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Supramolecular hydrogel of kanamycin selectively sequesters 16S rRNA†

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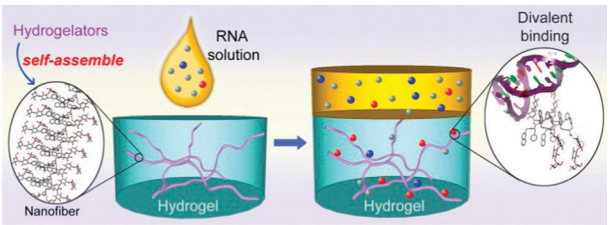
As the first example of hydrogelator derived from aminoglycoside antibiotics, the hydrogel of kanamycin indicates that the hydrogel of aminoglycosides preserve the specific interaction with their macromolecular targets (*e.g.*, 16S rRNA), thus illustrating a simple approach to explore and identify possible biological targets of supramolecular nanofibers/hydrogels.

This communication reports the first hydrogelator derived from an aminoglycoside antibiotic (*e.g.*, kanamycin[1]) and the resultant hydrogel for sequestering 16S rRNA selectively *via* divalent interaction. Hydrogelators are small molecules that self-assemble in water to yield supramolecular nanofibers to imbibe water and to afford hydrogels.[2] The early development of hydrogelators,[3] as a curiosity of physical organic chemistry,[4] has demonstrated that carefully balanced hydrophobic interactions and hydrogen bonding induce the aggregation of small molecules in water to form nanofibers and afford hydrogels. After that, the successful integration of biofunctional or bioactive molecules into the hydrogelators[5] has led to rapid development of supramolecular hydrogels[6,7] as a new class of biomaterials that show precise control on molecular structures and supramolecular organization.[2] For example, the hydrogelator of vancomycin has exhibited high activities towards vancomycin resistance enterococci (VRE).[8,9] Although a vast pool of bioactive molecules, ranging from peptides[10] to clinically used drug molecules,[9,11] are able to serve as building blocks for constructing supramolecular assemblies with a superior effectiveness that is unattainable by monomeric epitopes or individual drug molecules, there is little exploration on the self-assembly of functional small molecules in water, partially due to the lack of a general approach to promote molecular self-assembly and to verify the interactions between the supramolecular assemblies and their target(s).

We hypothesized that the conversion of a functional molecule to a hydrogelator offers a simple way to create the self-assembly of the functional molecule and to verify the interactions between the nanofibers and the potential targets. Scheme 1 illustrates the concept. The self-assembly of the hydrogelators leads to the formation of supramolecular nanofibers, which entangle to result in a network, stop the flow of water, and afford a hydrogel. Thus, hydrogelation can serve as a simple visual assay to indicate the self-assembly of small molecules in water. After the hydrogel reaches the steady state, it is possible to add the solution containing the potential target and appropriate control molecules on the top of the hydrogel. The gel phase is in contact with the solution, but the two phases remain immiscible. While the nanofibers remain in the gel phase, the target and control molecules, however, can enter the gel phase by diffusing through the pores of the network. The specific interaction of the nanofibers with the targets would retain more target molecules than the control molecules in the gel phase. Since it is easy to separate the gel phase from the solution phase by aspiration of the supernatant, the analysis of the remaining molecules in the solution phases (*i.e.*, the supernatant) should be able to identify the target molecules sequestered in the hydrogel due to the specific interaction between the target molecules and the supramolecular nanofibers.



Scheme 1 Supramolecular nanofibers sequester the potential targets (*e.g.*, 16S rRNA (in red)) in the gel phase.

To demonstrate the concept illustrated in Scheme 1, we prepared the hydrogelator of kanamycin (**1**) by attaching multiple phenylalanine residues to the 6′-*N* amino group of kanamycin A, which is a member of aminoglycoside antibiotics that bind 16S rRNA in ribosome to inhibit protein synthesis and act as bactericides.[1] We found that **1** not only acts as an effective hydrogelator at neutral pH with critical gelation concentrations, cgc, at 0.3 wt%, but the hydrogel of **1** also selectively sequesters 16S rRNA—the receptor of kanamycin A. Fluorescent titration confirms two molecules of **1** bind to the same site on the receptor (*i.e.*, A-site of 16S rRNA,[1,12]). This work not only indicates that the hydrogel of aminoglycosides preserve the specific interaction with their macromolecular targets (*e.g.*, 16S rRNA[1]), but also illustrates a feasible approach for exploring molecular self-assembly in water and screening the binding of the resulting supramolecular nanofibers/hydrogels to the potential target(s).

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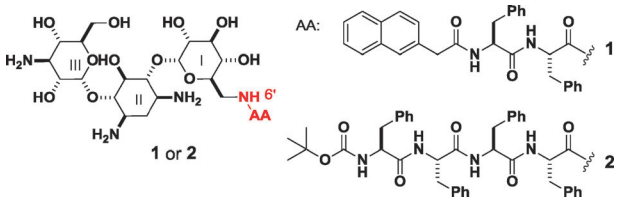

Scheme 2 Structures of hydrogelators based on kanamycin A.

Scheme 2 shows the structure of the hydrogelators of kanamycin A, **1** and **2**. The synthesis of **1** is straightforward. Being activated by *N*-hydroxysuccinimide (NHS), 2-(naphthalen-2-yl)acetamido)-3-phenylpropanamido)-3-phenylpropanoic acid (NapFF)[13] reacts with kanamycin in a mixture of acetone and water (pH ~ 8.0) for 24 h to afford the crude product, which undergoes chromatographic purification to give **1** as a white powder. Typically, 2 mg of **1** dissolves in 0.4 mL of water at pH 2.0 to result in a clear solution, which turns into a transparent hydrogel (gel I) after the pH of the solution was adjusted to 7.0. The transparent hydrogel easily changes back to a clear solution when the pH is lowered to less than 6.5 or the temperature is increased to higher than 46 °C. The hydrogel of **2** (Gel II) is formed similarly. The resulting transparent hydrogels (Fig. 1A and B) are stable at room temperature for months without visible changes.

To study the nanostructures or morphology of the hydrogels, we obtained their transmission electron micrographs (TEM) (Fig. 1C and D), which show small fibrils with the diameters from 50 nm to 60 nm. The elongated small fibrils (longer than 5 μm) tend to tangle with each other and form a dense network, which serves as the matrices of gels I and II. According to the dynamic frequency sweep of gel I, the values of the storage moduli (G') exceed the loss moduli (G'') by a factor of about 5 (Fig. S2†), indicating that the sample behaves like a solid.[7] The value of G' of the sample is higher than 600 Pa, indicating that the sample is viscoelastic. Both the values of G' and G'' of the hydrogel exhibit weak dependence with frequency (from 0.1 to 100 rad s^{-1}), suggesting that the matrices of the gel have a good tolerance to external force.

We used circular dichroism (CD) and fluorescent spectroscopy to investigate the molecular arrangement of the small molecule in gel I (Fig. S1†). A positive band near 196 nm (ππ* transition) and a negative peak at about 208 nm (nπ* transition) reveal the β-sheet like conformation of the peptidic backbones. The broad peak centered at 246 nm (ππ* transition of phenyl groups), two small peaks at 286 nm (ππ* transition of naphthyl aromatics) and 296 nm (nπ* transition of naphthyl aromatics) originate from the aromatic parts of the molecules, which adopt helical arrangements induced

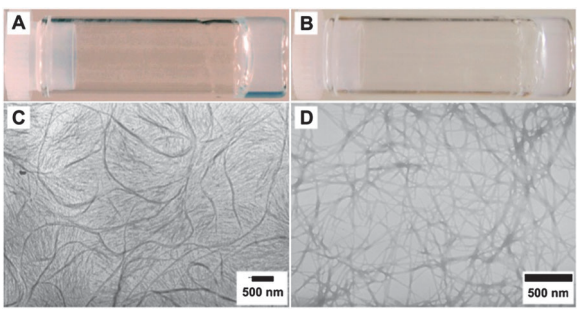

Fig. 1 Optical images of (A) gel I formed by **1** (0.5 wt%, pH 7.0); (B) gel II formed by **2** (1.0 wt%, pH 7.0); TEM of (C) gel I and (D) gel II.

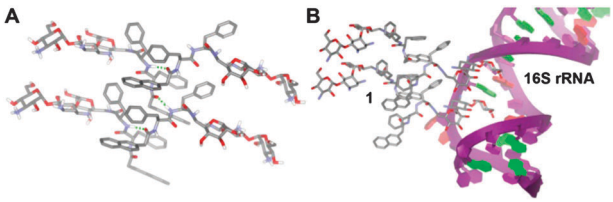

Fig. 2 (A) A possible molecular arrangement of **1** in the gel phase (some hydrogen bonds shown as the dot lines). (B) A hypothetical binding between two molecules of **1** and their receptor (A-site of 16S rRNA) derived from the crystal structure of kanamycin A.[12]

by the peptidic backbone and kanamycin A. The emission spectrum of **1** (Fig. S1†) shows a peak centered at 336 nm in solution and at 342 nm in the gel phase, indicating the monomeric naphthalene moieties. The additional peaks at 381 and 400 nm suggest strong interactions between the aromatic parts (naphthyl and phenyl aromatics) of the molecules, which is consistent with its self-assembled nanofibers observed in the TEM image. Based on the above information and the crystal structure of kanamycin A[12] and NapFF,[13] we propose a possible molecular arrangement of **1** in the gel phase. As shown in Fig. 2, the NapFF motif plays an important role for the formation of supramolecular structures: the π–π interactions of aromatic rings and the intermolecular hydrogen bonds between peptide bonds help **1** self-assemble into a β-sheet like chain. Part of the kanamycin A sticks out to the aqueous environment and forms hydrogen bonds with water molecules and other kanamycin molecules to help in the formation of nanofibers. Although other modes of molecular arrangements may exist in the gel phase, the current evidences indicate that the arrangement in Fig. 2A likely dominates.

It is well established that kanamycin A bound to the A-site of 16S rRNA to lead to the misreading of mRNA and the death of the bacteria.[1,12,14] To prove further that **1** binds to the A-site of 16S rRNA, we add a commercial oligonucleotide, 5′-UUGCGUCACACCGGUGAAGUCGC-3′ (*i.e.*, A-site of 16S rRNA[12]), to a solution of **1** and observed the change of fluorescence upon binding. We choose to evaluate the binding in PBS buffer to minimize the influence of the self-quenching of the naphthalene of **1** in the gel phase. Fig. 3A clearly shows that the intensity of the fluorescent peak at around 336 nm decreases after the addition of the oligonucleotide. The fluorescent peak almost disappears after the addition of 7.33 equivalents of the oligonucleotide. Based on this fluorescent titration (Fig. 3A),[15] the calculated binding number is 1.97 and the association constant

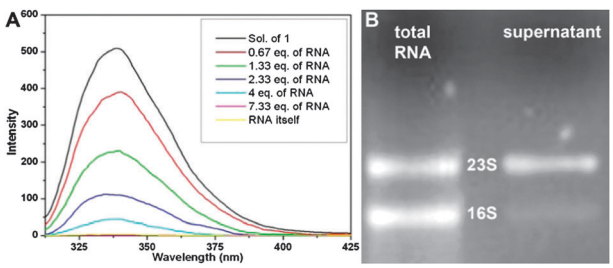

Fig. 3 (A) Fluorescence spectra of solution of **1** (1 μg mL^{-1}) and mixtures of **1** with different equivalents of oligonucleotide (A site of 16S rRNA) in PBS buffer solution. (B) Gel electrophoresis of 16S rRNA and 23S rRNA in the extract before and after incubation with hydrogel of **1** for 4 h.

is 4.0×10^9 M between **1** and the A-site of 16S rRNA. This result indicates two molecules of **1** bind to the same A-site of 16S rRNA, which agrees with the recently reported crystal structures of the interaction between the ribosomal subunit and aminoglycoside antibiotics[12,14] that reveals two aminoglycosides to bind to the same A site of 16S rRNA.[12] The association constant is much higher than the binding constant of kanamycin with 16S rRNA (5.6×10^5 M),[16] suggesting cooperative binding.

To verify whether the hydrogel of **1** is able to bind selectively to the 16S rRNA, we extracted total RNAs from *E. coli* and added the solution containing total RNAs on the top of the hydrogel of **1**. Gel electrophoresis shows that 5S, 16S and 23S rRNA are the major components of the total RNAs (Fig. S2†), in which the initial ratio of 16S : 23S : 5S rRNA is about 1 : 1 : 8. The large quantity of 5S rRNA in the total RNA, together with 23S rRNA, serves as a useful control. After being incubated with the hydrogel of **1** for 4 h, the amounts of RNAs in the supernatant decrease due to the absorption of RNA in the hydrogel of **1**, as indicated by gel electrophoresis†. Quantitative analysis of the RNA bands in Fig. 3B confirms that 20% of 16S rRNA and 70% of 23S rRNA remain in the supernatant. Despite the initial amount of 5S rRNA being about eight times of that of 16S rRNA (or 23S rRNA), 70% of 5S rRNA remains in the supernatant. This result suggests that the hydrogel of **1** selectively sequesters 16S rRNA. Because only 30% of 5S sRNA† undergoes uptake by the hydrogel of **1** (though the size of 5S rRNA is much smaller and the quantity of 5S rRNA in the total RNA much larger than those of 16S rRNA), the observed selectivity is unlikely to arise from the smaller size of 16S rRNA compared to 23S rRNA. Rather, the above results clearly indicate that **1** binds to the A-site of 16S rRNA, thus leading to the hydrogel of **1** binding selectively to 16S rRNA. If the hydrogel of **1** bound extracted RNAs *via* only electrostatic attractions, the hydrogel of **1** would bind 23S rRNA that carries the most negative charges among the extracted RNAs. Therefore, the hydrogel of **1** selectively binds 16S rRNA, which also indicates that the derivatization at the N6′ position is unlikely to completely eliminate the binding of **1** to the A-site of 16S rRNA. Thus, we suggest that the self-assembled molecules of **1** to bind with 16S rRNA as shown in Fig. 2B, which is a possible interaction of **1** with the A-site of 16S rRNA derived from the binding of kanamycin to the RNA groove without involving the ammonium N6′.[12] Although the suggested model in Fig. 2B agrees with the 2 : 1 stoichiometry (between **1** and the A-site of 16S rRNA) obtained from Fig. 3A, the atomistic description of the interactions between **1** and the A-site of 16S rRNA has yet to be established.

In summary, we have designed and synthesized novel molecular hydrogelators based on kanamycin A and demonstrated a feasible approach to explore the potential targets of supramolecular nanofibers/hydrogels. One potential practical application of this hydrogel of kanamycin would be the enrichment of 16S rRNA from cell lysates for typing bacteria. However, the selectivity of the gel towards 16S remains to be improved, possibly by designing and screening new kanaymicin containing hydrogelators. The principle illustrated in this work should help identify new targets of supramolecular nanostructures,[17] which may lead to new insights or phenomena that are inaccessible by an individual molecule.[18] Our future works will focus on the modifications of other amino-functions of kanamycin A and other aminoglycosides.

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