture of diluted *S. aureus* in 1 min.

bacterium *S. aureus* as shown in figure 6b. In addition, bacteria capture by Gm-MNPs was studied as a function of time. Although one hour incubation GM-FMNPs with bacteria could reach maximum capture effect, we could also find a few bacteria even after 10 min mixing through optical microscopy. Furthermore, the aggregation of GM-FMNPs and bacteria could be attracted. The diluted *S. aureus* with the concentration as low as 0.5×10^3 cfu/mL can be separated from the solution by the core-shell MNPs in 1 min as shown in (Figure 7).

It is known that GM could be uptaken by susceptible gram positive bacteria such as *S. aureus* strain and kill the bacterium under the regulation of membrane potential and electrochemical gradient [23,24]. The uptake of gentamicin by *S. aureus* is reported involving of ionic adherence to the cell surface and subsequently binding to a membrane aerobic energization complex [25]. Phospholiplids and teichoic acid on the surface of gram positive bacteria were supposed to be the initial binding site for aminoglycoside antibiotic [20,24]. Further quantitative analysis of the capture efficiency and detection limitation is under study.

Conclusions

In conclusion, by conjugating antibiotic gentamicin to silica coated magnetic nanoprobes, we have demonstrated an efficient and fast way for preconcentration and capture of gram positive bacterium *S. aureus*. FMNPs (diameter is 25 ± 8 nm), comprised of a magnetic core (Fe_3O_4) and fluorescent shell (SiO_2), are successfully conjugated with Gm, an aminoglycoside antibiotic. In this study, the interactions between *S. aureus* and engineered MNPs with and without Gm bioconjugation are investigated. *S. aureus* cells ($\sim0.5\times10^3$ cfu mL^{-1}) can be magnetically captured within 1 min by Gm-MNPs from 10 mL of solution under an external magnetic field of 0.2 T. TEM results clearly shows the FMNPs with GM conjugation that likely binds on the surface of *S. aureus*, whereas FMNPs without Gm are inert towards the bacteria. Our results also indicate that the GM-conjugated nanoparticles can capture and kill gram-negative bacteria, e.g. *E. coli*, in a short period. The Gm-MNP can be used as a multifunctional platform at nanoscale for rapid capture of bacteria.

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