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A supramolecular hydrogel for spatial-temporal release of auxin to promote plant root growth†

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Short peptide-based hydrogels have attracted extensive research interests in drug delivery because of their responsive properties. So far, most drug molecules have been conjugated with short peptides *via* an amide bond, restricting the release of the native drug molecules. In this study, we demonstrated the effectiveness of an auxin-based hydrogelator linked by a hydrolysable ester bond. Hydrogel I, formed by the gelator (NAA-G′FFY) linked with an ester bond, was able to release 1-naphthaleneacetic acid (NAA), whereas hydrogel II, formed by the gelator without an ester bond (NAA-GFFY), was not. By mixing NAA-G′FFY with Fmoc-GFFY to form a two-component hydrogel, the spatial and temporal release of NAA was achieved, promoting on-site auxin responses including primary root elongation and lateral root formation in the model plant *Arabidopsis thaliana*. The strategy of using a hydrolysable ester bond to connect drug molecules and self-assembling peptides could lead to the development of supramolecular hydrogels with more controllable drug release profiles.

The phytohormone auxin regulates nearly all aspects of plant development, including embryogenesis,[1,2] phyllotaxis,[3] vascular development,[4,5] lateral root emergence,[6–9] and gravitropism.[5,10] At relatively high concentrations, auxin can inhibit plant growth, acting as a herbicide.[11] At picomolar to nanomolar concentrations, auxin promotes primary root elongation[12] and adventitious root emergence.[13] So far, synthetic auxins other than indole-3-acetic acid (IAA) have been widely used in both scientific studies and agricultural and horticultural practices. The synthetic auxin 1-naphthalene acetic acid (NAA) is popular because of its relative stability and its lipophilicity, which allows it to freely enter the plant cell. However, because of the rapid diffusion of NAA in agarose hydrogel, the most commonly used technique for physically encapsulating NAA in an agarose hydrogel or hydrogel beads cannot fulfil the need for the spatial release of NAA for plant research. It remains a challenge to develop materials for the spatial-temporal release of auxin for research use.

Supramolecular hydrogels of short peptides have been widely used for sensing,[14–18] immune modulation,[19–24] cell fate control,[25–30] and drug release.[31–35] They are formed by the self-assembly of short peptides *via* noncovalent interactions[36–40] (*e.g.*, hydrogen bonds, aromatic and hydrophobic interactions, and charge interaction). For their application in drug release, supramolecular hydrogels can serve as physical carriers for the slow release of encapsulated drugs.[41–44] An alternative drug release mechanism is to covalently connect peptides and drug molecules through degradable chemical bonds to afford a carrier-free drug delivery system.[31,32,34,35,45] In such a system, the drug loading can be very high and controllable, and the drug release is constant and sustained. Besides, the kinetics of peptide self-assembly is highly important to tune the rheology of resulting hydrogels, thus providing a versatile strategy to control drug release.[46,47] Therefore, supramolecular hydrogels of drug-peptide amphiphiles have been widely used for the delivery of anticancer,[31,48,49] anti-inflammatory,[24,50,51] and anti-oxidative drugs.[52] In general, short peptides need to be modified with aromatic capping groups to construct supramolecular hydrogelators, including the widely used naphthalenic and fluorenyl groups.[53,54] NAA also possesses a naphthalene group that can be used to modify short peptides to generate supramolecular hydrogelators, and the resulting hydrogels may be applied for the spatial-temporal release of auxin for plant growth studies.

To verify our hypothesis, we chose 1-naphthylacetic acid (NAA) as an aromatic capping group for the short peptides. We designed and synthesized the compounds NAA-G′FFY and NAA-GFFY (compounds **1** and **2**, respectively, Fig. 1A). In compound **1**, we used a hydrolysable ester bond to connect the NAA and

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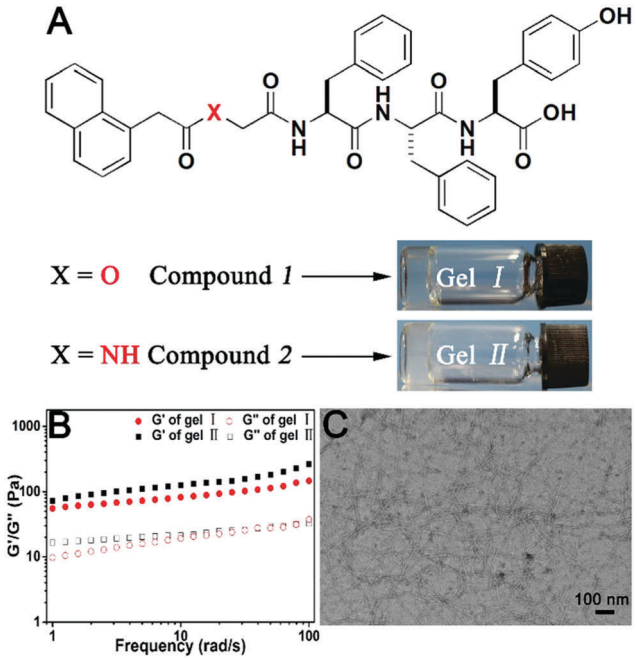

Fig. 1 (A) Chemical structures and optical images of supramolecular hydrogels of compounds **1** and **2** formed by a heating–cooling process, (B) dynamic frequency sweep of the Gels I and II with 0.3 wt% of compound at the strain value of 0.1%, and (C) TEM image of Gel I with 0.3 wt% of compound **1**.

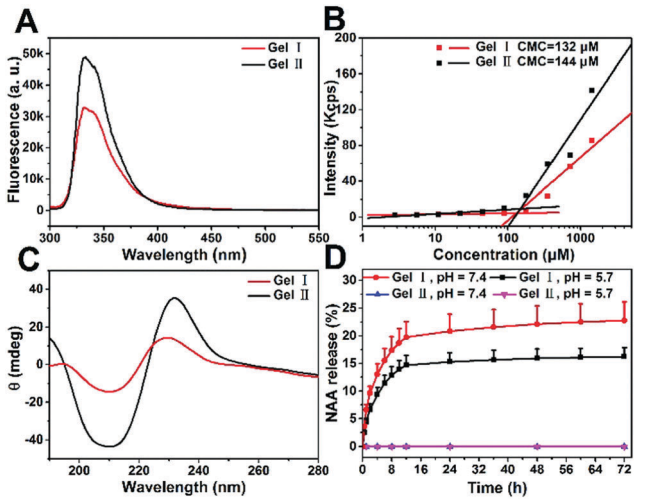

Fig. 2 (A) Fluorescence spectra of two gels at concentration of 0.3 wt%, (B) critical aggregation concentration (CAC) values of compounds **1** and **2**, (C) circular dichroism (CD) spectra of Gel I and Gel II at 0.3 wt%, (D) release profile of NAA from the Gel I and Gel II in the presence of PBS buffer (pH = 7.4) and 1/2 Murashige and Skoog (1/2 MS) medium (pH = 5.7).

the peptide. In the presence of changes in environmental conditions, such as pH, the NAA-G′FFY could slowly release NAA *via* ester bond hydrolysis. For control compound **2**, NAA was conjugated with the peptide *via* an amide bond, and therefore, NAA could only be released by enzymatic digestion. The two compounds were prepared by the standard Fmoc solid-phase peptide synthesis (SPPS) and then purified by high-performance liquid chromatography (HPLC). As shown in Fig. 1A, after a heating–cooling process, NAA-G′FFY formed a transparent gel (Gel I) at a concentration of 0.3 wt% in phosphate-buffered saline (PBS, pH = 7.4), whereas under the same conditions, NAA-GFFY formed an opaque gel (Gel II), which became transparent overnight.

We first tested the mechanical properties of both gels using a rheometer. The results of a dynamic frequency sweep showed that the value of the dynamic storage moduli (G') of each gel was greater than its corresponding value of the dynamic loss moduli (G'') (Fig. 1B). The ratio of G'/G'' for Gel I was approximately 6.75, and that for Gel II was approximately 4, suggesting that both gels were mechanically weak. We then investigated the morphology of the nanostructures of the two gels by transmission electron microscopy (TEM). As shown in Fig. 1C and Fig. S7 (ESI†), we observed networks of nanofibers, and the fibers were entangled with each other to form three-dimensional networks. The nanofibers in both gels were uniform, and the diameter was approximately 7 and 11 nm for Gel I and Gel II, respectively. We also recorded fluorescence spectra of both hydrogels. Both hydrogels exhibited similar fluorescence peaks centered at 333 nm but with different intensities of 32 717 and 49 025 a.u. for Gel I and Gel II, respectively (Fig. 2A). The fluorescence intensity of Gel I was lower than that of Gel II, implying compound **1** had a slightly better self-assembly property than compound **2**. The critical aggregation concentration (CAC) values of compounds **1** and **2** were 132 and 144 μM, respectively (Fig. 2B), also suggesting a slightly better self-assembly property of compound **1** than compound **2**. The circular dichroism (CD) spectra (Fig. 2C) suggested that the two peptides adopted β-sheet-like conformation in the gels, as indicated by a positive peak near 195 nm and a negative trough at 210–220 nm. The CD signal of compound **2** was stronger than that of compound **1** owing to the additional hydrogen bonding provided by the amide bond between NAA and the peptide.

Before testing the hydrogel *in planta*, we studied the release profile of NAA from Gel I in contact with PBS (pH = 7.4) or 1/2 Murashige and Skoog (1/2 MS) medium (pH = 5.7) used for plant growth.[55] As shown in Fig. 2D, Gel I was able to release free NAA into both PBS and 1/2 MS, and the release rate decreased over time at both pH. In contrast, Gel II failed to release free NAA at either pH. Assuming that the injection volume was 20 μL, we measured the three-day accumulated amount of NAA released from the gel at pH = 5.7. The calculated value was approximately 641 M, which was much higher than the order of magnitude required for plant growth.[56] We therefore mixed physiological concentrations of compound **1** with a complementary component, Fmoc-GFFY, producing a two component gel that could be tested on Arabidopsis. A serial dilution of NAA-G′FFY into Fmoc-GFFY was done with a total concentration of 0.3 wt% in 300 μL of PBS (Table S1, ESI†). The five coassembly gels were evaluated for their rheology and the release profile of NAA. The presence of Fmoc-GFFY did not significantly change the rheology of Gel I in three out of five concentrations tested (Fig. S8, ESI†), and the coassembly gels shared similar trends among themselves and with Gel I in NAA release (Fig. S9, ESI†).

The effect of the hydrogel on plant growth was then evaluated in Arabidopsis seedlings. To see how primary root elongation

and lateral root formation could be affected by the addition of the hydrogel close to the primary root, we first punched holes (5.5 mm in diameter) in the 1/2 MS medium, and injected 20 μL of the hydrogels containing various concentrations of NAA-G′FFY, or control gels/solutions, into the holes. Then we transfer Arabidopsis seedlings (Col-0 as the wild-type, and *ProDR5rev:GFP* as the auxin response marker line) vertically grown on 1/2 MS medium to the new plates, carefully placing their primary roots alongside the holes The seedlings were then grown vertically for 3 more days under the same conditions (Fig. S10 and S12, ESI†). Phenotypes were documented with a scanner (Epson Perfection V33), or a stereoscope (Leica 165FC, Germany) equipped with a CCD camera at the same time daily for three days. We found out that Gel I-4 was the most efficient in promoting primary root elongation (Fig. S10 and S11, ESI†). As expected, higher concentrations of compound **1** led to more lateral root production (Fig. S12 and S13, ESI†). Therefore, we considered Gel I-4 as the optimal concentration and used it for further analysis.

The auxin response in the primary root, indicative of the *in vivo* NAA concentration, was monitored with an auxin response marker *ProDR5rev:GFP* (Fig. 3). We designed 6 control conditions as follows. Gel II-4, made from compound **2**, served as a negative control for NAA release. To compare the Gel I-4 against pure NAA, three controls were prepared: the calculated amount of NAA contained in Gel I-4 was added in either PBS (NAA in PBS) or 0.1% Agar (NAA in Agar) and injected into the holes, or added to the whole plate (NAA evenly distributed). Fmoc-GFFY was also injected as a negative control (control). Finally, 1/2 MS, with an empty hole in the medium, was included as blank. As expected, GFP intensities were high in NAA in Agar and NAA in PBS, and low in Gel II-4, control, and blank. GFP intensities on Gel I-4 and NAA evenly distributed were in between, with Gel I-4 higher than the latter, indicative of the successful release of NAA at an optimal concentration by Gel I-4. Temporal release of NAA was also analyzed (Fig. S14, ESI†). Both NAA in PBS and NAA in Agar immediately induced auxin response to a high level that sustained over time, suggesting un-controlled release of NAA. In contrast, Gel I-4 gradually induced auxin response in the root of *ProDR5rev:GFP* over time. Combined, the *in vivo* observation confirmed that Gel I-4 successfully released NAA both spatially and temporally (Fig. S14, ESI†).

We then analyzed the physiological effects of the hydrogel by observing the primary root elongation and lateral root formation under the same conditions. Compared with Gel II-4, control, and blank, NAA in PBS, and NAA in Agar strongly inhibited primary root elongation and promoted lateral root emergence (Fig. 4). As expected, NAA evenly distributed slightly promoted

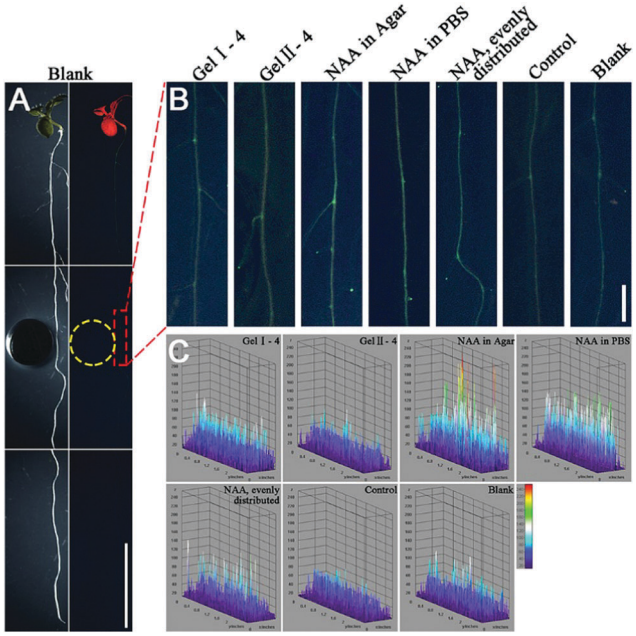

Fig. 3 (A) *In vivo* auxin response illustrated by GFP fluorescence in the auxin-responsive marker line *ProDR5rev:GFP*. Seven-day-old Arabidopsis seedlings, vertically grown on 1/2 MS medium, were transferred to new 1/2 MS medium containing different forms of the hydrogel or control gels as indicated in (B) and grown vertically for another 3 days. The yellow dotted circle outlines the area in the medium filled with the hydrogels. GFP fluorescence was captured with a fluorescent stereoscope (Leica 165FC) equipped with a CCD camera at day 10. (B) Images of the primary root sections adjacent to the hydrogels were enlarged to show the differences in GFP fluorescence, indicative of auxin response, among the 7 conditions. (C) Spatial distribution and intensities of GFP fluorescence was converted to 3-D surface plots for each image in (B) with the thermal LUT plug-in in ImageJ. Bar = 1 cm in (A) and 1 mm in (B).

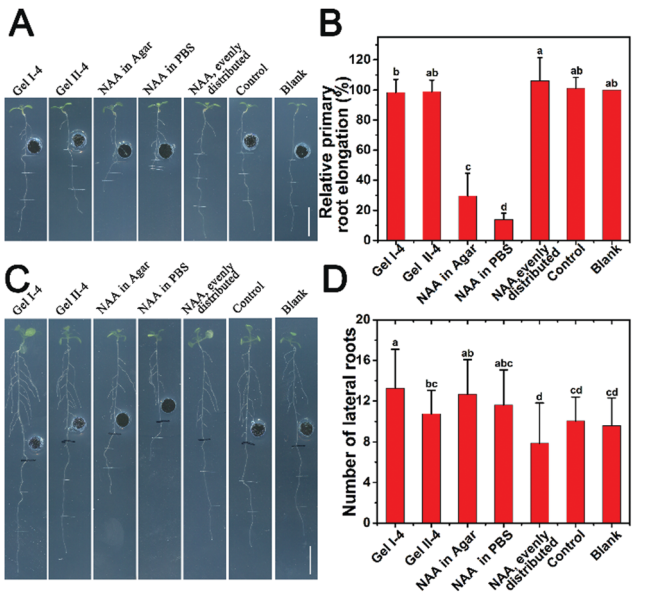

Fig. 4 (A) Four-day-old vertically-grown Arabidopsis seedlings (Col-0) were transferred to new plates and grown vertically for 3 days in the presence of Gel I-4, Gel II-4, NAA in 0.1% Agar, NAA in PBS, NAA (evenly distributed), control, and blank, respectively. Seedlings were scanned at day 7 with a scanner (Epson Perfection V33). (B) Statistical analysis of relative primary root elongation (%), as compared with blank (set as 100%), over the 3 days. The data shown are mean ± SD (n = 16). (C) Seven-day-old vertically-grown Arabidopsis seedlings (Col-0) were transferred to new plates and grown vertically for 3 days in the presence of Gel I-4, Gel II-4, NAA in 0.1% Agar, NAA in PBS, NAA (evenly distributed), control, blank, respectively. Seedlings were scanned at day 10. (D) Quantification of numbers of lateral roots of 10-day-old seedlings. The data shown are mean ± SD (n = 15). Bar = 1 cm in (A and C). One-way ANOVA was done with SPSS in (B and D).

primary root elongation and slightly repressed lateral root formation. Gel I-4 did not affect primary root elongation; however it induced both lateral root emergence and elongation, resulting in the largest root system among all treatments.

In summary, we demonstrated that an auxin-based supramolecular hydrogel connected by a hydrolysable ester bond has a good capacity to slowly release NAA into the environment, while the control connected by an amide bond cannot release NAA. The spatial and temporal release of NAA could be a useful new tool in the study of auxin-induced plant growth and development. Furthermore, there are many drug molecules bearing free carboxylic acid groups that could be connected with self-assembling peptides for their delivery. The strategy of using a hydrolysable ester bond to connect drug molecules and peptides could ultimately lead to nanomedicines with a faster and more controllable drug release profile, which may be beneficial for the treatment of diseases.

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Conflicts of interest

There are no conflicts to declare.

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