Supporting Information

A highly efficient dual-diazonium reagent for protein crosslinking and construction of a virus-based gel

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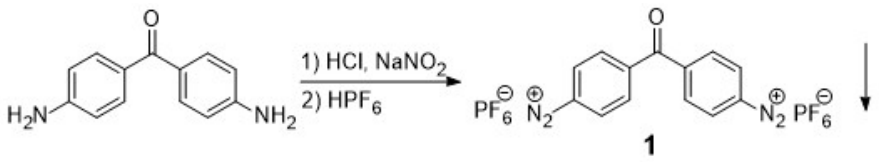
1. Reagents and instruments

All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiment without further purification. ^{1}H NMR，^{13}C NMR, ^{31}P NMR and ^{19}F NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks (DMSO-d_6 = 2.50 ppm). ^{1}H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as the following: d (doublet), h (hepta). The UV-visible spectra were recorded on a UV-3600 UV-VIS-NIR-spectrophotometer (SHIMADZU, Japan). Protein mass spectrum was recorded on Bruker Autoflex III TOF/TOF200.

2. Synthesis of linker 1



1

4,4′-Diaminobenzophenone (1.06 g, 5.0 mmol) was dissolved in 15 mL concentrated HCl and 30 mL H_2O and cooled down to -5 ºC. The water solution of $NaNO_2$ (2.77 g, 40.1 mmol) was slowly added to the mixture at -5-0 ºC. After 1 h reaction, 60% HPF_6 in water (2 mL, 13.7 mmol) was added at 0 ºC and stirred for 1 h. The product were collected by filtration and washed with ice-cold water, yielding a light yellow solid of **1** (1.20 g, 45.6%).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.69 (d, J = 8.8 Hz, 4H), 8.14 (d, J = 8.8 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.1, 133.1, 128.6, 115.1, 94.2. ^{31}P NMR (162 MHz, DMSO-d_6) δ -144.19 (h, $J_{P\text{-}F}$ = 710.6 Hz). ^{19}F NMR (376 MHz, DMSO- d_6) δ -70.08 (d, $J_{P\text{-}F}$ = 710.6 Hz).

3. UV-Vis absorbance analysis

The solubility of the diazonium reagent 1: 1 was dissolved in PBS (50 mM, pH 7.4) with different concentrations (10, 20, 30, 40, 50, 60, 80 or 100 μM). The solution was monitored by UV-Vis spectrophotometry from 250 to 600 nm. The concentration-dependent absorbance at 275 nm was linear, which implied the good water-solubility of **1** at the test concentration (Fig. S1).

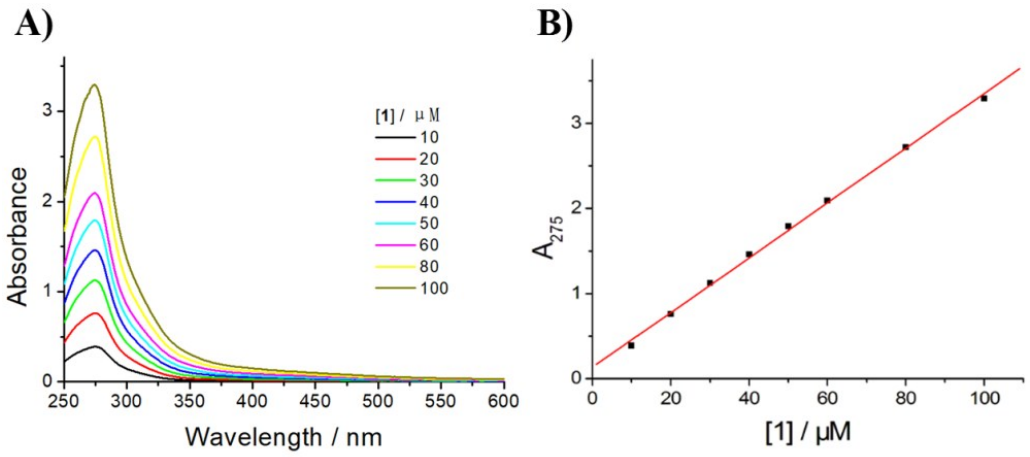


Fig. S1 (A) The absorption at 275 nm of **1** of different concentrations in PBS buffer (50 mM, pH 7.4). (B) Linear regression curve fitted according to the absorbance (R = 0.998).

Reaction of 1 with Tyr-containing molecule: To 2 mL phosphate buffer (20 mM, pH 7.4, containing 50% CH_3CN) was added 2 μL **1** (20 mM stock solution in DMSO) and 4 μLFmoc-Tyr-OH (100 mM stock solution in DMSO). The progress of the reaction was monitored by UV-Vis spectrophotometry from 250 to 600 nm (Fig. S2). For kinetics test, 20 μM **1** and Fmoc-Tyr-OH (400 μM) were reacted in phosphate buffer and the absorption at 350 nm was recorded per second.

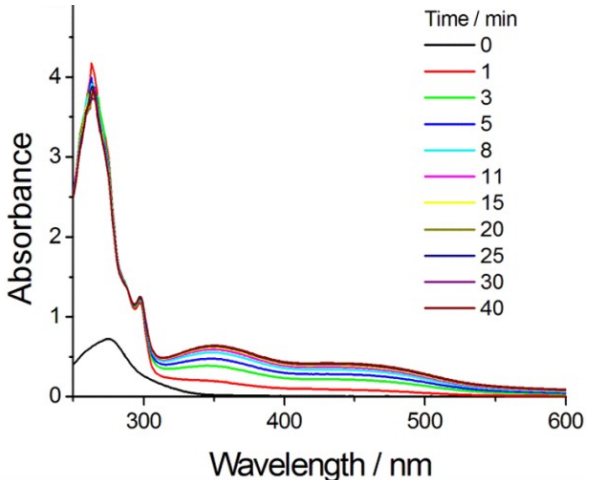


Fig. S2 Time-dependent UV-Vis absorbance spectra of **1** (20 μM) upon treatment with Fmoc-Tyr-OH(200 μM) in phosphate buffer (50 mM, pH 7.4, containing 50% CH_3CN) at room temperature. The reaction time is indicated in the inset.

4. TMV and TMV CP purification

Tobacco leaves infected by *tobacco mosaic virus* were homogenized with the pestle in the precooled mortar on ice and dissolved in 25 mL 0.2 M phosphate buffer (pH 7.0) and 250 μL *β*-mercaptoethanol (1%, v/v). The tobacco lysates were collected by two-layer screen cloth to remove excess tobacco debris. The clear lysates were further transferred into a new sterilized tube and supplemented with 8% *n*-butyl alcohol, which was fully mixed for 15 min with magnetic stirrers to completely eliminate chlorophyll. The lysates without chlorophyll would get light yellow and was further supplemented with 1 g NaCl (4%, w/v) and 1 g PEG 6000 (4%, w/v). The mixture was stirred on ice to precipitate virus particles. After 6 hours of stirring, the precipitate was collected by centrifugation at 10,000×g for 20 min and fully dissolved with 5 mL 0.01 M phosphate buffer (pH 7.0) for 2 hours. The dissolved suspension was centrifugated at 10,000×g for 20 minutes to remove the precipitate. The pelleted supernatant was then stirred again with 0.2 g NaCl (4%, w/v) and 0.2 g PEG6000 (4%, w/v) overnight on ice for precipitation. The precipitate was dissolved again with 2 mL 0.01 M phosphate buffer (pH 7.0) on ice for 6 hours. After centrifugation at 10,000×g for 5 min, the supernatant was the final virus suspension, whose purity can be verified by the specific ratio of A_{280}/A_{260} (0.84) and A_{260}/A_{248} (1.09). The virus concentration

was determined according to the formula $C_{(mg/mL)}=A_{260}/3.1$. The purified virus suspension was stored at 4 °C for hydrogel preparation.

The TMV capsid protein (TMV CP) was purified according to the following procedure. 1 mL TMV virus suspension (20 mg/mL) was diluted to 5 mg/mL with 0.01 M phosphate buffer (pH 7.0). The dilution was then supplemented with two volumes of glacial acetic acid and fully shaken at 4 °C for 2 h. After full mixing, an equal volume of sterile water was added and transferred into a dialysis bag to precipitate TMV CP. After three to five times of changing water, TMV CP precipitate accumulated up to the maximum. The TMV CP precipitate was obtained by spinning down at 12,000 rpm for 30 min and dissolved with 50 μL 0.1 M phosphate buffer (pH 7.0). The concentration was determined according to the formula $C_{(mg/mL)}=A_{282}/1.27$. The purified TMV CP was stored at 4 °C for protein crosslinking.

5. TMV capsid protein crosslinking with 1

The TMV capsid protein (TMV CP) crosslinking reaction was performed with 1 mg/mL TMV CP with various concentrations of **1**. Linker **1** was freshly dissolved in DMSO as 100 mM stock solution. The stock solution was then diluted to a gradient concentration (25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.2, mM). The reaction was performed by mixing 90 μL TMV CP (1 μg/μL) and 10 μL linker **1** dilutions (25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.2, mM). The reaction was incubated at 37 ℃ for 30 min. After reaction, samples were mixed with 2×SDS loading buffer and then boiled at 95 ℃ for 5 min. The boiled samples were then cooled down to ambient temperature and spinned down at 12,000 rpm for 5 min. 10 μL supernatant was loaded into 12% SDS-PAGE for gel electrophoresis. The PAGE gel was stained with Coomassie blue R250 and imaged with Quantity One software (Bio-Rad).

To identify how many tyrosines in TMV CP proteins could react with **1**, we performed the reaction by mixing 99 μL TMV CP (1 μg/μL) and 1 μL linker **1** (62.5 mM). The reaction was incubated at 37 ℃ for 30 min and the reaction mixture was

then transferred into 0.5 mL 3K centrifugal filters to remove excess linker **1** and washed by 5 times with water. The treated samples were then analyzed with MALDI-TOF.

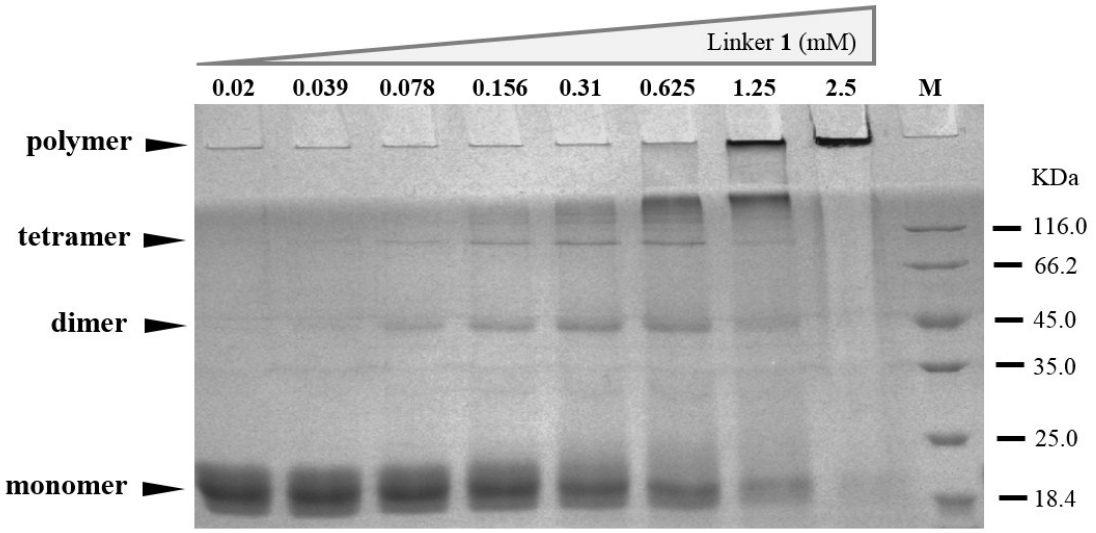


Fig. S3 SDS-PAGE of TMV CP (1 mg/mL) crosslinking with various concentrations of linker **1**. The reaction was performed in 0.1 M phosphate buffer (PB, pH 7.0) at 37 °C for 30 min.

6. TMV virus crosslinking with 1

The TMV virus crosslinking reaction was performed with 1 mg/mL TMV suspension with various concentrations of **1**. Linker **1** was freshly dissolved in DMSO as 100 mM stock solution. The stock solution was then diluted to a gradient concentration (50, 25, 12.5, 6.25, 3.12, 1.56, mM). The reaction was performed by mixing 90 μL TMV solution (1 μg/μL) and 10 μL linker **1** dilutions (50, 25, 12.5, 6.25, 3.12, 1.56, mM). The reaction was incubated at 37 ℃ for 30 min. After reaction, the virus mixture was then transferred into 0.5 mL 3K centrifugal filters to remove excess linker **1** and washed by 5 times with water. The washed samples were then mixed with 2×SDS loading buffer and then boiled at 95 ℃ for 5 min. The boiled samples were then cooled down to ambient temperature and centrifugated at 12,000 rpm for 5 min. 10 μL supernatant was loaded into 12% SDS-PAGE for gel electrophoresis. The PAGE gel was stained with Coomassie blue R250 and imaged with Quantity One software (Bio-Rad).

7. TEM and SEM characterization

A 125 μL TMV solution (20 mg/mL) was mixed with a 375 μL 0.1 M phosphate buffer (PB, pH 7.0) containing 50 μL **1** stock solution (0.39 mM, 1.56 mM or 6.25 mM). After reaction, a drop of the reaction solution was dropped onto a carbon-coated copper grid. After 2 min, reaction mixture was removed with the filter paper. Then, the copper grid was immersed with a 2% uranyl acetate solution for 2 min. The sample was imaged by the JEM-2100F transmission electron microscope operated at 200 kV and equipped with an Oxford EDX analyzer. The images were processed with the Image J software. For SEM analysis, the hydrogel was made by 5 mg/mL TMV and 2.5 mM **1** at 37℃ for 30 min. Then, the hydrogel was firstly cold-dried and loaded onto the sample vessel. The sample was visualized using a HITACHI S-4700 Field Emission SEM.

8. Optimization of TMV crosslinking with 1 for hydrogel preparation

TMV hydrogel was made by gently mixing of **1** solution and TMV solution in a glass vial. To optimize conditions for hydrogel preparation, we tried different TMV concentrations (0~5 mg/mL), **1** concentrations (0~10 mM), reaction temperatures (4℃, 25℃, 37℃, 50℃) and pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5). The reaction time was kept at 30 min so that TMV solution will get solidified with darker color (Fig. S4-S7).

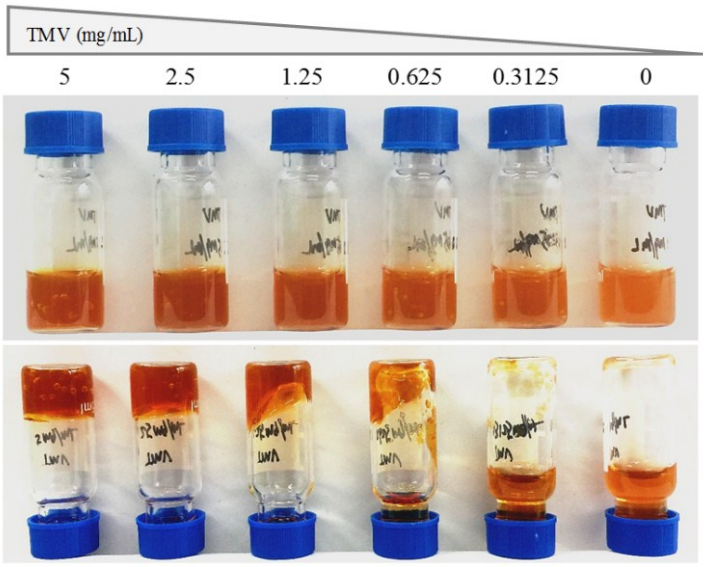


Fig. S4 Effects of TMV concentration on virus gelation in 0.1 M phosphate buffer (PB, pH 7.0) at 37℃. The concentration of crosslinker **1** was 10 mM.

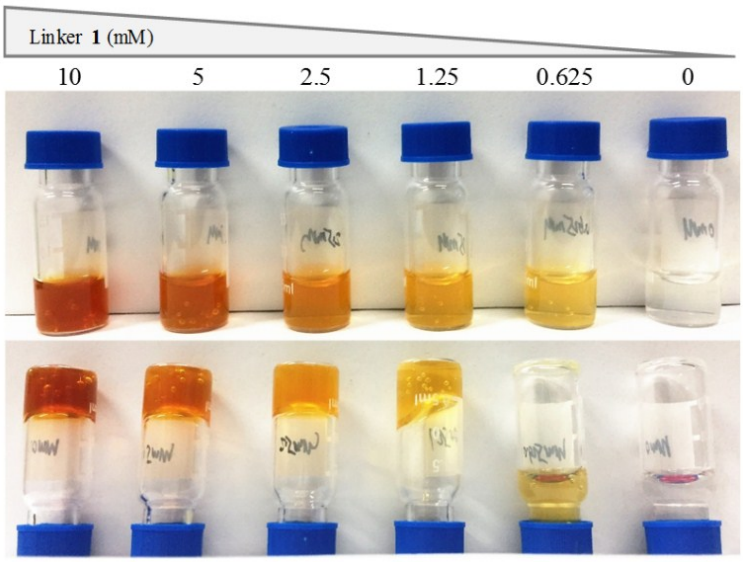


Fig. S5 Effects of linker **1** concentration on virus gelation in 0.1 M phosphate buffer (PB, pH 7.0) at 37℃. The concentration of TMV was 2.5 mg/mL.

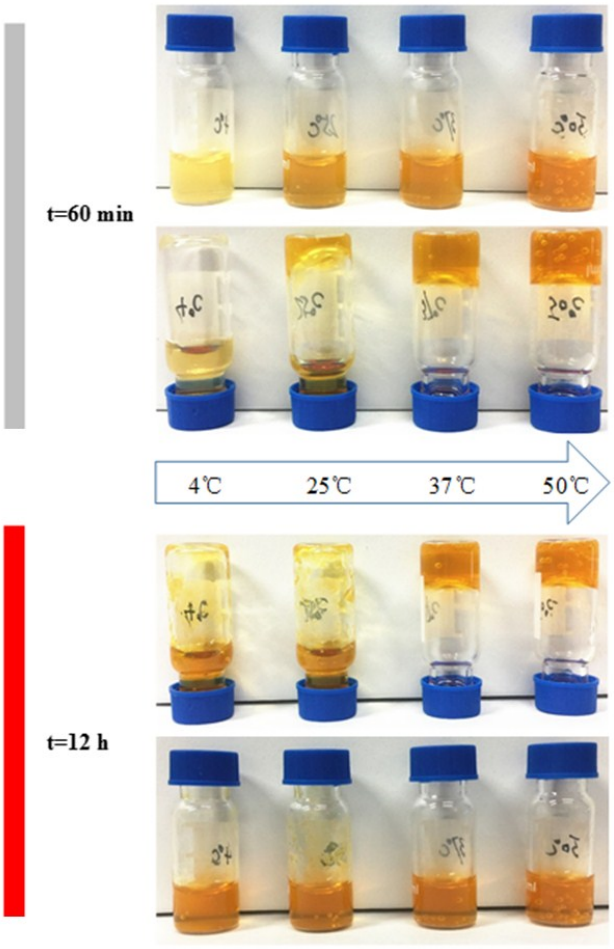


Fig. S6 Effects of incubation temperature on virus gelation in 0.1 M phosphate buffer (PB, pH 7.0). The concentration of crosslinker **1** and TMV was 2.5 mM and 2.5 mg/mL, respectively.

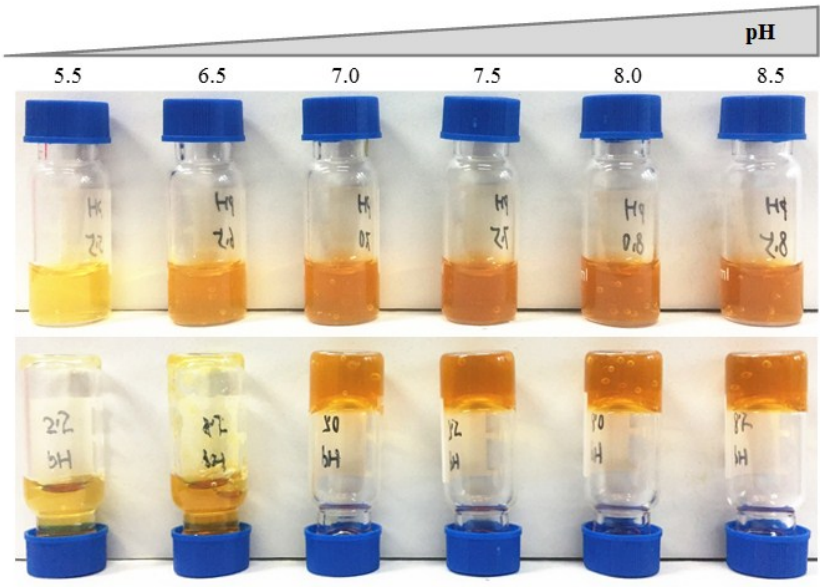


Fig. S7 Effects of pH on virus gelation in 0.1 M phosphate buffer (PB, pH 7.0) at 37℃. The concentration of crosslinker and TMV was 2.5 mM and 2.5 mg/mL, respectively.

9. Degradation of virus-based hydrogel

To understand the TMV hydrogel formation, we used various concentrations of SDS (0.5% and 5%, w/v) to damage the TMV structure before crosslinking with **1**. The working concentration of crosslinker **1** and TMV were 10 mM and 2.5 mg/mL, respectively. Gelation changes could be observed after incubation at 37 ℃ for 30 min. On the other hand, we used **1** to crosslink pure proteins like caseins and lysozymes to detect whether protein gelation occurred. The concentration of **1** and protein were 10 mM and 2.5 mg/mL, respectively. The reaction was performed at 37 ℃ for 30 min.

To degrade virus hydrogel with chemical agents, virus gel degradation was performed by $Na_2S_2O_4$ at various concentrations in 0.1 M phosphate buffer (PB, pH 7.0) at 37 ℃ for different time. The concentration of linker **1** and TMV were 10 mM and 2.5 mg/mL, respectively. On the indicated time, the sample was slowly shaken up and down to scatter the gel. Meanwhile, TMV hydrogel from 2.5 mM **1** and 2.5 mg/mL TMV was made at respective 37 ℃ and 50 ℃ for 30 min to acquire different gel strength. These two types of TMV hydrogels were tested to detect the effect of gel strength on degradation time using different concentrations of $Na_2S_2O_4$ in 0.1 M phosphate buffer (PB, pH 7.0) at 37 ℃.

On the other hand, we dropped 50 μL gelation reaction mixture onto the glass slide and made it solidified as the virus film at 37 ℃ in 30 min. After hydrogel film was formed, 50 μL $Na_2S_2O_4$ at various concentrations (50 mM, 0.5 M, 1 M) was transferred onto the center of the film and further incubated at 37 ℃ for 30 min. The color change of virus hydrogel film could be observed to detect the effect of gel thickness on gel degradation speed of $Na_2S_2O_4$ (Fig. S8-S12).

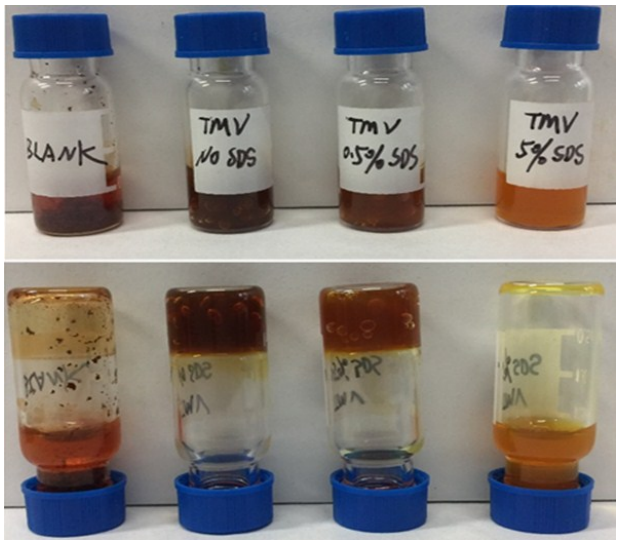


Fig. S8 Crosslinking of TMV disassembly by SDS. The concentration of crosslinker **1** and TMV was 10 mM and 2.5 mg/mL, respectively. The final SDS concentration was 0.5% (w/v) and 5% (w/v), respectively. Before crosslinking, 2.5 mg/mL TMV solution was firstly disassembled by SDS and incubated for 10 min. The TMV disassembly was then treated with crosslinker **1**.

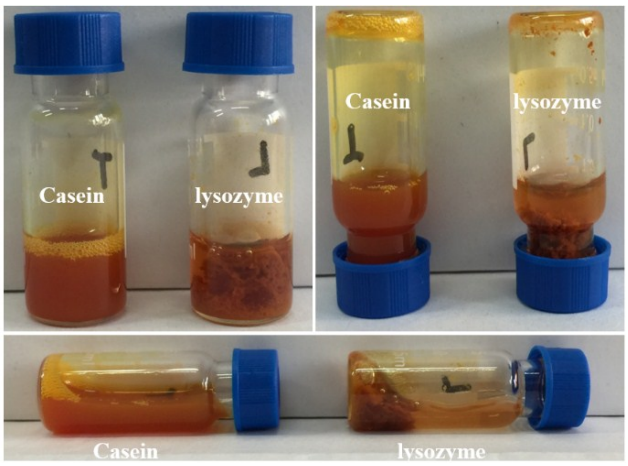


Fig. S9 Casein and lysozyme crosslinking in 0.1 M phosphate buffer (PB, pH 7.0) at 37 ℃. The concentration of crosslinker **1** and protein was 10 mM and 2.5 mg/mL, respectively.

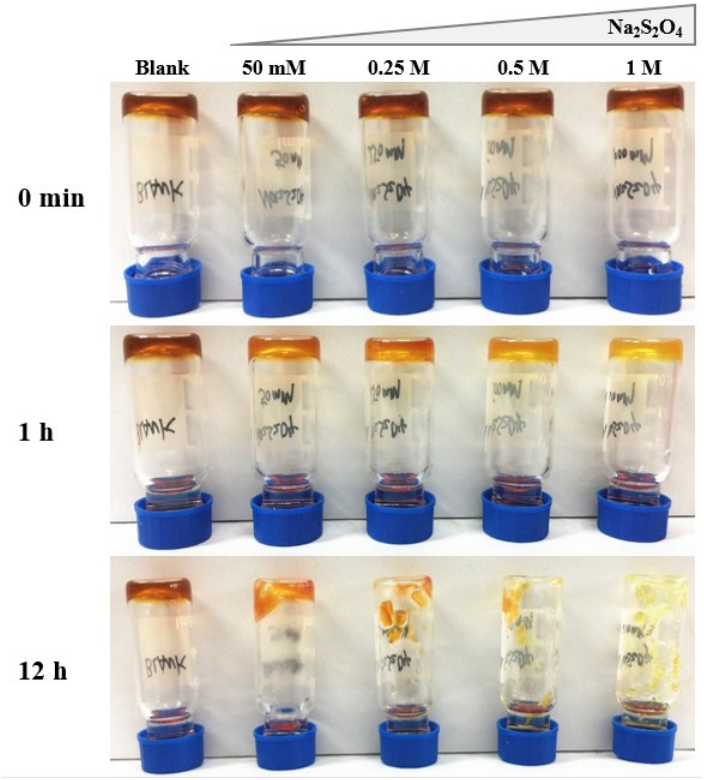


Fig. S10 Degradation of virus-based hydrogel by $Na_2S_2O_4$. The gel was prepared by 10 mM **1** and 2.5 mg/mL TMV. The degradation reaction was performed in phosphate buffer (PB, pH 7.0) at 37 °C for 1 h and 12 h with various concentrations of $Na_2S_2O_4$. After this, sample was gently shaken up and down to scatter the gel.

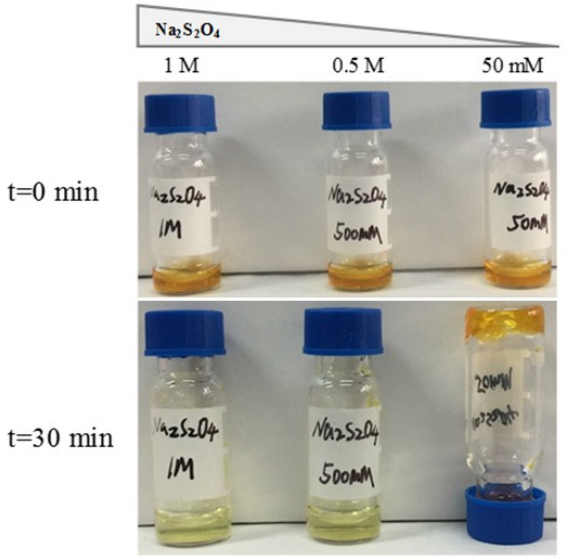


Fig. S11 Chemical degradation of TMV gel made at 37 ℃ by $Na_2S_2O_4$. Virus gel was constructed from 2.5 mM linker **1** and 2.5 mg/mL TMV at 37 ℃. The virus gel was then degraded by $Na_2S_2O_4$ at various concentrations in 0.1 M phosphate buffer (PB, pH 7.0) at 37 ℃.

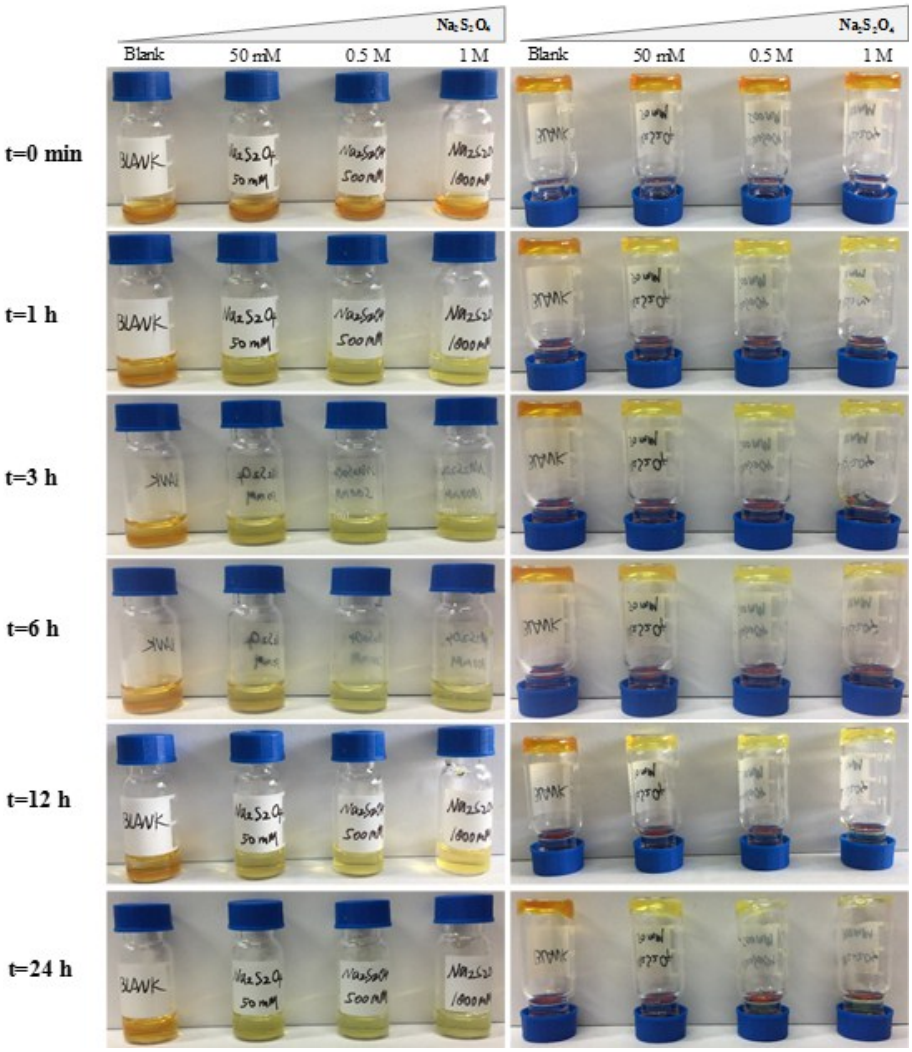


Fig. S12 Chemical degradation of TMV gel made at 50 ℃ by $Na_2S_2O_4$. Virus gel was constructed from 2.5 mM linker **1** and 2.5 mg/mL TMV at 50 ℃. The virus gel was then degraded by $Na_2S_2O_4$ at various concentrations in 0.1 M phosphate buffer (PB, pH 7.0) at 37 ℃.

10. Preparation of drug-loading hydrogel and its in vitro drug release

To load the drug camptothecin (CPT) into TMV hydrogel, we firstly dissolved CPT in DMSO as the stock solution C (2 mM). Before CPT addition, 50 μL **1** (100 mM solution in DMSO) was then mixed gently with 300 μL phosphate buffer (PB, 100 mM, pH 7.0) to form solution A. The TMV solution (20 mg/mL) was diluted into 125 μL as solution B. 350 μL solution A was firstly mixed with 25 μL solution C and then added into 125 μL TMV solution (20 mg/mL). After incubation for 30 minutes at 37 ℃, TMV-CPT hydrogel containing 100 μM CPT was formed.

In order to detect whether TMV-CPT hydrogel could release CPT, we placed TMV hydrogel into dialysis bag, which was immersed into a tube containing 50 mL 100 mM phosphate buffer (PB, pH 7.5). At the indicated time, 2 mL dialysis liquid was transferred into the quartz cell to detect the emission fluorescence intensity at 450 nm (The excitation wavelength were 370 nm). In the same way, the standard curve was established according to the linear correlation relationship between CPT concentration and fluorescence intensity. The release efficiency of CPT drug was calculated according to the linear regression equation ($y = 340.99x + 6.4197$, $R^2 = 0.9978$), where y denotes the fluorescence absorbance at 370 nm and x indicates the CPT concentration (μM). The equation of release efficiency for CPT drug is $C_{(\text{Intensity of dialysis liquid})}/2*100\%$ (Fig. S13-S16).

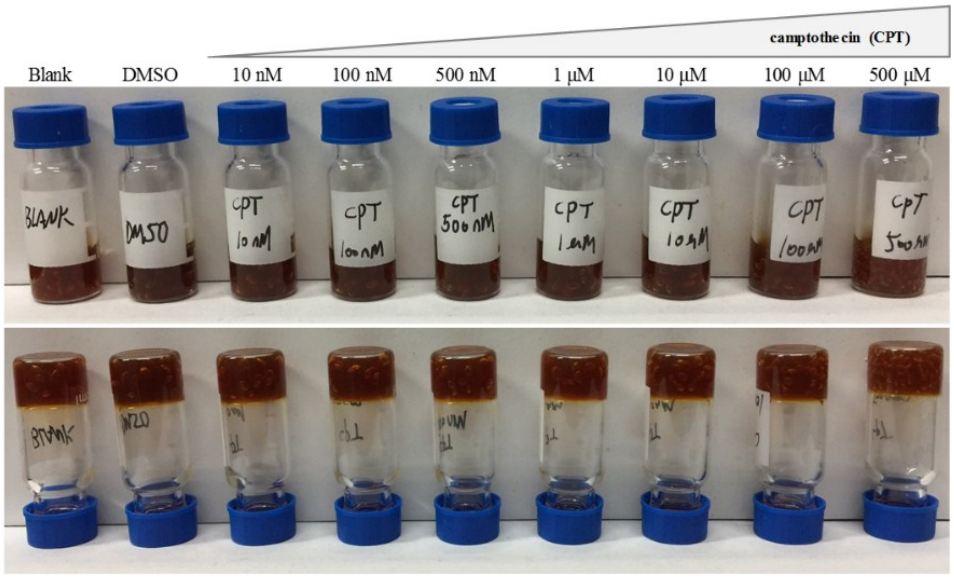


Fig. S13 Virus-based hydrogel loaded with camptothecin (CPT) at various concentrations. The concentration of linker **1** and TMV was 10 mM and 2.5 mg/mL, respectively. CPT was dissolved in DMSO and mixed with 0.1 M phosphate buffer (pH 7.0) containing **1**. The reaction was performed at 37 ℃ for 30 min.

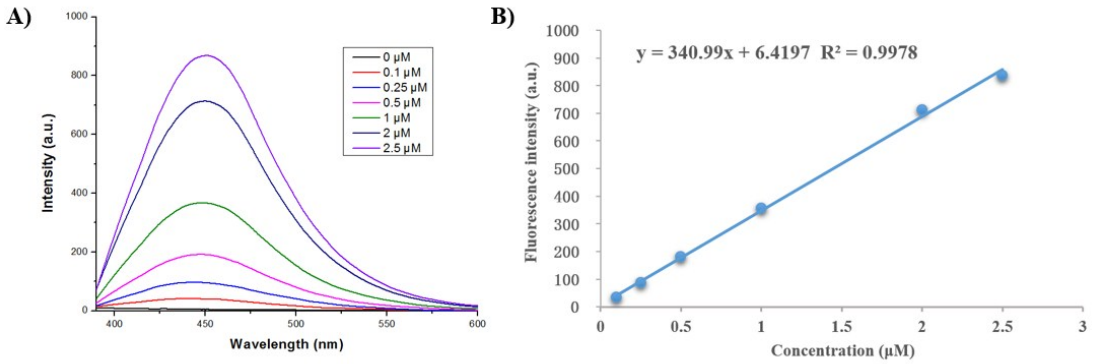


Fig. S14 Standard curve of CPT concentration and fluorescence intensity. (A) CPT was diluted into various concentrations with 0.1 M PB buffer (pH 7.5) containing 5% DMSO. The excitation wavelength was 370 nm. (B) Equation of linear regression was established according to the relationship between CPT concentration and fluorescence intensity.

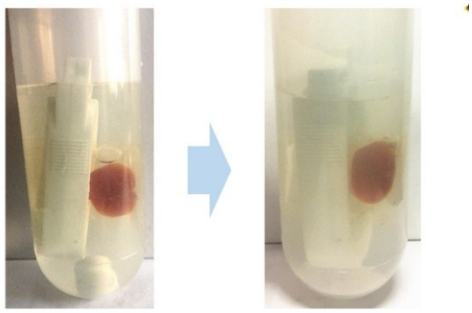


Fig. S15 Images of CPT releasing of TMV hydrogel containing CPT using dialysis bag in a tube.

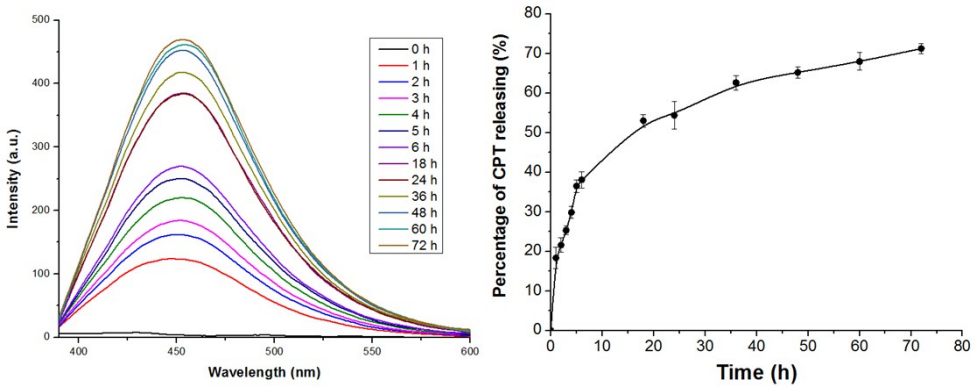


Fig. S16 Time-dependent CPT releasing from TMV hydrogels containing 100 μM CPT. TMV hydrogels containing 100 μM CPT was sealed into the dialysis bag immersed into a tube containing 50 mL 100 mM phosphate buffer (PB, pH 7.5). Left figure, 2 mL dialysis liquid was transferred into the quartz cell to detect the time-dependent fluorescence intensity at 450 nm (excitation at 370 nm). Right figure, time-dependent CPT releasing percentage from the drug-loaded hydrogel.

11. Biosafety evaluation of the hydrogel on tobacco plants

To study the biosafety of the hydrogel on tobacco plants, two ways were used to inoculate TMV hydrogels on tobacco leaves. The first method was to spray TMV hydrogels onto the surface of tobacco leaves. All the tobacco leaves were sprayed with the 500 μL reaction mixture (10 mM **1**, 2.5 mg/mL TMV) and waited to be solidified at 37℃ for 30 min. The second method was to smear the TMV-based hydrogels onto the surface of tobacco leaves. After gelation on tobacco leaves, these treated tobacco plants were cultured at 25℃ for several days. 50 μg/mL TMV inoculation using carborundum was set as the positive control. After 5 days, the virus circle on the surface of tobacco leaves could be observed when virus infected the leaves (Fig. S17, S18).

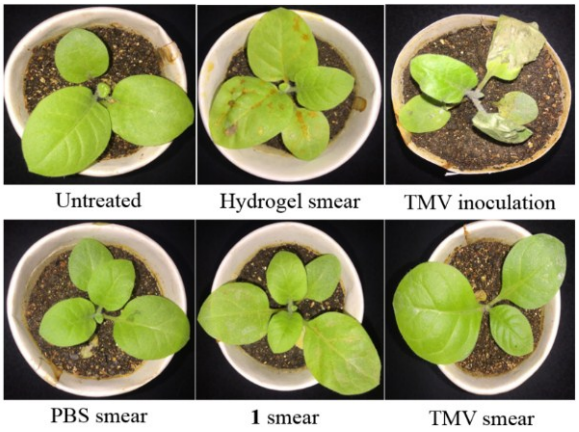


Fig. S17 Effects of TMV hydrogel smearing on tobacco leaves. Untreated tobacco plant was set as the blank control. Negative control included PBS-treatment and **1**-treatment tobacco plants. The positive control was treated with 50 μg/mL TMV using carborundum. 2.5 mg/mL TMV suspension and 2.5 mg/mL TMV hydrogel were directly smeared around the leaves. Images of tobacco plants were taken after five days.

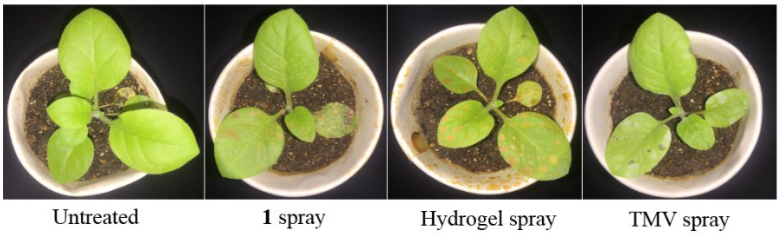
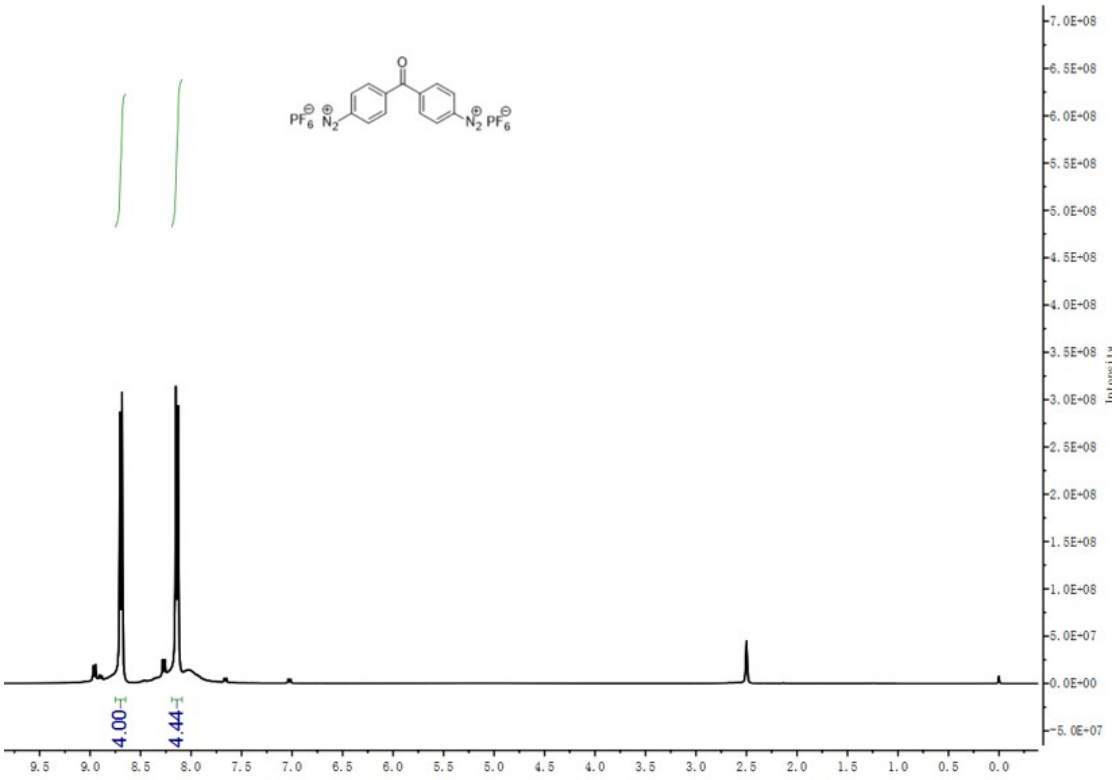


Fig. S18 Effects of TMV hydrogel spraying on tobacco leaves. Untreated tobacco plant was set as the blank control and tobacco plant treated with **1** was set as the negative control. 2.5 mg/mL TMV suspension and 2.5 mg/mL TMV hydrogel were directly sprayed onto the leaves, respectively. Images of tobacco plants were taken after five days.

12. Supporting figures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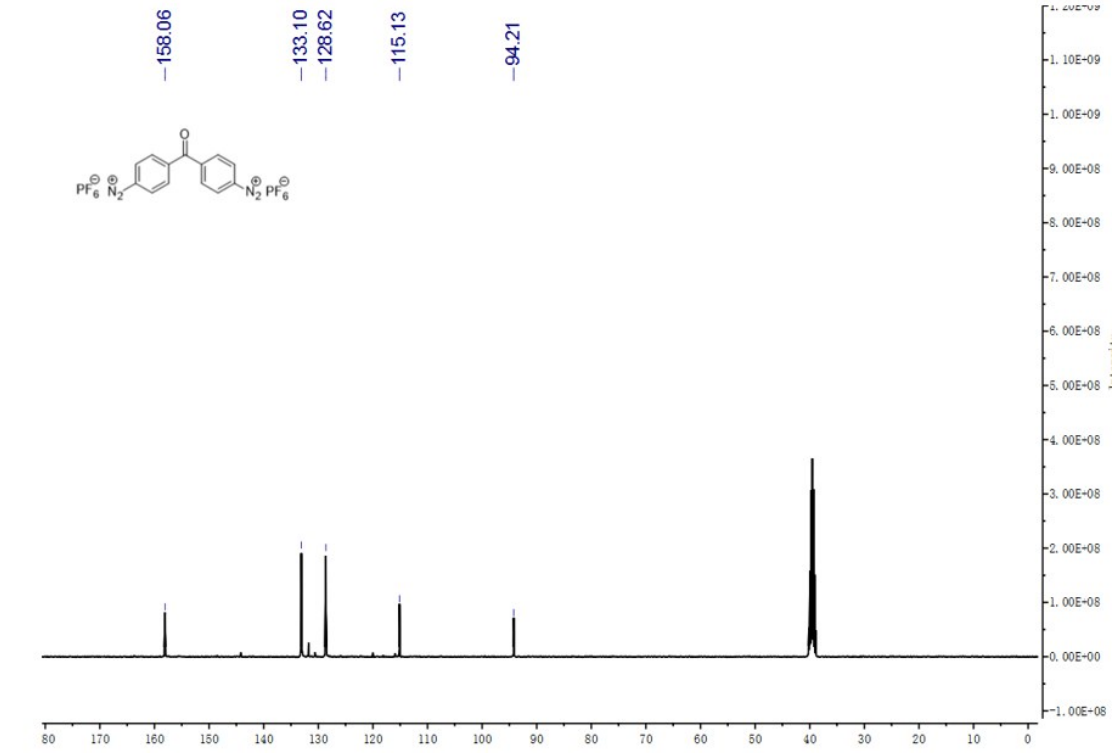
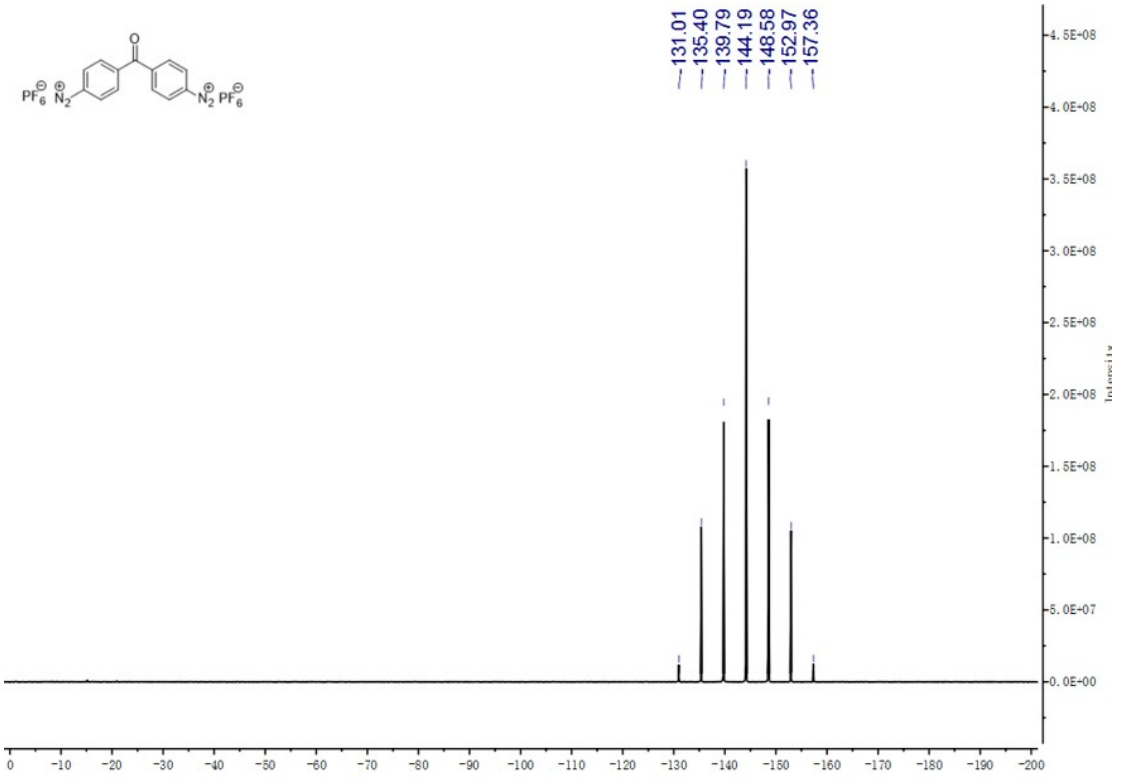
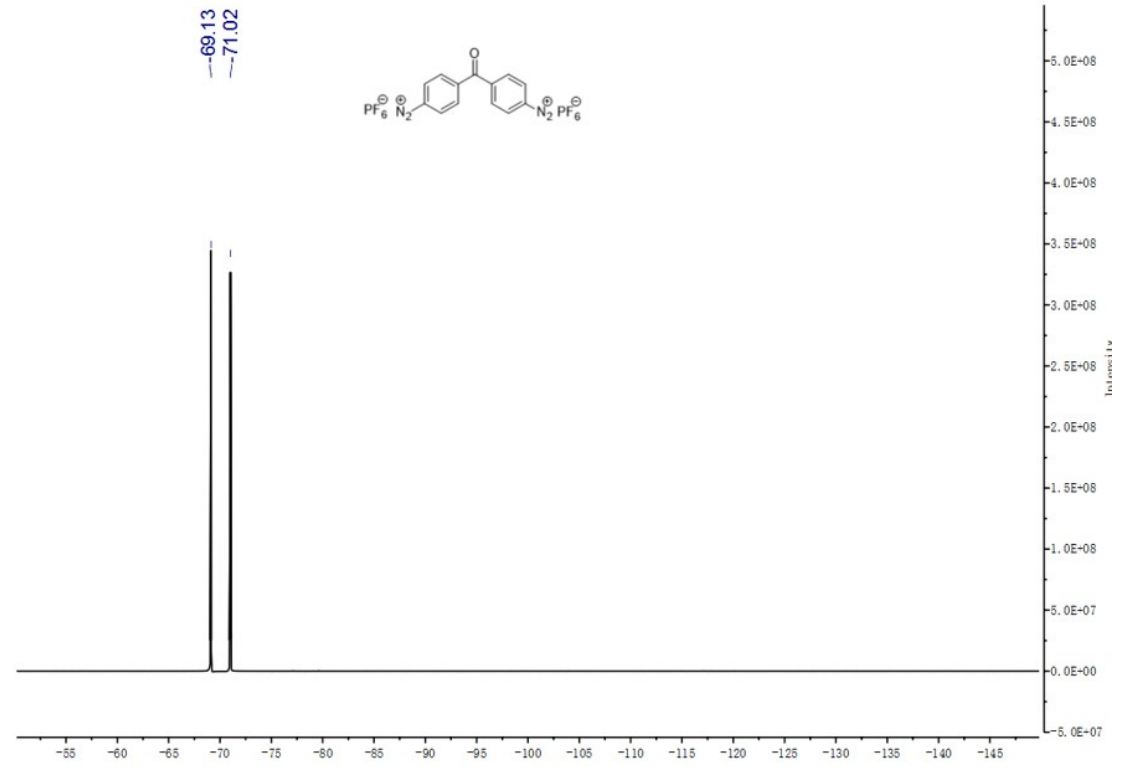
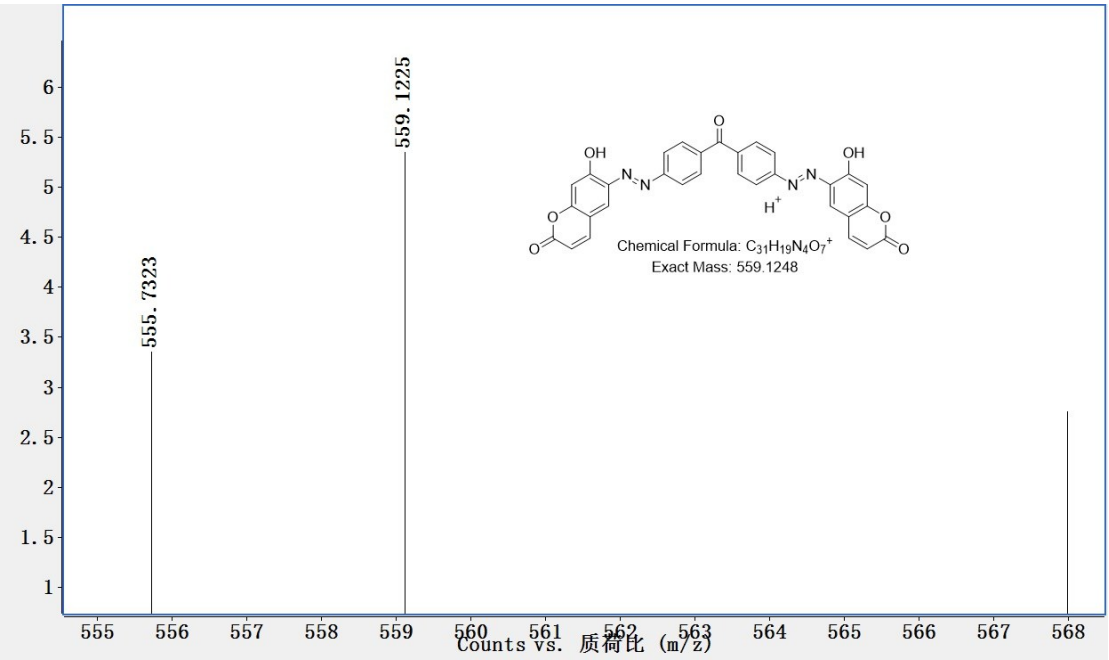

158.06
133.10
128.62
115.13
94.21

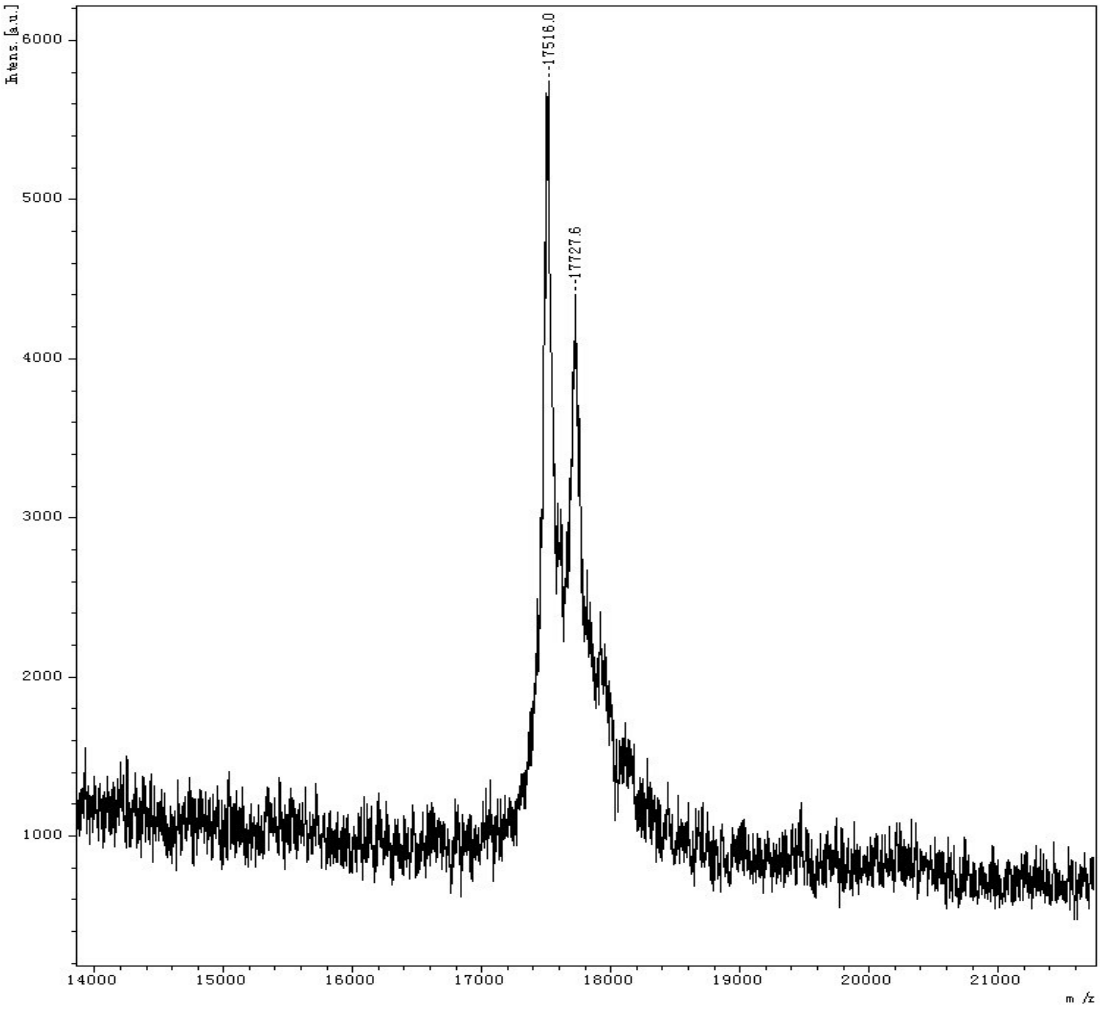




NMR spectra of **1** (^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR).



High resolution mass spectrum of **1** after reaction with 7-hydroxycoumarin.



Mass spectrum of TMV capsid protein reacting with linker **1**.