Electronic Supporting Information for

Synthesis of amphiphilic polysuccinimide star copolymers for responsive delivery in plants

Mingsheng Chen,[a,b] Shaun P. Jensen,[c] Megan R. Hill,[a] Gloria Moore,[c] Zhenli He,[b*]
Brent S. Sumerlin[a*]

[a] George & Josephine Butler Polymer Research Laboratory, Center for Macromolecular Science & Engineering, Department of Chemistry, University of Florida, Gainesville, Florida, USA,

E-mail: sumerlin@chem.ufl.edu;

[b] Indian River Research and Education Center, University of Florida, Fort Pierce, Florida, USA,

E-mail:zhe@ufl.edu;

[c] Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, FL, USA

EXPERIMENTAL METHODS

Materials. L-Aspartic acid 4-benzyl ester and 1-naphthaleneacetic acid were purchased from Alfa Aesar. 2,4,6-triaminopyrimidine (97%) was purchased from Acros, and triphosgene was purchased from TCI America. Hydrogen bromide (33 wt% solution in glacial acetic acid) was obtained from Sigma-Adrich. Tetrahydrofuran (THF), *n*-hexane, dioxane, and ethyl ether were treated with calcium hydride and distilled prior to use. The benzyl adenine (BA), Murashige and Skoog basal salt mixture (MS salt), claforan (cefotaxime), myo-inositol, and plant cell viability assay kit were obtained from Sigma-Aldrich and used as received unless otherwise noted. All other materials were purchased from VWR or Fisher unless otherwise noted.

Methods. ^{1}H NMR spectroscopy was performed using a Varian Mercury spectrometer (300 or 500 MHz) with deuterated dimethyl sulfoxide (DMSO-d_6) or D_2O as the solvents. Molecular weight and molecular weight distribution (M_w/M_n) were determined by size exclusion chromatography (SEC) in *N*,*N*-dimethylacetamide (DMAc) with 50 mM LiCl at 50 °C and a flow rate of 1.0 mL min^{-1} (Agilent isocratic pump, degasser, and autosampler, columns: PLgel 5 μm guard + two ViscoGel I-series G3078 mixed bed columns: molecular weight range 0−20 × 10^3 and 0−100 × 10^4 g mol−1). $M_{n,SEC}$ and $Đ$ were determined by conventional PS calibration. IR spectra were collected using a Bruker Vertex 80v with a Pike GladiATR stage using the Opus 6.5 software package. Transmission electron microscopy (TEM) was recorded with a Hitachi H7000 microscope operating at 100 kV. A freshly glow discharged (Pelco easiGlow™, Ted Pella, Inc.) A Formvar coated 400-mesh nickel grid was placed on a 0.5 mg/mL drop of solution. The grid was dried at room temperature before investigation. Dynamic light scattering (DLS) analysis was conducted at 25 ^{o}C on a Zetasizer Nano-ZS (Malvern).

Synthesis of *N*-carboxyanhydride of *β*-benzyl L-aspartate. The synthesis of the *N*-carboxyanhydride of β-benzyl L-aspartate (Asp-NCA) was conducted as follows. A suspension of L-aspartic acid 4-benzyl ester (7.0 g, 31 mmol) in THF (200 mL) was heated to 40 °C in an argon atmosphere, and triphosgene (4.5 g, 15 mmol) was added. When the solution became transparent, the mixture was precipitated in anhydrous hexane. The crude product was further purified by recrystallization from a mixture of anhydrous *n*-hexane/THF (×3), and the remaining solvents were removed by vacuum at room temperature. Yield, 82%. ^{1}H NMR (DMSO, 300 MHz): δ_H (ppm) = 2.8-3.2 (m, -C***H***$_2$CHN), 4.69 (s, -C***H***N), 5.12 (s, C***H***$_2$Ar), 7.37 (s, Ar-***H***), 9.0 (s, N***H***).

Synthesis of poly(benzyl-L-ASP). 2,4,6-Triaminopyrimidine (132 mg, 1.02 mmol) was dissolved in anhydrous dioxane (150 mL) and stirred for 30 min. Asp-NCA (7.9 g, 32 mmol) dissolved in 50 mL anhydrous dioxane was added to the TAPM solution. The reaction mixture was stirred for 36 h at 30 ± 5 °C and subsequently precipitated in diethyl ether to obtain poly(benzyl-L-ASP) (PBLA). M_n= 2.6×10^4 g/mol, M_w/M_n=1.2. Yield, 90%. ^{1}H NMR (DMSO, 300 MHz): δ_H (ppm) = 2.5-2.9 (CHC***H***$_2$C), 4.6 (C***H***NH), 4.75 (CC***H***C), 5.1 (ArC***H***$_2$), 7.3 (Ar***H***), 8.2 (N***H***, N***H***$_2$).

Deprotection of poly(benzyl-L-ASP). PBLA (2.0 g) was dissolved in trifluoroacetic acid (TFA, 20 mL), 8 equiv. HBr (33 wt% solution in glacial acetic acid) was added, and the mixture was stirred for 1-2 h. Diethyl ether was used to precipitate PASP, and the precipitate was dried under vacuum. Yield, 70%. ^{1}H NMR (DMSO, 300 MHz): δ_H (ppm) = 2.8 (CHC***H***$_2$C), 4.6 (C***H***CH$_2$C), 8.1 (N***H***).

Synthesis of PASP-*co*-PSI. Thionyl chloride (2.27 mL, 31.29 mmol or 4.55 mL, 62.58 mmol or 9.09 mL, 125.16 mmol) was dissolved in dichloromethane (DCM, 100 mL), and tPASP (1.2 g) was added separately. The solution was refluxed at 60 °C for 18 h. Rotary evaporation was used to remove the solvent. A mixture of DCM (100 mL) and pyridine (1.2 equiv.) was added and the resultant solution was refluxed at 60 °C for 18 h. The solvent was removed, and the product was dissolved in *N*,*N*-dimethylformamide (DMF) before being precipitated into diethyl ether to obtain (PASP$_{32}$-*co*-PSI$_{11}$)$_3$, (PASP$_{26}$-*co*-PSI$_{17}$)$_3$, and (PASP$_{17}$-*co*-PSI$_{26}$)$_3$,were obtained. Yield,

70%, 67% and 62%. ^{1}H NMR (DMSO, 300 MHz): δ_H (ppm) = 2.8 (CHC***H***$_2$C), 4.6 (NHC***H***CH$_2$), 5.36 (CC***H***CH$_2$C), 8.15 (N***H***).

Self-assembly and characterization of PASP-*co*-PSI nanoparticles. (PASP$_{32}$-*co*-PSI$_{11}$)$_3$, (PASP$_{26}$-*co*-PSI$_{17}$)$_3$, and (PASP$_{17}$-*co*-PSI$_{26}$)$_3$, were dissolved in DMSO (1 mL) separately and then dropped into DI water (10 mL) slowly under continuous stirring for 4 h. The PASP-*co*-PSI nanoparticle solutions were obtained for TEM and DLS measurement. The nanoparticle solutions were dropped onto nickel grids with air-drying at room temperature before investigation.

Encapsulation and release of 1-naphthaleneacetic acid from nanoparticles. For all copolymers, PASP-*co*-PSI (30 mg) and 1-naphthaleneacetic acid (NAA, 25 mg) were dissolved in DMSO (5 mL) with stirring, and then deionized (DI) water (15 mL) was added. After 5 h of continuous stirring, PASP-*co*-PSI, was assembled into NAA-loaded nanoparticles. The solution was transferred into a new dialysis bag (MWCO = 3500 D) and dialyzed against DI water for 24 h to remove free NAA. The nanoparticle solution was withdrawn and transferred into new dialysis bags. These bags were soaked in phosphate buffered saline (PBS) at different pH (pH 7.0, 8.5) in beakers with continuous stirring. At predetermined time intervals, 5 mL of the external solution was removed and replenished with an equal volume of fresh PBS for analysis. The amount of released and total NAA was measured by UV spectroscopy at wavelength of 282 nm with a standard calibration curve of NAA.

Hydrolysis of PASP-*co*-PSI. $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ was chosen as a model to research the hydrolysis of PASP-*co*-PSI. $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$, was dissolved at pH = 8.5 and stirred for 48 h. Afterwards, the resultant polymers was isolated by dialysis and lyophilization and subsequently characterized by NMR and FTIR spectroscopy. PASP was dissolved in $NaHCO_3$ solution and isolated by dialysis and lyophilization to obtain PASPA as a control spectrum for FTIR.

Preparation of germination medium. FM stock (1.87 g Na_2EDTA, 1.39 g $FeSO_4 \bullet 7H_2O$ into 500 mL DI water; 5.00 mL), myo-insitol (50.00 mg), MS salts (2.15 g), and sucrose (15.00 g) were added to a sterile beaker. A 10 N NaOH solution was added to bring the pH to 5.7, and the volume was adjusted to 1.00 L. Agar (7.00 g) was added to the medium and melted for 30 min to obtain the final germination medium.

Preparation of citrus seeds. Germination medium (12 mL) was added into sterile glass culture tubes. Healthy, viable citrus seeds from grapefruit (*Citrus paradise* Macf. cv Duncan) and sweet orange (*Citrus sinensis* (L.) Osb. cv Pineapple) were selected, and the seed coats were removed. The seed kernels were kept moist at all times. Seeds were placed in an autoclaved beaker equipped with a stir bar and stirred in 300 mL of the following solutions for the predetermined time intervals: 70% ethanol (2 min), 10% NaClO (10 min), and sterile DI water rinses 3 times (2 min).

Culture of citrus seeds. One seed was placed into each germination medium-filled culture tube and the tubes were placed in racks. The racks were wrapped with plastic wrap and doubly

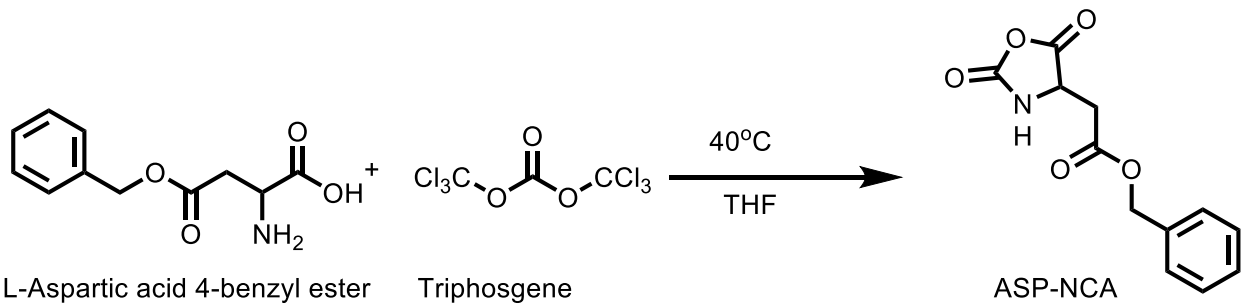
wrapped with aluminum foil in order to minimize light exposure. Finally, the enclosed test tube racks with seeds were put on the bottom shelf of a growth chamber for 35 days, when the etiolated seedlings were used for toxicity screening.

Preparation of the MSBC medium. GM stock (20 mg Glycine, 50 mg Nicotinic Acid, 100 mg Pyridoxine HCl, 100 mg Thiamine HCl into 500 mL; 10 mL), MS salts (4.30 g), sucrose (30.00 g), myo-inositol (100 mg), and BA (2 mg) were dissolved in DI water. After adjusting the pH to 5.7, additional DI water was added to bring the volume to 1.00 L. Agar (8.00 g) was added, and the solution was autoclaved for 25 min. After cooling, 1 mL of filtered and sterilized (500 g/L) Claforan stock was added to obtain 500 mg/L MSBC medium. The medium was transferred into sterilized culture dishes, and different concentrations of $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ were added before solidification, and poly(L-aspartic acid)$_{50}$-block-poly(L-leucine)$_{50}$-block-poly(L-lysine)$_{50}$ was used as control with a final concentration of 242 μg/mL.

Toxicity assessment by tissue culture. 1-2 cm segments of etiolated citrus plants were placed on MSBC medium-filled culture dish with their respective treatments. These dishes were then transferred into a growth chamber with 12 h of alternating light and dark for 2 weeks, which together with the media induce shoot regeneration and chlorophyll production. Living (green) and dead (white) segments were counted after predetermined times.

Toxicity assessment by dual color fluorescent staining system. Citrus leaves were used to evaluate toxicity according to the protocol of the Plant Cell Viability Assay Kit (Sigma) without

modification. Citrus leaves were incubated in 1 mg/mL $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ solution for 1 h at room temperature. Samples were rinsed (×3) and then incubated with staining solution for 2 min. Dead citrus leaves were used as a negative control.



Scheme S1. Synthesis of Asp-NCA.

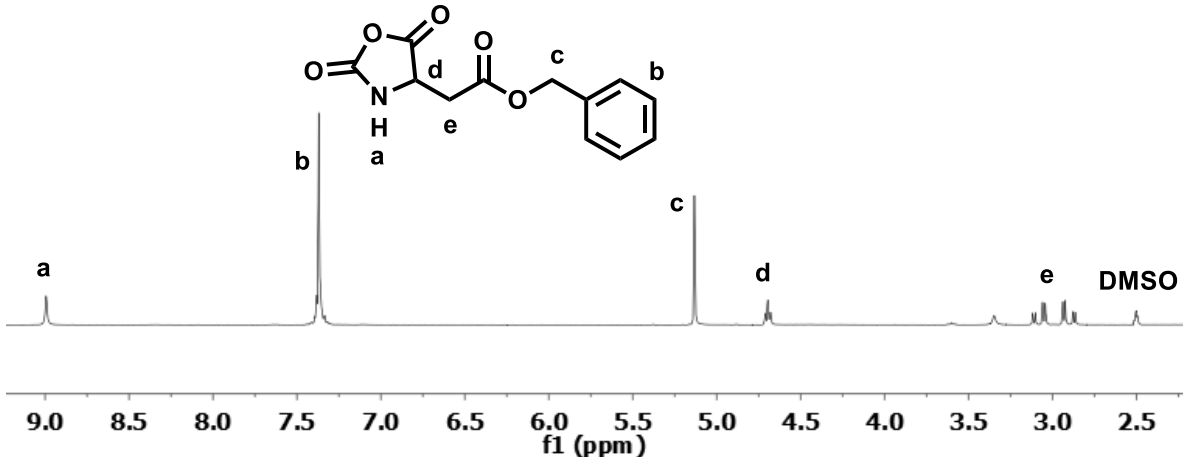


Figure S1. ^{1}H NMR spectrum of ASP-NCA.

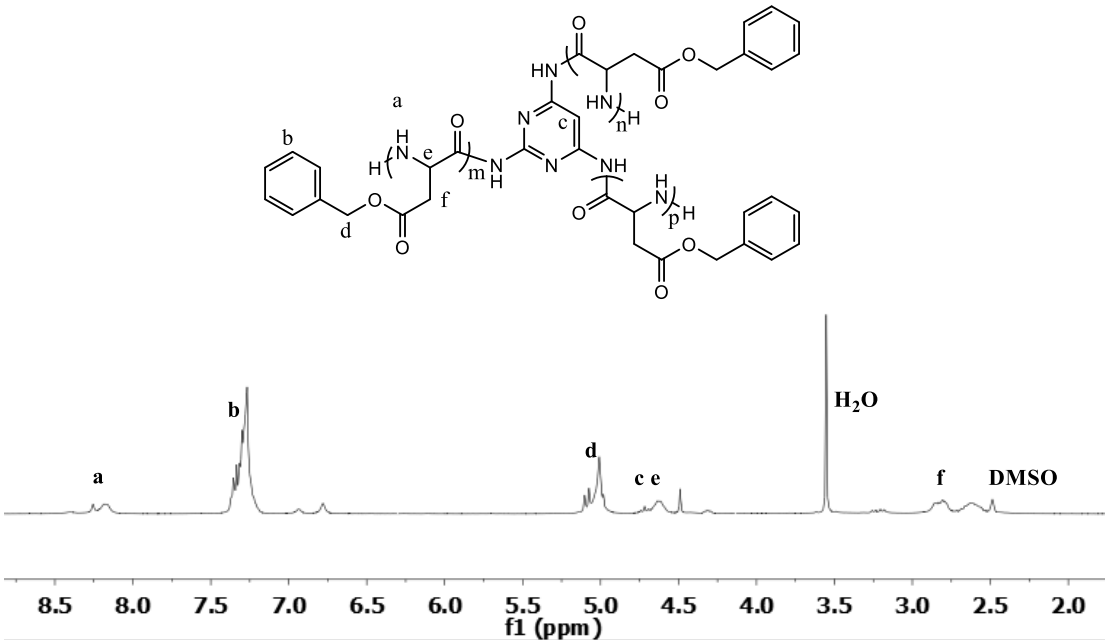


Figure S2. ^{1}H NMR spectrum of PBLA.

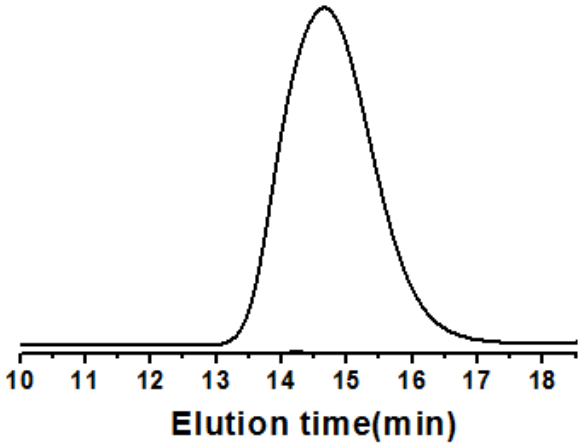


Figure S3. Gel permeation chromatography trace for the PBLA precursor (M_n= 26,600 g/mol, M_w/M_n = 1.2)

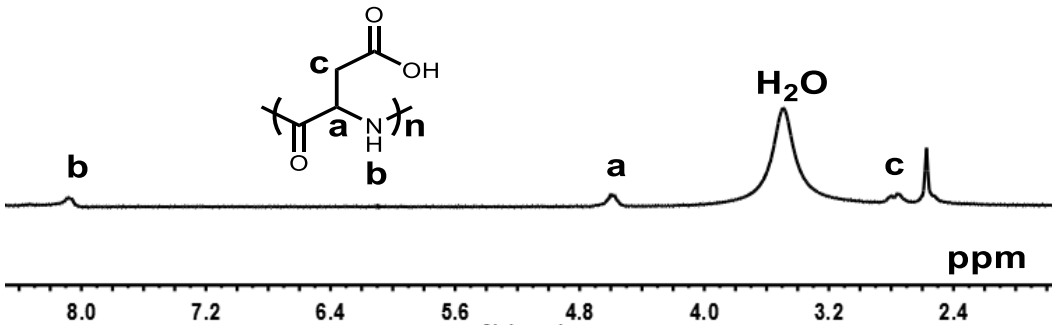


Figure S4. ^{1}H NMR spectrum of PASP.

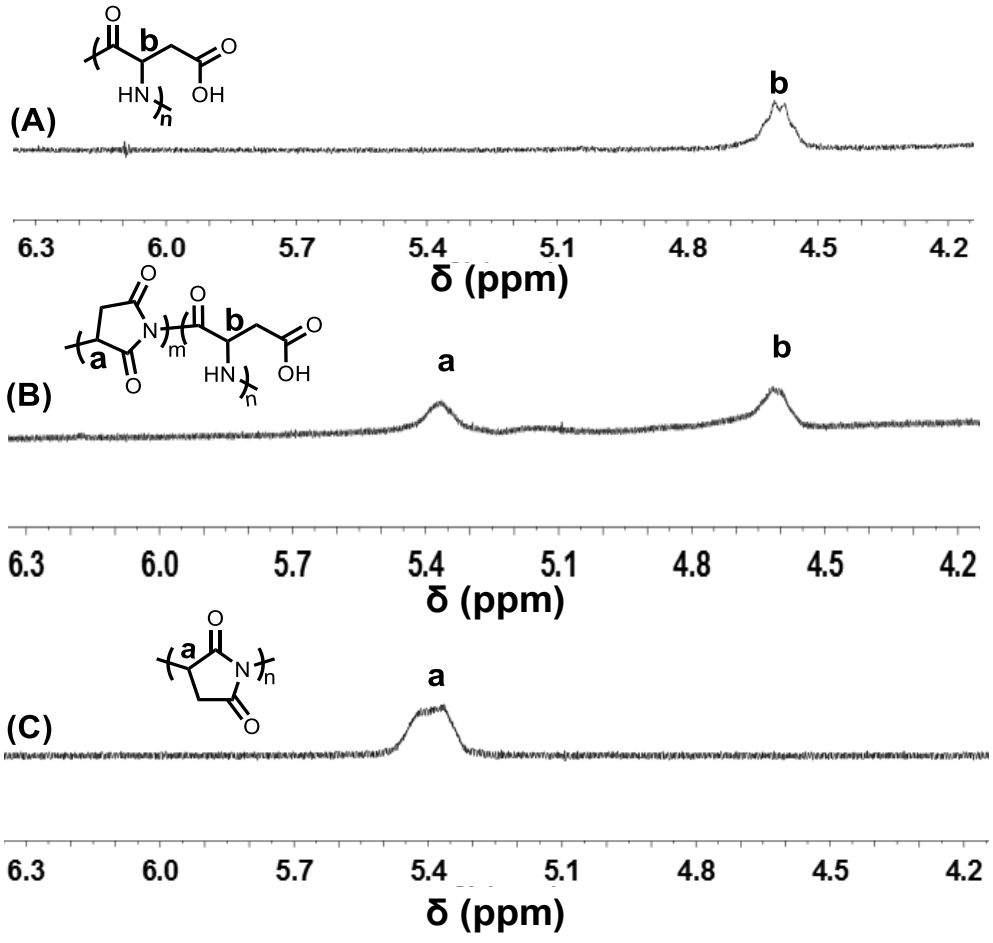


Fig. S5 ^{1}H NMR spectra of (A) poly(aspartic acid) (PASP), (B) (poly(aspartic acid)$_{26}$-*co*-polysuccinimide$_{17}$)$_3$ (PASP$_{26}$-*co*-PSI$_{17}$)$_3$ and (C) polysuccinimide (PSI).

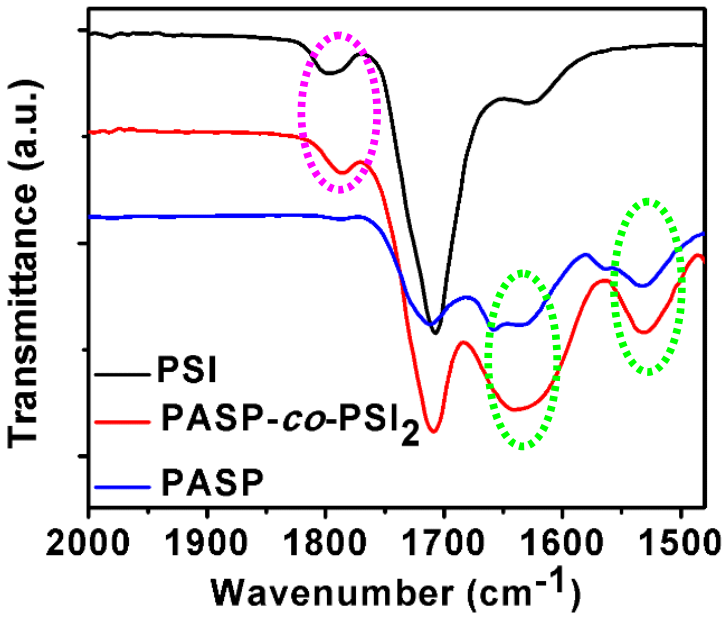


Fig. S6 FT-IR spectrum of polysuccinimide (PSI), $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ (PASP-*co*-PSI_2), and poly(aspartic acid) (PASP).

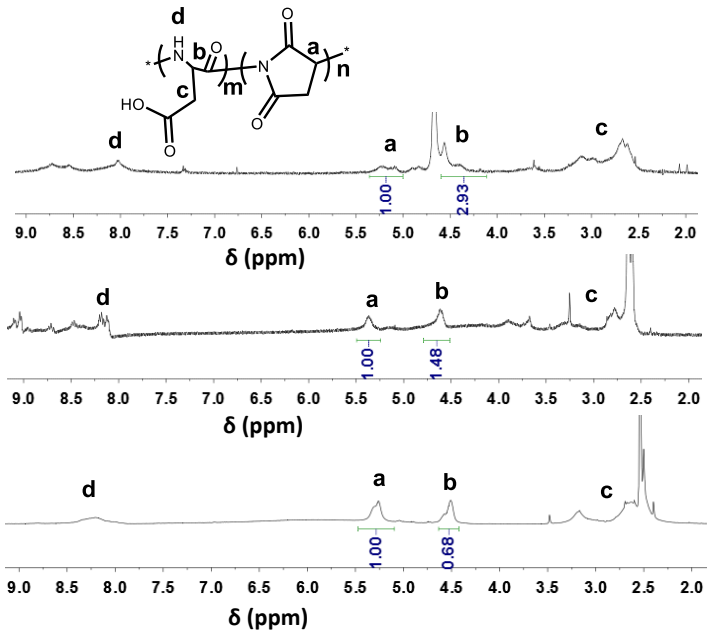


Figure S7. ^{1}H NMR spectrum of $(PASP_{32}\text{-}co\text{-}PSI_{11})_3$ (25% PSI), $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ (40% PSI), and $(PASP_{17}\text{-}co\text{-}PSI_{26})_3$ (60% PSI).

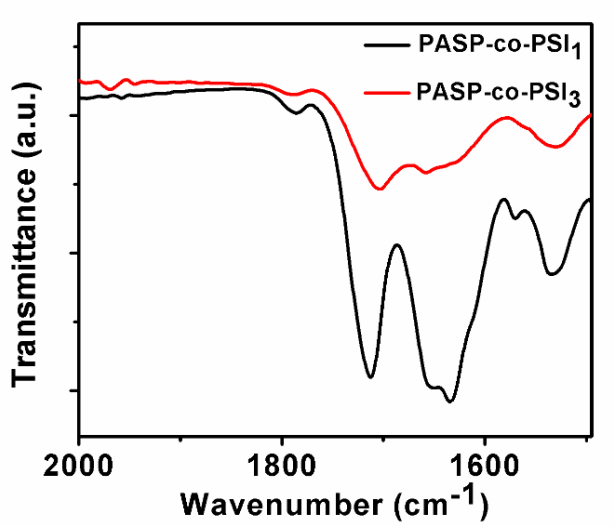


Figure S8. FT-IR spectrum of (a) PASP-*co*-PSI_1 $(PASP_{32}$-*co*-$PSI_{11})_3$ and PASP-*co*-PSI_3 $(PASP_{26}$-*co*-$PSI_{17})_3$.

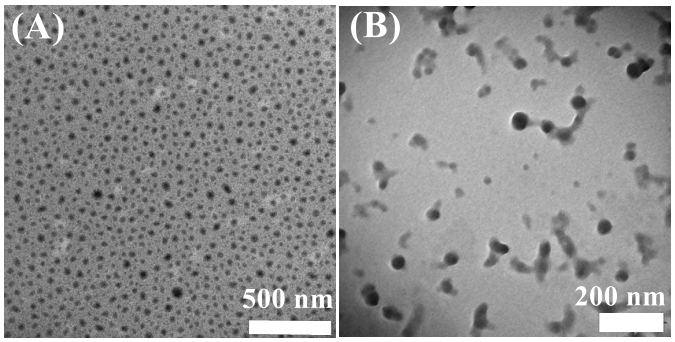


Figure S9. Transmission electron microscope (TEM) of $(PASP_{32}$-*co*-$PSI_{11})_3$ (A) and $(PASP_{17}$-*co*-$PSI_{26})_3$.

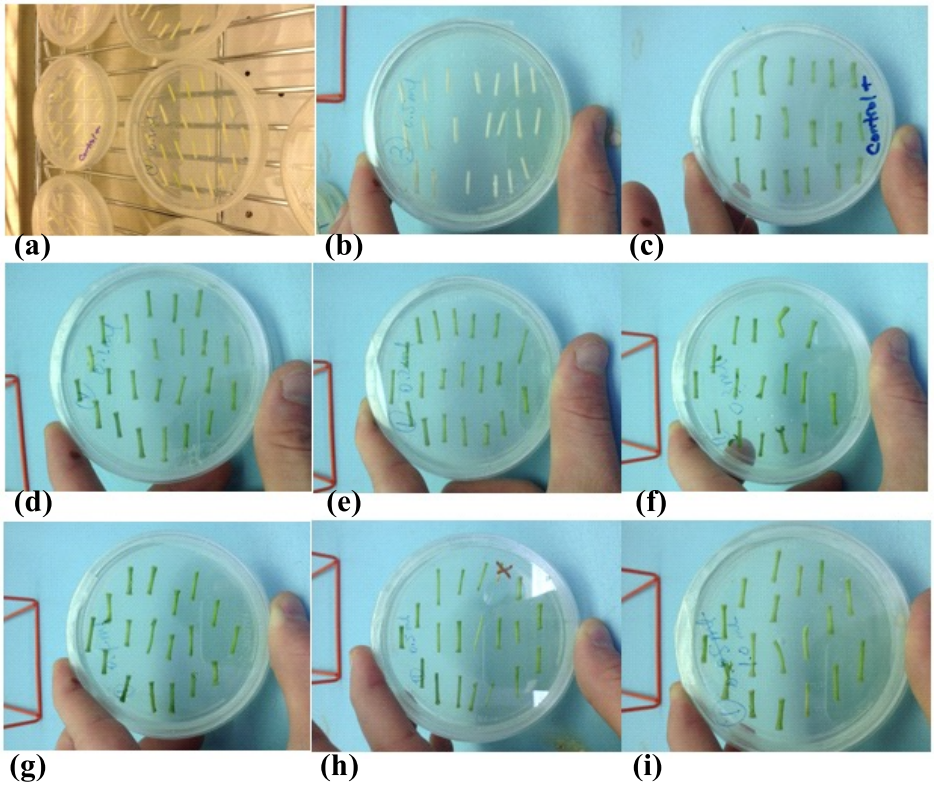


Figure S10. Toxicity assessment of $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$, by citrus tissue culture. (a) Seeding citrus tissues onto plates for lighting culture; (b) Positive control, poly(L-aspartic acid)$_{50}$-*b*-poly(L-leucine)$_{50}$-*b*-poly(L-lysine)$_{50}$ of 99 μg/mL and higher were found to was added elicit complete tissue death; (c) Negative control, nothing was added to MSBC medium, all explants should survive; (d)-(i) citrus tissues treated with different concentration random $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$.