

From Structural to Functional Materials: *a Green Way to Produce Functional Biopolymers Based on Polypeptides*



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Green synthesis catalyzed by proteases

Renewable polymeric materials produced from natural bioresources are fascinating alternatives to chemical products derived from petroleum that can fulfill the needs of future sustainable societies. Biodegradability and/or recyclability are key features for the development of sustainable materials to meet this requirement. The United Nations (UN) adopted 17 sustainable development goals (SDGs) in 2015 to achieve sustainable development by 2030. These SDGs aim to tackle an urgent call for action by all countries in a global partnership, and most of them relate to environmental issues. To realize these criteria, developing an environmentally benign manufacturing process and producing novel biobased polymeric materials to replace petroleum-based materials are inevitably important. Various biopolymers derived from natural resources, such as poly(lactic acid), polyhydroxyalkanoate, and cellulose, have extensively been applied to practical use in commercial products. Polypeptides are another attractive biopolymer in terms of versatility in the molecular design of amino acid sequences. A rational choice of amino acid residues in polypeptide sequences offers a desired functionality. Polypeptides are conventionally prepared by chemical synthesis, such as solid phase peptide synthesis and ring-opening polymerization of amino acid *N*-carboxyanhydride (NCA), or biosynthesis in host microbes as a form of protein, which generally suffers from tedious purification steps.

We have pursued ideal synthetic methods for functional biopolymers, including polypeptides, through engineered pathways utilizing natural machinery. Enzymatic synthesis of polypeptides, named chemoenzymatic polymerization, can be used to build peptide bonds with the aid of proteases, which naturally cleave the peptide bonds in proteins (Figure 1). An appropriate combination of proteases and amino acid monomers enables us to synthesize various types of polypeptides.¹⁻² Chemoenzymatic polymerization possesses tremendous advantages over traditional synthetic methods. The polymerization, which generally involves just mixing amino acid monomers with a protease, proceeds in aqueous buffer solutions instead of organic solvents. Because of the substrate specificity of proteases, the resulting polypeptides have regio- and stereoselectively well-defined structures. The leaving group is only a small alcohol molecule such as ethanol during polymerization, which can achieve excellent atom economy compared with conventional chemical syntheses using condensing agents. Collectively, chemoenzymatic polymerization offers green, facile synthesis of polypeptides with distinct structures. We have previously polymerized a variety of amino acid monomers, mostly in ester forms, via chemoenzymatic polymerization to provide polypeptides with a wide range of functional groups. In addition to amino acid monomers, oligopeptide ester derivatives are also candidate materials for polymerizable monomers. Di- or tripeptides with appropriate



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amino acid combinations offer complex periodic sequences through chemoenzymatic polymerization. Our targets are functional polypeptides for a wide range of applications, from biomimetic structural materials to bioactive peptides for biotechnology.

Proteases cleave amide bonds of specific proteins. The catalytic center in proteases attacks an amide bond to form an activated tetrahedral intermediate followed by hydrolytic reaction, resulting in scission of proteins. If we can exploit the reverse reaction of enzymatic hydrolysis, polymerization of amino

acids proceeds in a chemoselective manner regulated by spatial information for the catalytic pocket in enzymes.³ Although the equilibrium for enzymatic hydrolysis is biased toward cleavage, moderate activation of amino acids by modification with ester groups on the C-terminus kinetically facilitates protease-mediated formation of the tetrahedral intermediate and subsequent aminolysis reaction. The catalytic pocket in proteins consists of a series of subsites, each of which has distinct specificity to amino acid substrates. We harnessed chemoenzymatic polymerization by selecting

a reasonable combination of substrate-specific proteins and amino acid monomers, which was assisted by theoretical predictions using several techniques, including molecular dynamics simulations.⁴ Even an amino acid that mismatches the substrate specificity of proteases can be incorporated into polypeptides when it is inserted into an oligopeptide monomer with an elaborate sequence design to mitigate the mismatch. This technique motivates us to design and synthesize novel artificial polypeptides with more complicated sequences for further functionalization.

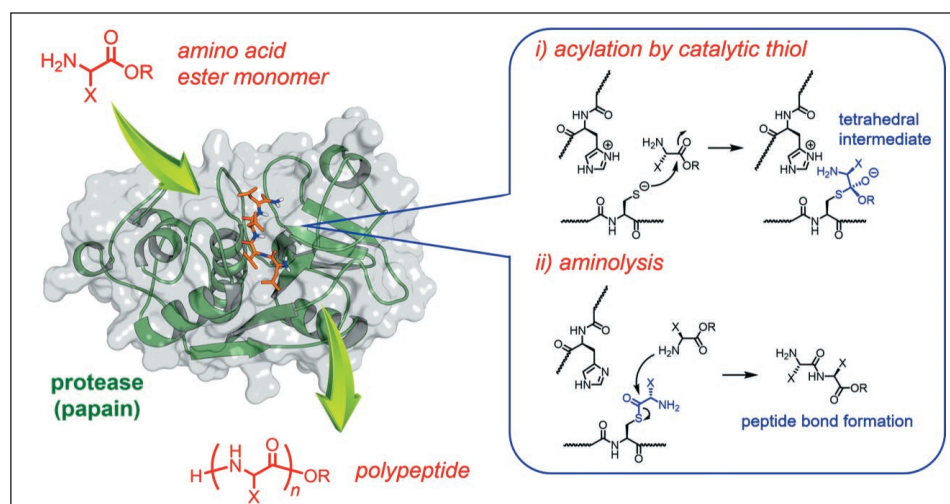


Figure 1. Chemoenzymatic polymerization catalyzed by proteases (papain). The amino acid ester monomer is activated by acylation with a catalytic cysteine residue in papain to produce a tetrahedral intermediate, which subsequently undergoes aminolysis with another amino acid ester monomer.

Structural proteins

Proteins and polypeptides play critical roles in living bodies. Versatile functions of proteins are determined by sequential variety, which are assembled into functional higher-order structures, and proteins are roughly categorized into structural proteins and globular functional proteins such as enzymes. In particular, structural proteins often possess long repetitive sequences to fold into specific higher-order structures that build up supportive frameworks in body tissues.⁵ To mimic such repetitive sequences as found in silk fibroin, collagen, elastin, and resilin, our synthetic technique for designing and controlling periodic sequences via chemoenzymatic polymerization is useful to offer artificial biomimetic polypeptide materials with physical and physiological functionality. We designed polypeptides containing specific short peptide motifs that are thought to govern the physical properties of structural proteins

to mimic their functionality using artificial polypeptide materials. The chemoenzymatic polymerization of amino acids or short oligopeptide motifs can be used to realize the facile synthesis of biomimetic artificial polypeptides for these structural proteins.

Silk proteins are produced by some animals, such as silkworms and spiders, to construct silk fibers for multiple tasks. Spider silk exhibits excellent mechanical properties that occasionally compete with those of artificial high strength fiber or even steel.⁶ A dragline silk, which is the most well-studied type of spider silk, primarily consists of major ampullate spidroins (MaSp) and shows high strength and toughness.⁷ Polyalanine motifs repeatedly appear in the amino acid sequences of MaSp proteins and form β -sheet crystallites in silk fibers. The β -sheet nanocrystals align along the fiber axis during the spinning process, resulting in the high tensile strength of the silk fiber. On the other hand, glycine-rich complex motifs

alternate with polyalanine motifs in the highly repeating domain, which are responsible for the extensibility of silk fibers. We have focused on these motifs to assemble multiblock polypeptides that possess a sequence similar to that of spider dragline silk proteins (Figure 2).⁸ Chemoenzymatic polymerization of alanine ethyl ester was performed in the presence of papain, a cysteine protease with broad substrate specificity, in phosphate buffer at mild temperature, and polyalanine was obtained as a precipitate within an hour. Structural analysis revealed that the obtained polyalanine spontaneously forms a β -sheet structure similar to that of natural spider silks. Similarly, the glycine-rich motif in spider silk proteins was imitated by a random sequence of glycine and leucine obtained by papain-catalyzed copolymerization of these amino acid monomers. Ligation of the resulting polypeptide motifs with condensing agents afforded a specific amino acid sequence in which crystalline polyalanine

and amorphous poly(glycine-*random*-leucine) motifs are tandemly flanked. For a crystalline/random composition ratio that is similar to that of natural spider silk, the multiblock polypeptide was found to exhibit a secondary structure containing β -sheet crystallites and the ability to simultaneously form nanofibers. A great advantage of the chemoenzymatic synthesis approach used to construct spider silk-mimetic sequences is easy tuning of the polypeptide motifs. Random polypeptides of glycine with other amino acids, such as serine and tyrosine, are also available for an amorphous motif, and reactive hydroxy side groups of these motifs can be exploited for further modification or cross linking.

Another example of the chemoenzymatic synthesis of biomimetic polypeptides is elastin. Elastin repeatedly contains a valine-proline-glycine-valine-glycine (ValProGlyValGly) motif in the hydrophobic region of its amino acid sequence, and the high elastic property of elastin arises from the tandem sequence of these motifs with cross linking.⁹ The ValProGlyValGly motifs in the elastin sequence undergo a temperature-induced reversible phase transition above a transition temperature, which has tremendous potential for use in thermoresponsive biomaterials.¹⁰ To construct the repetitive sequence of elastin, solid phase peptide synthesis is generally used for the synthesis of elastin-mimetic polymers. We utilized a chemoenzymatic polymerization technique to synthesize a repetitive sequence containing the thermoresponsive motif of elastin (Figure 3).¹¹ Papain-catalyzed copolymerization of ValProGly tripeptide and ValGly dipeptide ester monomers in aqueous buffer affords a polypeptide with an elastin-mimetic sequence. The polypeptide with a tandem ValProGly-*random*-ValGly sequence showed a temperature-dependent structural transition. Interestingly, when the valine residue was substituted with a glycine residue, the resulting analogous sequence of GlyProGly-*random*-ValGly did not show any temperature-dependent structural transition. Such a trivial difference in sequences drastically changes the physical properties of the polypeptides, indicating the significant importance of specific motifs that structurally and physically determine the properties of proteins.

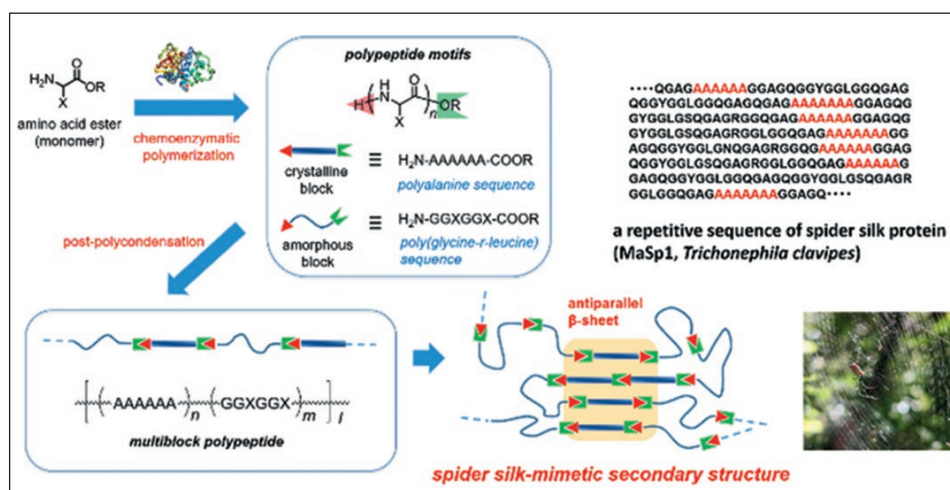


Figure 2. Synthesis of a multiblock polypeptide mimicking a repetitive sequence of spider silk proteins via chemoenzymatic polymerization. Reproduced from Ref. 8 with permission from the American Chemical Society.

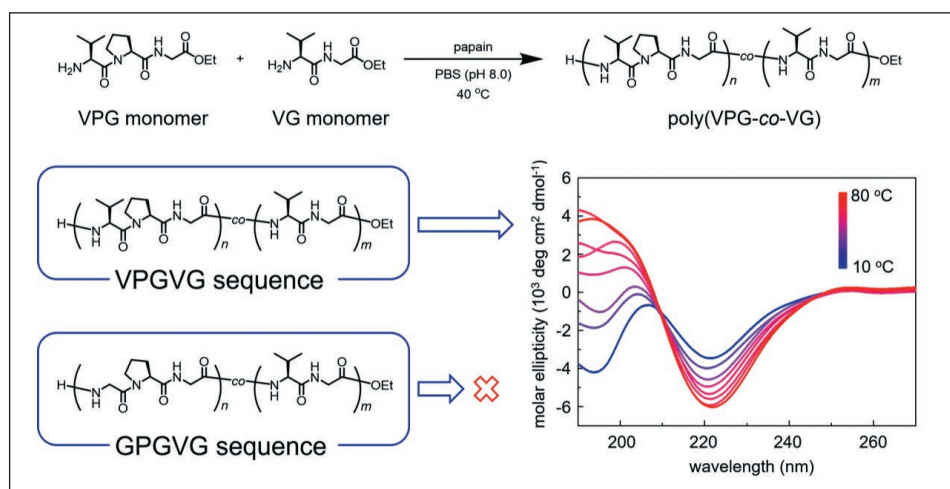


Figure 3. Papain-catalyzed polymerization of VPG and VG monomers for the synthesis of elastin-mimetic polypeptide. The polypeptide possessing VPGVG repetitive motifs exhibits a reversible structural transition in a temperature-dependent manner, as shown by the circular dichroism (CD) spectra. Reproduced from Ref. 11 with permission from the Royal Society of Chemistry.

that simple. Thanks to the substrate specificity, we can chemoselectively polymerize natural amino acid monomers by chemoenzymatic polymerization. However, unnatural amino acid monomers are tightly excluded during the enzymatic reaction. Our initial attempt to polymerize unnatural amino acid esters in the presence of various proteases was not satisfactory: the amount of unnatural amino acids that can be incorporated into polypeptide sequences is quite limited,¹² which motivated us to design unnatural amino acid-containing oligopeptide ester monomers. Sandwiching the unnatural amino acids amid natural amino acids allows proteases to recognize such species in the substrate pocket, leading to successful polymerization of these oligopeptide monomers (Figure 4).¹³⁻¹⁵ A typical unnatural amino acid, 2-aminoisobutyric acid (Aib), is an α,α -disubstituted amino acid with bulky side groups. Introduction of Aib residues in polypeptides is known to strongly induce a helical conformation due to the bulky structure. We prepared a tripeptide ethyl ester with an alanine-Aib-alanine (AlaAibAla) sequence for the monomer for chemoenzymatic polymerization.¹⁵ Neither Aib nor a Aib-containing dipeptide monomer was found to be able to polymerize in the presence of papain because the Aib unit has a poor affinity for papain. In contrast, the papain-catalyzed polymerization of the Aib-containing tripeptide monomer afforded a polypeptide that periodically contains Aib every three residues. The resulting polypeptide adopts an α -helix conformation, whereas polyalanine with no Aib units shows a β -strand structure in circular dichroism spectroscopic analysis. We expanded this “tripeptide” strategy to various types of unnatural amino acids from *N*-alkyl amino acids to monomer units of synthetic polyamides such as nylon and aramide polymers. The periodic introduction of nylon units (ω -aminoalkanoic acid) provides polypeptides with melting behavior below their decomposition temperature, promising improvement in the processability of polypeptide materials with thermal plasticity.¹³ On the other hand, aromatic monomers can also be inserted in polypeptides via chemoenzymatic polymerization of tripeptide monomers containing 4-aminobenzoic acid residues.¹⁴ Fusing the polypeptide backbone with an aromatic structure can increase the thermal stability of polypeptides, which is reminiscent of the thermal properties of synthetic aromatic polyamides such as Kevlar. Notably, periodic introduction of such an aromatic structure distinct from natural polypeptide forms, is important for improving the physical properties. The random introduction of 4-aminobenzoic acid in the polypeptide backbone was found to conversely deteriorate the thermal stability. Therefore, the introduction of unnatural amino acids shows synergy with secondary structures derived from periodic

sequences to improve the physical properties of polypeptide materials.

Functional polypeptides for plant modification

Aligning functional side groups of polypeptides by rationally designed sequences can lead to assembly into specific higher-order structures with unique functionality. Such

polypeptides have been applied to pharmaceutical and biomedical fields, including drug delivery systems, due to their physiological functions. In particular, our interest lies in plant-based sustainable bioproduction of bulk polymeric materials. Material production using plants has been studied in a broad range of fields, such as drug discovery, energy production, food production, and materials synthesis.

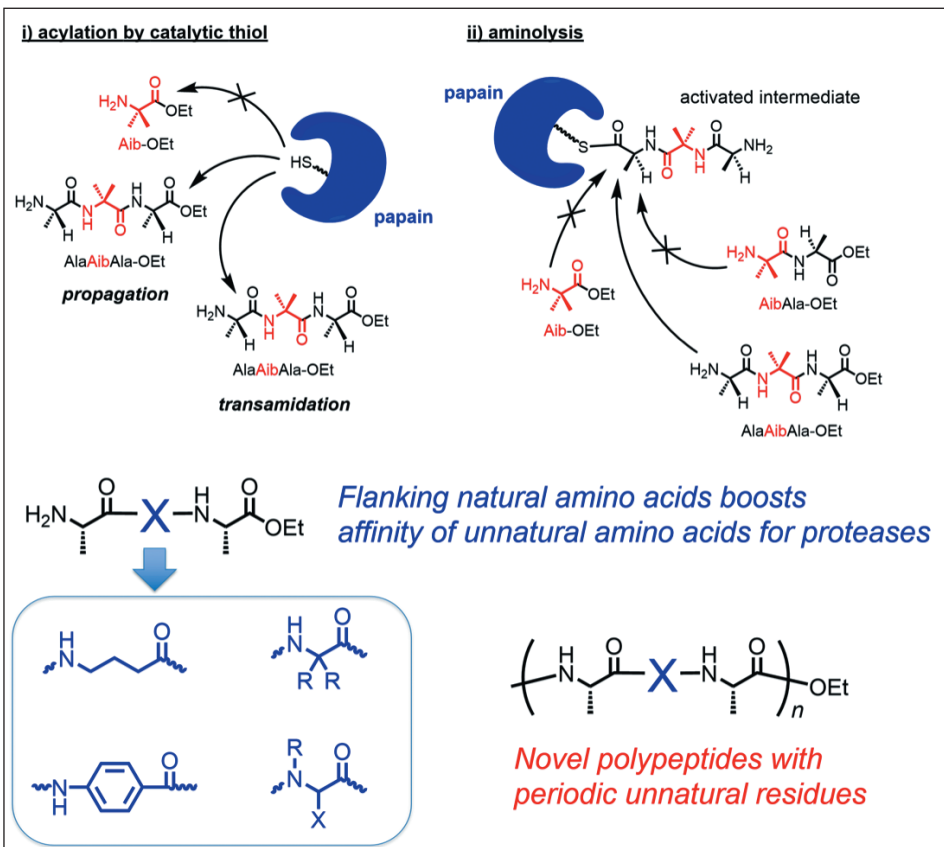


Figure 4. 2-Aminoisobutyric acid (Aib), an unnatural amino acid showing a poor affinity for proteases, can be recognized in the catalytic center of papain by “sandwiching” with natural amino acids. Various unnatural amino acids can be introduced in periodic sequences via chemoenzymatic polymerization using tripeptide esters flanking natural amino acids. Reproduced from Ref. 15 with permission from the Royal Society of Chemistry.

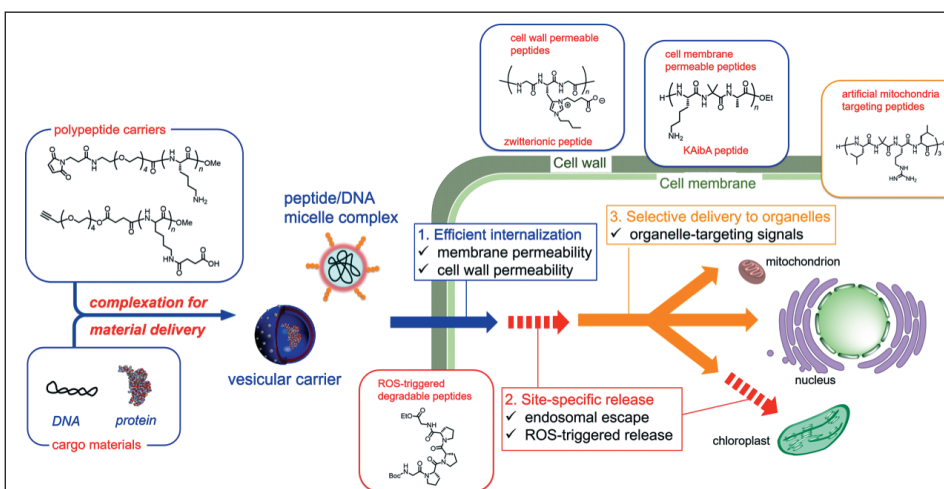


Figure 5. Material delivery into plant cells mediated by peptide carriers. Cationic polypeptides are used as carriers to complex with cargo materials (DNA, proteins), and the complex is further functionalized by various peptides to overcome several barriers for internalization into desired organelles.

Gene delivery is a powerful biotechnology for engineering plants based on modifying plant organelles (nucleus, chloroplasts, and mitochondria) to acquire desired traits for material production. To date, plant modification has been mainly achieved via agrobacterium-mediated or biolistic transformation, which suffers from several limitations for plant species and organelles. We have utilized functional polypeptides synthesized by chemoenzymatic polymerization as tools to modify plants (Figure 5). Exogenous genes, in the form of plasmid DNA (pDNA), double-stranded DNA (dsDNA), or RNA, electrostatically interact with a cationic peptide carrier to form a peptide/DNA complex.¹⁶ The peptide carrier consists of two or more functional sequences, including cationic DNA condensing domains, cell-penetrating peptides (CPPs), and organellar transit

peptides, to efficiently translocate cargoes into plant cells. The peptide/DNA complex is typically delivered into plant cells to engineer organelles by immersing plants (tissues) into the complex solution or direct infiltration using a syringe. We have rationally designed functional polypeptides that individually match requirements for overcoming barriers for internalization into target organelles that we aim to modify. For this purpose, chemoenzymatic synthesis is a useful technique to synthesize such functional sequences.

As a sophisticated carrier that enables DNA cargoes to enter plant cells, we developed lysine-based cationic peptides ligated with a terminal-functionalized oligo(ethylene glycol) (Figure 6).¹⁷ By mixing the cationic peptide with DNA, a micellar complex immediately forms by electrostatic interaction, and the resulting

micelle complex exhibits reactive functional groups such as maleimide and alkyne on its surface. We can exploit the surface reactive groups to further modify the micelle complex with another functional peptide or a peptide cocktail with multiple functionalities. The thiol-maleimide click reaction using cysteine-terminated functional peptides easily postfunctionalizes the micelle complex at the surface after the complexation process, and the types of functions and the reaction degree can be tuned on demand. Our fundamental experiments reveal that postfunctionalization with CPP doubles the gene delivery efficiency of a functionalized peptide/DNA complex compared with a nonfunctionalized micelle complex in a model plant (*Arabidopsis thaliana*).

Furthermore, when the complex is modified with two peptides, CPP and endosome disrupting peptide (EDP), at an optimized ratio of 1 to 1, the gene delivery efficiency is further increased.¹⁸ The doubly functionalized complex with CPP and EDP can effectively deliver the DNA cargo into cells via CPP-mediated endocytosis and subsequently release it from the endosome to the cytosol. This success in improving delivery efficiency strongly implies that multiple functionalizations to address each barrier during the internalization process are essential to achieve high gene delivery efficiency.

The most important function for internalization into cells is the membrane penetration property. CPPs have been developed as biological tools to deliver biomolecules into cells, initially for animal cells. We screened known CPPs originally used for material delivery into animal cells for internalization into plant cells to assess their availability for plant modification.¹⁹ Among the three categories of CPPs, namely, cationic, hydrophobic, and amphiphilic peptides, some amphiphilic CPPs were found to show a high internalization ability for model plant cells, although we found no remarkable correlation between the type/sequence of CPPs and the plant species that was used. In particular, no CPP was found that could be adapted to all plant species. The screening results for the CPP library motivated us to develop a novel artificial CPP suitable for versatile use across all plant species and tissue types. The most suitable candidate CPP in the peptide library was found to be an amphiphilic peptide adopting a helical conformation, which is assumed to be the key feature to penetrate cell membranes. We attempted to introduce a bulky nonproteinogenic Aib

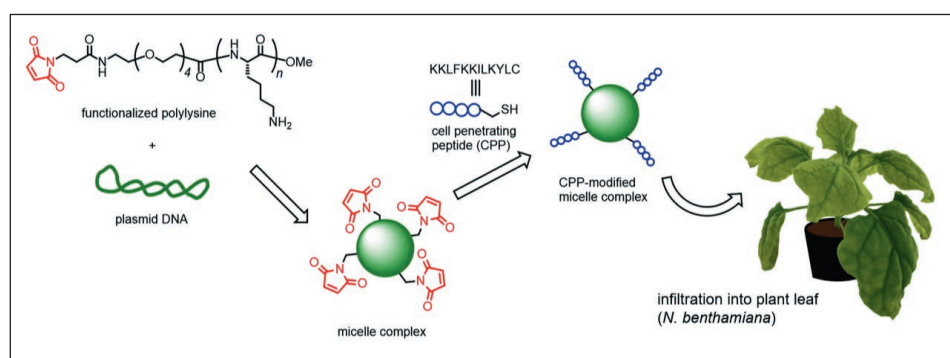


Figure 6. Infiltration of the peptide/DNA micelle complex into plant cells. The micelle complex, which displays reactive functional groups (maleimides) on its surface, can be postfunctionalized with various functional peptides, including cell penetrating peptides and organellar transit peptides, via a click reaction.

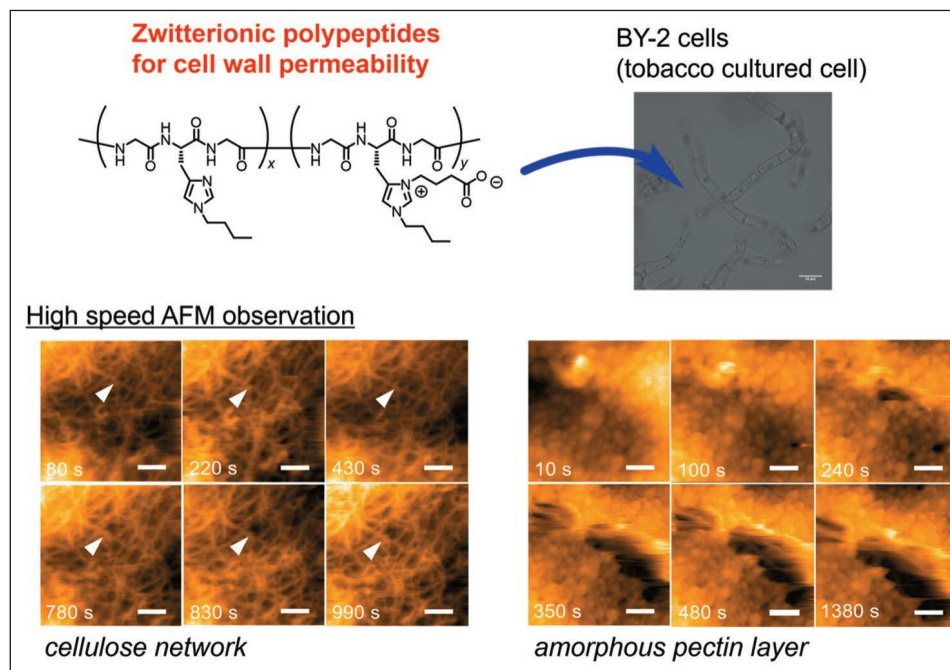


Figure 7. Zwitterionic polypeptides for dissociation of cellulose networks in cell walls. High-speed atomic force microscopy reveals that polypeptide treatment of cultured plant cells leads to partial dissociation of the cellulose network and amorphous pectin layer in cell walls. Bars show 50 nm. Reproduced from Ref. 23 with permission from the American Chemical Society.



residue into the sequence of CPP because it is known to strongly induce helix structures. Novel periodic peptides were synthesized by the chemoenzymatic polymerization of tripeptide esters containing cationic lysine and Aib residues.²⁰ The resulting peptide with a repetitive sequence of the LysAibAla motif, designated the KAibA peptide, exhibited not only excellent membrane permeability but also long-term stability in plant cells compared with conventional amphiphilic or cationic CPPs. The KAibA peptide was applicable to various plants from model plants such as *A. thaliana* and tobacco (*Nicotiana benthamiana*) and crops such as rice (*Oryza sativa*) to practical plants

such as kenaf (*Hibiscus cannabinus*, a fast-growing tall plant offering high-strength fibers) regardless of the type of tissue, including leaves and calli.

Plant cells, unlike animal cells, have a cell wall in addition to a cell membrane. Cell walls are a relatively rigid tissue with hierarchical, dense network structures consisting mainly of cellulose and other polysaccharides, such as hemicellulose and pectin.²¹⁻²²

Therefore, in contrast to cell membranes with dynamic lipid-layered structures, cell walls afford a major physical barrier for the transport of materials into plant cells. We focused on the cellulose network in cell walls and attempted to dissociate the network to maximize the penetration efficiency of the peptide/DNA complex through the cell wall. Cellulose, which suffers from poor processability due to its low solubility and nonmelting property, is known to dissolve in certain ionic liquids. Imidazolium-type ionic liquids with high hydrogen bond accepting ability interact and cleave the intermolecular hydrogen bonds of cellulose, resulting in complete dissolution of cellulose. We introduced such an imidazolium zwitterion structure similar to cellulose-dissolving ionic liquids into periodic polypeptides via chemoenzymatic polymerization (Figure 7).²³ A tripeptide ester consisting of histidine flanked with glycine residues was polymerized in the presence of papain, and imidazole side groups of the resulting periodic polypeptide were converted to a zwitterionic structure. Based on in vitro experiments, bundles of cellulose nanocrystals derived from tunicates are found to be dissociated into smaller cellulose crystallites by treatment with zwitterionic polypeptides under mild conditions. Cultured tobacco plant cells (BY-2 cells) were also treated with zwitterionic polypeptides to investigate the effect of the polypeptide on the cellulose network in the cell wall. The zwitterionic polypeptide interacts with both the cellulose network and the amorphous pectin layers of BY-2 cells at a low

polypeptide concentration, which leads to the formation of large pores. Compared with the ionic liquid that dissolves cellulose, zwitterionic polypeptide shows almost no cytotoxicity at the efficacious concentration. We are trying to improve the material delivery efficiency into plant cells using such a novel “cell-wall permeable peptide” that helps polypeptide carriers penetrate cell walls.

Conclusion

Based on multifaceted approaches to produce polypeptide materials, we have developed protease-catalyzed green synthesis of polypeptide materials.²⁴ Designing suitable amino acid sequences to construct specific secondary to higher-order structures that provide desired functionality allows us to synthesize various polypeptides that are expected to be widely applicable ranging from

structural to functional materials. A simple protocol for chemoenzymatic polymerization leads to cost-effective, large-scale production of functional polypeptide materials. Eco-friendly chemical synthesis using enzymes has huge potential to replace the existing materials derived from exhaustible resources. From the perspective of energy reduction, enzymatic synthesis will also contribute to sustainable material production because it proceeds at mild temperatures. Our goal is to establish an innovative material manufacturing system that enables carbon-neutral cycles for a sustainable society. The novel synthetic method based on chemoenzymatic polymerization will be a key technology that plays an important role in the material ecocycle system. Recent advancements using enzyme-utilized synthesis will shed light on the potential for innovative materials based on polypeptides. ◆

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