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Surface Modification of Tobacco Mosaic Virus with "Click" Chemistry

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Cu^{I}-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction,[1,2] renascent of the well-known Huisgen reaction,[3] has recently flourished with applications in organic synthesis, drug discovery, polymer and materials science, and biotechnology.[4–7] The high reaction yield, simple reaction and purification conditions, wide range of solvent and pH stabilities, and functional group tolerance make the CuAAC reaction a prototypical "click chemistry",[8] ideal for incorporating functionalities onto desired scaffolds. Over the years it has been widely employed to construct and functionalize polymeric and polyvalent display systems, including polymers,[9–16] dendrimers,[17,18] nanoparticles,[19] and surfaces,[20–22] where an extremely high reaction efficiency for every unit reaction is desirable.[23,24] In particular, as organic azides and alkynes are almost unreactive with biomolecules and water, CuAAC reactions have been employed in derivatizing biomacromolecules,[25,26] viruses,[27–29] and cells[30–32] with high efficacy under mild reaction conditions.

Recently tyrosine residues have been considered as a particularly attractive target for chemoselective modification of proteins because of its subabundant distribution. Francis and coworkers have reported a number of transformations, ranging from the Mannich-type reaction to a transition metal mediated allylation reaction to a diazonium-coupling reaction, which can efficiently target the phenolic group of tyrosine residues at physiological conditions.[33–36] To overcome the sluggish reactivity with electron-enriched diazonium salts, a sequential reduction/oxidation/Diels–Alder reaction was developed to break the limitation of functionalities being incorporated.[36] In this communication, we report that CuAAC reactions can be combined with a diazonium-coupling reaction to quantitatively functionalize tyrosine residues with a wide array of starting materials.

Tobacco Mosaic Virus (TMV) is a classic example of rodlike plant viruses consisting of 2130 identical protein subunits arranged helically around genomic single RNA strand. The length of TMV, that is, 300 nm, is defined by the encapsulated genomic RNA that stabilizes the coat protein assembly. The polar outer and inner surfaces of TMV have been exploited as templates to grow metal or metal oxide nanowires,[36–43] and conductive polymers have been coated on 1D assembled TMV to produce conductive nanowires.[44,45] TMV based materials have recently shown great potential with applications in nanoelectronics and energy harvesting devices.[46–48] In addition, it has been reported that tyrosine residues (Y139) of TMV are viable for chemical ligation using the electrophilic substitution reaction at the *ortho* position of the phenol ring with diazonium salts.[17] This reaction is very efficient, yet has two distinct disadvantages for broader applications. First, it is difficult to synthesize desired starting materials; and second, the reaction is not compatible with acid-labile functional groups and suitable for electron-deficient anilines only. To embrace the structural diversity of various starting materials, TMV offers an ideal polyvalent display system which allows us to test the efficiency of CuAAC reaction in combining with the tyrosine ligation reaction.

As shown in Scheme 1, TMV was first treated with the diazonium salt generated from 3-ethynylaniline **1** in situ adapted from the protocol reported by Francis and co-workers.[36] MALDI-TOF MS analysis indicated that $>95\%$ of the capsid monomers were converted into alkyne derivatives **2** (Figure 1) despite the absence of a strong electron withdrawing group in the diazonium reagent.[49] Encouraged by this result, the CuAAC reactions between **2** and azides were explored. For bioconjugation reactions using CuAAC, the Cu^{I} catalysts are either generated directly by addition of Cu^{I} salts,[28,31] or in situ from soluble Cu^{II} sources and a reducing agent, such as a copper wire, phosphines, thiols, or ascorbate.[27] Multidentate heterocyclic ligands are often required for enhancing the reaction efficiency.[27–29] Upon screening a series of reaction conditions, we found that the combination of $CuSO_4$/sodium ascorbate (NaAsc) gave the best results. Whereas it is destructive to most other protein complex systems,[27] ascorbate is evidently benign to TMV and has no impact on its structural integrity.

3-Azido-7-hydroxy-coumarin **a** was first employed as the azido counterpart in the reaction, which could be easily monitored by UV-visible absorption at 340 nm (Figure 1B).[50] As a general protocol, **2** (2 mg mL^{-1}) and **a** (3 mM) were added to a solution of $CuSO_4$ (1 mM) and NaAsc (2 mM) in Tris buffer (10 mM, pH 7.8) with 20% DMSO (used to increase the solubility of the azide component). After incubation for 18 h at room temperature, the viral particles were separated from the small molecules by sucrose gradient sedimentation. The integrity of TMV was confirmed by TEM and size-exclusion chromatography (SEC) analysis (data not shown). A strong absorption at 340 nm indicated the successful attachment of coumarin motifs (Figure 1B). MALDI-TOF MS analysis indicated a near quantitative transformation of surface alkynes to triazoles as shown in Figure 1A.

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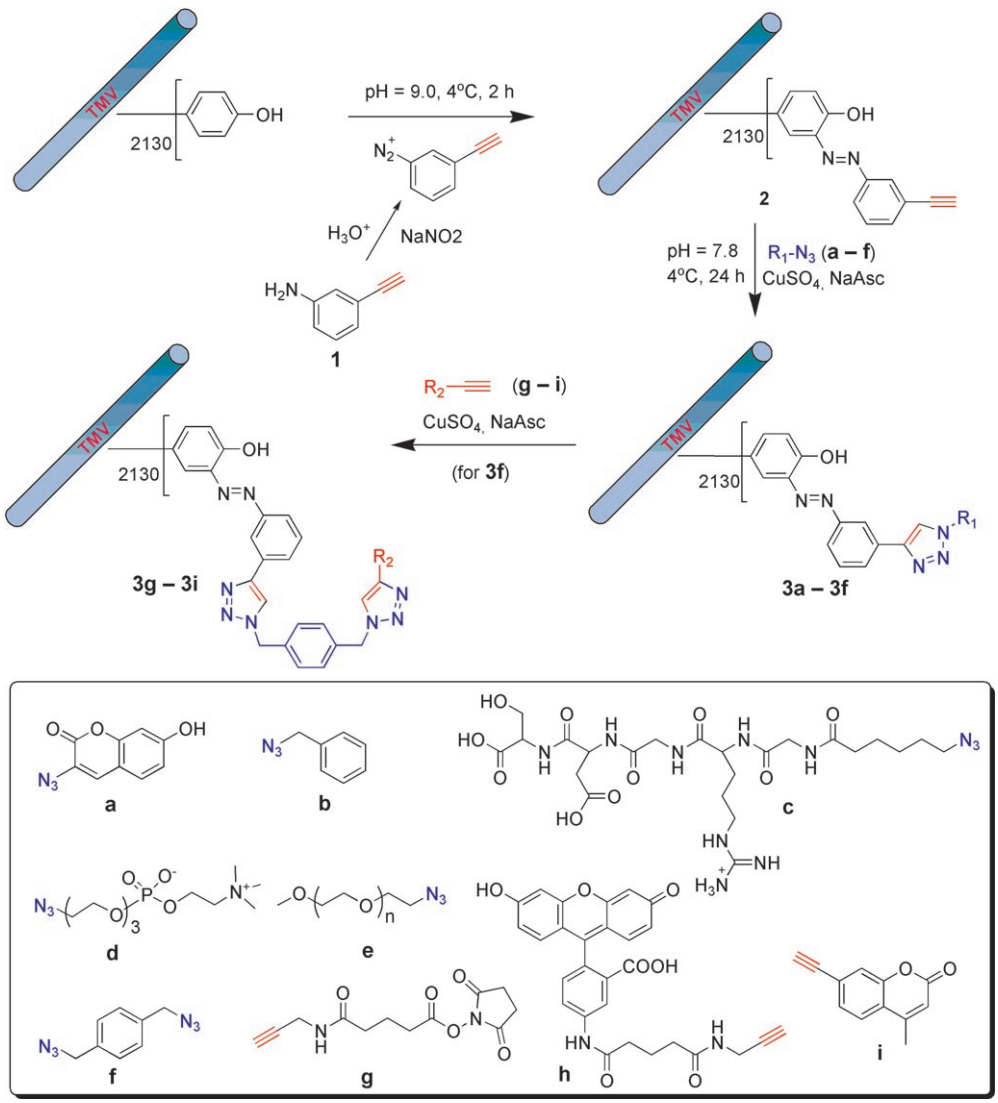

Scheme 1. Bioconjugation of TMV by means of CuAAC reactions.

Using the same protocol, a wide selection of azido compounds, ranging from small molecules to peptides to polymers (**a**–**e**), were coupled onto the TMV surface, all with high reaction efficiencies as confirmed by MALDI-TOF MS (Figure 1 A). Furthermore, when **2** was treated with a 1:1 mixture of two structurally distinctive azides, **c** and **d** (biologically relevant groups), the final product displayed almost equal amounts of the two motifs (Figure 1 C). The unequal peak heights are a property of the instrument itself. This result highlights that CuAAC reaction efficiency is independent of the structure of the starting materials. This makes CuAAC reaction very attractive for the site-directed incorporation of structurally-distinctive functionalities throughout a simple reaction protocol, which will have a beneficial impact on future cell binding studies. The attachment of **e** to the surface of TMV required the use of a larger excess of copper catalyst. One explanation for this requirement is the high binding affinity of copper to the polyethylene glycol backbone, therefore lowering the efficiency of the catalyst. The attachment of this polymer to the viral particle is monitored by MALDI-TOF MS, as can be seen in Figure 1 A. The poor resolution of the modified peak can be attributed to the poor ionizability of the polymer modified protein. The conversion of **2** to the product **3 e** is estimated based on the comparison of peak heights of **2** (at ~17 662 *m/z*) and TMV (at ~17 534 *m/z*) in the spectrum. The disappearance of the peak of **2** (related to the peak of TMV) implies the complete consumption of the starting materials and a near quantitative reaction.

The direct incorporation of an azide to tyrosines using 3-azidobenzenediazonium was attempted without success, likely due to the weak reactivity. We therefore performed the conjugation of 1,4-bis-azidomethylbenzene **f** to the surface of TMV to anchor an azide handle to the particle (**3 f**). This new product provides the opportunity to do a second CuAAC reaction using small molecules with terminal-alkyne functionality (**g**–**i**, Scheme 1), thus further broadening the scope of possible small molecules and polymers used for TMV derivatization. Again, TEM and SEC showed that TMV particles were intact after the sequential CuAAC reaction (Figures 2 B and 2 C). For **3 g** and **3 h**, the transformations were very efficient based on the MALDI-TOF MS analysis. Both mass spectra show the disappearance of the starting material peak, **3 f** (~17 848 *m/z*), and the emergence of new peaks of products (for **3 g**, 17 931 *m/z* and 18 037 *m/z* corresponding to the acid and the NHS-ester, respectively (the acid peak results from the dissociation of the product in solution or during the ionization process). The lack of reactivity of fluorogenic coumarin **i** is likely due to its low solubility in water whereas a considerably high quantity of starting materials **3 f** still remained in the reaction mixture.[51] It should be noted that in both steps of CuAAC reactions, the concentration of the small molecular reactants were as low as 0.5 mM without losing the coupling efficiency.

To explore potential applications of TMV as a multivalent scaffold[52] to probe cellular response, this tyrosine-ligation/CuAAC conjugation protocol was employed to program the surface properties of TMV with a variety of groups that promote or inhibit cell binding. NIH-3T3 fibroblast was employed in our preliminary study because of its sensitivity towards sur-

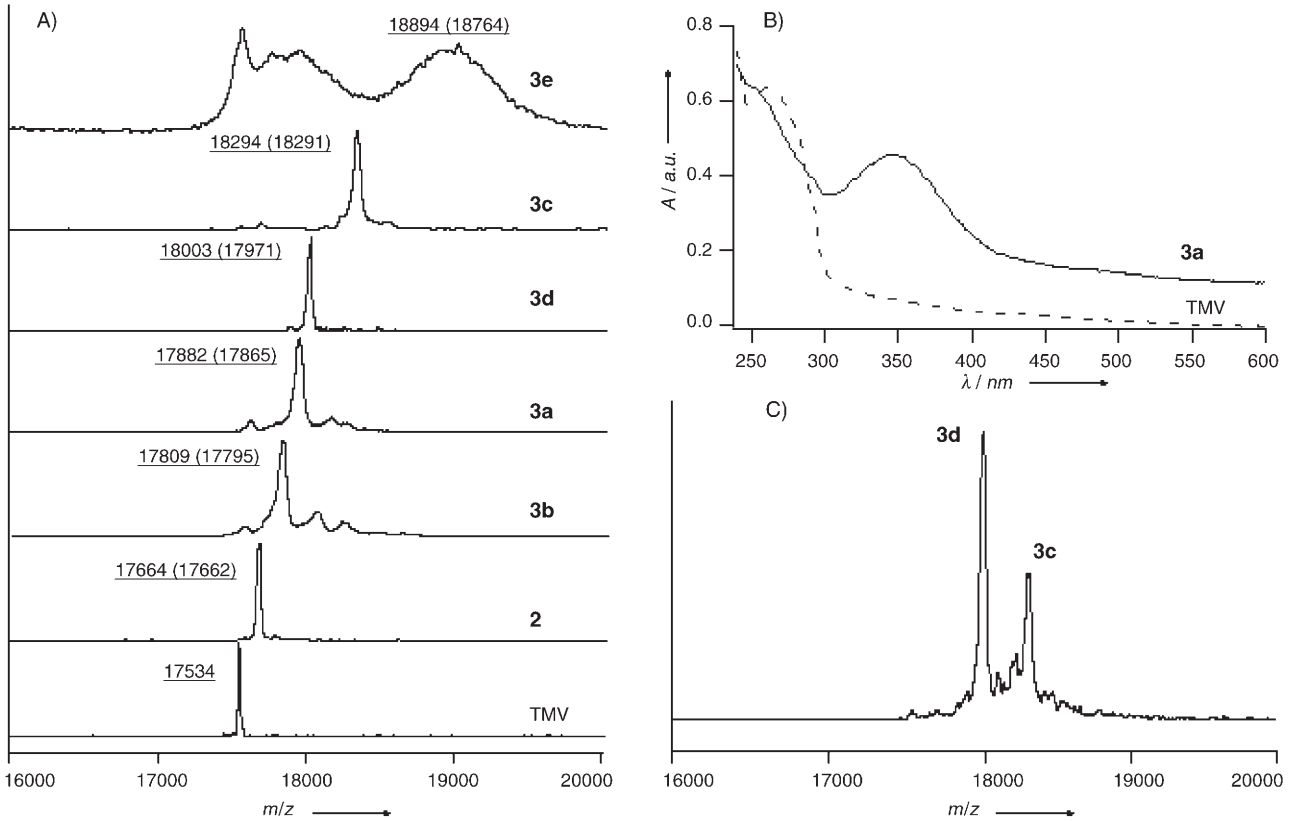

Figure 1. A) MALDI-TOF MS spectra of the subunit protein of native TMV (17534 *m/z*), acetylene-TMV **2** and CuAAC reaction products **3a**–**3e**. B) UV-Vis analysis of TMV and coumarin product **3a.** C) MALDI-TOF MS of dual modification of TMV with a 1:1 mixture of **c** and **d** as starting materials. The numbers in parentheses refer to the expected masses.

face chemistry and the distinctive cell morphology during the adhering process. Native TMV, **2c**, and **2e** were deposited on silane coated glass surfaces and coverage of viral particles was checked by atomic force microscopy (data not shown). Each sample was seeded with 1×10^4 NIH-3T3 fibroblast cells. In one hour, cells formed stress fibers on glass coated with TMV and **2c**. After 5 h, little to no cells was observed on **2e** coated glass whereas cells had attached and spread on surfaces coated with **2c**. Similar results were observed with 24 h cultures (Figures 3A–C). Cell counts on each surface indicated a significant increase in cell attachment and survival on RGD modified TMV coated slides as compared to native TMV coated slides (Figure 3D). The result demonstrates the feasibility of modulating cell behavior grown on TMV-based scaffolds modified by CuAAC reaction. Future studies will focus on the use of a variety of ligands that affect the binding properties of a multitude of cell lines with the end goal being the optimization of conditions (bionanoparticle, peptide, and surface) for all different cell lines.

Using aryldiazoniums to catch the *ortho*-position of phenolic group of tyrosines is a well-known method for protein bioconjugation. However, this reaction has two distinct disadvantages. First, synthesis of desired starting materials is difficult; and second, the reaction is only suitable for electron-deficient anilines, which dramatically impedes its potential applications. Herein, we demonstrate that an alkyne group can be quantitatively attached to tyrosine residues by diazonium-coupling and a sequential CuAAC reaction with azides can efficiently conjugate a wide range of compounds to the surface of TMV. These azides have shown to retain their functions after attachment to the rodlike viral scaffold as shown in the cell binding study. Future work is focused on using this unique scaffold with various other peptides and multiple cell lines. The mild and biofriendly conditions of CuAAC reaction ensure the compatibility of this bioconjugation strategy. Current efforts are focused on the exploration of this method to other protein targets.

Experimental Section

General: All reagents were used as received. The virus was prepared as previously described.[45] Compounds **a**–**i** were synthesized according to literature protocols.[14,27,50,51] Unless otherwise noted, "buffer" refers to 10 mM potassium phosphate buffer, pH 8.0. Sucrose gradient ultracentrifugation separation of virus samples was performed on a 20 mL gradient (made of 40% (*w*/*w*) sucrose solution in buffer, frozen at $-20\,^{\circ}C$, and thawed before use) with centrifugation at 90000 *g* for 2 h with a Beckman SW41 rotor using a Beckman Optima™ L90K Ultracentrifuge. The concentration of unmodified virus was measured by absorbance at 260 nm; 0.1 mg mL^{-1} of TMV gives a standard absorbance of 0.3.[53] Modified virus concentrations were measured using a modified Lowry protein assay kit (Pierce). The molecular weight of a single subunit of wild-type TMV is 17,534 Daltons.

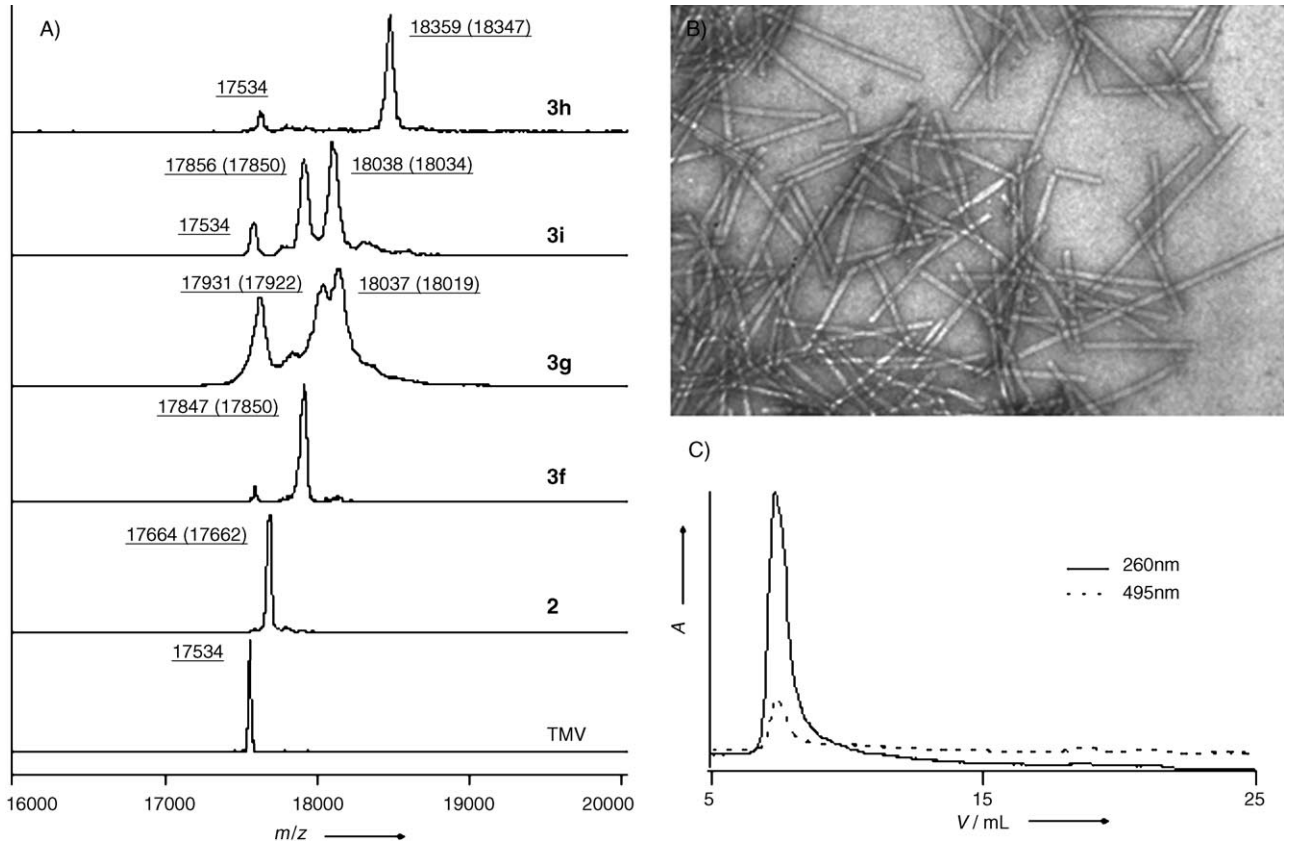

Figure 2. A) MALDI-TOF MS spectra of the subunit protein of CuAAC products **3 f**–**i**. B) TEM image and C) SEC diagram of **3 h** confirming that TMV is still intact after multiple rounds of conjugations. The numbers in parentheses refer to the expected masses.

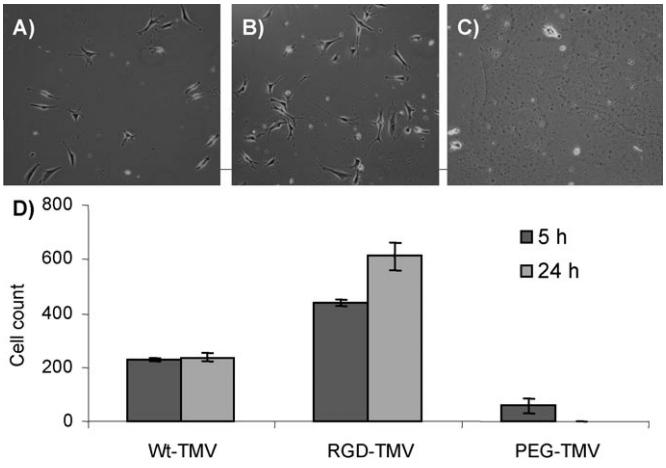

Figure 3. Optical images of NIH-3T3 cells grown on A) native TMV, B) **2 c** and C) **2 e** coated glass slides. D) The counting of adhered cells on above surfaces after growth of 5 h and 24 h.

Analysis: For MALDI-MS analysis, viruses were denatured by adding guanidine hydrochloride (6 μL, 6 M) to the sample (24 μL) and mixing for 5 min at room temperature. Denatured proteins were spotted on MTP 384 massive target plate using Millipore ZipTips$_{\mu C18}$® tips to remove excess salts and assist the binding of protein to the sinapic acid matrix. MALDI-MS analysis was performed using a Bruker Ultra-Flex I TOF/TOF mass spectrometer. TEM analysis was performed by depositing 20 μL of sample onto 300-mesh carbon coated copper grid for 2 min. The grids was then stained with 2% uranyl acetate and viewed with a JEOL 100 CX II transmission electron microscope. FPLC analysis was performed on an AKTA explorer (GE Amersham Pharmacia Biotech) instrument, using a Superose-6 size-exclusion column. Buffer used was 50 mM potassium phosphate buffer (pH 7.0) with 150 mM NaCl. Intact particles showed an elution volume of approximately 7.5 mL and broken particles eluted further downstream at 20 to 25 mL.

General protocol of CuAAC bioconjugation: The synthesis of **2** was modified from the protocol reported by Francis et al.[36] For standard CuAAC reactions, azide (100 mM, 55 μL) and a solution **2** (15 mg mL^{-1}, 265 μL) were mixed with Tris buffer (10 mM, 660 μL, pH 8.0). Then solutions of $CuSO_4$ (100 mM, 10 μL) and NaAsc (200 mM, 10 μL) were added and the mixture was incubated at room temperature for 18 h. The reaction mixture was purified via a 10–50% sucrose gradient from which the light scattering region was collected. The modified virus was then pelleted using ultracentrifugation at 160000 *g* for 2.5 h. The pellet was dissolved in buffer and characterized by UV-Vis, FPLC, MALDI-TOF MS, and TEM.

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