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ARTICLE TYPE

Visualized detection of vancomycin by supramolecular hydrogelations

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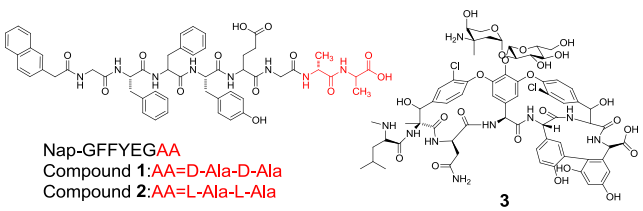
The specific binding between self-assembling peptide Nap-GFFYEG^{D}A^{D}A and the antibiotics vancomycin leads to supramolecular hydrogelations. This sol-gel phase transition can be easily identified by naked eyes. Therefore, it may be developed into a versatile method to detect vancomycin in remote place and in house.

Antibiotics, the most important medicine in treating bacteria infectious diseases, have been widely used in the world since its occurrence and made great contributions in clinical.[1] However, the abuse of antibiotics may lead to antimicrobial resistance[2] and other adverse health effects such as nephrotoxicity and ototoxicity. Vancomycin (VAN), a powerful broad spectrum antibiotic, is a third-line drug and often used as the last defense against the superstrain.[3] Unfortunately, vancomycin resistant bacteria have been found worldwide since the first report in Japan.[4] Moreover, vancomycin is extremely ototoxic and nephrotoxic compared to other antibiotics.[5] Therefore, it is of great significance to develop analytical techniques for detecting antibiotics residues such as vancomycin. In the past few decades, several analytical techniques have been developed, including microbiological assay,[6]immunoassay[7] and instrument[8] analysis. Although these techniques exhibit both high sensitivity and accuracy in detecting antibiotics, they are expensive, time-consuming, operation complicated and not suitable for real-time and on-site analysis. Thus, the invention of a cost-effective, rapid and simple method for detecting antibiotics remains a considerable challenge.



Scheme 1. Chemical structures of Nap-GFFYEG-D-Ala-D-Ala (**1**), Nap-GFFYEG-L-Ala-L-Ala (**2**), and Vancomycin (**3**).

Supramolecular hydrogels based on peptides have been demonstrated as promising nano-materials due to their excellent properties, such as the ease of design and synthesis, biocompatibility, degradability and fast response to external stimuli.[9] They have shown great potential in drug delivery,[10] regenerative medicine,[11] immune boosting,[12] and analyte detection,[13] etc. The sol-gel and gel-sol transitions of peptide hydrogels can be triggered by pH or temperature changes, photo-irradiation, and enzymes. Such kind of phase transition can be easily recognized by naked eyes, which have been developed into a novel method for analyte detection. For instance, gel-sol or sol-gel phase transitions have been applied for the detection of enzymes,[14] glucose,[15] metal ions,[16] melamine,[17] etc. Compared to routine detecting techniques and methods, there is no need to utilize any expensive equipment or to transport samples to a laboratory for this new method. These advantageous properties make the fast, real-time, and on-site analysis of analytes possible, which is especially useful for in house and remote place detections.

It's well known that VAN binds to the terminal D-Ala-D-Ala of peptidoglycan precursors with specificity and high affinity, thereby preventing their integration into the bacterial cell wall and resulting in the cell's lysis.[18] Besides being used in the treatment of diseases, the ligand-receptor interactions between vancomycin and D-Ala-D-Ala derivative in aqueous solution has been well established. Xu and co-workers have reported on a supramolecular hydrogels based on *N*-(fluorenyl-9-methoxycarbonyl)-D-Ala-D-Ala, which exhibited gel-sol transition upon binding to vancomycin via a ligand-receptor interaction.[19] In addition, they have also reported that the addition of vancomycin into the mechanically weak hydrogels of a derivative of D-Ala-D-Ala leaded to the increase of the storage modulus of the hydrogel.[20] Illuminated by this strategy and the intrinsic properties of the self-assembled peptide systems, we opted to design a peptide based D-Ala-D-Ala derivative with self-assembling properties for the detection of vancomycin. We envisioned that the derivative may self-assemble into short nanofibers but not hydrogels because of the relatively weak inter-fiber interactions. Upon the addition of the vancomycin, the ligand-receptor interaction between D-Ala-D-Ala and vancomycin may increase the inter-fiber interaction and therefore cross-link the nanofibers, leading to a sol-gel phase transition. Such a kind of phase transition might be developed into a suitable method for the detection of vancomycin.

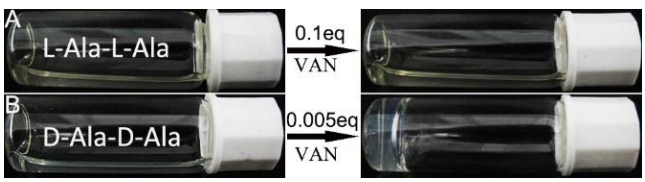


Fig. 1.Optical images of the solutions of A) Nap-GFFYEG^{D}A^{D}A and B) Nap-GFFYEG^{L}A^{L}A without (or with) VAN in PBS. The temperature was 25 ºC.

In order to test our hypothesis, we designed two peptides, Nap-GFFYEG-D-Ala-D-Ala (**1**, Scheme 1) and its enantiomer, Nap-GFFYEG-L-Ala-L-Ala (**2**, Scheme 1). Many derivatives contains the moieties FF or FFY have been verified as molecular hydrogelators with excellent self-assembly properties.[21] The D-Ala-D-Ala moiety can binds to its receptor (vancomycin, VAN). We assumed that our designed peptides might self-assemble into short nanofibrs with good water solubility, but cannot form hydrodgels without vancomycin. We firstly prepared Nap-GFFYEG-D-Ala-D-Ala and Nap-GFFYEG-L-Ala-L-Ala by standard solid phase peptide synthesis (SPPS). The pure compounds were achieved by reverse phase high performance liquid chromatography (HPLC).

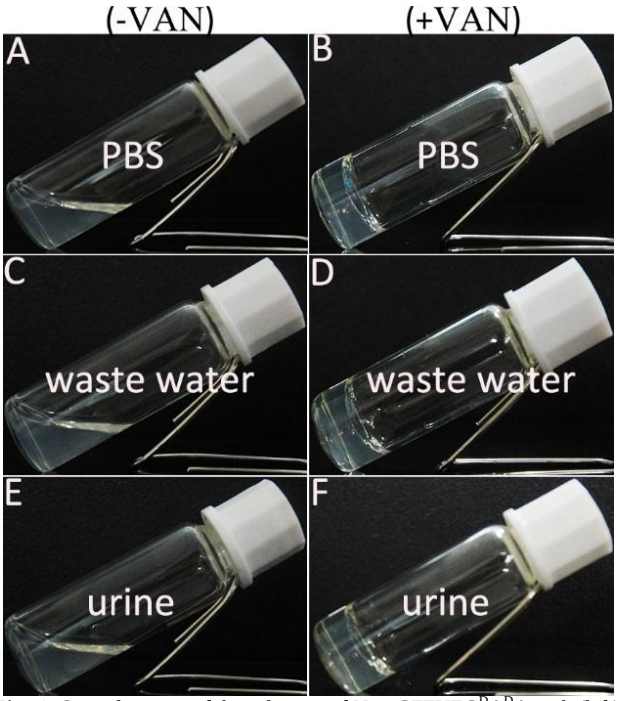


Fig. 2. Optical images of the solutions of Nap-GFFYEG^{D}A^{D}A with (left) or without (right) VAN in PBS (A and B), waste water (C and D), and urine (E and F). The temperature was 25 ^{o}C.

After obtaining the compounds, we studied the self-assembly property of them in the presence or absence of VAN. We found that both peptides could form clear solutions in the phosphate buffer saline (PBS, pH = 7.4) solution at a concentration of 1.5 wt%, suggesting that the peptides have good water-dispersity and would not form gels themselves at this concentration. We then tested whether they could form hydrogels or not after the addition of different equivalents of VAN. As shown in Fig. 1B, the solution of **1** formed a clear gel within 5 minutes after addition of 0.005 equiv VAN (PBS, pH = 7.4), and it took a less time of about 30 seconds for hydrogelations when the concentration of VAN was higher than 0.005 equiv. Whereas, the solution of **2** could not form gels even in the presence of 0.1 equiv. of VAN (Fig. 1A). These results clearly demonstrated the success of our design. The minimum equiv. of VAN needed for gelation was about 0.005 in 0.01M PBS. Such value would be smaller when decreasing the pH value or increasing the ionic strength of the buffer (Fig. S8, S9, and S10). For example, it was 0.0025 or 0.0017 equiv. of VAN (~30 μg/mL) needed for gelations in the pH 5.5 or 0.05 M of PBS buffer solution. The minimum equiv. of VAN needed to gelate the PBS solution of the peptide (1.5 wt%, 0.05 M PBS, pH=5.5) can be lower to about 0.001 (20 μg/mL) at 25 ^{o}C (Fig. S11). Such sol-gel phase transition can be easily recognized by the naked eyes, which might be developed into a useful technique to detect VAN without any equipment.

Due to the intensive use of antibiotics for human (domestic and hospital use), veterinary and agriculture purposes, these compounds are continuously released into the environment without metabolism or in conjugate forms. It has been reported by many studies that the occurrence of antibiotic residues in water sources including municipal wastewater effluents and surface waters.[22] We then tested whether our method could be applied to detect VAN in wastewater effluents and biological fluids such as urine. As shown in Fig. 3C, the mixture of PBS solution **1** and the waste water without VAN would not form a gel. In the presence of VAN, a gel (Fig. 3D) would quickly form within 30 seconds when the concentration of vancomycin was higher than 0.005 equiv. Similar results was observed in the urine samples (Fig. 3E and F), and the lowest detection concentration of VAN was also about 0.005 equiv (0.1mg.mL^{-1}). Although the detection limit of our method might be not as low as other methods, our method could be easily identified by naked eyes and there is no need to separate VAN from samples or send the samples to a laboratory and then measure them by equipments.

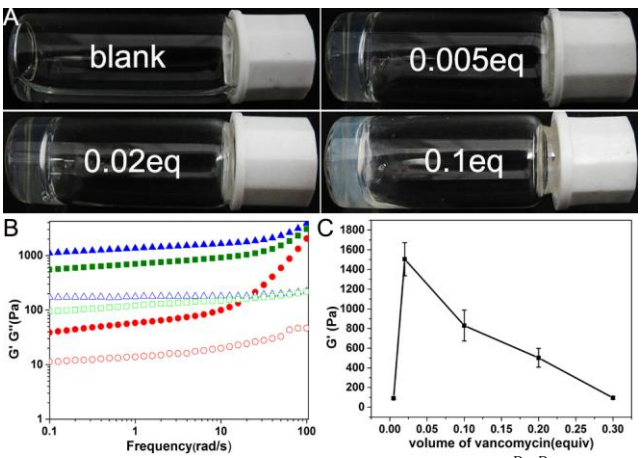


Fig. 3. A) Optical images of the solutions of Nap-GFFYEG^{D}A^{D}A with 0, 0.005, 0.02, 0.1 equiv of vancomycin in PBS, respectively. B) Dynamic frequency sweep at the strain of 0.5% of the hydrogels with addition of 0.005 (circles), 0.02 (triangles), 0.1(squares) equiv. of vancomycin (filled symbols: G' and open symbols: G''). C) The G' value in the mode of dynamic frequency sweep at the frequency of 10 rad/s and strain of 0.5% of the gels with different equivalents of vancomycin. The temperature was 25 ^{o}C.

We then utilized a rheometer to investigate the mechanical property of hydrogels (Fig. S5, S6, S7). The mixed solutions containing **1** and different amounts of VAN were directly transferred to the rheometer. We then successively performed the dynamic time, strain, and frequency sweep. As shown in Fig. 3B, the value of storage moduli (G', elasticity) of all hydrogels was bigger than that of their corresponding loss moduli (G'', viscosity), suggesting that all samples behaved as viscoelastic materials. The G' values of the hydrogels became bigger with the increased amount of VAN when the concentration of VAN was

lower than 0.02 equiv. Once over 0.02 equiv., the G' values turned to decrease (Fig. 3C). These results were consistent with the optical images of the gels. As shown in Fig. 3A, the gels were very transparent under 0.02 equiv.. Once exceeded 0.02 equiv., the gels turned to be opaque.

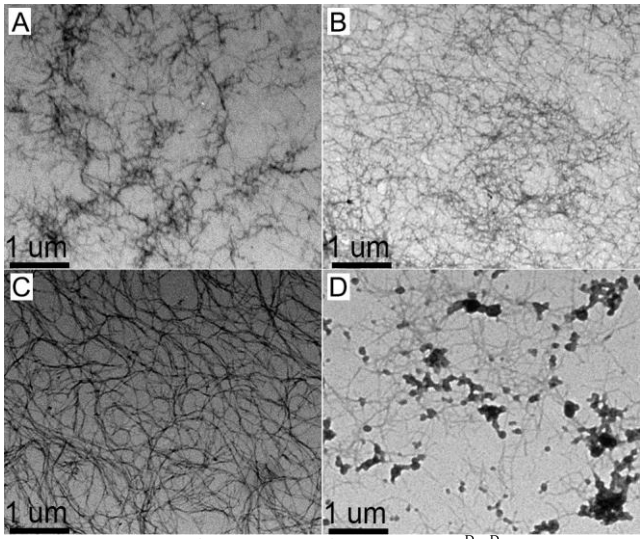


Fig. 4. TEM images of (A) solution of Nap-GFFYEG^{D}A^{D}A and gels with different equiv. of vancomycin: (B) 0.005 equiv., (C) 0.02 equiv., and (D) 0.1 equiv.

We also characterized the morphology of the self-assembled nanostructures in the solution and the hydrogels with different equivalent of VAN by transmission electron microscopy (TEM). As shown in Fig. 4A, compound **1** itself self-assembled into short fibers with low density of cross-linking points. With the addition of VAN, the fibers grew into longer and bigger with high density of cross-linking points (Fig. 4B and 4C). However, aggregated structures appeared when the concentration was higher than 0.1 equiv. (Fig. 4D). These observations were consistent with the results observed by optical images and mechanical properties of the gels. These results also suggested that VAN help to bring the peptides together and extend the supramolecular chains to form longer fibers and their bundles. However, the excess amount of VAN disrupted the supramolecular interactions, thus resulting in partial aggregations.

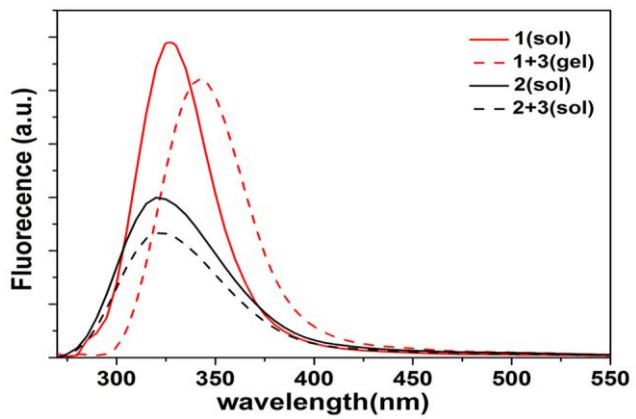


Fig. 5. Fluorescent spectra (λ_{ex}= 272 nm) of **1** (sol), **1**+**3** (gel), **2** (sol) and **2**+**3** (sol).

To further understand the molecular arrangements in the hydrogels, we measured emission spectra of the PBS solutions of **1** or **2** with and without VAN. As shown in Fig. 5, both the PBS solutions of **1** and of **2** exhibited distinct peaks centered at about 328 nm, suggesting monomeric naphthalene moieties in the solutions. In the presence of VAN, the peak showed a red shift to about 358 nm for **1**+**3**(gel), indicating that naphthyl groups stacked more efficiently. However, the emission spectrum of the solution of **2** showed little difference in the presence of VAN. The results also suggested that VAN helped to extend the supramolecular chains and the formation of hydrogels through the specific interaction between VAN and the dipeptide D-Ala-D-Ala.

Based on the above measurements, we proposed a plausible molecular interaction between **1** and VAN in the gels. As indicated in Fig. 6, the peptide self-assembled to short fibers due to the hydrogen bonds and the π-π interactions. With the increasing amount of VAN, VAN cross-linked the short fibers via the specific ligand-receptor interaction and hydrogen bonds. Therefore, the short fibers grew into longer ones or their bundles, resulting in hydrogelation.

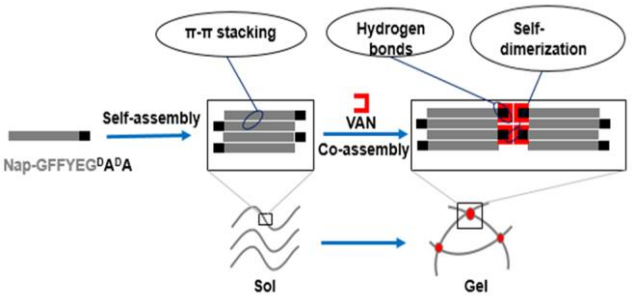


Fig. 6. Possible molecular arrangements.

In conclusion, we reported a supramolecular hydrogel-based system for the visualized detection of VAN. This simple method is applicable for detecting VAN in waste water effluent and urine by naked eye. Vancomycin is generally used in human to treat bacteria by oral administration or i.v. injection, and the therapeutic window of VAN is 20-5 μg/mL. Immunoenzymatic techniques and chromatographic methods are generally used to detect the concentration of VAN in biological fluids, and their detection limit can be low to 0.005 μg/mL (others are 2-5 μg/mL).[23] The detection limit of our method is about 20 μg/mL in 0.05 M of PBS solution at pH 5.5. Although the detection limit of our method might be not as low as other techniques using complicated equipments, it may be a potential candidate for the rapid, simple, real-time and on-site screening of vancomycin in waste water effluents and biological fluids in house and in remote places. One limitation of our method is that the gelation will be greatly affected by both the pH value and the ionic strength. For relatively accurate detection of VAN in the sample, the pH value requires to be adjusted to a certain value and the high ionic strength buffer solution of peptide (e.g. 0.1M PBS) is needed to be used to avoid the dilution effect of the addition of the sample. Our further step is to lower the detection limit of our method or apply our method for the detection of bacteria, because the addition of bacteria might disrupt the binding between VAN and the peptide that might lead to gel-

sol phase transition.

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Notes and references

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