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# Photosensitizer and vancomycin-conjugated novel multifunctional magnetic particles as photoinactivation agents for selective killing of pathogenic bacteria†

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**Novel multifunctional magnetic particles (MMPs) conjugated with photosensitizer and vancomycin were fabricated by surface modification of $Fe_3O_4$ particles. The capacities to target, capture and inactivate pathogenic bacteria and good biocompatibility suggest that the MMPs have great potentials as photodynamic inactivation agents for serious bacterial contamination.**

Infectious diseases caused by bacteria are one of the world's most important health challenges. In particular, infections caused by antibiotic-resistant bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin (VAN)-resistant *Enterococcus* (VRE) are considered to be one of the main causes of death among hospital-acquired infectious diseases and have been responsible for thousands of deaths.[1–3] However, limited progress has been made in developing new antibiotics to treat these bacteria.[4] Therefore, an alternative method for killing the pathogenic bacteria is one of the most urgent challenges in medical biotechnology.

Among the alternative methods reported for pathogenic bacteria, photodynamic inactivation (PDI) has been proposed as an alternative bactericidal method to combat antibiotic-resistant pathogenic microbes.[1,5,6] PDI is based on the principle that a nontoxic dye, known as a photosensitizer (PS), can be preferentially in close proximity to the bacteria and subsequently activated by irradiation of an appropriate wavelength to generate microorganisms *via* oxidative stress to cell membranes and other cellular components.[7]

Recently, functionalized magnetic particles with PS or VAN for pathogenic bacteria have been reported.[8–11] However, their inactivation efficiency is still inadequate to get the optimized inactivation effect.

Herein, we report multifunctional magnetic particles (MMPs) conjugated with PS and VAN as PDI agents for targeting to bacteria with VAN, capturing or removing from contaminated sites with magnetic particles, and selective killing of pathogenic bacteria with PS.

Fig. 1a shows a schematic representation of the synthetic procedure to prepare the MMPs. The MMPs were based on $Fe_3O_4$ particles with a PS, [5,15-bisphenyl-10,20-bis(4-methoxy-carbonylphenyl)-porphyrin] platinum (t-PtCP), to allow separation of the captured bacteria from the contaminated sites and complete removal of the PS after PDI. And it was also functionalized with VAN used as a targeting molecule to deliver the MMPs to the bacterial cell membrane. The VAN, a glycopeptide antibiotic, binds through hydrogen bonds to D-Ala-D-Ala moieties in the peptidoglycan of the cell wall of bacteria. Previous studies have also demonstrated that VAN-combined particles are capable of selectively capturing not only Gram positive bacteria but also VAN-resistant bacteria.[8,9]

Fig. 1b shows that the $Fe_3O_4$ particles have good size uniformity as previously reported.[12] The high-magnification image indicates that each particle is composed of many small

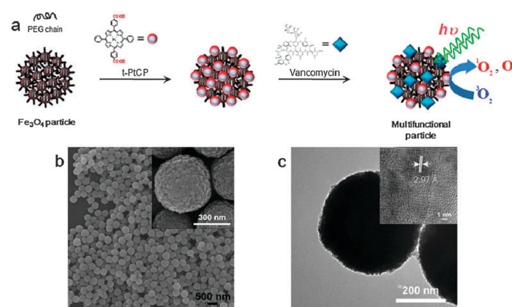


**Fig. 1** Schematic representation of MMPs (a) and morphology and crystal structure of the $Fe_3O_4$ particles: (b) FE-SEM and (c) TEM micrographs of the $Fe_3O_4$ particles.

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† Electronic supplementary information (ESI) available: Preparation of the $Fe_3O_4$ particles, photosensitizer and vancomycin conjugation to $Fe_3O_4$ particles, characterization of the MMPs, detection of singlet oxygen, diffusion distance of singlet oxygen in aqueous solution, photodynamic killing of pathogenic bacteria, cytotoxicity assessment of the MMPs, the coverage amount of t-PtCP and vancomycin on particles, detection capability of MMPs. See DOI: 10.1039/c2cc17766h
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nanoparticles, 12–17 nm in diameter (inset of Fig. 1b). A more detailed crystal structure is obtained with the HR-TEM image that is recorded at the edge of a spherical submicron particle (Fig. 1c). The inset in Fig. 1c shows that the assembled small nanoparticles have regular parallel lattice fringes. The interlayer distance of the lattice fringe is estimated to be ~2.97 Å, which is comparable to those of the (220) planes in the inverse spinel-structure of magnetite nanoparticles.[13] The average size of particles is estimated to be approximately 400 nm with a distribution of ±19 nm (Fig. S1, ESI†). And the powder XRD pattern of the $Fe_3O_4$ particles confirmed that the particles were of crystalline cubic inverse spinel $Fe_3O_4$ structure (JCPDS, card 19-0629) (Fig. S2, ESI†).

To understand the bonding nature of the carboxyl groups of t-PtCP and VAN and the Fe ions of the $Fe_3O_4$ particle, the FT-IR spectra of all samples were compared as shown in Fig. S3a and S3b (ESI†). Based on this result, it is concluded that the carboxyl terminal groups of t-PtCP form the Fe–carboxylate complexation as a result of chemical coordination bonding between the Fe ions and the carboxylate, and some of them also exist in a protonated carboxyl state. The IR spectrum of VAN bonded to $Fe_3O_4$ also shows a similar result after the interface bonding reaction. The amount of the conjugated t-PtCP and VAN was estimated by UV-visible adsorption spectroscopy. $1.1 \times 10^7$ t-PtCP molecules and $3.34 \times 10^5$ VAN were approximately conjugated onto the surface of each $Fe_3O_4$ particle (ESI†). Fig. S3c (ESI†) indicates that magnetization decreases as the surface modification reaction goes from pure $Fe_3O_4$ to MMPs@bacteria, which is due to the diamagnetic contribution of the various organic compounds bonded to the surface of the $Fe_3O_4$ particle. In particular, it is noted that the bacteria bonded sample of MMPs@bacteria still has a high enough magnetization to be applied as the targeting and separating agent. The photophysical and photochemical properties of the MMPs were also investigated. The emission bands of the MMPs in THF were slightly shifted to the blue region compared with the t-PtCP free in THF solution (Fig. S4, ESI†). These blue shifts suggest that the surface-anchored t-PtCP moieties on the magnetic particle may form π–π stackings that induce stronger interaction for the *y*-polarized transition dipoles.[14,15]

Fig. 2 verifies the generation of singlet oxygen from the MMPs in THF solution. It is well known that the phosphorescence band generated by the relaxation of the excited singlet oxygen is normally located at 1190–1350 nm.[16] The singlet oxygen lifetime is estimated to be ~20 μs from the single exponential fitting of the MMPs in THF solution, which is in agreement with the lifetime of singlet oxygen from other PS in THF solution.[17] Therefore, such fabricated MMPs are suitable as a multifunctional medium for various photodynamic applications that utilize ROS, including singlet oxygen.

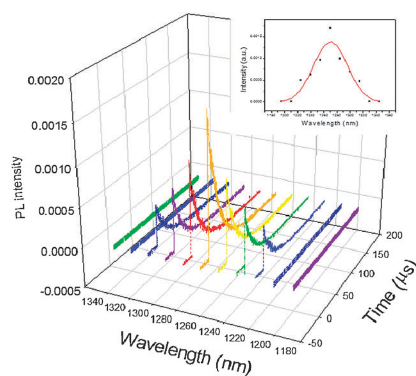


**Fig. 2** Time and wavelength resolved singlet oxygen phosphorescence decay signals of the MMPs in THF. The inset shows the emission spectrum of the singlet oxygen generated by the MMPs in THF.

In addition, we investigated the capturing capability of MMPs for several pathogenic bacterial strains. Table S1 and Fig. S6 (ESI†) present the capturing capacities of MMPs for eight bacterial strains: clinical isolates of MRSA and VRE, *Enterococcus faecalis* (*E. faecalis*), *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Escherichia coli* O157:H7:K$^-$ (*E. coli*), *Salmonella typhimurium* (*S. typhimurium*). As shown in the second column of Table S1 (ESI†), the capturing capacity of MMPs was 84.84 ± 1.70% ($10^6$ cfu $ml^{-1}$) for Gram-positive bacteria and 48.48 ± 1.79% ($10^5$ cfu $ml^{-1}$) for Gram-negative bacteria. This result shows that our MMPs have the capacity of capturing pathogenic bacteria, although the capacity of capturing Gram-negative bacteria like *E. coli* and *S. typhimurium* is poor among these bacterial strains.

Fig. 3 and Fig. S5 (ESI†) present TEM images of bacteria with MMPs followed by magnetic separation. The cell membrane of the bacteria appeared to be fully covered by the MMPs. It clearly indicates that the MMPs were capable of recognizing and binding to the cell membrane of the bacteria due to the binding ability of VAN against the bacterial cell membrane (Table S1, ESI†). And the optimum number of MMPs is approximately estimated to be $2.50 \times 10^7$ MMPs for capturing of $10^6$ bacteria (ESI†).

To examine whether MMPs and VAN without irradiation might affect the inactivation of bacteria, the experiments were performed under complete darkness conditions to prevent the bactericidal effects caused by the generated $^1O_2$ from the photo-excited PS. The number of bacteria after incubation was not reduced, indicating that they did not affect the survival rate of bacteria (third and fourth columns of Table S1 (ESI†)). In addition, the captured fractions of the $Fe_3O_4$@van particles were irradiated to determine the effect of laser irradiation on the survival rate, but the number of bacteria did not change, which meant that the laser irradiation itself did not kill the bacteria without t-PtCP (fifth column of Table S1, ESI†).

We further examined the photo-killing effect of MMPs under irradiation with a laser at 510 nm with 6.1 mW $cm^{-2}$ of power density and the survival rates of eight different bacteria were monitored as shown in Fig. 4. After the incubation time of 2 h for capturing, the captured Gram-positive bacteria including the

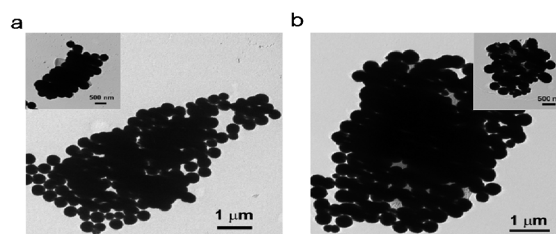


**Fig. 3** TEM images of (a) *E. coli* O157:H7:K$^-$, (b) *S. aureus*; insets show the TEM images that represent a single cell of each bacterium.

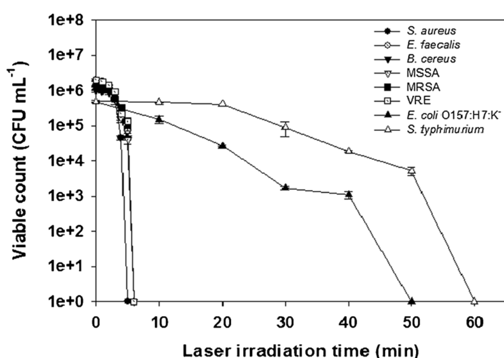


**Fig. 4** Photodynamic killing kinetics of Gram-negative bacteria (*E. coli* and *S. typhimurium*) and Gram-positive bacteria (*E. faecalis*, *S. aureus*, *B. cereus*, MSSA, MRSA, and VRE) by the MMPs.

antibiotic-resistant bacteria with MMPs were completely killed in less than 6 min of irradiation time, whereas it took almost 1 h of irradiation time to completely inactivate the Gram-negative bacteria. This result is consistent with a previous report on photodynamic treatment, which found that Gram-positive bacteria were more susceptible to the photodynamic effect than Gram-negative bacteria.[18] In detail, Gram-negative bacteria contain an additional membrane layer in the cell wall, which is a specific component of Gram-negative bacteria's outer membrane, lipopolysaccharide (LPS), which quenches reactive oxygen species generated by PS.[19,20] In other words, this meant that the Gram-positive bacteria can be selectively killed by our MMPs and the MMPs are highly effective as a photo-inactivating agent for pathogenic bacteria.

Furthermore, we investigated the cytotoxicity of MMPs itself on mammalian cells, because it is essential to evaluate the potential for the bio-application of MMPs as shown in Fig. S8 (ESI†). The result indicated that the MMPs have non-cytotoxicity and good biocompatibility on L-929 cells. Hence our MMPs are expected to be safe for other PDI application.

According to our knowledge, the time required for the complete inactivation of Gram-positive bacteria upon irradiation is less than the previous reports.[21–30] In addition, we used a low concentration of the PS (0.35 μM) and a very low dosage of light (6.1 mW $cm^{-2}$). Such high inactivation efficiency of bacteria by the MMPs fabricated in this study implies that the noticeable proximity effect of the PS on the bacterial cell surface is due to the strong binding with VAN (Fig. S7, ESI†). Because, the reactive oxygen species (ROS) generated by PS are highly volatile and, in particular, the singlet oxygen species which is known to be the most destructive element among ROS can penetrate approximately 219 nm in aqueous solution (ESI†), the PS that are distant from bacteria are not effective for killing.[31,32]

In conclusion, we have demonstrated that our MMPs have good biocompatibility and have the capacities to target, capture, and inactivate several pathogenic bacteria including MRSA and VRE under irradiation with 510 nm light. The main purpose of this study is to show the synthesis of the multi-functional magnetic particles and the enhancement of the functionality for *in vitro* photodynamic inactivation (PDI) of bacteria. In particular, since these nanoparticles have a high value of saturation magnetization and superparamagnetism, they have high capacity for the separation of the functional magnetic nanoparticles from the solvent mixture by magnetic field. Therefore, such a multifunctional particle system can be applied to environmental industry fields such as water disinfection system because of an efficient photodynamic inactivation (PDI) effect and easy withdrawal by magnetic field.

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