Ratiometric fluorescence detection of pathogenic bacteria based on dual-recognition nanoprobes with controllable G-quadruplex release

Yizhong Shen,[a,b] Tingting Wu,[b] Huanhuan Chen,[b] Yingwang Ye,[*b] and Jing-Juan Xu[*a]

[a] State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, 210023, China

[b] School of Food & Biological Engineering, Key Laboratory for Agricultural Products Processing of Anhui Province, Hefei University of Technology, Hefei 230009, China

*Corresponding authors. E-mails: xujj@nju.edu.cn (J.-J. Xu),

yeyw04@mails.ucas.ac.cn (Y. Ye)

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1. Experimental sections

Chemical and Biological Reagents. The chemical and biological reagents applied in this work were purchased from suppliers without any pretreatments. Citric acid was obtained from Maclean Biochemistry Co., Ltd. (Shanghai, China). Polyethylenimine (PEI, MW = 1800), vancomycin hydrochloride (Van), Ethylene glycol-bis(succinic acid *N*-hydroxysuccinimide ester) (EGS) were purchased from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA). Potassium chloride (KCl) and tris-hydrochloride buffer (Tris-HCl) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). *N*-methyl mesoporphyrin IX (NMM) was bought from Frontier Scientific Inc. (Logan, Utah, USA). Plate count agar and tryptic soy broth (TSB) medium were obtained from Beijing Aoboxing Biotech., Co., Ltd. (Beijing, China). The involved bacteria (*i.e.*, MRSA, *Listeria monocytogenes*, *Vibrio parahemolyticus*, and *Cronobacter*) used in this work were obtained from Guangdong Institute of Microbiology (Guangzhou, China). D.I. water applied throughout was acquired from the Milli-Q system (Millipore, USA, 18.2 MΩ resistivity) for preparing all solutions. DNA molecular beacon sequence: 5'-CTGGGAGGGAGGGAGGGAAAAAACACAGAAA**ATGCGGTTGGTTGCGGTTGGGCATGATGTATTTCTGTGTTTT**TTCCC-$(CH_2)_6$-NH_2-3' (MB, the bold portion is MRSA recognition aptamer, and the underlined portion is the G-rich DNA sequence) and amutated molecular beacon sequence: 5'-CTGGGAGGGAAAAAGGGAAAAAACACAGAAA**ATGCGGTTGGTTGCGGTTGGGCATGATGTATTTCTGTGTTTT**TTCCC-$(CH_2)_6$-NH_2-3' (mMB, the blue

portion is the mutated bases in the G-rich DNA segment), was synthesized and purified with HPLC by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Instrumentations. A LS55 Fluorescence spectrophotometer (Perkin Elmer, USA) was used to gather fluorescence spectra. A TU-1901 UV-Vis spectrophotometer (PERSEE, Beijing, China) was applied to collect the absorption spectra. A JEOL-2100 transmission electron microscope (TEM, JEOL Ltd., Japan) was used to adopt TEM image. A XD-3 X-ray diffractometer (Beijing Purkinje General Instrument Co., Ltd., China) was applied to obtain X-ray powder diffraction (XRD). A thermo ES-CALAB 250XI X-ray photoelectron spectrometer (XPS, Thermo Scientific, USA) was employed to acquire elemental information. A 90 Plus/BI-MAS equipment (Brook haven, USA) was exploited to collect the data of dynamic light scattering (DLS) and zeta potential. A PerkinElmer Frontier Fourier-transform spectrometer (USA) was employed to collect the FT-IR spectra. A Zeiss LSM800 Laser confocal microscope (Germany) was applied to obtain the images of all bacteria. A PHS-3C pH meter (Leici, Shanghai, China) was utilized to adjust the pH of solution.

Preparation of Cdots. PEI-functionalized Cdots was prepared through one-pot hydrothermal method by reference to previous report with minor modifications.[1] Briefly, PEI (~ 0.05 g) and citric acid (~ 0.25 g) were dissolved into 4.0 mL of D.I. water under vigorous stirring. Next, the colorless mixture was rapidly transferred into a hydrothermal reactor (25.0 mL) for heating to ~ 110.0 °C in an electric thermostatic drier. After ~ 2.0 h incubation, the mixture was naturally cooled down to room

temperature. At this point, the crude pale yellow Cdots solution was successfully prepared, which could be further purified with methanol and D.I. water under 15000 rpm centrifuging. By repeating it three times, the precipitate was transferred into a vacuum freeze-drying equipment, and the drying PEI-functionalized Cdots was ultimately obtained for further use.

Conjugation of Dual-Recognition Van-MB-Cdots. The dual-recognition Van-MB-Cdots was conjugated by means of crossing-linker EGS. In brief, 1.0 mg/mL of Cdots, 100.0 μM of MB, 25.0 μM of Van, and 0.5 mM EGS (dissolved in DMSO) were mixed in Tris-HCl buffer (pH = 7.6, 20.0 mM), and then the mixture solution was transferred into a 37.0 ℃ constant temperature incubator for ~ 1.0 h shaking. After that, the resulting solution was rapidly transferred into a 30 kDa Millipore to centrifuge (6000.0 rpm) and washed with Tris-HCl buffer (pH = 7.6, 20.0 mM) for three times, finally achieving the purified dual-recognition Van-MB-Cdots. In order to ensure the MB formation on the surface of Cdots, the above conjugation was heated to 95 °C for 5.0 min and then slowly cooled to room temperature for storing at 4.0 ℃ in dark. Likewise, the single-recognition MB-Cdots was also prepared by employing crossing-linker EGS as the same conditions. The concentrations of the dual-recognition Van-MB-Cdots and single-recognition MB-Cdots were determined by Cdots, whose concentration could be calculated by its OD at 360 nm.

Bacteria Culture and Counting. MRSA and other bacteria used in this work were acquired from Guangdong Institute of Microbiology, which were activated in tryptic soy broth (TSB) medium by continuous shaking overnight at 37.0 °C. Subsequently, ~

10.0 μL of each above bacteria was further activated for another 6.0 h, and then centrifuged (8000.0 rpm/2.0 min) and washed with Tris-HCl buffer (pH = 7.6, 20.0 mM). According to the classic agar plate-counting method, we carried out the colony counting on the plates after overnight incubation at 37.0 °C, which was then utilized to calculate the amounts of colony-forming units per milliliter (CFU/mL), thereby determining the concentration of each bacteria.

Visualization Imaging of MRSA. The sterilized cell slides were firstly placed into a 24-well plate, and then 1.0 mL TSB medium containing 1% (V/V) fresh MRSA was added into each well for overnight incubation in a 37.0 °C incubator. After that, the above medium was carefully removed from each well and washed by Tris-HCl buffer (pH = 7.6, 20.0 mM) three times. In this case, these bacteria were further incubated in 1.0 mL Tris-HCl buffer (pH = 7.6, 20.0 mM) containing the four indicated reagents and randomly divided into four groups such as group 1: Cdots (0.5 mg/mL); group 2: NMM (8.5 μM); group 3: MB-Cdots (0.5 mg/mL, determined by Cdots) + NMM (8.5 μM) + K^+ (100.0 mM); and Van-MB-Cdots (0.5 mg/mL, determined by Cdots) + NMM (8.5 μM) + K^+ (100.0 mM), respectively. After 30.0 min incubation in a 37.0 °C incubator, the above Tris-HCl buffer was carefully removed from each well and all bacteria were washed with fresh Tris-HCl buffer (pH = 7.6, 20.0 mM) three times. At this point, the bacteria in the cell slides were taken out from each well and performed the fluorescence imaging on a Zeiss LSM800 laser confocal microscope equipped with a 405 nm laser, and collected the blue fluorescence in the channel of 440 ± 10 nm as well as red fluorescence in the channel of 610 ± 10 nm.

Ratiometric Fluorescent Response of Van-MB-Cdots Coupled with NMM toward MRSA in Aqueous Solution. Upon 37.0 °C, the dual-recognition conjugation of Van-MB-Cdots (3.0 μg/mL, determined by Cdots) in Tris-HCl buffer (pH = 7.6, 20.0 mM, 100.0 mM of K^+, 2.0 μM of NMM) was adequately incubated with fresh MRSA at the different concentrations of 0, 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/mL for 30.0 min, respectively. After that, all of these test samples were naturally cooled down to room temperature, and their dual-emission fluorescence spectra were collected with a single-excitation wavelength at 390 nm. Moreover, upon 37.0 °C, the dual-recognition conjugation of Van-MB-Cdots (3.0 μg/mL, determined by Cdots) in Tris-HCl buffer (pH = 7.6, 20.0 mM, 100.0 mM of K^+, 2.0 μM of NMM) was also adequately incubated with fresh MRSA, *L. monocytogenes*, V. parahaemolyticus, and *Cronobacter* at the same concentration of 10^8 CFU/mL, respectively. Afterward, all of these test samples were naturally cooled down to room temperature, and their dual-emission fluorescence spectra were also acquired with a single-excitation wavelength at 390 nm.

Profiling the Fouling Levels of MRSA in Real Food Samples. The commercial tea drink and drinking water, randomly acquired from the supermarket in Hefei, China without further pretreatments, were chosen as the representative real food matrixes for revealing the practicability of the dual-recognition Van-MB-Cdots in the presence of NMM to report the fouling levels of MRSA with the standard addition method. In detail, all of these test samples were firstly adjusted to pH = 7.6 with Tris-HCl buffer (20.0 mM, 100.0 mM of K^+, 2.0 μM of NMM). Afterward, the dual-recognition Van-

MB-Cdots (3.0 μg/mL, determined by Cdots) and the different fouling levels of MRSA at 10^3, 10^4, and 10^5 were added into all of the test samples for 30.0 min incubation at 37.0 °C. After that, all of these test samples were excited with the wavelength of 390 nm for collecting the dual-emission fluorescence intensities of each sample at ~ 440 nm and ~ 605 nm to achieve the quantitative analysis of each sample.

2. Supplementary Figures

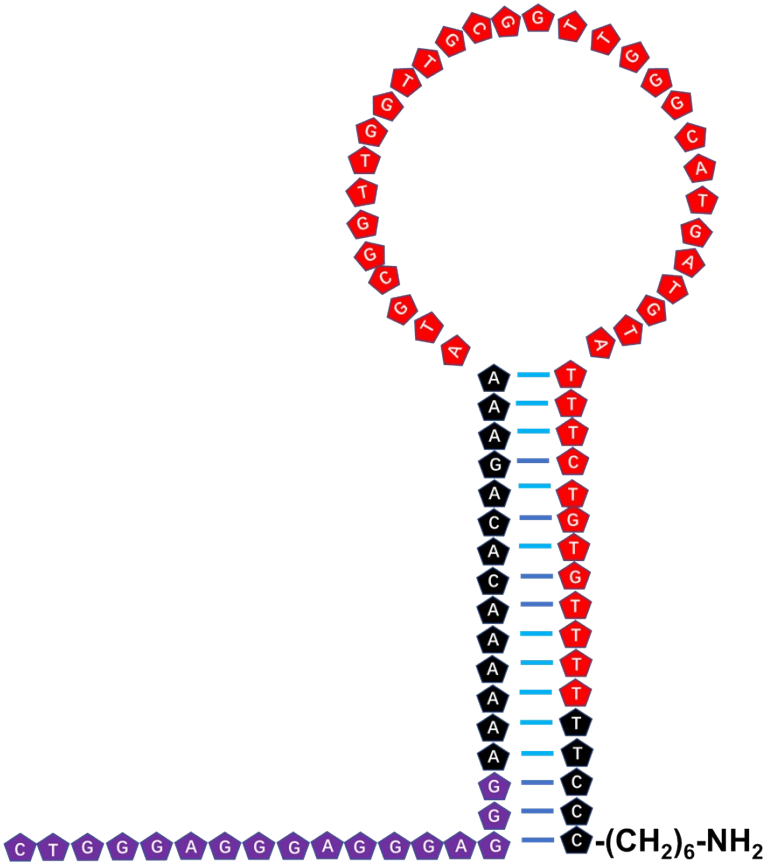

Fig. S1. Illustration of MB structure.

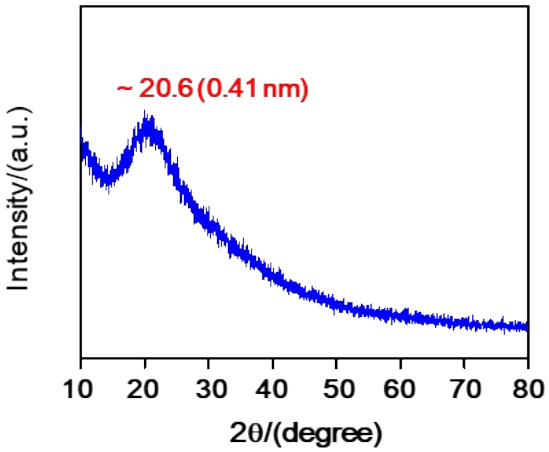

Fig. S2. XRD pattern of the as-prepared Cdots.

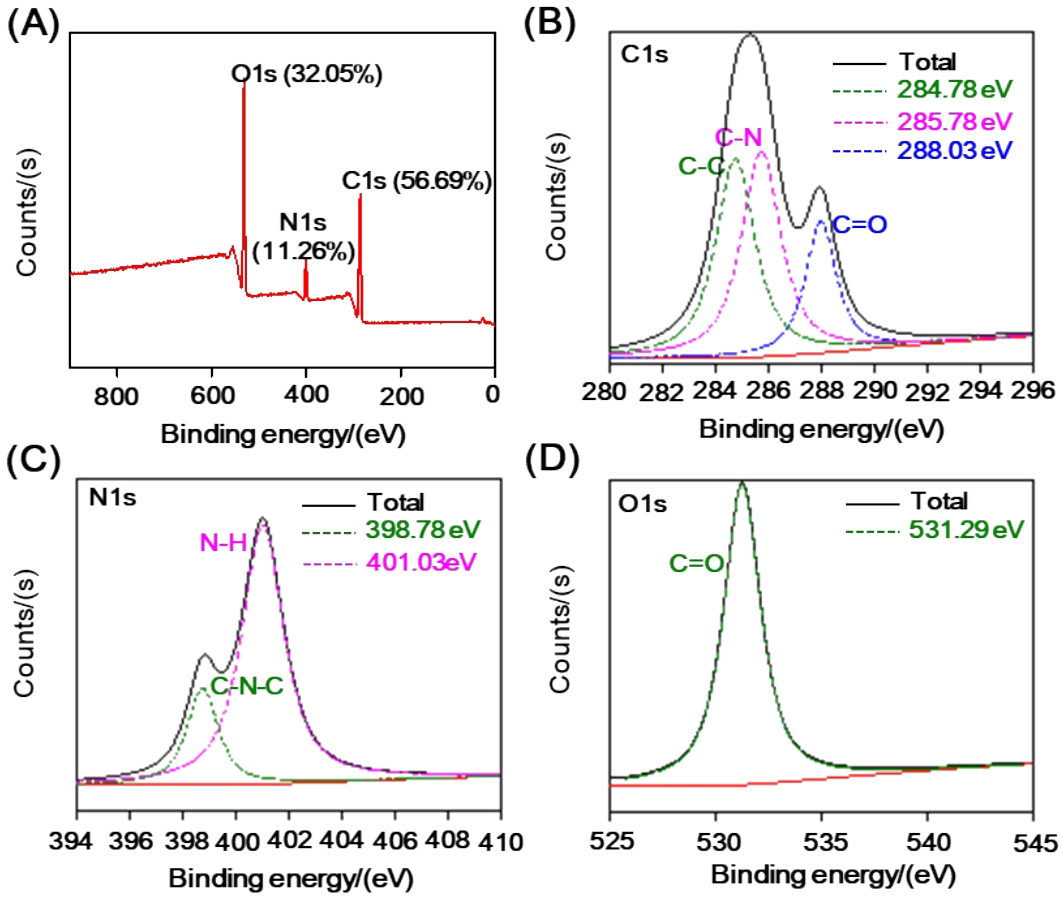

Fig. S3. XPS analysis of the as-prepared Cdots. (A) XPS, (B) C1s, (C) N1s, and (D) O1s spectra of the as-synthetized Cdots.

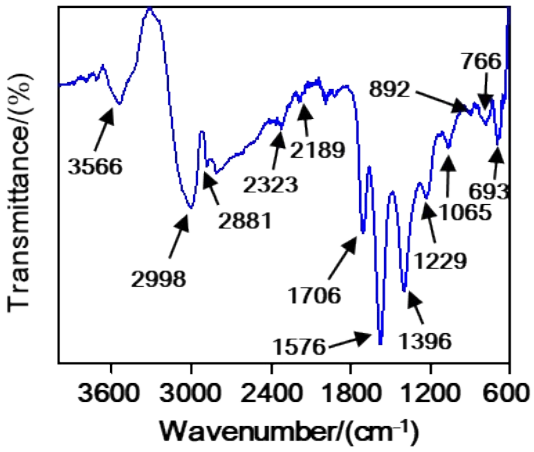

Fig. S4. FT-IR spectrum of the as-prepared Cdots.

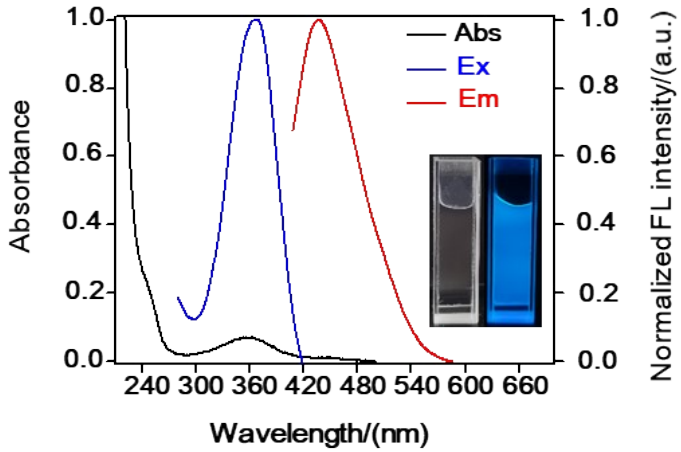

Fig. S5. Absorption, fluorescence emission and excitation spectra of the as-prepared Cdots in D.I. water. Inset: the photographs of the as-synthetized Cdots before (left) and after (right) excitation of a 365 nm UV lamp.

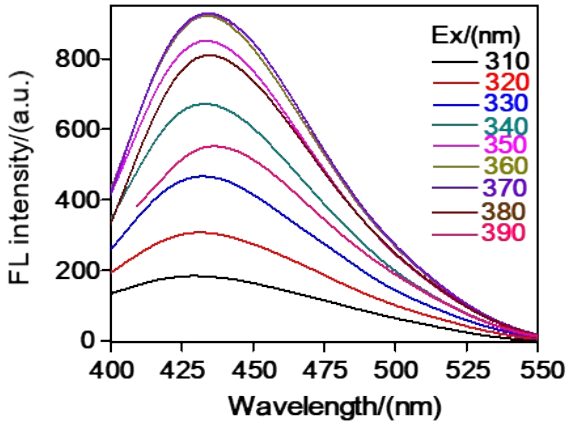

Fig. S6. Fluorescence emission spectra of the as-synthetized Cdots in D.I. water at different excitation wavelengths.

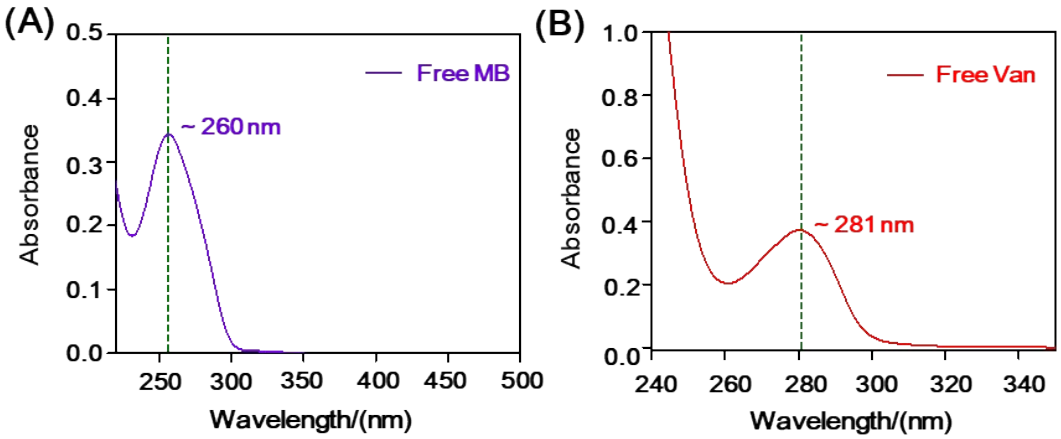

Fig. S7. Absorption of free MB (A) and Van (B) in Tris-HCl buffer (pH = 7.6, 20.0 mM).

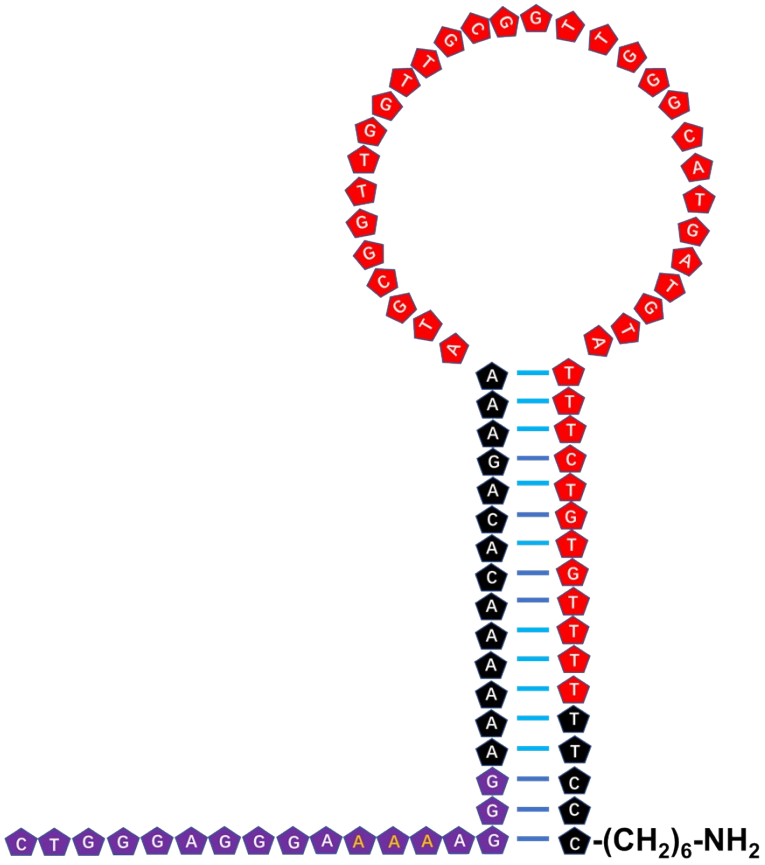

Fig. S8. Illustration of mMB structure.

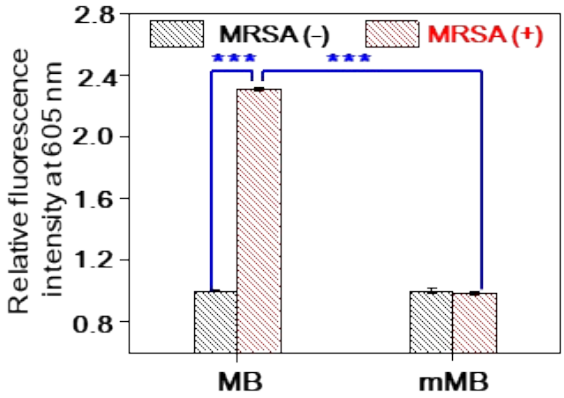

Fig. S9. Quantitative comparison of the fluorescence intensity of NMM + MB and NMM + mMB at 605 nm before (-) and after (+) incubation with MRSA in Tris-HCl buffer (20.0 mM, 100.0 mM of K^+) for 30.0 min. The concentrations of NMM, MB, and mMB were 2.0 μM. (***$P < 0.001$). Error bar expressed the deviation of three measurements.

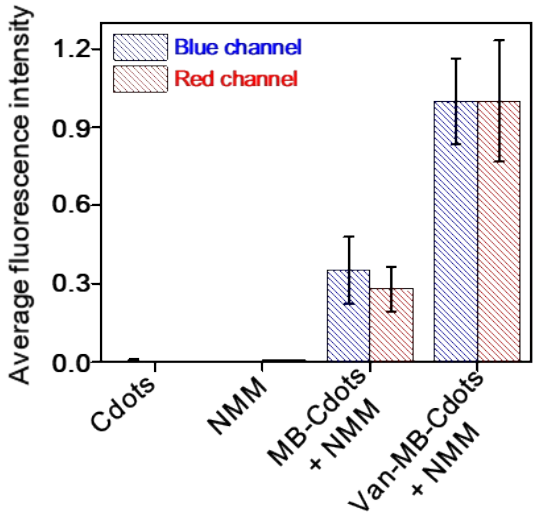

Fig. S10. Quantification comparison of the average fluorescence intensities inside different treated MRSA at red channel and blue channel as shown in Fig. 3 with ImageJ software.

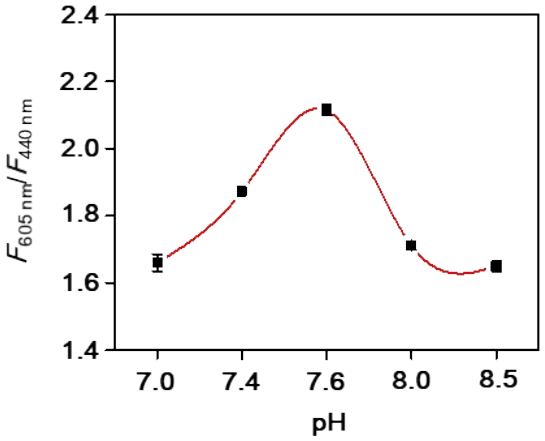

Fig. S11. Effect of pH on the fluorescence intensity ratio $F_{605\ nm}/F_{440\ nm}$ of dual-recognition Van-MB-Cdots (3.0 μg/mL, determined by Cdots) together with 10^8 CFU/mL of MRSA in Tris-HCl buffer (20.0 mM, 100.0 mM of K^+, 2.0 μM of NMM). The fluorescence data were obtained with the single-excitation wavelength of 390 nm. Error bar expressed the deviation of three measurements.

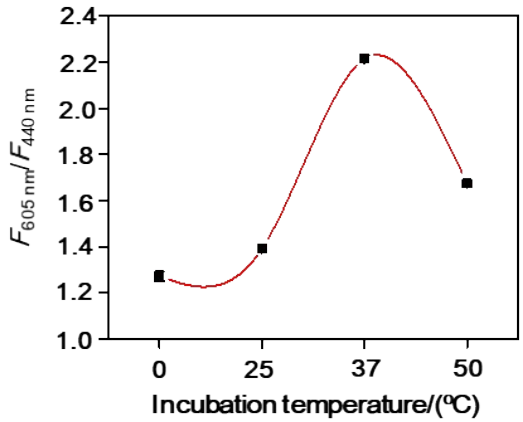

Fig. S12. Effect of incubation temperature on the fluorescence intensity ratio $F_{605\ nm}/F_{440\ nm}$ of dual-recognition Van-MB-Cdots (3.0 μg/mL, determined by Cdots) together with 10^8 CFU/mL of MRSA in Tris-HCl buffer (20.0 mM, 100.0 mM of K^+, 2.0 μM of NMM). The fluorescence data were obtained with the single-excitation wavelength of 390 nm. Error bar expressed the deviation of three measurements.

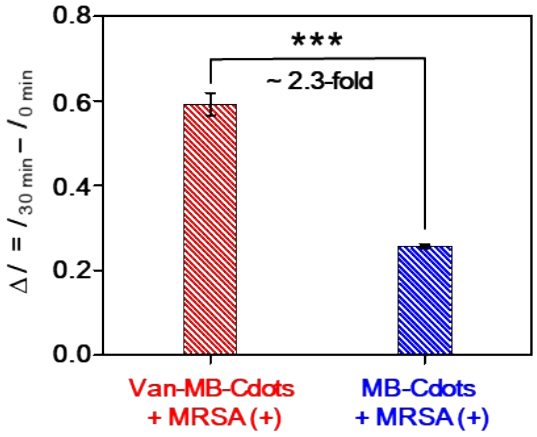

Fig. S13. Quantitative comparison of the enhancement extent (ΔI) of fluorescence intensity ratio ($I = F_{605\,nm}/F_{440\,nm}$) of NMM-coupled Van-MB-Cdots and NMM-coupled MB-Cdots before ($I_{0\,min}$) and after ($I_{30\,min}$) incubation with MRSA for 30.0 min as shown in Fig. 4A. (***$P < 0.001$). Error bar expressed the deviation of three measurements.

3. Supplementary Tables

Table S1. Comparison of the analytical parameters of different detection methods for the assay of MRSA.

Analytical methods	Detection limit (CFU/mL)	Linear range (CFU/mL)	*Ref.*
Colorimetry	10^6	–	2
Chemiluminescence	100	10^2 – 10^7	3
Electrochemistry	845	10^3 – 10^5	4
Flow cytometry	40	1.5×10^2 – 1.5×10^6	5
Fluorimetry	30	10^2 – 10^6	6
Fluorimetry	300	10^3 – 10^7	7
Fluorimetry	8.2	27 – 10^5	This work

Table S2. Ratiometric fluorescence assay of NMM-coupled Van-MB-Cdots for MRSA in real samples.

Samples	Found	Spiked (CFU/mL)	Measured/ (CFU/mL)	Recovery/ (%, n=5)	RSD/ (%, n=5)
Tea drink	ND	1.00×10^3	1.04×10^3	104.00	1.82
	ND	1.00×10^4	0.94×10^4	94.00	1.09
	ND	1.00×10^5	0.95×10^5	95.00	3.00
Drinking water	ND	1.00×10^3	1.05×10^3	105.00	1.99
	ND	1.00×10^4	0.94×10^4	94.00	3.19
	ND	1.00×10^5	1.01×10^5	101.00	4.18

4. References

[1] J.-Y. Li, Y. Liu, Q.-W. Shu, J.-M. Liang, F. Zhang, X.-P. Chen, X.-Y. Deng, M. T. Swihart and K.-J. Tan, *Langmuir.*, 2017, **33**, 1043.

[2] S. Currie, F. J. Shariatzadeh, H. Singh, S. Logsetty and S. Liu, *ACS Appl. Mater.*

Interfaces, 2020, **12**, 45859.

[3] M.-P. N. Bui, J. Brockgreitens and A. Abbas, *Adv. Healthc. Mater.*, 2018, **7**, 1701506.

[4] C. R. Nemr, S. J. Smith, W. Liu, A. H. Mepham, R. M. Mohamadi, M. Labib, and S. O. Kelley, *Anal. Chem.*, 2019, **91**, 2847.

[5] Y. Wang, Y. He, S. Bhattacharyya, S. Lu and Z. Fu, *Anal. Chem.*, 2020, **92**, 3340.

[6] Y. Ning, L. Zou, Q. Gao, J. Hu and F. Lu, *Microchimica Acta*, 2018, **185** 183.

[7] Y. Ning, Q. Gao, X. Q. Zhang, K. Wei and L. L. Chen, *J. Biomol. Screen*, 2016, **21**, 851.