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Communication

Solid-Phase Synthesis of Megamolecules

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ABSTRACT: This paper presents a solid-phase strategy to efficiently assemble multiprotein scaffolds—known as megamolecules—without the need for protecting groups and with precisely defined nanoscale architectures. The megamolecules are assembled through sequential reactions of linkers that present irreversible inhibitors for enzymes and fusion proteins containing the enzyme domains. Here, a fusion protein containing an N-terminal cutinase and a C-terminal SnapTag domain react with an ethyl *p*-nitrophenyl phosphonate (pNPP) or a chloro-pyrimidine (CP) group, respectively, to give covalent products. By starting with resin beads that are functionalized with benzylguanine, a series of reactions lead to linear, branched, and dendritic structures that are released from the solid support by addition of TEV protease and that have sizes up to approximately 25 nm.

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Megamolecules are modular structures that are assembled by the reaction of fusion proteins and linkers.[1] The reactions occur between an enzyme domain in the fusion with a covalent inhibitor and have the benefit that they are rapid, proceed in high yield, and are highly selective.[2] We previously reported the solution-phase synthesis of precisely defined linear and cyclic structures with molecular weights up to 300 kDa and dimensions of approximately 20 nm,[3] and we also reported the use of megamolecule scaffolds to organize fluorescent proteins for studies of energy transfer.[4] However, the preparation of the megamolecules required size exclusion chromatography (SEC) to purify the intermediates after each reaction step and made it difficult to efficiently prepare larger numbers of structures. Here, we describe a solid-phase synthesis method that enables the rapid assembly of megamolecules.

The syntheses we demonstrate are based on the reaction of the serine esterase cutinase (C) with an ethyl *p*-nitrophenyl phosphonate (pNPP) group, which covalently inhibits the enzyme through esterification of the Ser120 active site residue[5] and of SnapTag (S) with a chloro-pyrimidine (CP) group that reacts with the catalytic Cys145 residue in the enzyme (Figure 1A).[6,7]

We initiate the solid-phase synthesis by treating highly cross-linked agarose (4%) magnetic resin beads that are functionalized with benzylguanine (BG) groups[8] with a cutinase-SnapTag fusion protein (referred to as CS), which results in the attachment of the SnapTag domain to the bead.

We then wash the beads with buffer and add a bifunctional linker having one CP and one pNPP group (Figure 1B). In this reaction, the free cutinase domain anchored to the bead reacts with the linker, which results in a terminal CP group that is available for a subsequent coupling step with another CS protein. This cycle—the addition of a fusion protein followed by a linker—can be repeated to synthesize megamolecules of increasing length.

We applied this strategy to synthesize a pentameric megamolecule, and which required nine individual reactions. However, the first step in the synthesis used a fusion protein that included a TEV protease sequence (ENLYFQG) in the linker that connected the two protein domains (we refer to this fusion as C*S) (Figure S2).[9] The fusion protein used in subsequent steps omitted the protease sequence in the linker and is referred to as CS (Figure 2A). This strategy allowed us to treat the beads with TEV protease at the end of the synthesis to release megamolecules from the solid support and then determine the purity of the intermediates and final product. In the formation of the tetrameric structure (C−SC−SC−S) and the pentameric structure (C−SC−SC−SC−S), we capped the scaffold with a monovalent SnapTag domain, which prevented further elaboration.

Figure 2B shows sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the unpurified intermediates and products in the construction of the linear pentamer. We also confirmed the masses of these products by electrospray ionization mass spectrometry (ESI-MS) (Figure 2C, Table S2). We can infer from the highest density band in each gel that the addition of TEV protease at the conclusion of the synthesis selectively cleaves the linear scaffold from the solid support, liberating a single megamolecule in high purity. Further, imaging by transmission electron microscopy (TEM) reveals species of 19 nm (Figure 2D, Figure S6) and 25 nm (Figure 2E, Figure S7) in length for the tetramer and pentameric products, respectively. The range of structures observed by TEM reveal that the molecules are not rigid, but have conformational flexibility, and is consistent with previous work that demonstrates that linear megamolecules are conformationally flexible.[4]

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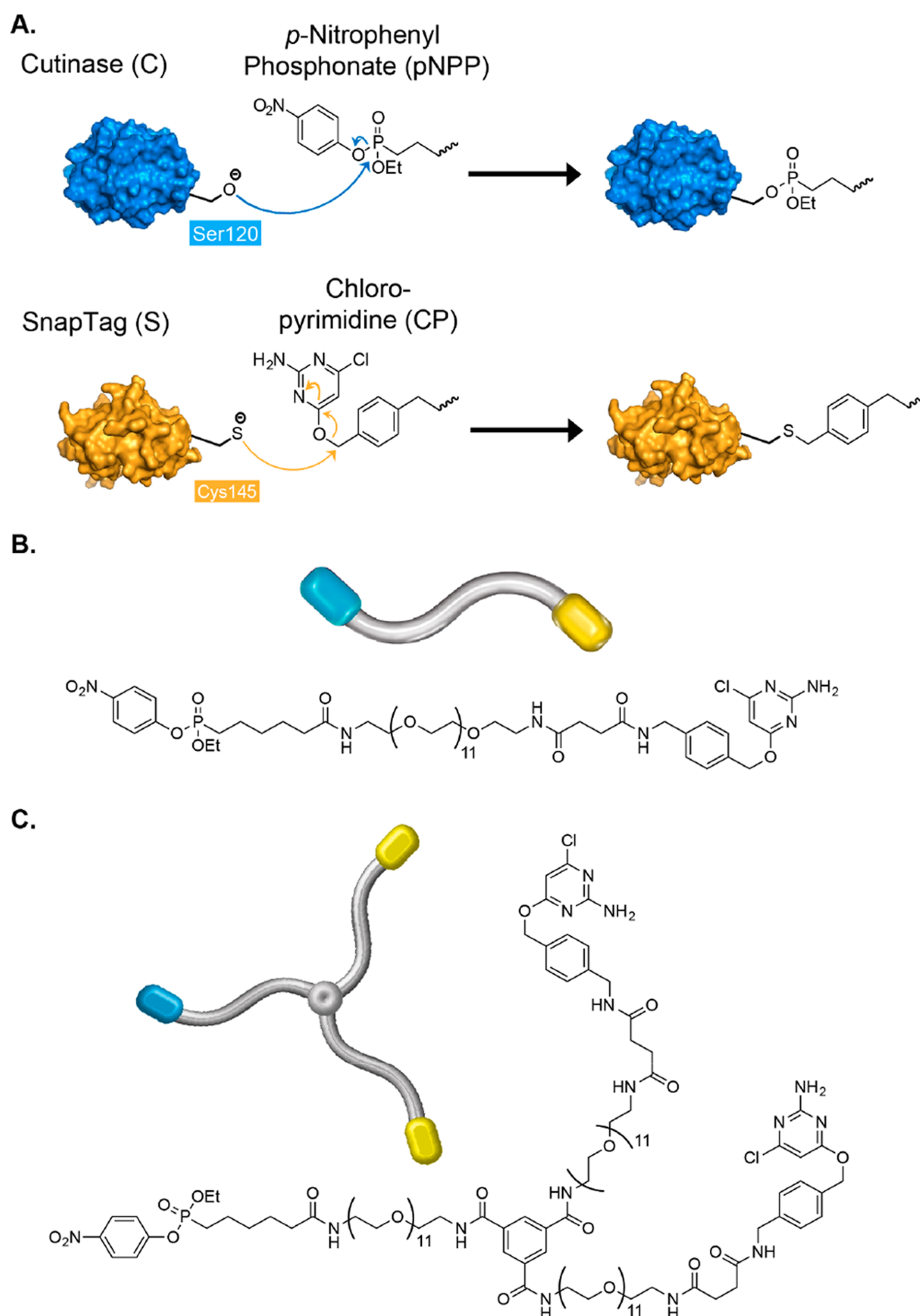

Figure 1. Enzymatic reactions and linkers. (A) *Top*: Cutinase reacting with a *p*-nitrophenyl phosphonate (pNPP) group. *Bottom:* SnapTag irreversibly reacting with a chloro-pyrimidine (CP) inhibitor. (B) Bifunctional linker having an ethylene glycol (EG) backbone with pNPP (blue) and a CP (yellow) groups. (C) Trifunctional linker terminated in one pNPP and two CP covalent inhibitors with an EG backbone.

One important aspect of this solid-phase synthesis method is that it does not require the use of protecting groups and deprotection steps,[1,3,4,10] which are necessary in the solid-phase synthesis of DNA,[11,12] peptides,[13,14] oligosaccharides,[15,16] and other molecules,[17−19] though we recognize there are a larger number of monomers used in those syntheses. This is because the covalent inhibitors on the linkers are poorly reactive with functional groups found on the surface of proteins and only react efficiently when they are present in the active site of the target enzyme. Indeed, this "self-activation" confers a high level of specificity in the reactions and eliminates the need for protecting groups.[20]

Recently, Howarth and co-workers constructed protein scaffolds using a similar approach on a solid support.[21] However, the molecules were anchored to the support noncovalently, which can result in molecules washing off of the support between reactions. Additionally, the method is limited to the assembly of linear structures, as the domains are connected by isopeptide bonds.[22] We next demonstrate that megamolecules can overcome this restriction by using linkers that give rise to branched megamolecules and, by extension, a range of more complex structures.

We synthesized a heterotrifunctional linker that contains a terminal pNPP and two terminal CP ligands, and which is described in the Supporting Information (Figure 1C, Figure S1). We treated the beads with C*S and then added the trifunctional linker to branch the scaffold, doubling the number of reaction sites per megamolecule chain. We then added an

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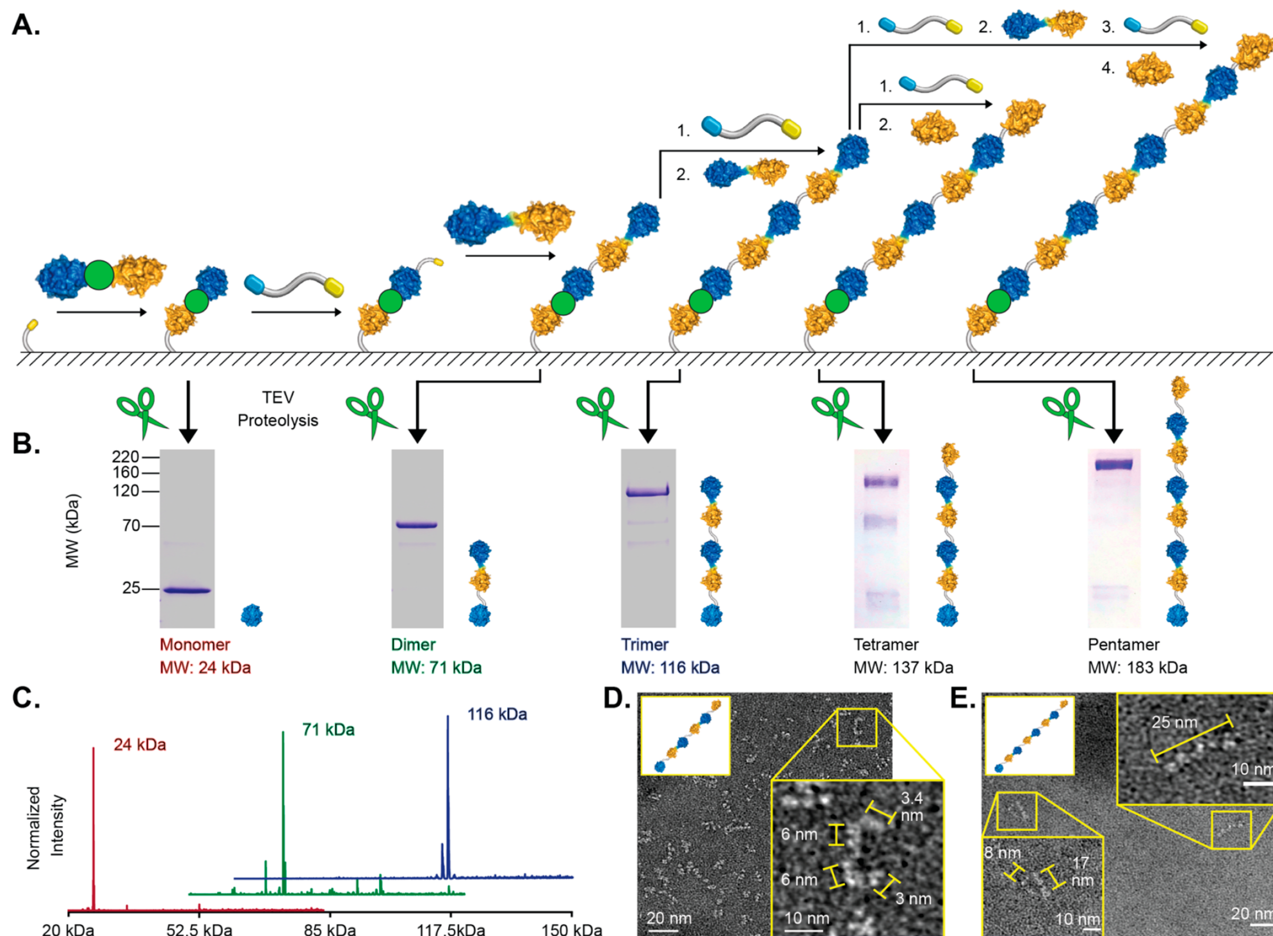

Figure 2. Solid-phase synthesis of linear megamolecules on a resin bead. (A) C*S is added to the BG functional group (yellow) on the agarose resin where SnapTag (yellow fusion domain) is pulled down to the surface. After washing, cutinase (blue fusion domain) is irreversibly reacted with a heterobifunctional linker (blue end, pNPP; yellow end, CP). The beads are washed, and the next protein (CS) is added to the linear scaffold. The construct is cleaved from the resin by proteolysis (green scissors, TEV protease) at the TEV protease site on the first bound protein (green circle). (B) SDS-PAGE characterization. (C) ESI-MS data. (D) TEM of the linear tetramer with insets of schemes and a selected image. (E) TEM of the linear pentamer with insets.

excess of CS to react with the two pendant CP groups on the beads, thus forming a first generation (G1) dendron. We continued the synthesis by adding 2 equiv of trifunctional linker to again double the number of reactive sites per chain and then finished the construction by adding 4 equivalents of the monomeric SnapTag domain. This resulted in a second generation (G2) dendron (Figure 3A). Analysis of the crude products by SDS-PAGE in Figure 3B reveals bands that migrate with the expected mobility of the constructs in the gel. The molecular weights calculated for each megamolecule are also in agreement with those obtained by mass spectrometry (Figure 3C, Table S2).

We note that while we expanded the number of active sites on the megamolecule through branching, we did not observe side products arising from intermolecular reactions between neighboring chains. This demonstrates the orthogonality and specificity of the enzyme−inhibitor reactions used in our megamolecule synthesis.

We characterized the size of the branched structures with TEM, taking into consideration that negative staining and drying procedures used during grid preparation often induce morphological changes to the molecules such as reducing their apparent size (Figure 3D).[23,24]

We also asked whether there was a relationship between the purity of the product and the loading density of the first fusion protein on the resin. Because the branch point would increase the size of the megamolecule, steric hindrance between neighboring strands could become significant for higher synthesis densities. We found this to be the case. We diluted the synthesis sites by introducing monomeric SnapTag protein during the coupling of the C*S fusion protein, and found that as more monomeric SnapTag is added, there was a decrease in the amount of product formed, but a corresponding improvement in the purity for the full-length product. This suggests that adjusting the functional surface density of the bead minimized steric congestion between scaffolds as the structures were extended (Figure S4).

Megamolecules are a new class of structures that are exciting because they are very large yet atomically perfectly defined. The modular synthesis allows the incorporation of a variety of functional domains, including organic/inorganic molecules, protein domains, and nucleic acids, among other groups. We believe that the controlled fabrication of megamolecule scaffolds is relevant to the field of self-assembled viral nanoparticles,[25−28] enzyme prodrug therapy,[29,30] and analogues of therapeutic antibodies. The ability to prepare candidate structures with solid phase approaches will expand

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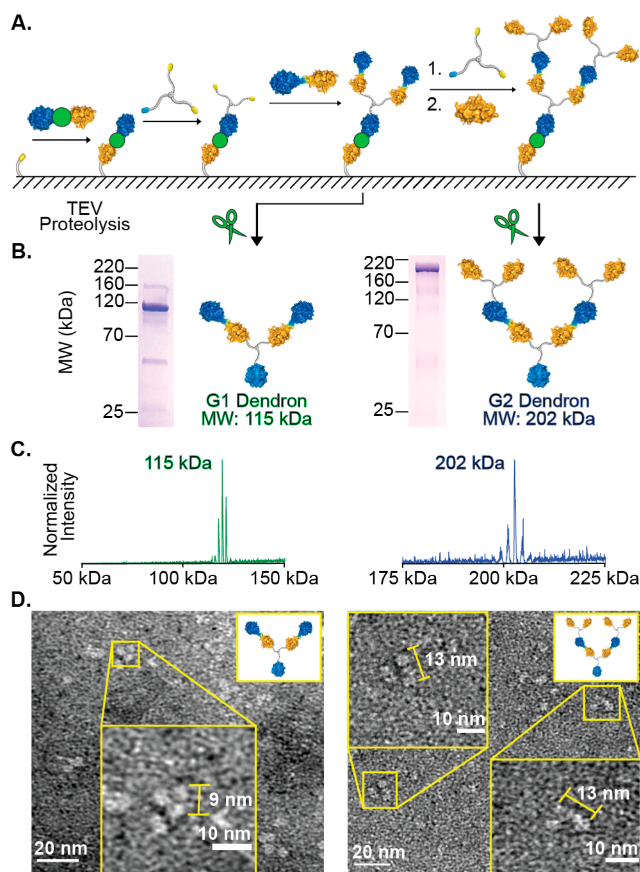

Figure 3. Construction of branched megamolecules. (A) Scheme for the synthesis of dendrons using a heterotrifunctional linker. (B) SDS-PAGE characterization of the panel directly from the crude reaction mixture and without purification by chromatography. (C) ESI-MS spectra of a G1 dendron and a G2 dendron. (D) TEM characterization of the structures with insets of schemes and selected images.

the rate at which megamolecules can be tested and will increase their development for a variety of applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b12003.

Synthesis, characterization, and experimental methods for the solid-phase synthesis of megamolecules (PDF)

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Notes

The authors declare no competing financial interest.

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