CHAPTER ELEVEN

Expression of Cellulolytic Enzyme as a Fusion Protein That Reacts Specifically With a Polymeric Scaffold

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Abstract

The formation of higher-order assemblies of multiple proteins or enzymes is a general mechanism to achieve more sophisticated biological function in biological systems. For example, cellulosomes are large complexes consisting of multiple cellulolytic enzymes that rely on the concerted actions of different enzymes built onto a common protein scaffold to facilitate the breakdown of the polymeric substrate, cellulose. One strategy for mimicking these highly effective nanomachines may involve the use of synthetic scaffolds that can react to and organize multiple engineered enzymes to promote synergistic action between the enzymes on the scaffold. As an example of the earlier strategy, we describe here an approach for the expression of cellulolytic enzymes with a serine esterase tag, and the rapid reaction between the tag and the end-functionalized polymers to form enzyme–polymer–enzyme multienzyme conjugates. In principle, this general and versatile supramolecular approach may be used to organize specific

Methods in Enzymology, Volume 590
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2016.12.003

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cellulolytic enzymes onto synthetic scaffolds to form multienzyme complexes to potentially work in synergy for enhanced biological activities. Best reaction conditions, good activities of the armored cellulolytic enzymes and the design of optimal protein linker in the fusion protein are discussed in detail. If other reactive tags are included on the enzyme in future, multiple types of synergistic enzymes may be positioned at specific sites on a designed polymer scaffold that mimics the complex structure and enhanced function of natural cellulosomes. This type of nanoarmoring of multiple enzymes on a nanoscale might also enhance enzyme stability, when compared to the unprotected enzymes.

1. INTRODUCTION

The successful synthesis and characterization of fusion proteins assembled onto a polymer nanoscaffold at defined locations/geometries, as a test case for the assembly of large, catalytic supramolecular assemblies, are described here. Recent discoveries in chemical biology have revealed that higher-order assemblies of multiple intracellular proteins, also known as "signalosomes," are capable of rather sophisticated, machine-like function in signal transmission and transduction (Bienz, 2014; Wu, 2013). Formation of higher-order protein assemblies may be a general mechanism for cells to achieve and regulate specific biological functions (Norris et al., 2007; Wilson & Gitai, 2013). Interestingly, extracellular proteins and enzymes can also form higher order, structured assemblies or machines in order to accomplish more challenging tasks (Amar et al., 2008; Norris et al., 2007).

Cellulosome, for example, is a multienzyme complex comprising of a noncatalytic protein scaffold (scaffoldin) onto which a number of cellulolytic enzymes are anchored and organized into a large enzyme assembly (Bayer, Belaich, Shoham, & Lamed, 2004; Fontes & Gilbert, 2010). The presence of similar and different multiple catalytic units in the cellulosomes make them function in a concerted fashion to catalyze the conversion of highly complex, insoluble, lignocellulose polymeric substrates into soluble sugars, which is an arduous and challenging task for individual cellulolytic enzymes (Moraïs et al., 2012). The enhanced functions of multiple copies of the same enzyme and different enzymes that are present in the cellulosome make it a formidable biocatalyst to match, where the function of one enzyme could depend on the function of another. The product from one enzyme, for example, might generate the necessary reactive site for another enzyme and thus function in a concerted fashion before the

biocatalyst is desorbed from the substrate surface. Mimicking such highly efficient, multicatalytic nanomachines is of considerate importance in developing new and more efficient green processes for applications in a variety of enzyme-based technologies (Mitsuzawa et al., 2009; Moraïs et al., 2012; Wilson, 2009).

However, the design and scale-up of large enzyme/protein/scaffold structures such as scaffoldins to serve as a framework to attach specific enzymes is challenging, but one promising alternative approach is the use of synthetic polymers or nanoparticles as scaffolds to organize multiple, synergistic enzymes into higher-order complexes. This can be done by specific high affinity binding or by selective chemical conjugation of the polymer scaffold to particular sites on the enzymes (Kamat et al., 2013; Sun & Chen, 2016; Zhang et al., 2013; Zheng et al., 2015). The use of polymer scaffold may have certain advantages for the assembly of multiple enzymes into functional biocatalysts over naturally occurring scaffoldins.

Numerous methods, for example, have been employed to prepare a variety of protein–polymer conjugates as described in this volume, or protein–nanoparticle conjugates in the previous volume of this series (Vol. 571, 2016) in support of this strategy, and these approaches include but not limited to: (1) the utilization of free reactive thiol, carboxyl, or amine groups that are naturally present on enzymes and proteins for coupling to specific reactive groups placed on the polymer/nanoparticle (Dieterich et al., 2007; Nilsson, Kiessling, & Raines, 2000; Rabuka, Rush, deHart, Wu, & Bertozzi, 2012); (2) incorporation of unnatural amino acids into the primary sequence of the enzyme for site-specific coupling to the scaffold (de Graaf, Kooijman, Hennink, & Mastrobattista, 2009; Dieterich et al., 2007); or (3) the use of a fusion protein carrying a protein or enzymatic tag that can covalently conjugate with multiple desired moieties on the scaffold and provides a good synthetic control to form large assemblies of protein–polymer conjugates of specific size, shape, or structure (Los et al., 2008; Rabuka et al., 2012; Sunbul & Yin, 2009; van Vught, Pieters, & Breukink, 2014). These nanoarmored enzymes in the supramolecular complex may also be more stable than individual enzymes due to the presence of polymeric scaffold surrounding the enzymes.

In this chapter, we outline the strategy of engineering a cellulase–cutinase fusion protein, and the attachment of the fusion protein to a synthetic, water-soluble polymer where the two ends of the polymer are functionalized with a chemical moiety that reacts irreversibly with

cutinase. Together, these form cellulase–cutinase–polymer–cutinase–cellulase biocatalysts in an optimized enzyme to polymer molar ratio (e.g., 2:1) or they form cellulase–cutinase–polymer complexes if polymer is used in a large excess, under complete synthetic control. These supramolecular enzyme–polymer complexes are fully active, further verifying the success of the earlier strategy.

The strategy of using fusion proteins with enzymatic tag that reacts with synthetic scaffolds containing suicide inhibitors positioned at strategic locations on the polymer scaffold confers many advantages in building artificial analogs of multienzyme assemblies (Sunbul & Yin, 2009; van Vught et al., 2014). The reaction, for example, specifically takes place at the catalytic site of the enzyme, resulting in the formation of highly specific, efficient, and stable covalent adducts between the fusion protein and the scaffold. Being selective and irreversible, the resultant complexes may not require subsequent purification steps, which can be especially challenging for the preparation of large protein–scaffold complexes (Hodneland, Lee, Min, & Mrksich, 2002). Other advantages include: (1) the use of end-functionalized polymers with the suicide inhibitors attached at specific location which can have different or distinct architectures such as linear, branched, star, or comb type to systematically explore the polymer scaffold structure and enzyme arrangement on the function of the multienzyme complex (González-Toro & Thayumanavan, 2013; McRae et al., 2012; Tao et al., 2009a, 2009b) and (2) the use of mild conditions in aqueous media for the coupling of the fusion protein with the polymer scaffold, as compared to other chemical reactions (Nwe & Brechbiel, 2009). The challenge associated with the use of such fusion proteins is to express the protein of interest with specific tags, without decreasing enzymatic activity of the corresponding fusion protein.

We have used the recombinant DNA technology to form the desired fusion protein, and the technology involves manipulation of DNA at the molecular level, along with rational design of the fusion construct, and using optimal conditions for the fusion protein production. Work in this area by numerous groups have provided effective solutions in expressing engineered proteins with full retention of functionality (Lambertz et al., 2014; Miller & Baxter, 1981). We expressed target proteins with an enzymatic tag that reacts specifically, irreversibly, and rapidly with synthetic scaffolds and these were shown to facilitate a modular design, good adaptability, and in certain cases, orientation of the target proteins on the scaffolds (Gauthier & Klok, 2010; Hodneland et al., 2002; Thordarson,

Le Droumaguet, & Velonia, 2006). Controlling the enzyme position, orientation, and composition of the catalytic complex is important in optimizing function.

Herein, we demonstrate the use of cutinase, a highly stable enzyme, that can be fused to a variety of target enzymes by recombinant DNA technology, as a bridge to attach cellulase to the polymer scaffold (Fig. 1). The cutinase tag is a 22 kDa serine esterase, consisting of a Ser–His–Asp catalytic triad (Martinez, De Geus, Lauwereys, Matthyssens, & Cambillau, 1992). The catalytic serine residue at the active site of the enzyme forms an irreversible covalent conjugate with phosphonate-functionalized oligomers or polymers (Bonasio et al., 2007; Dijkstra et al., 2008; Galbiati et al., 2015; Kwon, Han, Karatan, Mrksich, & Kay, 2004; Mannesse et al., 1995; Modica, Skarpathiotis, & Mrksich, 2012).

We discuss the design of a fusion protein comprising of cutinase tag with our target enzyme, a cellulase (Cel48F, family 48), from *Clostridium cellulolyticum* cellulosome (Fontes & Gilbert, 2010; Parsiegla et al., 2000) and the method to prepare functionally active Cel48F-cutinase fusion protein that can be site specifically linked with phosphonate-functionalized polymers (Fig. 1). For simplicity, we only show the characterization and analysis performed on the reaction of the fusion proteins to bifunctionalized synthetic scaffolds. By precisely controlling the number of reactive groups on the polymers (e.g., in a brush-like polymers), one may tune the number of cellulolytic enzymes being conjugated to the scaffold. In the current design, the polymer is end-labeled with two phosphonate tags so that one or two enzyme–cutinase moieties would react with one polymer scaffold, under complete synthetic control.

Advantages associated with this approach are its modular nature to extend the strategy to numerous other enzymes and the flexibility of decorating the polymeric scaffold with different functional groups and the use of different scaffold geometries to produce a variety of geometrical structures for the assembly. Additionally, the present assembly design mimics the natural cellulosome where the dockerin domain (unit that binds to scaffoldin) is replaced by the cutinase domain and scaffoldin is replaced by the synthetic polymer. If other reactive tags (e.g., SNAP tag, Keppler et al., 2003; aldehyde tag, Rabuka et al., 2012; and dehalogenase tag, Los et al., 2008) are included for bioorthogonal conjugation, multiple types of synergistic enzymes may be positioned at specific sites along the designed polymeric scaffold to mimic the structure and function of natural cellulosomes (Fig. 2).

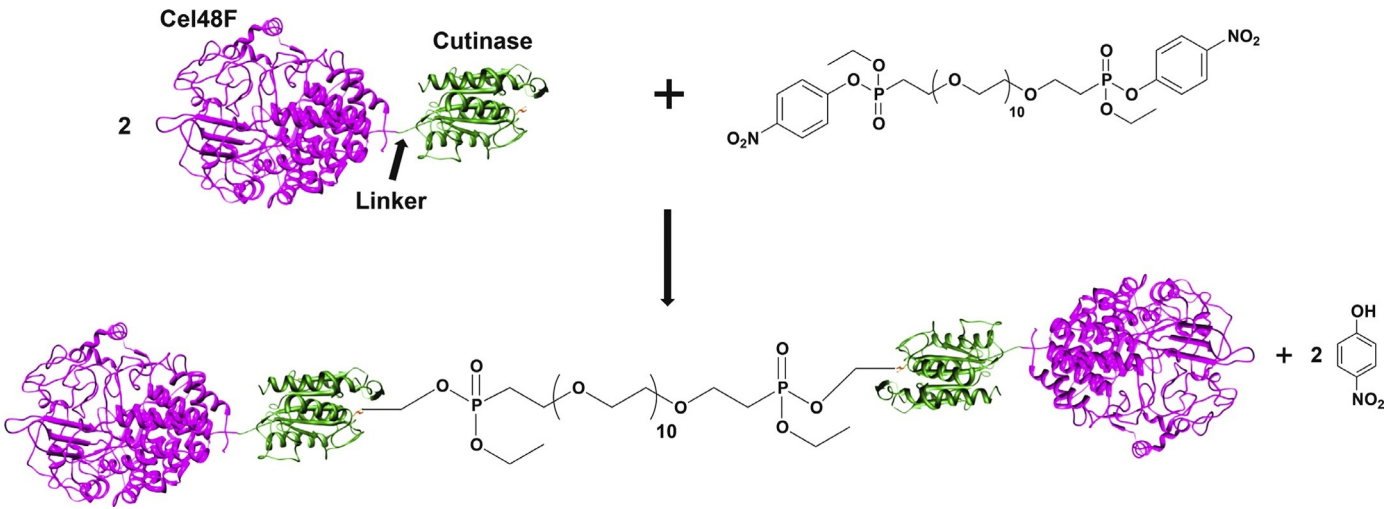

Fig. 1 Conjugation of cutinase-tagged Cel48F with bisphosphonate end-functionalized polymer. The fusion protein prepared from Cel48F (*pink*) and cutinase (*green*). A linker of (GGGGS) repeat, present within the two domains, provides the optimal flexibility to the two enzymes. The fusion protein reacts with the bifunctional ends of the polymer to form a protein–polymer–protein complex, with the release of *p*-nitrophenol.

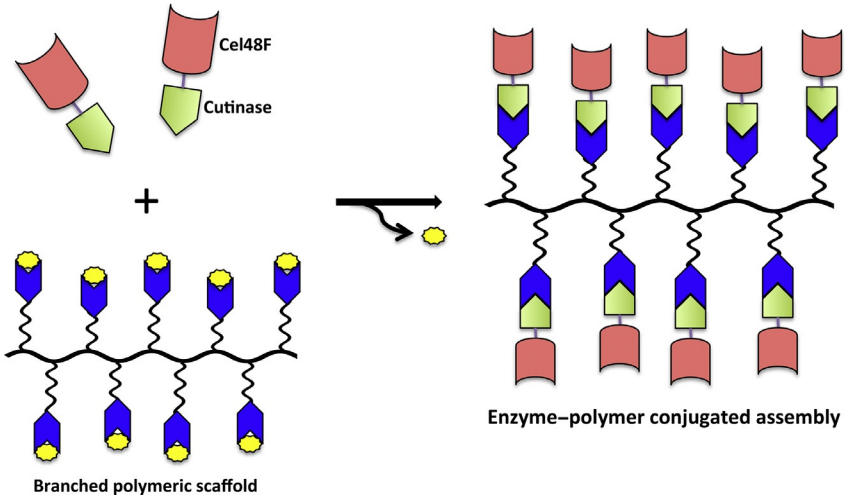

Fig. 2 Developing an artificial cellulosome by replacing a linear polymeric scaffold with a brush-like polymeric scaffold. Brush like polymers decorated with reactive moieties (shown as *blue ends*) can be tagged with cellulase–fusion proteins. The color generated by the release of the leaving groups (for example, *p*-nitrophenolate, in this specific case, shown as *yellow*) can be used to monitor the extent of conjugation in real time, as the conjugation reaction progresses.

2. MATERIALS

2.1 Personal Protective Equipments

Laboratory coat, nitrile gloves, safety glasses, fume hood, and UV blocking face shield. Properly labeled chemical waste receptacles and secondary containers, and biohazard sharps container are required as necessary.

2.2 Equipments

Heating block (Fisher Scientific Isotemp 125D), Incubator (New Brunswick Scientific), Benchtop mini centrifuge (Fisher Scientific accuSpin MicroR), 1.5 mL microcentrifuge tubes, 50 mL Falcon tubes, French Press, ÄKTA system (Amersham Biosciences), Magnetic stir bar, Stirrer, Gel electrophoresis apparatus, Gel imaging system with BioRad GelDoc system, Resource Q column (GE Healthcare #17-1177-01), Spectrophotometer, nutator mixer (Labnet Rocker 25), Micropipettors, Pipettor tips, dialysis bags, clips, precast gels, glass cuvettes, and amicon filters.

2.3 Reagents

Kanamycin, agar plates, isopropyl β-D-1-thiogalactopyranoside (IPTG), lysozyme, Terrific broth modified EZmix powder (Sigma), glycerol, Tris base, Tris maleate, sodium chloride, imidazole, calcium chloride, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), 2× Laemmli sample buffer (Bio-Rad Catalog #1610737), electrophoresis buffer, and Avicel (PH101, Fluka, Buchs, Switzerland). Origami 2 (DE3) chemically competent cells were purchased from EMD Biosciences. Ni-NTA agarose was purchased from Qiagen (Catalog #30230). Complete-Mini EDTA-free protease inhibitor tablets were purchased from Roche Applied Science.

3. METHODS

3.1 Molecular Cloning of Fusion Protein

The Cel48F-cutinase fusion protein was constructed by fusing the N-terminus of cutinase domain to the C-terminal end of catalytic domain of Cel48F, a cellulolytic enzyme (Fig. 1A). The newly constructed fusion protein has a C-terminal His tag with the two enzymes separated by a $(GGGGS)_2$ linker (Fig. 3). The N- and C-termini of Cel48F enzyme are opposite to the catalytic active site (Parsiegla et al., 2000), thus modification of either end would be suitable for conjugation. When cutinase is fused to a large cellulase domain, a flexible linker of (GGGGS) repeat at its N-terminus provides the optimal flexibility to the two enzymes, as compared to the construct without the (GGGGS) repeat. Compared to $(GGGGS)_1$, the cutinase activity is about an order of magnitude higher in the fusion protein with $(GGGGS)_2$ linker. Longer linkers of 3–4 repeats of (GGGGS) might further improve the cutinase activity within the fusion protein; however, care must be taken, as longer linkers are susceptible to proteolysis (Huang & Shusta, 2006) and may detach the enzyme Cel48F from the cutinase tag in the fusion protein or from the enzyme–polymer assembly.

The Cel48F plasmid was synthesized by GenScript Corporation, which was further ligated with DNA coding for the cutinase insert, as described previously (Modica et al., 2012). The product was cloned into pET26b vector via NdeI and XhoI, and the resulting plasmid (pCel48F-Cut) was transformed in Origami2 (DE3) strain. The transformed cells were placed on LB-agar plate supplemented with kanamycin (50 μg/mL) for 16–18 h at 37°C.

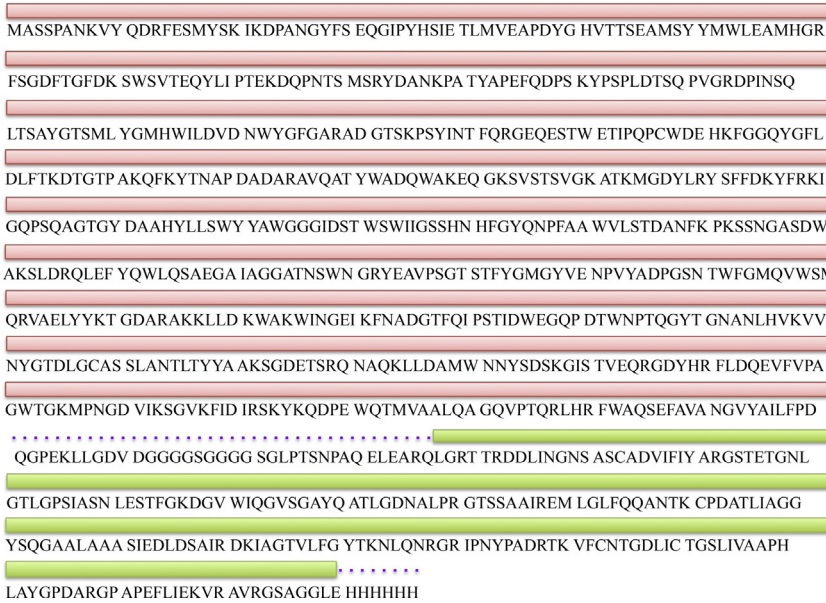

Fig. 3 Sequence of Cel48F-cutinase. The Cel48F (*red*) is linked with cutinase (*green*) by the $(GGGGS)_2$ linker.

3.2 Expression and Purification

Expression

1. Pick a single colony from the agar plate and suspend it in 50 mL Terrific Broth having Kanamycin (50 μg/mL) antibiotic. Allow it to grow for 12–16 h at 37°C with shaking at 225 rpm. This is referred to as seed culture.
2. Inoculate 1 L of autoclaved Terrific Broth with 10 mL of seed culture (1:100 dilution). Grow at 37°C with shaking at 225 rpm till it reaches OD = 0.6. Transfer the flask at 18°C and allow it to cool for an hour. Induce it with 20 μ*M* IPTG. Grow at 18°C with shaking at 225 rpm for 16 h.
3. Harvest cells by centrifuging at 4000 rpm for 40 min. Discard the supernatant and store the pellet at −20°C or use it immediately for further purification.

Useful tips

- The same seed culture can be used to prepare multiple batches, when expressed the same day. The cell pellets can be stored at −80°C for long term storage.

- Lower temperature and lower IPTG concentrations help in expression of Cel48F-cutinase as a soluble protein which otherwise is expressed in inclusion bodies. Once OD = 0.6 has been obtained, the flask should be cooled by using ice or by placing in a cold room, till the shaker reaches the desired temperature.

Purification

1. Resuspend the frozen pellet in 50 m*M* Tris, pH 8.0, 300 m*M* sodium chloride (lysis buffer). Add 3 mg lysozyme, 1 tablet of EDTA-free protease inhibitor, and 1 m*M* of PMSF. Incubate the cell suspension at room temperature for 20 min.
2. Lyse the cells using French press. Repeat the cell lysis cycle for four times. Remove the insoluble cell debris by centrifugation at 8000 rpm for 1 h at 4°C.
3. Preparation of affinity column: Take 2 mL Ni-NTA agarose resin in affinity column and decant the supernatant. Continue to wash the resin 3–5 times with lysis buffer to completely remove ethanol.
4. Load the lysate onto the affinity column and incubate with Ni-NTA agarose resin at 4°C for 1 h on a nutating mixer.
5. Wash the resin sequentially with 20 mL of lysis buffer with increasing concentrations of imidazole, ranging from 5 to 500 m*M*.
6. Check the fractions with SDS-PAGE (200 V, ~35 min).
7. Pool the fractions containing the target protein and dialyze against 1 L of dialysis buffer (20 m*M* Tris, pH 8.0) at 4°C.
8. Further purify the protein using Resource Q column on FPLC. Run a linear gradient (0–100%B) in 12 CV; 1.5 mL/fraction at a flow rate of 2 mL/min, using buffer A (20 m*M* Tris, pH 7.5) and buffer B (20 m*M* Tris, pH 7.5, 1 *M* NaCl).
9. Check the fractions on SDS-PAGE (an example is shown in Fig. 4).
10. Pool the fractions that contain the desired protein.

Useful tips

- If French press is not available, the cells can be lysed using Bug Buster solution, with sonication.
- The Ni-NTA beads can be washed, equilibrated, and incubated in a Falcon tube. Alternatively, His–Trap columns can be used with liquid chromatography system.
- Handling the protein at 4°C prevents its degradation and helps its long-term stability. The protein solution can be stored in 1 mL aliquots at −20°C. The aliquots should be thawed on ice or at 4°C.

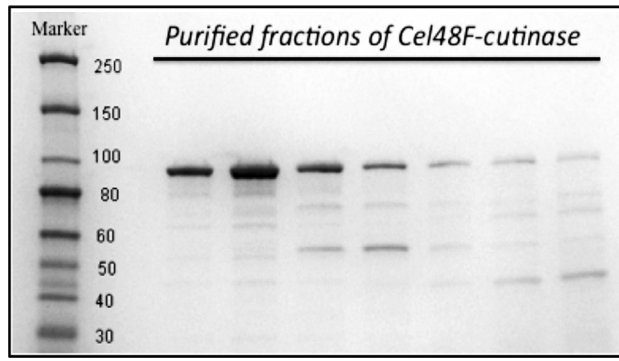

Fig. 4 SDS-PAGE gel of different fractions obtained after anion exchange of Cel48F-cutinase.

3.3 Conjugation of Cel48F-Cutinase

Conjugation of Cel48F with polymeric scaffold via the cutinase tag offers certain advantages including site specificity and formation of conjugates, which are resistant to hydrolysis. Apparently, one of the biggest challenges in conjugating polymers with cutinase-tagged fusion proteins is their access to the catalytic serine at cutinase active site. We investigated the conjugation of *p*-nitophenyl phosphonate-functionalized polymer with Cel48F-cutinase using UV–vis absorption spectroscopy. The rate constant of the reaction between the enzyme and the phosphonate polymer was obtained by following the release of *p*-nitrophenolate ($\lambda_{max} = 401$ nm) as a function of reaction time.

Kinetic study for conjugation

1. Prepare 4 μ*M* of Cel48F-cutinase in 20 m*M* Tris, pH 8, 100 m*M* NaCl.
2. Prepare 0.1 *M* stock solution of EG10 bisphosphonate polymer in DMSO. Dilute the stock solution to 200 μ*M* using the same buffer as above. At 1:50 ratio, the dimer formation is suppressed.
3. The polymer was added just before the UV analysis.
4. Monitor the release of *p*-nitrophenolate from the reaction at 401 nm using high throughput UV spectrophotometer for 15 min.

The resulting kinetic trace (Fig. 5) was fit to a pseudo first-order equation and the effective second-order rate constant was obtained by dividing these quantities by the concentration of excess ligand. The rate constant was $\sim 37\ M^{-1}\ s^{-1}$, confirming that cutinase domain in our engineered construct is capable of reacting with the phosphonate-functionalized polymers.

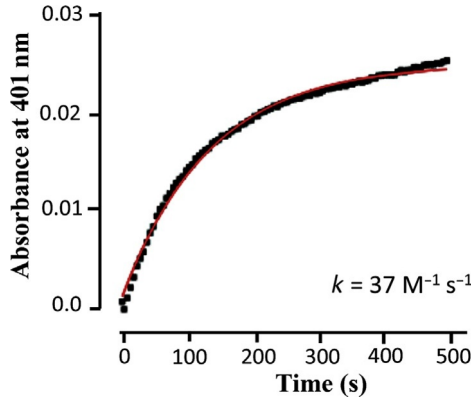

Fig. 5 Ligand functionalization of Cel48F-cutinase with EG10 bisphosphonate (polymer) and release of *p*-nitrophenolate as monitored by its absorbance at 401 nm. Data were fit to pseudo first-order kinetics (*solid line*) and divided by the concentration of excess species to yield the effective second-order rate constant.

Useful tips

- Amicon filters with 3 kDa cut-off were used for buffer exchange and caution should be taken to prevent protein aggregation during the buffer exchange.
- Hydrolysis of polymer, indicated by yellow color is observed even in the absence of protein. Thus, it is extremely important to perform a blank run (buffer with the polymer) simultaneously with the actual experiment, which is then subtracted from the data obtained from the actual run.
- The polymer should be added once everything is set. While adding the polymer, gently mix the polymer into the solution by pipetting the liquid up and down.
- A double beam UV–vis spectrophotometer or a high throughput UV–vis spectrophotometer eases data analysis.

Finding optimized conditions for the conjugation reaction

We further verified the conjugation of cutinase fusion protein with bifunctionalized polymer by varying the concentration of the polymer. We expected Cel48F-cutinase to conjugate at the two ends of the polymer linker and assemble into a dimeric assembly at low mole ratios while single site attachment is expected when the polymer is in excess.

1. Incubate 500 μL protein (5 m*M* Tris maleate, pH 6, 100 m*M* NaCl) with EG10 polymer at 25 and 37°C at different *E*:*L* ratio: 1:0, 1:1, 1:2, 2:1, 4:1, 1:10, 1:20.

2. Aliquot 50 μL of samples after 6, 24, 48, and 72 h.
3. Prepare 1:1 dilution of sample with Laemmli buffer.
4. Heat the samples at 70°C for 5 min.
5. Load 25 μL to the mini-protean gel and run at 200 V for 35 min.

SDS-PAGE analysis indicated that dimeric protein assemblies were formed at different protein: polymer ratios (Fig. 6). The lanes at 1:1, 1:2, 2:1, 4:1 ratios show the presence of an additional band of higher molecular weight, that is attributed to the dimeric protein–polymer conjugate (E_2L). When a higher concentration of polymer was used, no dimerization was observed (as in the case of 1:10 and 1:20) (data not shown). Theoretically, two moles of enzyme should react with one mole of bifunctionalized polymer so that the optimized mole ratio is 2:1. However, presumably due to some hydrolysis of the phosphonate moiety in solution, we observed an optimal ratio of 1:1, where a slightly higher concentration of polymer is required to facilitate a better yield for dimers. No dimerization was observed in the absence of polymer (lane 1:0), demonstrating that the dimer formation is a result of site-specific conjugation with the linker, resulting in the formation of enzyme–linker or enzyme–linker–enzyme conjugates.

Useful tips

- The protein solution can be diluted to the appropriate concentration and the same diluted solution can be used for all the samples by keeping the enzyme concentration constant.

3.4 Cellulase Activity Assay of the Fusion Protein

As we fused a cutinase tag onto the cellulase, it would be necessary to confirm the functionality of the protein, in particular its hydrolytic activity to breakdown cellulose. The activity of the Cel48F enzyme can be determined by high performance liquid chromatography (HPLC) analysis of the reaction products or by ferricyanide assay, using Avicel as a substrate. Avicel is hydrolyzed into simple sugars including glucose, which reacts with ferricyanide, and ferrous ammonium sulfate to give Prussian blue color. The color intensity was monitored at 690 nm, which is proportional to the amount of soluble sugars released upon Avicel hydrolysis (Park & Johnson, 1949).

Cellulase activity assay

1. Perform buffer exchange of Cel48F-cutinase with 20 m*M* Tris maleate, pH 6.0, 100 m*M* NaCl, 1 m*M* $CaCl_2$ using amicon filter (mol. wt. cut-off 30 kDa).
2. Incubate the protein with EG10 polymer at 1:1 ratio.

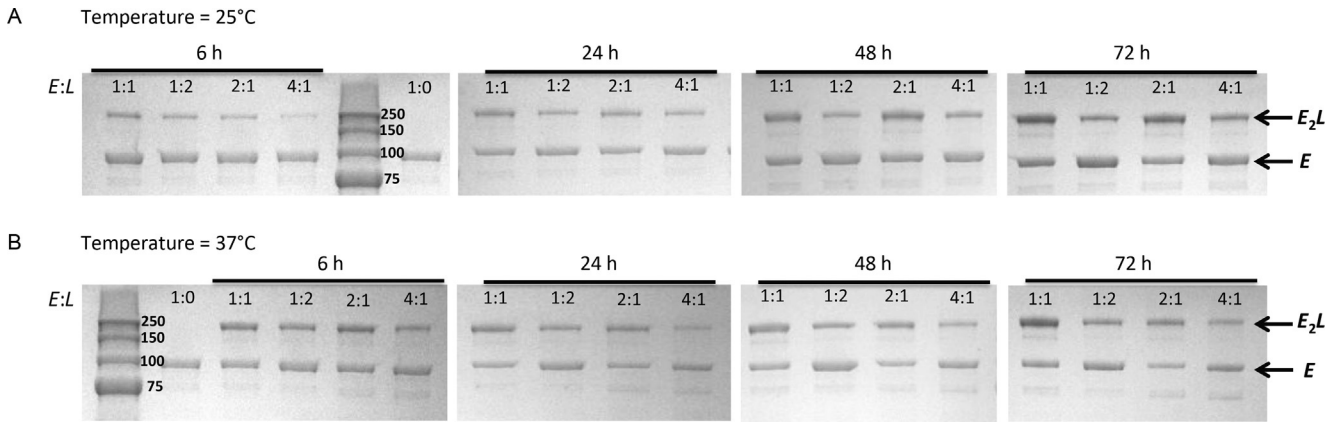

Fig. 6 SDS-PAGE analysis of Cel48F-cutinase with bifunctional polymer. The samples were incubated at different enzyme: linker (*E*:*L*) ratios for 6, 24, 48, and 72 h. The dimerization (E_2L) was observed at pH 6.0, at (A) 25°C and (B) 37°C. Gel images were cropped and resized for publication purpose only.

3. Incubate 1 μ*M* of protein in free and conjugated state with 4 mL of microcrystalline cellulose (Avicel PH101, Fluka, Buchs, Switzerland) at 20 mg/mL in 20 m*M* Tris maleate, pH 6.0, 1 m*M* $CaCl_2$ at 37°C.
4. Aliquot 0.6 mL at 0, 1, 5, and 24 h, centrifuge, and transfer the supernatant to a new 1.5 mL Eppendorf tube. Examine for soluble reducing sugars using ferricyanide assay.

Fig. 7 compares the absorbance at 690 nm at different time intervals by the same amount of proteins, either in the free state or in the conjugated state with bifunctionalized polymer. The hydrolytic activity of Cel48F is retained upon conjugation (red squares) and is comparable to that of the free enzyme (black diamonds).

Useful tips

- Set up the reaction in a falcon tube, which allows better mixing of cellulose.
- Cellulose tends to settle at the bottom of the tube. Before withdrawing the sample, gently mix the contents of the tube to ensure resuspension of any settled cellulose.
- The withdrawn sample is collected in a microcentrifuge tube. Centrifuge the tubes long enough so that Avicel forms a pellet at the bottom of the

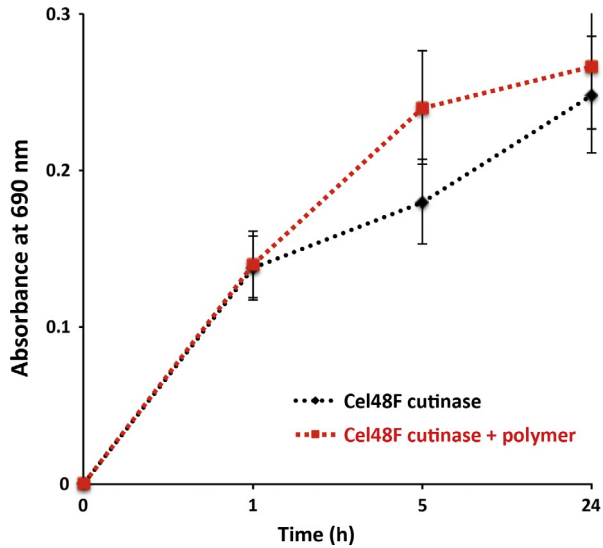

Fig. 7 Avicel hydrolysis by Cel48F-cutinase in free state (*black diamonds*) or conjugated with the polymer (1:1, *red squares*). Sugar release was followed by the ferricyanide assay after incubation at 37°C. The final enzyme concentration was 1 μ*M* in both free and conjugated states.

tube. Carefully transfer the clear supernatant to a new microcentrifuge tube. If the Avicel particles are being withdrawn with the pipette, it is advised to centrifuge the sample again.

4. FUTURE OUTLOOK

This chapter describes a strategy to create cutinase-tagged cellulase that is capable of conjugating with functionalized polymers to form higher-order enzyme–polymer nanoarmored complexes. The conjugated enzyme (Cel48F–cutinase–polymer) activity is comparable to that of its free form. The method may be easily adapted to include other enzymes, proteins, or polypeptide tags. Attaching other types of cellulases that work synergistically with Cel48F enzyme on the same scaffold may lead to enhanced enzyme activities/stabilities for cellulose to sugar conversion. Developing brush-like polymers having multiple reactive chain ends that can be used to conjugate with differently tagged cellulases, using bioorthogonal chemistry, will be interesting. The approach described here may ultimately lead to the preparation of multicatalytic nanomachines that can act in concert to carry out complex sequences of enzymatic reactions in a highly effective way.

ACKNOWLEDGMENTS

Y.L. acknowledges supports from the US Department of Energy, Office of Basic Energy Sciences (DE-SC0005039) and the US National Science Foundation (DMR-1150742).

REFERENCES

Amar, P., Legent, G., Thellier, M., Ripoll, C., Bernot, G., Nystrom, T., et al. (2008). A stochastic automaton shows how enzyme assemblies may contribute to metabolic efficiency. *BMC Systems Biology*, *2*(1), 1–13.

Bayer, E. A., Belaich, J. P., Shoham, Y., & Lamed, R. (2004). The cellulosomes: Multienzyme machines for degradation of plant cell wall polysaccharides. *Annual Review of Microbiology*, *58*, 521–554.

Bienz, M. (2014). Signalosome assembly by domains undergoing dynamic head-to-tail polymerization. *Trends in Biochemical Sciences*, *39*(10), 487–495.

Bonasio, R., Carman, C. V., Kim, E., Sage, P. T., Love, K. R., Mempel, T. R., et al. (2007). Specific and covalent labeling of a membrane protein with organic fluorochromes and quantum dots. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(37), 14753–14758.

de Graaf, A. J., Kooijman, M., Hennink, W. E., & Mastrobattista, E. (2009). Nonnatural amino acids for site-specific protein conjugation. *Bioconjugate Chemistry*, *20*(7), 1281–1295.

Dieterich, D. C., Lee, J. J., Link, A. J., Graumann, J., Tirrell, D. A., & Schuman, E. M. (2007). Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging. *Nature Protocols*, *2*(3), 532–540.

Dijkstra, H. P., Sprong, H., Aerts, B. N. H., Kruithof, C. A., Egmond, M. R., & Klein Gebbink, R. J. M. (2008). Selective and diagnostic labelling of serine hydrolases with reactive phosphonate inhibitors. *Organic & Biomolecular Chemistry*, *6*(3), 523–531.

Fontes, C. M., & Gilbert, H. J. (2010). Cellulosomes: Highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. *Annual Review of Biochemistry*, *79*, 655–681.

Galbiati, E., Cassani, M., Verderio, P., Martegani, E., Colombo, M., Tortora, P., et al. (2015). Peptide-nanoparticle ligation mediated by cutinase fusion for the development of cancer cell-targeted nanoconjugates. *Bioconjugate Chemistry*, *26*(4), 680–689.

Gauthier, M. A., & Klok, H.-A. (2010). Polymer–protein conjugates: An enzymatic activity perspective. *Polymer Chemistry*, *1*(9), 1352–1373.

González-Toro, D. C., & Thayumanavan, S. (2013). Advances in polymer and polymeric nanostructures for protein conjugation. *European Polymer Journal*, *49*(10), 2906–2918.

Hodneland, C. D., Lee, Y. S., Min, D. H., & Mrksich, M. (2002). Selective immobilization of proteins to self-assembled monolayers presenting active site-directed capture ligands. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(8), 5048–5052.

Huang, D., & Shusta, E. V. (2006). A yeast platform for the production of single-chain antibody-green fluorescent protein fusions. *Applied and Environmental Microbiology*, *72*(12), 7748–7759.

Kamat, R. K., Ma, W., Yang, Y., Zhang, Y., Wang, C., Kumar, C. V., et al. (2013). Adsorption and hydrolytic activity of the polycatalytic cellulase nanocomplex on cellulose. *ACS Applied Materials & Interfaces*, *5*(17), 8486–8494.

Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., & Johnsson, K. (2003). A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nature Biotechnology*, *21*(1), 86–89.

Kwon, Y., Han, Z., Karatan, E., Mrksich, M., & Kay, B. K. (2004). Antibody arrays prepared by cutinase-mediated immobilization on self-assembled monolayers. *Analytical Chemistry*, *76*(19), 5713–5720.

Lambertz, C., Garvey, M., Klinger, J., Heesel, D., Klose, H., Fischer, R., et al. (2014). Challenges and advances in the heterologous expression of cellulolytic enzymes: A review. *Biotechnology for Biofuels*, *7*, 135.

Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., et al. (2008). HaloTag: A novel protein labeling technology for cell imaging and protein analysis. *ACS Chemical Biology*, *3*(6), 373–382.

Mannesse, M. L., Boots, J. W., Dijkman, R., Slotboom, A. J., van der Hijden, H. T., Egmond, M. R., et al. (1995). Phosphonate analogues of triacylglycerols are potent inhibitors of lipase. *Biochimica et Biophysica Acta*, *1259*(1), 56–64.

Martinez, C., De Geus, P., Lauwereys, M., Matthyssens, G., & Cambillau, C. (1992). Fusarium solani cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. *Nature*, *356*(6370), 615–618.

McRae, S., Chen, X., Kratz, K., Samanta, D., Henchey, E., Schneider, S., et al. (2012). Pentafluorophenyl ester-functionalized phosphorylcholine polymers: Preparation of linear, two-arm, and grafted polymer–protein conjugates. *Biomacromolecules*, *13*(7), 2099–2109.

Miller, W. L., & Baxter, J. D. (1981). Synthesis of biologically active proteins by recombinant DNA technology. *Drug Development Research*, *1*(4), 435–454.

Mitsuzawa, S., Kagawa, H., Li, Y., Chan, S. L., Paavola, C. D., & Trent, J. D. (2009). The rosettazyme: A synthetic cellulosome. *Journal of Biotechnology*, *143*(2), 139–144.

Modica, J. A., Skarpathiotis, S., & Mrksich, M. (2012). Modular assembly of protein building blocks to create precisely-defined megamolecules. *Chembiochem: A European Journal of Chemical Biology*, *13*(16), 2331–2334.

Moraïs, S., Morag, E., Barak, Y., Goldman, D., Hadar, Y., Lamed, R., et al. (2012). Deconstruction of lignocellulosecbe into soluble sugars by native and designer cellulosomes. *MBio*, *3*(6), e00508–e00512.

Nilsson, B. L., Kiessling, L. L., & Raines, R. T. (2000). Staudinger ligation: A peptide from a thioester and azide. *Organic Letters*, *2*(13), 1939–1941.
Norris, V., den Blaauwen, T., Cabin-Flaman, A., Doi, R. H., Harshey, R., Janniere, L., et al. (2007). Functional taxonomy of bacterial hyperstructures. *Microbiology and Molecular Biology Reviews*, *71*, 230–253.
Nwe, K., & Brechbiel, M. W. (2009). Growing applications of "Click Chemistry" for bioconjugation in contemporary biomedical research. *Cancer Biotherapy & Radiopharmaceuticals*, *24*(3), 289–302.
Park, J. T., & Johnson, M. J. (1949). A submicrodetermination of glucose. *The Journal of Biological Chemistry*, *181*(1), 149–151.
Parsiegla, G., Reverbel-Leroy, C., Tardif, C., Belaich, J. P., Driguez, H., & Haser, R. (2000). Crystal structures of the cellulase Cel48F in complex with inhibitors and substrates give insights into its processive action. *Biochemistry*, *39*(37), 11238–11246.
Rabuka, D., Rush, J. S., deHart, G. W., Wu, P., & Bertozzi, C. R. (2012). Site-specific chemical protein conjugation using genetically encoded aldehyde tags. *Nature Protocols*, *7*(6), 1052–1067.
Sun, Q., & Chen, W. (2016). HaloTag mediated artificial cellulosome assembly on a rolling circle amplification DNA template for efficient cellulose hydrolysis. *Chemical Communications (Cambridge, England)*, *52*(40), 6701–6704.
Sunbul, M., & Yin, J. (2009). Site specific protein labeling by enzymatic posttranslational modification. *Organic & Biomolecular Chemistry*, *7*(17), 3361–3371.
Tao, L., Kaddis, C. S., Loo, R. R. O., Grover, G. N., Loo, J. A., & Maynard, H. D. (2009a). Synthesis of maleimide-end functionalized star polymers and multimeric protein–polymer conjugates. *Macromolecules*, *42*(21), 8028–8033.
Tao, L., Kaddis, C. S., Loo, R. R. O., Grover, G. N., Loo, J. A., & Maynard, H. D. (2009b). Synthetic approach to homodimeric protein-polymer conjugates. *Chemical Communications (Cambridge, England)*, (16), 2148–2150.
Thordarson, P., Le Droumaguet, B., & Velonia, K. (2006). Well-defined protein–polymer conjugates—Synthesis and potential applications. *Applied Microbiology and Biotechnology*, *73*(2), 243–254.
van Vught, R., Pieters, R. J., & Breukink, E. (2014). Site-specific functionalization of proteins and their applications to therapeutic antibodies. *Computational and Structural Biotechnology Journal*, *9*, e201402001.
Wilson, D. B. (2009). Cellulases and biofuels. *Current Opinion in Biotechnology*, *20*(3), 295–299.
Wilson, M. Z., & Gitai, Z. (2013). Beyond the cytoskeleton: Mesoscale assemblies and their function in spatial organization. *Current Opinion in Microbiology*, *16*(2), 177–183.
Wu, H. (2013). Higher-order assemblies in a new paradigm of signal transduction. *Cell*, *153*(2), 287–292.
Zhang, Y., Yang, Y., Ma, W., Guo, J., Lin, Y., & Wang, C. (2013). Uniform magnetic core/shell microspheres functionalized with Ni2 + − iminodiacetic acid for one step purification and immobilization of His-tagged enzymes. *ACS Applied Materials & Interfaces*, *5*(7), 2626–2633.
Zheng, J., Li, Y., Sun, Y., Yang, Y., Ding, Y., Lin, Y., et al. (2015). A generic magnetic microsphere platform with "clickable" ligands for purification and immobilization of targeted proteins. *ACS Applied Materials & Interfaces*, *7*(13), 7241–7250.