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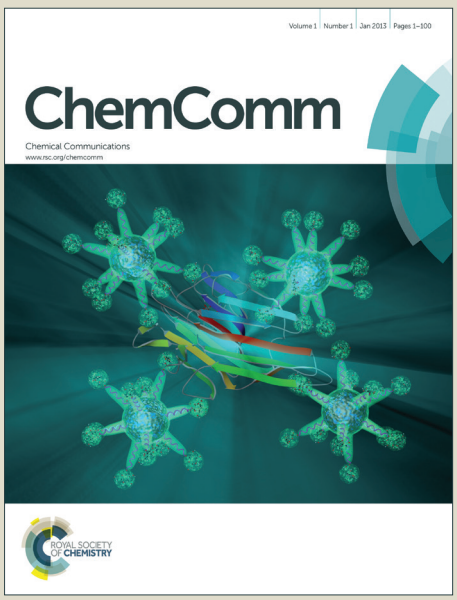

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ARTICLE TYPE

Interfacial Self-assembly Leads to Formation of Fluorescent Nanoparticles for Simultaneous Bacterial Detection and Inhibition

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Here we reported on self-assembling vancomycin derivatives for bacterial detection and inhibition simultaneously.

Self-assembly prevails in nature.[1] Taken the advantages of self-assembly, monomeric molecules can form functional structures such as lipid bilayers and cell skeletons. By mimicking these self-assembly systems in nature, researchers have developed many smart self-assembly systems that are dynamic, dissipative, or reversible.[2] Recently, great efforts have been devoted for the development of biofunctional self-assembly materials.[3] For instance, Smith and co-workers have rationally developed self-assembled multivalent systems for high affinity bindings.[4] Stupp and co-workers have developed self-assembled nanofibers with high density of bioactive ligands with improved activities.[5] Huang and co-workers have developed supramolecular materials for bacterial cell agglutinations.[6] Despite great advances have been made towards engineering sophisticated self-assembled biomaterials in bulk solutions, the self-assembly at biosurfaces and biointerfaces is largely unexplored.

Biosurfaces and biointerfaces are ubiquitous in biology. They serve as natural barriers to separate biosystems with surroundings. Meanwhile, they contain various receptors for cell-extracellular matrix or inter-cellular communications. Due to their unique physical and chemical features, such surfaces could also lead to distinct self-assembly behaviors from the bulk systems. Recently, Tiller and co-workers have reported on a phenomenon of 'surface-induced hydrogelation', in which they found small molecules could self-assemble into gels on attracting surfaces at concentrations much lower than their minimum gelation concentrations in bulk solutions.[7] This strategy has been applied by our group for surface functionalization of hydrophobic PCL nanofibers to improve their *in vivo* performance[8] and for platelet aggregation inhibition.[9] In this work, we showed that by using the "surface-induced self-assembly" strategy, we could successfully form nanostructures at bacterial surface with fluorescence turn on property and high density of an antibiotics, vancomycin (Van), for simultaneous bacterial detection and inhibition.

Van was an important antibiotics for the treatment of gram positive bacterial infection.[10] It can tightly bind to gram positive bacterial cell wall peptides, leading to the inhibition of bacterial cell wall synthesis and bacterial death. However, it loses the high binding affinity to vancomycin resistance enterococci (VRE) because VRE has mutated the terminal dipeptide from D-Ala-D-Ala to D-Ala-D-Lactate.[11] In order to overcome this serious problem, multivalent Van derivatives have been developed, including self-assembled small molecular Van derivatives and polymers bearing multiple Van.[12] For instance, Xu and co-workers reported on the first example of molecular hydrogelator of a Pyren-Van conjugate,[13] which could efficiently inhibit both gram positive bacterial and VRE due to the enhancement of local concentration of the antibiotics.[14] Additionally, 4-nitro-2,1,3-benzoxadiazole (NBD) is known as an environment-sensitive fluorophore, which shows weak fluorescence in polar and protic environments but emits intensely in hydrophobic surroundings.[15] Therefore, we opted to develop NBD-Van conjugates that could self-assemble at the bacterial surface through 'surface-induced self-assembly' to synergistically combine the advantages of self-assembled multivalency of Van and environment-sensitive fluorescence property of NBD. This strategy might be applied for bacterial detection and inhibition simultaneously.

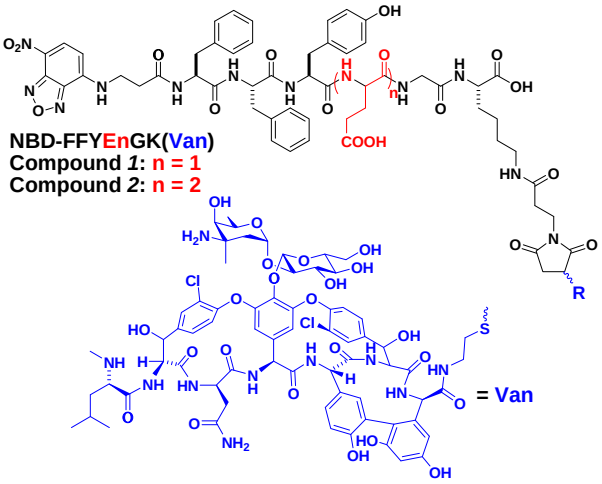

Fig. 1. Chemical structures of NBD-Vancomycin (Van) conjugates (***1***: NBD-FFYEGK(Van) and ***2***: NBD- FFYEEGK(Van)

In order to test our hypothesis, we designed two NBD-Van conjugates, NBD-FFYEGK(Van) (***1***) and NBD-FFYEEGK(Van) (***2***) (Fig. 1). Many peptide derivatives based on FF or FFY with aromatic capping groups have been demonstrated as molecular hydrogelators with excellent self-assembly property.[16] Therefore, peptide derivatives based on NBD-FFY might also have good self-assembly abilities. The number of glutamic acid (E) could tune the self-assembly

property of the conjugates. The synthetic strategy for ***1*** and ***2*** was similar, which was shown in Scheme S-1.

We firstly studied their self-assembly behaviours by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The DLS results indicated that the critical micelle concentration (CMC) for ***1*** and ***2*** was 75 and 190 μg/mL, respectively (Fig. S-16). The smaller CMC value of ***1*** indicated that ***1*** with only one hydrophilic E had a better self-assembly ability than ***2*** with two Es. We also obtained the TEM images of phosphate buffer saline (PBS, pH = 7.4) solutions of ***1*** and ***2*** at different concentrations. The results in Fig. S-16A and S-16B showed that solution of ***1*** or ***2*** exhibited nanoparticles when their concentration was higher than their respective CMC value. For example, we observed nanoparticles with diameter of about 400 and 90 nm for solution of ***1*** and ***2*** at the concentration of 200 μg/mL, respectively (Fig. S-16A and S-16B). While for solution of ***1*** or ***2*** below their corresponding CMC value, they exhibited amorphous morphologies (Fig. S-17). The size of the nanoparticles observed in TEM images was consistent with the results determined by DLS (Fig. S-16C and S-16D).

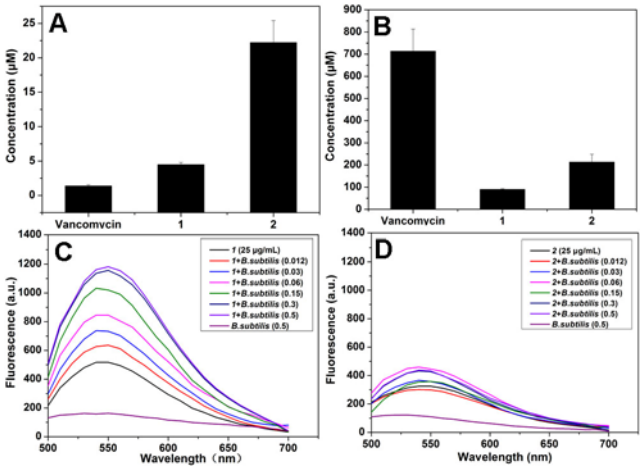

Fig. 2. Minimum inhibition concentration to A) *B.subtilis* and B) *E. faecalis*, emission spectra of PBS solutions (pH = 7.4) of C) ***1*** and D) ***2*** in the presence of different OD values of *B. subtilis* ($\lambda_{exc.}$ = 480 nm)

We then studied the bacteria inhibition capacity of ***1*** and ***2***. Two bacteria strains were chosen as model organisms, Van sensitive strain of *Bacillus subtilis* (ATCC 33677, *B. subtilis*) and Van resistant enterococci (VRE) of *Enterococcus faecalis* (VanB genotype, ATCC 51299, *E. faecalis*). The anti-bacteria activity of ***1*** and ***2*** was investigated by the standard broth microdilution assay. As shown in Fig. 2A, for *B. subtilis*, the minimum inhibition concentration (MIC) of ***1*** was about 4.5 μM, which was similar to the parent Van molecule (1.3 μM). However, the MIC of ***2*** (22.4 μM) was about 17 times higher than that of parent Van molecule (Fig. 2A). Fig. 2B indicated that both ***1*** and ***2*** exhibited more effective antimicrobial activities against *E. faecalis* than the parent Van molecule. The MIC of ***1***, ***2***, and the parent Van molecule was 90, 213, and 728 μM, respectively. Compared with Van, the antimicrobial activity against *E. faecalis* was increased for 7 times by ***1*** while only 2.3 times by ***2***. We proposed that the better antibacterial activities of ***1*** over ***2*** might be due to its stronger self-assembly ability on bacterial surfaces.

We then monitored the fluorescence response of solutions of ***1*** and ***2*** to the bacteria, respectively. As shown in Fig. 2C and 2D, the fluorescence response of solutions of ***1*** and ***2*** to the bacteria was also different. The fluorescence intensity of ***1*** increased gradually with the addition of more and more bacteria (OD value from 0 to 0.5, *B. subtilis* in Fig. 2C and *E. faecalis* in Fig. S-18A). Since the concentrations of ***1*** used (25 and 50 μg/mL for *B. subtilis* and *E. faecalis*) were lower than the CMC value of ***1***, the fluorescence enhancement of ***1*** to both bacteria was probably due to the enrichment of ***1*** on bacteria, resulting in its self-assembly and fluorescence turn-on of the environment-sensitive fluorophore NBD. There were almost no or slight fluorescence responses of ***2*** to the addition of both kinds of bacteria (Fig. 2D and Fig. S-18 B).

In order to understand the different fluorescence responses of ***1*** and ***2*** to the bacteria, we separated the bacteria from the solutions of ***1*** and ***2*** to determine their concentrations at the surface of bacteria. As shown in Fig. S-19A and S-19B, more amounts of compounds were observed on bacteria when higher concentrations of compounds were used. The enrichments of ***1*** and ***2*** on bacteria were probably due to the surface-induced self-assembly *via* specific interactions between Van and terminal peptides at the surface of bacteria. Upon incubation *B. subtilis* with ***1*** and ***2*** at a same concentration of 25 μg/mL, the amount of ***1*** on *B. subtilis* was 430 μg/mL, higher than the CMC of ***1*** (75 μg/mL). While the amount of ***2*** was 220 μg/mL, only slightly higher than the CMC of ***2*** (190 μg/mL). Similar results were observed by incubating *E. faecalis* with ***1*** and ***2*** at 50 μg/mL and the amounts of ***1*** and ***2*** on the *E. faecalis* were 390 and 180 μg/mL, respectively. Since the concentration of ***1*** on both bacteria was higher than its CMC value, compound ***1*** would therefore self-assemble and form nanostructures on bacteria. The formation of self-assembling nanostructures on bacteria made NBD emitting more intensely, leading to the fluorescence intensity increase of ***1***. While the concentration of ***2*** on both bacteria was close to its CMC value and thus there were no big differences in fluorescence intensity for ***2*** with or without bacteria.

We obtained the confocal fluorescence microscopy and TEM images of bacteria treated with ***1*** or ***2***. The bacteria were incubated in a culture media containing ***1*** or ***2***. The bacteria were then separated by centrifuge, washed with PBS for three times, re-suspended, and stained with nile blue (10 μg/mL) for observations. As shown in Fig. 3, bright yellow fluorescence of fluorescent NBD was observed from both *B. subtilis* (Fig. 3A) and *E. faecalis* (Fig. 3C) with ***1***, indicating its presence at the surface of bacteria. The nile blue had been widely applied to stain self-assembled nanostructures.[17] The results in Fig. 3B and 3C showed bright red fluorescence of nile blue from *B. subtilis* and *E. faecalis*, respectively, further suggesting the presence of self-assembled nanostructures of ***1*** at the surface of both bacterial. However, for both bacterial treated with ***2*** at the same conditions, we observed much weaker yellow fluorescence from NBD and red fluorescence from nile blue (Fig. 3E-3H). We observed nanoparticles at the surface of *B. subtilis* (Fig. 3I) and *E. faecalis* (Fig. 3J) treated with 25 and 50 μg/mL of ***1***, respectively. Moreover, the size of

nanoparticles on *B. subtilis* (about 200 nm) was bigger than that on *E. faecalis* (about 50 nm). However, it was hard to observe nanoparticles on both bacteria treated with ***2*** (Fig. 3K and 3L). For bacteria without any treatments, their surfaces were smooth (Fig. S-22). These observations in confocal fluorescence microscopy and TEM images correlated well with those of fluorescence response of both compounds to bacteria and observed amounts of both compounds on bacteria.

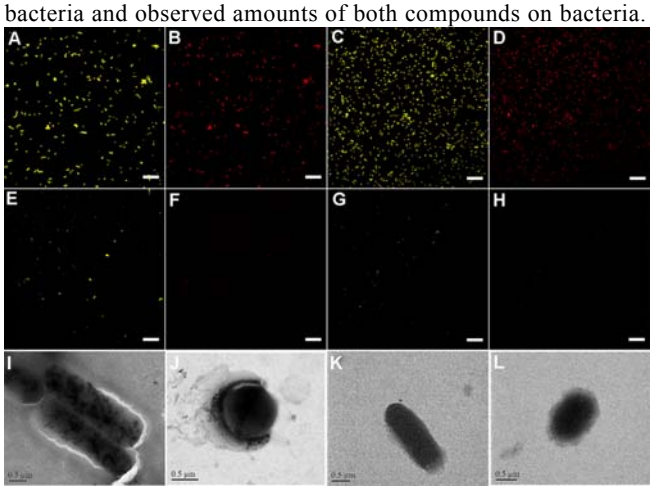

Fig. 3. Confocal fluorescence images of bacteria treated with different compounds. A and B) *B. subtilis* with 25 μg/mL of ***1*** and then with nile blue, C and D) *E. faecalis* with 50 μg/mL of ***1*** and then with nile blue, E and F) *B. subtilis* with 25 μg/mL of ***2*** and then with nile blue, and G and H) *E. faecalis* with 50 μg/mL of ***2*** and then with nile blue ($\lambda_{exc.}$ = 490 and 630 nm for NBD and nile blue, respectively. Scale bars in A-H represent 10 μm), and TEM images of I) *B. subtilis* with 25 μg/mL of ***1***, J) *E. faecalis* with 50 μg/mL of ***1***, K) *B. subtilis* with 25 μg/mL of ***2***, L) *E. faecalis* with 50 μg/mL of ***2***

This was first example of bacterial surface-induced self-assembly at which specific peptide-antibiotics interaction could initiate the self-assembly of an environment-sensitively fluorescent NBD-Van conjugate. This process could be applied for simultaneous detection and inhibition of bacteria. We believed that our study could stimulate the development of other examples of surface-induced self-assemblies at surfaces of cells, biomacromolecules, viruses, etc., which would ultimately lead to the development of novel self-assembling biomaterials and novel strategies to control the fate of biological individuals.

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Notes and references

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†Electronic Supplementary Information (ESI) available: [Synthesis and characterization, chemical structures, CMC value, confocal images, and TEM images]. See DOI: 10.1039/b000000x/

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