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Synthesis of amphiphilic polysuccinimide star copolymers for responsive delivery in plants

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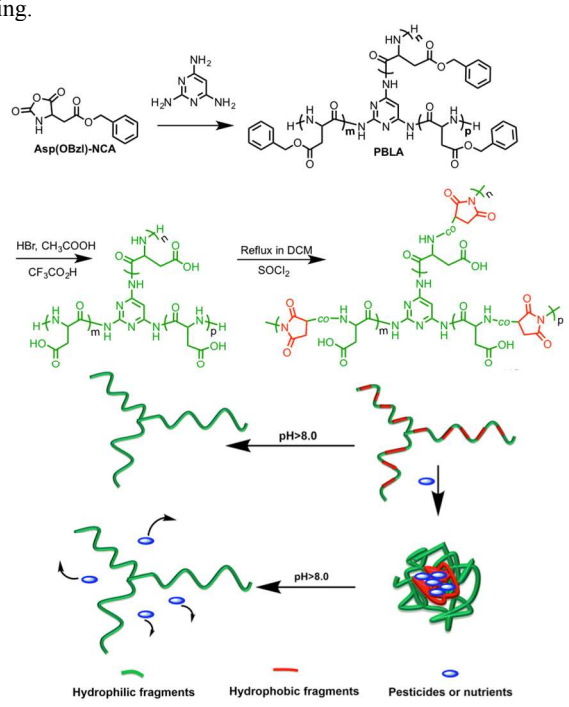
While polymeric nanocarriers are widely used in medicine for controlled release and site-specific delivery, few reports have applied such delivery methods within agriculture, despite the urgent need for specific delivery of pesticides and nutrients. We report the synthesis of stimuli-responsive and biodegradable polymeric nanocarriers designed for delivery to the phloem of plants and describe methods employed to evaluate their toxicity in plant cells.

Due to their biocompatibility and biomimetic properties, polypeptides are widely used in medicine and biology, including drug delivery,[1] gene therapy,[2] and regenerative medicine.[3] Among the various polypeptides, poly(aspartic acid) (PASP) has often been considered because of its water-solubility and facile synthesis. PASP is typically prepared from the thermal condensation of L-aspartic acid, which leads to the precursor polymer, poly(succinimide) (PSI). PSI is a particularly useful polymer as it may be easily hydrolyzed to PASP under basic conditions or functionalized with hydrophilic amines to produce water-soluble and biocompatible PASP derivatives.[4,5] Due to its facile functionalization, response to alkaline pH, and biodegradability of its hydrolyzed products, we believe PSI holds great promise in the area of controlled delivery.[6] However, currently, the most common method for its synthesis occurs through step-growth polymerization, which leads to limited control over molecular weight, molecular weight distribution and architecture of the resulting polymer. Additionally, tuning the amphiphilicty of PSI by reacting with small molecule amines may have negative effects on the biodegradability and environmental impact of the resultant derivatized polymer.[7]

Stimuli-responsive materials have received tremendous interest for site- and rate-specific drug delivery by capitalizing on the ability to carefully construct systems capable of delivering a payload under specific microenvironment conditions. While most systems have been designed to utilize disease-site microenvironments in humans (*i.e.,* the acidic nature of a cancerous tumor, a high concentration of H_2O_2 in inflamed tissue, etc.),[8] the versatility of such materials leads to applications beyond the human body. For example, plant phloem, the vascular tissue responsible for transport of photosynthates and nutrients, has a higher pH (8.0-8.5) than the surrounding plant tissue.[9] Given the low efficiency of pesticide and nutrient delivery to plants in modern agriculture,[10] a system designed to deliver effective molecules specifically to the phloem could greatly enhance use efficiency and therefore reduce the amount of pesticides and nutrients currently required to maintain agriculture productivity. Extensive research on synthetic biomaterials over the last few decades has led to clear procedures to probe the potential toxicity of such materials in the human body.[11] Given the lack of research conducted in the area of responsive materials in agriculture, guidelines to evaluate toxicity of synthetic materials to plants are lacking.



Scheme 1 Synthesis and pH-responsive behavior of three-arm star copolymers of poly(aspartic acid-*co*-succinimide) and their subsequent hydrolysis to polyaspartate.

Herein, we report the synthesis of well-defined and controlled architecture PSI-based star polymers, demonstrate controlled release at elevated pH, and describe a novel method to evaluate the potential toxicity of polymers in plants. As opposed to most other methods for PSI synthesis, our approach yields polymers with controlled molecular weights *via* a chain-growth process. We describe a novel method to prepare an amphiphilic star polypeptide, poly(aspartic acid-*co*-succinimide) (PASP-*co*-PSI), through ring-opening polymerization[12] of a protected α-amino acid *N*-carboxyanhydride (NCA) and subsequent deprotection and post-polymerization modification to yield controlled molecular weight PSI-based copolymers. PSI is relatively hydrophobic, but it is readily hydrolyzed to hydrophilic polyaspartate (PASPA) at elevated pH. Therefore, we hypothesize that these polymers may respond to the alkaline nature of the phloem and may be used to construct an eventual pH-responsive delivery system in agriculture. Additionally, biodegradation to innocuous byproducts, a reported benefit of PASP and PSI,[13] is another important consideration when designing materials for agricultural delivery. We believe PASP-*co*-PSI copolymers may be used to prepare promising nanomaterials for agricultural applications, especially in addition to the establishment of a method to evaluate its potential toxicity in plant tissue.

Compared to linear polymers, star-shape polymers with three-dimensional globular structures have been widely investigated due to their unique properties, such as compact structures, and lower viscosities compared to their linear analogs,[14] which may facilitate their transportation within vasculature in controlled delivery systems.[15] The synthetic route for the preparation of star (poly(β-benzyl-L-aspartate)$_{43}$)$_3$ (PBLA$_{43}$)$_3$ is shown in Scheme 1. After polymerization of Asp(OBzl)-NCA with a trifunctional amine initiator, the resulting polymer (M_n= 26,600 g/mol, M_w/M_n= 1.2) was deprotected with HBr/CH_3COOH/CF_3COOH to afford (PASP$_{43}$)$_3$ (Scheme 1).[16] Finally, the star-PASP was reacted with thionyl chloride to partially ring close the units of aspartic acid to yield the desired succinimide units. The presence of both aspartic acid and succinimide units led to the resultant copolymers being amphiphilic.[17] Complete synthetic and characterization details for PBLA, PASP, and PASP-*co*-PSI are given in ESI†.

The partial ring closure of PASP to PSI was confirmed by ^{1}H NMR spectroscopy (Figure S5) and IR spectroscopy (Figure S6). The signals from the methylene units in PASP (NHC*H*CH_2) and in PSI (CC*H*CH_2C) are clearly visible at 4.6 and 5.4 ppm, respectively. Three different PASP-*co*-PSI copolymers were considered, for which integration of the PASP-*co*-PSI peaks corresponds to about 25% (PASP$_{32}$-*co*-PSI$_{11}$)$_3$, 40% (PASP$_{26}$-*co*-PSI$_{17}$)$_3$, and 60% (PASP$_{17}$-*co*-PSI$_{26}$)$_3$ of the units in each copolymer being in the ring-closed form (Figure S7). The partial ring closure of PASP to PSI was additionally confirmed by FTIR spectroscopy. The stretching vibration of ν(C=O) of the carboxylic groups, in the amides of PASP was present at 1710, 1640 and 1533 cm^{-1}.[18] After ring-closing, the imide peak of PSI was clearly visible at 1796 cm^{-1}, as with the thermally prepared PSI, confirming the presence of succinimide rings within the PASP-*co*-PSI (Figure S6 and S8).

With amphiphilic PASP-*co*-PSI successfully synthesized, self-assembly into well-defined nanoparticles was investigated. Transmission electron microscope (TEM) offered evidence of PASP-*co*-PSI spherical nanoparticles with an average size of 30, 40, and 60 nm for (PASP$_{32}$-*co*-PSI$_{11}$)$_3$, (PASP$_{26}$-*co*-PSI$_{17}$)$_3$, and (PASP$_{17}$-*co*-PSI$_{26}$)$_3$, respectively (Figures S9A, 1A and Figure S9B). Dynamic light scattering (DLS) analysis yielded *Z*-average hydrodynamic diameters of 75, 140, and 186 nm with a polydispersity index of 0.239, 0.127, and 0.163 (Figure 1B), indicating rather narrow distributions of particle sizes. The difference in sizes observed by TEM and DLS is likely attributed to the dehydration of the copolymer nanoparticles upon desorption onto the TEM grid and the formation of aggregates in aqueous solution.[19] Control of size is important during nanoparticle-facilitated delivery to plants, as the cell wall prevents large particles from being passed through.[20] However, the particles in the size range observed here are expected to be promising for delivery in plants. For example, recently, Numata and coworkers first reported that peptide carriers can be used to deliver genes into plant cells, with a size of pDNA/ peptide complexes below about 200 nm leading to good transfection efficiency.[21]

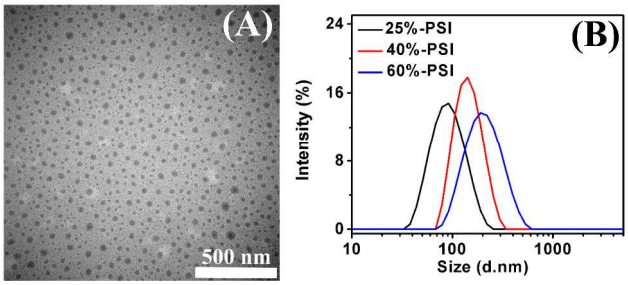


Fig. 1 (A) Transmission electron microscope (TEM) of (PASP$_{26}$-*co*-PSI$_{17}$)$_3$ and (B) dynamic light scattering (DLS) size distributions of PASP-*co*-PSI self-assemblies showing *Z*-average hydrodynamic diameters of: (PASP$_{32}$-*co*-PSI$_{11}$)$_3$, (25%-PSI) = 75 nm (PASP$_{26}$-*co*-PSI$_{17}$)$_3$, (40%-PSI) = 140 nm and (PASP$_{17}$-*co*-PSI$_{26}$)$_3$ (60%-PSI) = 186 nm.

Naphthaleneacetic acid (NAA) is a synthetic plant hormone in the auxin family[22] and is involved in many processes of live plant activity, such as cell elongation, division, and response to external environmental variety.[23] NAA has limited solubility in water and excellent fluorescence and UV absorption properties,[24] making it useful as a model pesticide to provide insight into the potential utility of PASP-*co*-PSI copolymers for controlled release in plants. As shown in Figure 2A, only minimal NAA release was observed for the PASP-*co*-PSI copolymer nanoparticles at neutral pH, suggesting the hydrophobic succinimide units are relatively stable under these conditions. On the other hand, when the pH was increased to 8.5 (*i.e.*, near the pH of the phloem), NAA release was significantly accelerated. These results are consistent with the pH-dependent hydrolysis of the hydrophobic PSI units to yield hydrophilic PASP units and subsequent nanoparticle disassembly. To confirm this, (PASP$_{26}$-*co*-PSI$_{17}$)$_3$ was dissolved at pH = 8.5 and allowed to age for 48 h. Afterwards, the resulting polymer was isolated by dialysis and lyophilization and subsequently characterized by NMR and FTIR

spectroscopy. The results (Figures 2B and C) were consistent with hydrolysis of the succinimide units, as evidenced by these spectra being nearly identical to those of polyaspartate homopolymer.

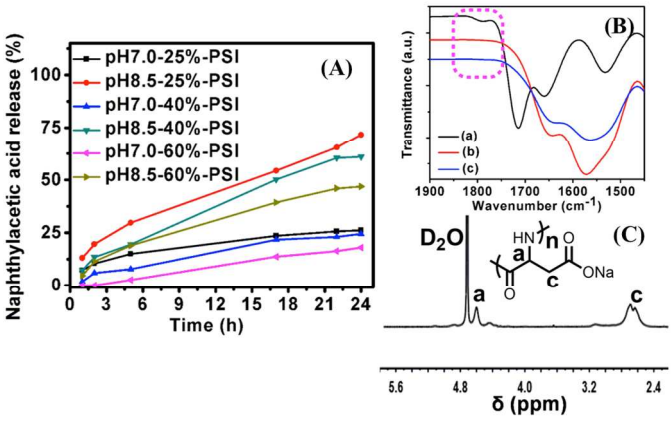


Fig. 2 (A) pH-Dependent release profile of 1-naphthaleneacetic acid from $(PASP_{32}\text{-}co\text{-}PSI_{11})_3$, (25% PSI), $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ (40% PSI) and $(PASP_{17}\text{-}co\text{-}PSI_{26})_3$ (60% PSI) nanoparticles; (B) FTIR spectrum of hydrolysate of $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$: (a) Control of $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$; (b) Control of polyaspartate; (c) $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ for 48 h at pH= 8.5; (C) ^{1}H NMR spectrum of poly$(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ after aging at pH 8.5 for 48 h.

While there are many established methods to evaluate the safety of polymeric materials in medicine, methods for toxicity evaluation in plant cells and tissues are much less developed. We developed a method based on plant tissue culture to evaluate the toxicity of polymers in plants.[6] Citrus seeds were planted on germination medium and were cultured in the dark at 25 ^oC for five weeks, causing the seedlings to become partially etiolated, or white, to reduce the potential interference of chlorophyll during subsequent fluorescence microscopy. The seedlings were cut into 1-2 cm fragments and placed on MSBC plates, which included specific concentrations of dissolved $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$. The seedlings were placed into a growth chamber with alternating light and dark (12 h each) for two weeks. The dead and living tissue segments were counted. As shown in Figure 3A, almost all citrus segments survived, even at high concentrations (*i.e.,* 240 μg/mL) of polymer, indicating $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ is relatively non-toxic to citrus plant tissue.

To further investigate the toxicity of $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$, we utilized a dual color fluorescent staining system designed for simultaneous visualization of viable and non-viable plant cells.[25] Viable cells have intact plasma membranes and intracellular esterases with the ability to enzymatically hydrolyze a fluorescein diacetate probe. The resultant fluorescent hydrolyates are polar compounds that cannot cross the plasma membrane, which leads to green fluorescence within the cytoplasm. On the other hand, propidium iodide can enter non-viable cells due to their damaged membranes, which leads to bright red fluorescence upon intercalation with DNA within the nucleus. As shown in Figure 3B, citrus leaves treated with $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ demonstrated the green color of fluorescein diacetate under blue light at 490nm/525nm Ex/Em (FITC), while showing no fluorescence under blue light at 570nm/590nm Ex/Em (Rho). Conversely, when dead citrus leaves were used as a positive control, very little green fluorescence from FITC was observed, while significant red fluorescence from the propidium iodide was clearly visible. These results offer further evidence that $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ is non-toxic at the concentrations considered.

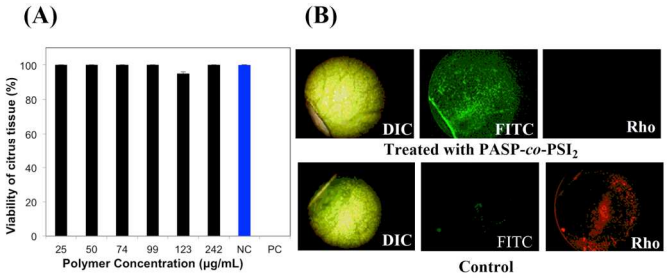


Fig. 3 Toxicity evaluation of $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ by (A) plant tissue culture (where NC = negative control (no polymer) and PC = positive control (complete tissue death induced by high concentrations of a toxicant) and (B) dual color fluorescent staining system. Top image shows the results of a live citrus leaf treated with $(PASP_{26}\text{-}co\text{-}PSI_{17})_3$ and the bottom image shows the results from analysis of a dead citrus leaf (Red areas indicate dead citrus cells and green areas indicate living citrus cells; DIC= Differential interference contrast; FITC= fluorescein isothiocyanate fluorescence setting; Rho= Rhodamine fluorescence setting.

In conclusion, compared to traditional methods involving the thermal condensation polymerization of aspartic acid to PSI and its subsequent partial hydrolysis to PASPA to produce amphiphilic polysuccinimide copolymers, a novel method using NCA ring-opening polymerization was employed. The star polymer product, PBLA, was produced with a controllable molecular weight and a narrow molecular weight distribution. After deprotection, the resultant polypeptides were converted to PSI-containing copolymers by partial ring closing of the aspartic acid units. The resultant amphiphilic star copolymers self-assembled into aggregates with the ability to incapsulate NAA, a common plant hormone, and showed rapid release at an increased pH, similar to conditions present in the phloem of plants. Furthermore, a novel method to assess the toxicity of polymers in plant cells and tissues was established. Because plant cells can not be reliably cultured, plant tissue culture and a dual color fluorescent staining system were utilized to evaluate the toxicity of amphiphilic polypeptide. The results showed limited toxicity of the synthesized polymers to plant tissue. Although the utility of controlled delivery systems has been widely proposed for the treatment of human diseases with the goal of reducing side effects and improving availability of the delivered drugs, similar delivery systems for pesticides and nutrients to plants have received much less attention. However, given the current low use efficiency of fertilizers and pesticides, modern agriculture could greatly benefit from a site-specific delivery system to reach targeted sites and reduce potential pollution caused by undelivered components. We believe this work has significant potential for phloem-limited release, and given the biodegradability and minimal toxicity of these polymers to plant

tissue and cells, other potential applications in agriculture can be envisioned.

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Notes and references

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Electronic Supplementary Information (ESI) available: Experimental details, characterization data, and additional figures. See DOI: 10.1039/b000000x/

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Table of Contents Graphic and Synopsis

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