The Potential of Bacterial Microcompartment Architectures for Phytonanotechnology

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Summary

The application of nanotechnology to plants, termed phytonanotechnology, has the potential to revolutionize plant research and agricultural production. Advancements in phytonanotechnology will allow for the time-controlled and target-specific release of bioactive compounds and agrochemicals to alter and optimize conventional plant production systems. A diverse range of engineered nanoparticles with unique physiochemical properties are currently being investigated to determine their suitability for plants. Improvements in crop yield, disease resistance, and nutrient and pesticide management are all possible using designed nanocarriers. However, despite these prospective benefits, research to thoroughly understand the precise activity, localization, and potential phytotoxicity of these nanoparticles within plant systems is required. Protein-based bacterial microcompartment shell proteins that self-assemble into spherical shells, nanotubes, and sheets, could be of immense value for phytonanotechnology due to their ease of manipulation, multifunctionality, rapid and efficient producibility, and biodegradability. In this review, we explore bacterial microcompartment-based architectures within the scope of phytonanotechnology.

Introduction

The field of phytonanotechnology is a new and promising area of research that involves the use of engineered nanoparticles (ENPs) to alter and/or enhance conventional plant systems (Hatami *et al.*, 2016; Wang *et al.*, 2016; Rodrigues *et al.*, 2017; Shang *et al.*, 2019; Jiang *et al.*, 2021; Agrawal *et al.*, 2022). Through "on-demand" and "on-command" targeted delivery of bioactive molecules and agrochemicals, ENPs could have an immense impact on significant global problems related to climate change and the scale of food production (Nair *et al.*, 2010). ENPs in phytonanotechnology are designed with key physiochemical features such as programmable surface area, highly-charged surface potential, enhanced reactivity, cargo binding domains, etc. that provide functionality as nanocarriers within plants (Cunningham *et al.*, 2018; Demirer *et al.*, 2019). These properties are dependent on the chemical composition, surface structure, size, morphology, and solubility (Wong *et al.*, 2016; Vega-Vásquez *et al.*, 2020). The surge in the use of nanoparticles in medicine and material fabrication has led to the advancement of novel medical therapies and industrial products that include microelectronics, semiconductors, catalysts and household products (Smith *et al.*, 2013; Neupane *et al.*, 2019; Vega-Vásquez *et al.*, 2020). In contrast, the expansion of nanotechnology to plant sciences is relatively new but has the capability to drastically improve conventional plant production systems.

Candidates for encapsulation and display that could be transported via nanoparticles in plants include fertilizers, pesticides, fungicides, herbicides, biomolecules (such as DNA and proteins) and other nutrients and growth enhancers. Currently, the use of these candidates directly (i.e., without nanoparticle-based delivery) relies on oversaturation during application and a large portion is lost due to runoff and degradation (Ladha *et al.*, 2005). For example, nitrogen fertilizer uptake efficiency by crops after application is estimated to be 30-50% of that applied (Tilman *et al.*, 2002). Prospective nanocarriers that lessen application waste by protecting the cargo, delivering it to a specific location, and releasing the cargo in accordance with the demands of the plant could have a significant influence

on efficiency of use. The transient expression of bioactive molecules transported by ENPs into plant systems will additionally allow for the incorporation of heterologous biological pathways and development of "smart" crops that are more robust and productive. While the field of phytonanotechnology is developing with the use of carriers such as single wall carbon nanotubes and other abiotic nanoparticles, recent structural insights into Bacterial Microcompartments (BMCs), proteinaceous organelles found in bacteria, suggest that the proteins of the BMC shell offer a promising biotic building system for phytonanotechnology. While there are several efforts underway to transfer the carboxysome, a BMC for CO_2 fixation, into plants (Price *et al.*, 2013; Zarzycki *et al.*, 2013; Atkinson *et al.*, 2016; Hanson *et al.*, 2016; Long *et al.*, 2018), here we focus on the potential use of the shell proteins common to all BMCs as a material for building nanocarriers for diverse applications in phytonanotechnology.

A Brief Summary of Characteristics of ENPs Currently Used in Phytonanotechnology

Numerous ENPs are currently under development for phytonanotechnology (Wang *et al.*, 2016; Jiang *et al.*, 2021; Agrawal *et al.*, 2022); these include liposomes (LPs) (Karny *et al.*, 2018), carbon nanotubes (CNTs) (Serag *et al.*, 2011; Lahiani *et al.*, 2013; Kwak *et al.*, 2019; Demirer *et al.*, 2020), mesoporous silica nanoparticles (MSNs) (Torney *et al.*, 2007; Martin-Ortigosa *et al.*, 2012; Chang *et al.*, 2013; Hussain *et al.*, 2013), gold nanoparticles (AuNPs) (Zhu *et al.*, 2012; Zhai *et al.*, 2014; Dan *et al.*, 2015), magnetic nanoparticles (MagNPs) (González-Melendi *et al.*, 2008; Huang *et al.*, 2011), metal oxide nanoparticles (MONPs) (Kurepa *et al.*, 2010; Larue *et al.*, 2012; Rastogi *et al.*, 2017), and quantum dots (QDs) (Etxeberria *et al.*, 2006; Koo *et al.*, 2015; Pang and Gong, 2019) (Table 1). Characterization of each type of nanoparticle, particularly their capacity for bioactive compound transport, direct uptake by plants, and toxicity to plants is of paramount importance. Certain ENPs are more applicable to specific tasks in phytonanotechnology based on their overall structure. For instance, LPs, CNTs, MSNs (and

BMCs), which can encapsulate as well as display cargo, are more versatile in their utilization as transporters while GNPs, MagNPs, MONPs, and QDs are relatively limited as they can only display cargo on their surfaces. However, the smaller sized AuNPs, MagNPs, MONPs, and QDs can often more readily translocate through subcellular barriers due to their minimal diameters. For example, the specific localization of MagNPs within plant tissue was shown to be directly manipulatable through use of magnetic gradients (González-Melendi *et al.*, 2008). The ability to encapsulate cargo is desired when transporting degradable materials or when a sustained release of a substrate is desired. For instance, it was reported that silica-based MSNs (ordered, honeycomb-like, porous nanoparticles), can be loaded with the pesticide avermectin, protecting the substance from photodegradation and slowing its diffusion into the surrounding microenvironment (Li *et al.*, 2007). MSNs have also been shown to be able to encapsulate and transport the enzyme urease for hydrolysis of urea; a potential technique for the controlled release of ammonia from urea fertilizer (Hossain *et al.*, 2008).

The use of ENPs for the delivery of plasmid DNA for genome incorporation or transient expression is of particular interest as traditional methods are inefficient and tend to damage plant tissue. Successful targeted delivery of plasmid DNA into plant tissues has been reported for several ENPs, including CNTs, MSNs, AuNPs, and QDs (Etxeberria *et al.*, 2006; González-Melendi *et al.*, 2008; Wu *et al.*, 2011; Martin-Ortigosa *et al.*, 2012). For example, CNTs, composed of cylinders of graphene, were grafted with chitosan and shown to be able to capture plasmid DNA and passively translocate through chloroplast membranes, with subsequent expression of the DNA within the chloroplast (Kwak *et al.*, 2019). Passive translocation was attributed to the high zeta potential on the exterior surface of the CNTs and the overall diameter of the tubes (~20 nm). CNTs have also be reported to be able to penetrate various subcellular membranes and target specific cellular substructures (Serag *et al.*, 2011).

Indeed, research to-date suggest that the interaction of ENPs with plant tissues and subcellular structures depends on the physiochemical properties of the nanoparticle. Of particular importance in

these interactions are size, surface area, and surface electrostatic potential (Wong *et al.*, 2016). Entry of ENPs into plants can occur via either aboveground tissues or organs (e.g., stigma, stomata, or leaf surface wounds) or belowground root tissues (e.g., 'direct' uptake through root hairs or root surface ruptures). To-date, only a few reports have detailed the 'direct' uptake and detection of ENPs into plants (Zhu *et al.*, 2008, 2012; Corredor *et al.*, 2009; Serag *et al.*, 2011; Slomberg and Schoenfisch, 2012; Hussain *et al.*, 2013; Zhai *et al.*, 2014; Dan *et al.*, 2015; Koo *et al.*, 2015; Wang *et al.*, 2016). Passive 'direct' uptake methods are appealing as they do not involve genetic modification of the plant, nor do they immediately damage the plant. However, the majority of plant-nanocarrier studies have relied on injection or particle bombardment for the incorporation of ENPs into plants or isolated plant cells or organelles (Wang *et al.*, 2016). Although effective, these methods tend to damage plant tissues, are labor intensive, and are impractical for widespread agricultural application of ENPs.

Once within plants, ENPs must overcome further translocation hurdles (Schwab *et al.*, 2016). Barriers such as the stomata, the Casparian strip, the cell wall, and the cell membrane all have size-exclusion thresholds for passage that must be met for uptake to occur. For instance, the cell wall of plant cells limit translocation to particles with diameters ~20 nm (Wang *et al.*, 2016). ENPs with diameters greater than 20 nm are likely to be blocked at this barrier. However, studies suggest a flexibility to these size exclusion limits depending on the chemical microenvironment around the cell wall and the specific physiochemical properties of the ENPs (Schwab *et al.*, 2016).

Beyond the cell wall, penetration of ENPs through the cell membrane has been demonstrated to be particularly dependent on the electrostatic surface potential of the particle (Zhu *et al.*, 2012; Wong *et al.*, 2016). The lipid exchange envelope penetration (LEEP) model surmises that the size and the zeta potential of nanoparticles has the greatest influence on passive uptake through the cell membrane (Wong *et al.*, 2016). A detailed report by Wong *et al.* (2016) reported that passive translocation of different ENPs through the chloroplast membrane occurred for particles with diameters below 30 nm

and high magnitude surface zeta potentials (above 30 mV or below -30 mV). An increased understanding of the requirements for efficient plant system transport to better traverse and overcome plant barriers will allow for the development of more functional and transportable ENPs.

Although ENPs show promise to revolutionize food and agrochemical production, the relatively new application of nanomaterials to the plant sciences necessitates considerable effort to assess possible hazards that ENPs may pose (Kranjc and Drobne, 2019). Adverse environmental effects and the capability for trophic transfer of toxic and stable ENPs in the food chain represent significant concerns (Rodrigues *et al.*, 2017; Kranjc and Drobne, 2019). Research on the toxicity and impact of nanomaterials on human/animal health have shown that adverse effects can result from exposure to certain ENPs. (Pietroiusti *et al.*, 2018). Reports have detailed inflammation and scarring of the lungs due to inhalation of multiwalled carbon nanotubes and carbon nanofibers, genotoxicity due to exposure to metal and metal oxide nanoparticles, and have identified several ENPs as potential carcinogens (Fu *et al.*, 2014; Kinaret *et al.*, 2017; Pietroiusti *et al.*, 2018). A wide range of factors, including plant species, application route, duration of exposure, and the specific physicochemical properties and composition of the ENP would all affect interactions with plants, toxicity, and transmissibility to the surrounding ecosystem (Ma *et al.*, 2010; Nair *et al.*, 2010; Jiang *et al.*, 2021). These factors along with ENP adsorption, translocation, localization, and bioaccumulation can contribute to both beneficial and adverse effects of the nanoparticle on plant systems (Kranjc and Drobne, 2019). The future widespread application of phytonanotechnology is dependent on the elimination of the toxic potential and a safe-by-design approach should be the guiding principle for the development of all ENPs.

Bacterial Microcompartment Shell Proteins: A New Building Block Set for Phytonanotechnology

A new candidate carrier for phytonanotechnology possessing the attributes that address many of the concerns surrounding abiotic nanoparticles are Bacterial Microcompartment (BMC)-based structures. BMCs have certain advantages that make them prime candidates for development within the field of phytonanotechnology (Fig. 1). In nature, BMCs are functionally diverse, semi-permeable, self-assembling protein organelles widely used by microorganisms to encapsulate biochemical pathways in order to isolate reactive enzymes or toxic intermediates from the cytosol of the cell (Kerfeld *et al.*, 2018; Sutter *et al.*, 2021). The basic architecture of the BMC membrane, the shell, is conserved across different bacterial phyla but the reaction pathways they encapsulate are varied (Kerfeld *et al.*, 2018; Sutter *et al.*, 2021). The most well studied BMC, the carboxysome, is found in all cyanobacteria and some chemoautotrophs where it functions to sequester carbonic anhydrase and Ribulose-1,5-bisphosphate carboxylase/oxygenase for CO_2 fixation (Kerfeld and Melnicki, 2016, 2016; Turmo *et al.*, 2017; Borden and Savage, 2021; Liu, 2022). Catabolic BMCs (also known as metabolosomes) include those that are involved in the metabolism of organic compounds such as ethanolamine, propanediol, fucose, and rhamnose (Bobik *et al.*, 1999; Kofoid *et al.*, 1999; Petit *et al.*, 2013; Erbilgin *et al.*, 2014). A recent phylogenetic survey identified more than 7000 BMC loci including 68 types/subtypes of metabolosomes, many of unknown function, highlighting their broad range of use by microorganisms (Melnicki *et al.*, 2021; Sutter *et al.*, 2021).

The BMC shell is composed of three types of protein building blocks: a hexamer protein (BMC-H), a trimer protein (BMC-T), and a pentamer protein (BMC-P) (Fig 2). The BMC-H component is the most abundant type in shell formations and consists of six arranged monomers [Pfam00936 domain] (Fig 2A). When assembled into a hexagonal oligomer, the BMC-H building block has a convex and concave side with a thickness of ~20 Å and a diameter of ~70 Å (Kerfeld *et al.*, 2005). BMC-T proteins consist of a tandem fusion of two Pfam00936 domains and the trimer resembles BMC-H hexamers Because of this, BMC-T trimers are referred to as “pseudohexamers” (Klein *et al.*, 2009) (Fig 2A).

Additionally, there are two subtypes of the BMC-T proteins: a single trimer form and a second form (BMC-T^{dp}) wherein two trimers dimerize across their concave faces (Klein *et al.*, 2009; Cai *et al.*, 2013). A pore, located at the center of BMC-H, BMC-T, and BMC-T^{dp} oligomers serves as a selective-permeable channel for metabolites (Kerfeld *et al.*, 2005; Klein *et al.*, 2009). At ~4-7 Å most pores are large enough to permit the passage of small molecules such as bicarbonate and carboxylic acids but small enough to exclude larger molecules. Pores of the BMC-T^{dp} trimer are larger at ~14 Å and are gated by absolutely conserved residues (Klein *et al.*, 2009; Cai *et al.*, 2013; Larsson *et al.*, 2017; Mallette and Kimber, 2017). The ability of BMC-T^{dp} proteins to dimerize across their concave surfaces results in an internal chamber between the interior and exterior of the shell. In general, selective permeability of small molecules is postulated to be determined by the polar residues lining the pore. This hypothesis is consistent with studies that demonstrate alteration of BMC function when these residues are mutated (Chowdhury *et al.*, 2015; Slininger Lee *et al.*, 2017). The BMC-P protein, which consists of a single monomer [Pfam03319 domain], forms a cyclical pentamer and serve to cap the vertices of the shell assemblies (Tanaka *et al.*, 2008; Sutter *et al.*, 2017). Shell formation without the BMC-P are possible and are referred to as "wiffle ball" shells as they possess ~47 Å holes at the vertices where the BMC-P would be (Sutter, McGuire, *et al.*, 2019; Kirst *et al.*, 2022) (Fig 2A). In addition to empty polyhedral shells, nanotube and sheet architectures have been discovered through the heterologous overexpression of certain BMC hexamers and BMC-T pseudohexamers; the sheets and nanotubes are composed of a single type of shell protein (Fig. 2C & D) (Kerfeld and Erbilgin, 2015; Noël *et al.*, 2016; Uddin *et al.*, 2018). However, not all hexamer and pseudohexamer BMC-T species can form nanotubes and sheets. The ability to form these structures is likely dependent on the specific amino acid interactions on the edges of these hexamers which, as of yet, have not been fully characterized. Once fully assembled, BMC shells have diameters ranging from 20 nm - 400 nm (depending on the BMC species type) and BMC-based nanotubes have diameters of 20 nm (Parsons *et al.*, 2010; Lassila *et al.*, 2014; Sutter *et al.*, 2016; Sutter, McGuire, *et al.*, 2019).

Engineering Cargo Display and Encapsulation on BMC Shells

Although the main function of carboxysomes and metabolosomes is the sequestration of enzymes and their reactive intermediates, a cardinal discovery of the last decade was that the formation of BMC shell membranes does not require cargo; empty shells can readily form (Parsons *et al.*, 2010; Lassila *et al.*, 2014; Sutter *et al.*, 2016; Hagen, Plegaria, *et al.*, 2018; Sutter, McGuire, *et al.*, 2019). Empty shells assembled without their corresponding cargo have been structurally characterized at atomic resolution with a combination of cryo-EM and x-ray crystallography, verifying their independent formation and structural stability (Sutter *et al.*, 2016, 2017; Sutter, Laughlin, *et al.*, 2019; Kalnins *et al.*, 2020). Moreover, these studies provided a structural blueprint of empty shells, detailing specific features like where the N- and C-termini of the shell proteins are positioned and the electrostatics of the interior and exterior. Detailed knowledge of the structural characteristics of these assemblies is foundational not only for understanding the role of the shell in native BMC function but additionally enables for the precise bioengineering of shell components and positioning of cargo to exterior and luminal surfaces. In these studies, the N- and C-termini of the shell proteins were shown to be located on the external surface of the assemblies. This informed designs for displaying proteins on the outside facets of shells and nanotubes (Kalnins *et al.*, 2020). Using both termini of a hexamer allows for the potential fusion of two distinct elements (one attached at each terminus) per monomer; for a total of twelve attachments points. The ability to display enzymatic cargo on the exterior of these architectures is beneficial, for example, to catalyze a reaction to accumulate a substantial concentration of substrate adjacent to the central protein pores for rapid diffusion to the interior. Likewise, identification of interior surface residues permits the swapping of sidechains for tailored interaction with cargo through electrostatic association. Ensuing development of circularly permuted hexamers that display the termini

on the luminal surface of a shell enabled encapsulation of fused cargo to the interior (Ferlez *et al.*, 2019).

Initial attempts at heterologous cargo loading of BMC shells utilized encapsulation peptides that are present in many BMCs, however, studies using this method reported meager loading efficiency (Lassila *et al.*, 2014; Aussignargues *et al.*, 2015; Wagner *et al.*, 2017; Hagen, Sutter, *et al.*, 2018). Since then, further progress has been made to modify the structural proteins to display and encapsulate cargo. Using the SpyTag/SpyCatcher split bacterial adhesion system (Veggiani *et al.*, 2016), the shortcomings of the encapsulation peptide strategy were circumvented and a new method was developed for encapsulation (Hagen, Sutter, *et al.*, 2018). This method relies on the formation of a covalent bond when the independent domains of the SpyTag and SpyCatcher interact. By modifying a region on the luminal side of the BMC-T component to include a loop containing the 1.4 kDa SpyTag element, encapsulation of heterologous cargo containing the 9 kDa SpyCatcher element to the interior surface was made possible. Loading and attachment of cargo via this adhesion system is possible *in vivo* through recombinant expression of the shell proteins along with expression of cargo proteins and *in vitro* via diffusion of cargo through the gaps of wiffle ball shells with subsequent pentamer "capping" to create fully encapsulated shells. Moreover, the "capping" strategy using pentamers that are functionalized with an affinity tag allows for purification of the full formed and loaded shells (Hagen, Plegaria, *et al.*, 2018).

Thus far, several model shell system platforms have been developed and include the PDU (propanediol utilization) BMC shells and HO (*Haliangium ochraceum*) BMC shells (Parsons *et al.*, 2008; Lassila *et al.*, 2014). Utilizing these and other BMC shell membranes, several synthetic nanoreactors have been constructed that include a PDU encapsulating ethanol bioreactor that is able to transform pyruvate to ethanol (Lawrence *et al.*, 2014), a *Citrobacter freundii* bacterial microcompartment-based system that can accumulate and store polyphosphate (Liang *et al.*, 2017), and a hydrogen-producing α-

carboxysome shell nanoreactor (Li *et al.*, 2020). Recently, employing the HO shell platform, a synthetic formate utilizing BMC was developed that encapsulated two distinct enzymes, the oxygen-sensitive glycyl radical enzyme pyruvate formate lyase and a phosphate acyltransferase (Kirst *et al.*, 2022). In this study, researchers were able to take advantage of the SpyTag/SpyCatcher system to attach one enzyme and the second was incorporated with the similarly functioning SnoopTag/SnoopCatcher split adhesion system. In another recent report that highlights the modifiability of BMC shell components, a synthetic hexagonal trimer protein was engineered through the tandem fusion of two domains of the BMC-H, creating cyclic trimers (designated BMC-H^2) that self-assemble to form icosahedral wiffle ball shells with gaps at the pentamer positions (Sutter, McGuire, *et al.*, 2019). The generated BMC-H^2 shell had a smaller diameter (25 nm) than the typical BMC-H-containing minimal shell (40 nm). This method of shell protein engineering opens the possibility of fusing two distinct hexamer domains together from disparate species to create unique cyclic trimer proteins containing two different sets of residues on their surfaces and on the periphery of the central pore; as compared to the repeated residues for typical homohexameric proteins.

In regard to the other BMC-based architectures, numerous different species of hexamers have been identified that can readily form nanotube and sheet structures but more research is needed to elucidate and take advantage of the subtle differences that dictate the differences in the resulting self-assembled morphologies (Parsons *et al.*, 2010; Lassila *et al.*, 2014; Pang *et al.*, 2014; Cai *et al.*, 2016; Noël *et al.*, 2016; Sutter *et al.*, 2016; Uddin *et al.*, 2018) (Fig. 3). Nanotube and sheet architectures have several distinct features relative to shells. For example, nanotubes are formed by a single type of protein (Parsons *et al.*, 2010; Noël *et al.*, 2016). While the diameter of nanotubes is in the range of that of shell structures (~20 nm), they can be micrometers in length (Fig. 3C, D) (Noël *et al.*, 2016). Likewise, BMC-based protein sheets are also composed of a single protein and can have planar dimensions that range in size up to micrometers (Fig. 3E, F) (Sutter *et al.*, 2016). Both sheets and tubes could serve as binding

surfaces to organize, immobilize, and pack closely together, large quantities of enzymes or other bioactive molecules or could additionally serve as structure-enhancing elements to modify the stability of plant cells. Given that these structures are composed of a single BMC element, their assembly is much simpler, and their formation doesn't depend on stoichiometric ratios of several different shell components as is the case for BMC-based shells. These structures are also highly modifiable. Slight deviations in how the hexamers of these architectures interact along their edges could, for example, result in nanotubes of varying diameters and rigidity or sheets with slight curvatures or repeating undulations across their surfaces that could be taken advantage of for bioengineering purposes.

Advantages of BMC Architectures for Phytonanotechnology

The recent advances in functionalizing BMC-based shell components demonstrate the future potential for use of BMC-based architectures as nanocarriers or scaffolds. The architectures being developed offer a wide range of applicability for phytonanotechnology. Because BMC-based architectures are composed entirely of protein, these structures are biodegradable, can be rapidly produced and modified for functionality *in vitro* (Fig. 1A), and are able to be expressed directly *in vivo* within the plants through transient expression or genome incorporation (Fig. 1B). Unlike carbon-, metal-, or lipid-based ENPs that are not genetically encoded, BMCs can readily be fine-tuned to "program" charge, hydrophobicity, surface structure, etc., through codon mutagenesis. In vitro assembly reactions for BMC-based architectures are typically conducted in neutral or slightly alkaline buffer solutions (Hagen, Plegaria, *et al.*, 2018), which is similar to the internal environment of plant cells that, under normal condition, have a cytosolic pH of 7.5 (Kader and Lindberg, 2010). The stability of these architectures in distinct plant cell compartments that possess more acidic pH environments is less certain, however, and their use in such compartments would need to be further investigated to elucidate the viability of their use there. Both synthetic BMC shells and nanotubes possess diameters

that meet or are close to the size exclusion limit requirements (~20 nm) of plant transport barriers. Because they are genetically encoded, it is relatively trivial to modify surface residues of BMC-based shells and nanotubes to tune the zeta potential, which has been shown to be important for passive membrane translocation via the LEEP mechanism (Fig. 3B & D) (Wong *et al.*, 2016). However, as of yet, the ability of translocation of BMC-based shells or nanotubes to translocate through membranes or between plant cell compartments via this mechanism has not been experimentally confirmed.

The functional diversity of catabolic BMCs found in nature underscores the adaptability of the BMC shell system for various biochemical pathways (Melnicki *et al.*, 2021; Sutter *et al.*, 2021). Bioinformatic analyses along with structural characterizations of BMC shell systems have demonstrated shared universal principles of shell building blocks that can be taken advantage of for the engineering of mix-and-match, function-specific structures (Sutter *et al.*, 2017, 2021). When further developed, this catalog of interchangeable building blocks provides an inventory of components to select from for precise engineering BMC architectures for phytonanotechnology. Engineering of the central pore present in the hexamer, trimers, and pseudohexamers can be explored to allow for selective uptake of substrates including electrons; metal centers have been introduced into pores (Aussignargues *et al.*, 2016; Plegaria *et al.*, 2020). Likewise, the pores may be of use for the controlled release of encapsulated substances. The redesign of pores could grant the controlled-uptake of nutrients and substrates to interact with encapsulated enzymes or the controlled-release of fertilizers, pesticides, or other agrochemicals that can interact directly to benefit the plant.

Future Prospects and Concluding Remarks

The development of green nanotechnology platforms, such as protein-based BMC architectures, and their application in the field of phytonanotechnology show immense potential. BMC-based shells, nanotubes, and sheets are promising for research in this field due to their ease of production,

multifunctionality, and biodegradability, however, the interactions between BMC structures and plant systems are yet to be explored. Studies that focus on these interactions will help to guide future development of BMC-based platforms in this field. Several methods are currently available for cargo loading onto BMCs but novel methods that permit the controlled, targeted release of cargo are necessary for the development of “smart” systems of transport. Incorporation of agriculture-relevant agrochemicals and biomolecules during future attachment experimentation will be important to assess feasibility in phytonanotechnology.

The expression of BMC-based architectures in plants is yet another interesting possible avenue for application of these platforms. Expression of nanotubes or sheets could potentially affect the rigidity and structural stability of plant cells or could serve as unique scaffolds for the attachment of enzymes, agrochemicals, or bioactive molecules. As with all ENPs, rigorous studies to elucidate possible plant toxicity and ecotoxicity related to direct uptake and expression of BMCs within plant systems must be conducted to ensure safe use and to establish social acceptance of the technology.

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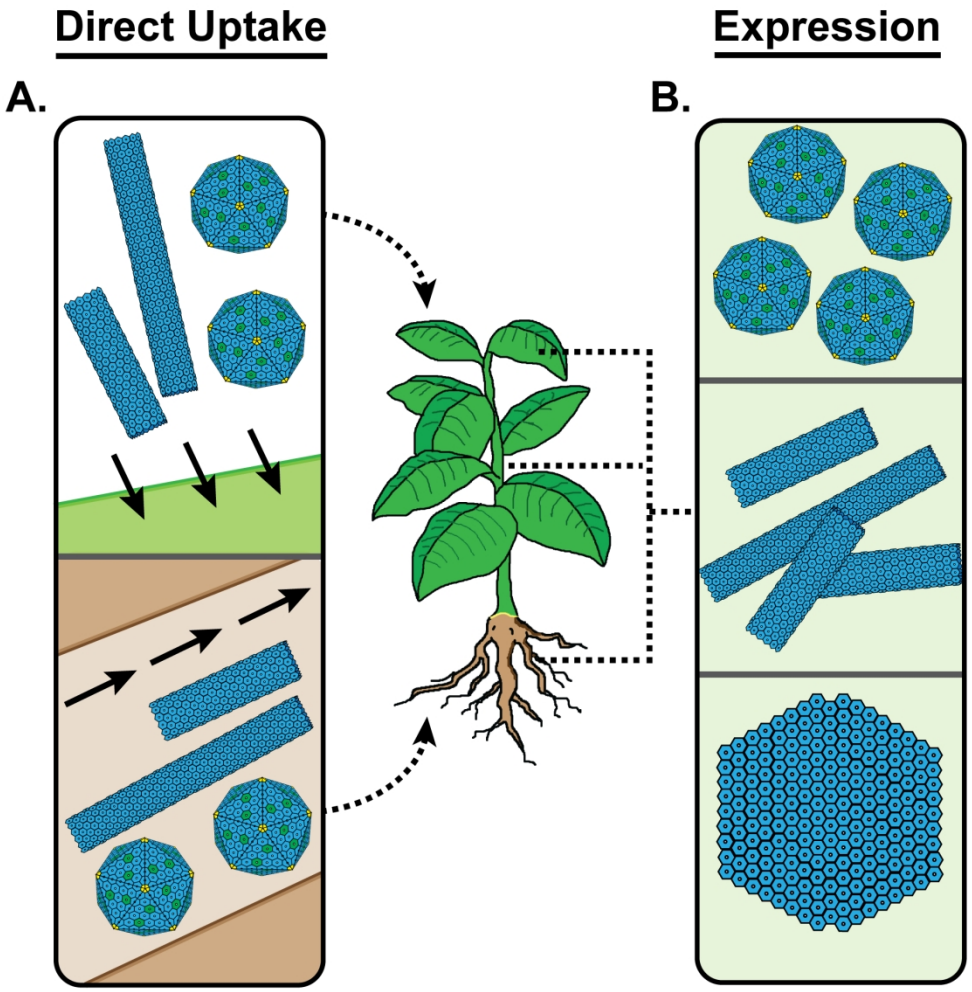

Figure 1. Direct uptake and expression of BMC-base architectures in plant systems. An overview of how BMC-based structures might be transported into and expressed within plant systems. BMC-based architectures have the potential to be used as shuttles for transporting biomaterials into plant systems or could serve a temporary purpose through transient expression in plant systems. The advantages of protein-based ENPs compared to non-biological ENPs include ease of manipulation, multifunctionality, rapid and efficient producibility, and biodegradability. A. Direct uptake of in vitro assembled BMC-based shells and nanotubes through leaves and roots. These structures have the capability to load and subsequently isolate or display cargo such as bioactive compounds, agrochemicals, and fertilizers for transport. B. Expression and assembly of encapsulating BMC shells, nanotubes, and 2D sheets in plant leaves and roots.

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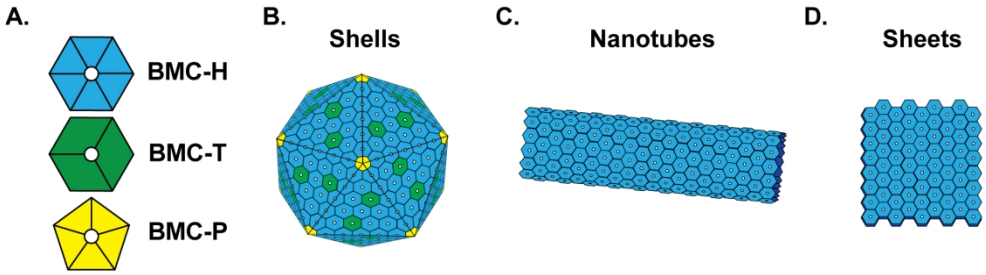

Figure 2. BMC-based components and architectures. A. Representation of BMC structural components. B. BMC shells are composed of protein hexamer (BMC-H), trimer (BMC-T), and pentamer (BMC-P) elements. The pentamers serve to cap the vertices of the protein shell. C-D. BMC-based nanotubes and sheets are composed solely of protein hexamers and pseudohexamers. Differences in residues along the edge periphery likely contribute to the formation of the distinct architectures.

429x119mm (118 x 118 DPI)

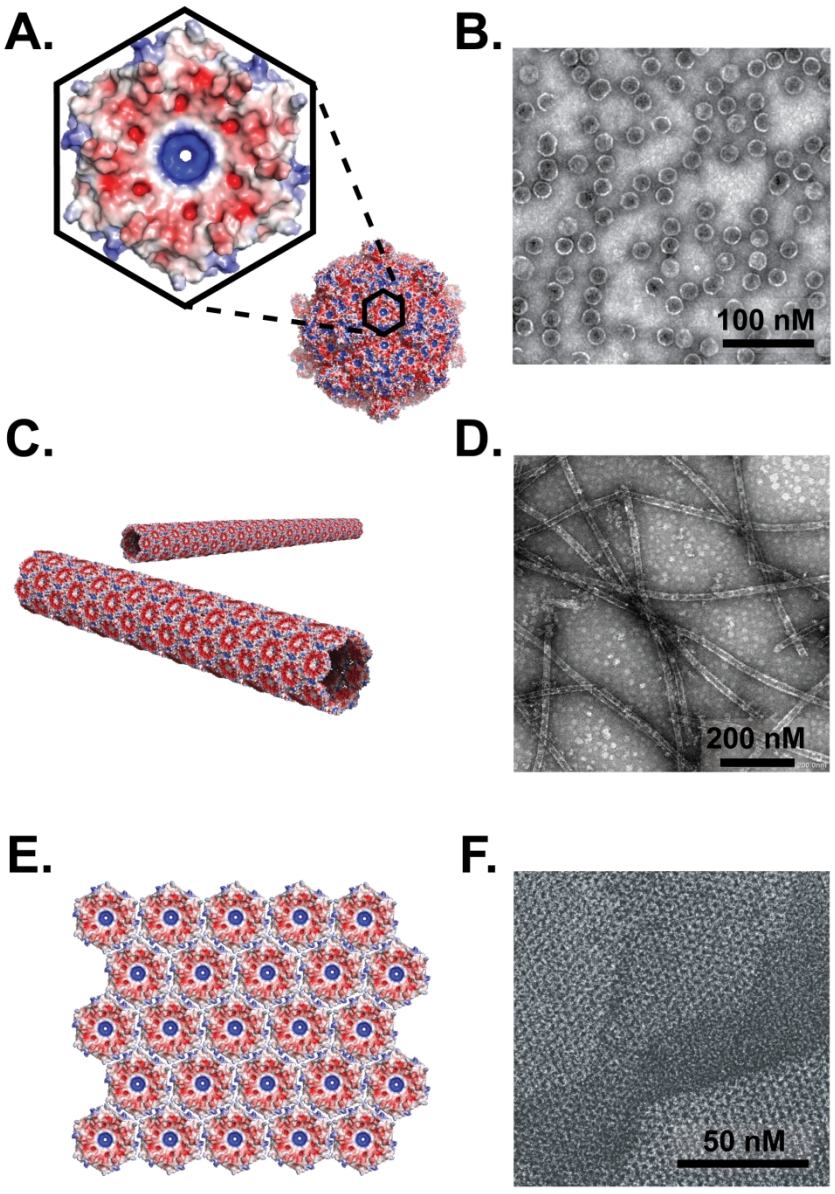

Figure 3. Models and electron microscopy images TEM of BMC-based architectures. A. Electrostatic model of synthetic beta-carboxysome shell (PDB 6OWG) with individual hexamer component outlined and (B) TEM image of minimal BMC shells on a grid. The central pore of the BMC-H and BMC-T components allow for the selective shuttling of molecules to the interior of shells. C. Electrostatic homology model of BMC-based nanotubes composed of the RmmH hexamer of M. smegmatis (Noël et al., 2016) and (D) TEM image of RmmH hexamer BMC-based nanotubes on a grid. E. Electrostatic representation of BMC-based sheets composed of the H. ochraceum BMC-H hexamer (PDB 5DJB) and (F) an unpublished TEM observation of BMC-T based sheets from Clostridium. BMC-based sheets have been shown in previous publications to be solely composed of BMC-H, however, as identified in this image, the pseudohexamer BMC-T can in certain cases also form sheets.

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Table 1. Nanoparticles utilized in phytonanotechnology experimentation

Nanoparticle	Size	Shape	Plant	Remarks	Ref
Liposomes	100 nm	Spherical	*S. lycopersicum*	Liposomes loaded with Fe and Mg restored acute nutrient deficiency	(Karny *et al.*, 2018)
CNTs	D x 0.8-50nm, L x 4 nm-1 um	Tube	*C. roseus* protoplasts	Penetration cell membrane and localize to nucleus, plastids, and vacuoles	(Serag *et al.*, 2011)
			N. benthamiana	Protection/transport of siRNA	(Demirer *et al.*, 2020)
			Soybean, barley, & wheat seeds	Penetration of seed coat	(Lahiani *et al.*, 2013)
			E. sativa, N. officinale, N. tabacum and *S. oleracea* & *A. thaliana* protoplasts	Transport/expression of DNA through the cell membrane	(Kwak *et al.*, 2019)
MSNs	2-600nm	Spherical	*N. tabacum*	Transportation of DNA and chemicals into isolated plant cells and intact leaves	(Torney *et al.*, 2007)
			A. thaliana	Delivery of foreign DNA into intact A. thaliana roots via direct uptake	(Chang *et al.*, 2013)
			Onion epidermis tissue	DNA delivery via gold-capped MSNs	(Martin-Ortigosa *et al.*, 2012)
			Wheat, lupin, *A. thaliana*	Uptake and distribution during seed germination	(Hussain *et al.*, 2013)
AuNPs	6-50 nm	Spherical	*A. thaliana*	AuNPs taken up by plant roots or shoots depending on charge	(Zhu *et al.*, 2012)
			P. deltoides	Uptake and translocation to roots, stems, and leaves	(Zhai *et al.*, 2014)
MagNPs	2-10nm	Spherical	*C. pepo*	Translocation of MagNPs in plant tissues	(González-Melendi *et al.*, 2008)
MONPs	3-200nm	Spherical	*A. thaliana*	TiO2 NPs Penetration of cell walls and accumulation within cell	(Kurepa *et al.*, 2010)
		Spherical	*T. aestivum*	TiO2 translocation and accumulation	(Larue *et al.*, 2012)
QDs	2-50nm	Spherical	*A. thaliana*	Uptake by roots and translocation to leaves	(Koo *et al.*, 2015)
			Sycamore cells	Endocytic uptake of QD particles	(Etxeberria *et al.*, 2006)