

Biologically Assembled Nanobiocatalysts

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Published online: 28 September 2012
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Abstract The use of nanostructures for enzyme immobilization is an attractive method to increase the overall activity and stability. Self-assembly of enzyme nanostructures has also been shown to provide similar beneficial effects with improved control at the molecular level. In this review paper, we highlighted the recent success in the use of biological assembly methods in creating highly active nanobiocatalysts.

Keywords Cascade reaction · DNA scaffold · Multienzyme · Protein scaffold · Carbon nanotubes

1 Introduction

In recent years, the application space for enzymes has increased significantly, and the current trend suggests that the use of biocatalysts will continue to grow at an ever-increasing pace. While the use of enzymes in many industries [1], is already fairly well-established, the thrust of today's research emphasizes a need for more efficient and sophisticated methods of using enzymes.

One of the most attractive features of using enzymes is the benefit to the environment. Compared to traditional chemical synthesis, methods that involve enzymes are shorter, produce less waste, and are more economical as enzymes boast high chemical and stereo selectivity [2]. Depending on the reactions, the best catalysts tend to involve exceedingly rare transition metals, such as gold and platinum, which add to the cost of production. In comparison, biocatalysts tend to be much cheaper and more sustainable.

However, continuous progress is needed in order to realize the full potential of enzymes. Enzymes are fragile and are inherently unstable; small changes in pH and temperature could lead to denaturation and loss of activity. Substrate transport could also limit the rate of reaction, particularly as multiple enzymes are involved. For these reasons, maintaining the proper orientation and stability are keys to achieving highly efficient synthetic enzymatic reactions. The active site must be exposed properly to incoming substrates and protected from inhibiting molecules and denaturation.

Enzyme immobilization has been used to increase the overall activity and stability. The use of nanostructures such as nanoporous media and carbon nanotubes [3] for immobilization is particularly attractive since the effective enzyme concentration can also be substantially improved due to the high surface area. Self-assembly of enzyme nanostructures or nanobiocatalysts has also been shown to provide similar beneficial effects while offering the potential for improved control at the molecular level. Currently, there are four general methods of creating nanobiocatalysts: physical adsorption onto nanomaterials, encapsulation and entrapment, chemical conjugation, and biological assembly. In this review paper, we have chosen to focus on biological assembly as this method offers

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several advantages. One major advantage is that biological assembly limits denaturation caused by immobilization as only properly folded enzymes are used. A proper orientation of the active site can also be controlled to maximize interactions with the substrate. Specific interactions and similar working environments allow the potential to directly immobilize enzymes from cell lysates, thereby reducing the need for costly purification techniques. The binding affinity can be easily fine-tuned by choosing the proper receptor/ligand pairs to guarantee strong and efficient immobilization or interaction.

In the following sections we discuss some biological assembly methods and their use in creating nanobiocatalysts.

2 DNA-Directed Immobilization

DNA is a promising template for immobilization of proteins [4–6], enzymes [7–9] and nanomaterials [10, 11], because of the nanoscale precision afforded by the specific base-pairing interactions. Site-selective, DNA-directed immobilization of different enzymes onto one-dimensional (1D) DNA templates has been achieved by using biotinylated enzymes and DNA-streptavidin (STV) conjugates as the adaptor (DNA-STV adaptors) [12]. After coupling to the DNA-STV adaptors, these single-stranded DNA-tagged enzymes were immobilized to surface-bound capture oligonucleotides by means of the unique specificity of base pairing (Fig. 1). To avoid enzyme denaturation by chemical biotinylation, *in vivo* biotinylation was employed to attach a single biotin molecule to the N-terminus biotin carboxy carrier protein tail of two recombinant enzymes, NAD(P)H:FMN oxidoreductase (NFOR) and luciferase (Luc) using biotin ligase. Direct assembly of the two enzymes onto a single DNA-template was achieved, resulting in an artificial bienzymic complex [13]. This

bienzymic complex produced threefold more light compared with the same two enzymes immobilized through random hybridization, demonstrating the importance of spatial precision in arranging two enzymes for proper substrate channeling.

The self-assembly of 2D DNA scaffold has also been explored for enzyme immobilization and organization [7]. A set of single-stranded DNA oligos that are partially complementary to each other was used to form either two hexagon or four hexagon-like structures with each hexagon containing a 10 bp overhanging DNA tether for hybridization (Fig. 2). The enzymes glucose oxidase (GOx) and horseradish peroxidase (HRP) chemically functionalized with different DNA oligos were attached onto two separate hexagons on the DNA scaffolds by hybridization with the complementary DNA tether in the hexagon structure. The overall activity of the enzyme cascade can be fine-tuned by controlling their relative position on the DNA scaffolds. The flexibility of creating more rigid, 2D hexagonal scaffolds offer the possibility of position-specific immobilization of multiple enzymes for the self-assembly of complex enzyme cascades.

Although enzymes have not been immobilized on more complex 3D structures, DNA has been used to create structures such as tetrahedrons, octahedrons and icosahedrons [14]. A 3D structure not only further increases rigidity and stability of the complex, but also allows complex arrangements of enzymes, which could directly affect the overall activity of enzyme cascades.

3 Affinity Tag/Binding Peptides

Synthetic protein scaffolds is a popular common method used to provide modular control over enzyme spacing [15, 16] and orientation [17]. The major advantage of this method

Fig. 1 Multiple enzymes have been immobilized onto 1D DNA templates by using biotinylated enzymes and DNA modified with STV adaptors

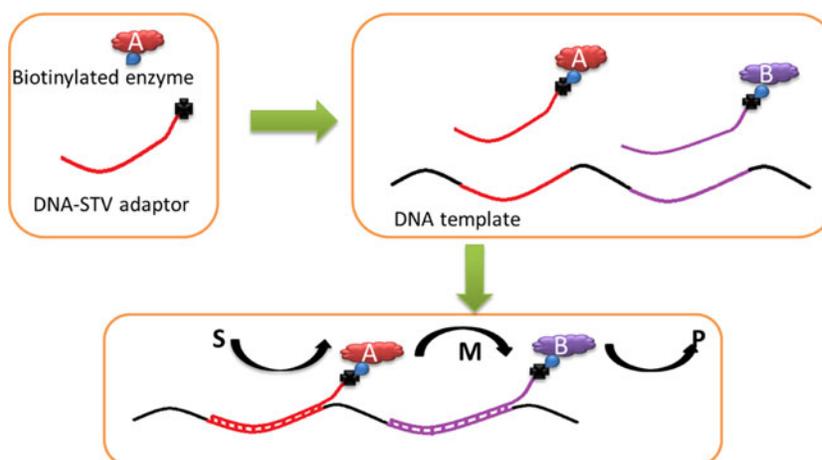
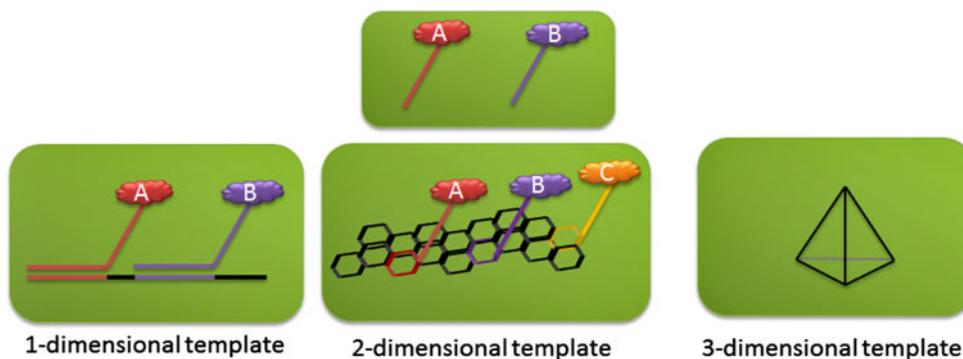


Fig. 2 DNA can be modified to create templates in multiple dimensions. 1 and 2D templates have already been used for biocatalysts



is the possibility to generate highly specific protein scaffolds or binding peptides for many materials of interest [18]. A wide variety of techniques, ranging from phage display to mRNA display, have been used to select for binders with the required affinity [16, 19, 20]. These synthetic scaffolds, when fused with enzymes either at the N- or C-terminus, allow direct immobilization onto different nanomaterials. In one example, a genetically linked alkaline phosphatase (AP)-gold binding peptide (GBP1) was self-immobilized onto micro-patterned gold substrates through the binding of GBP1 to gold [21]. The immobilized enzyme showed higher enzymatic activity per unit area. A similar result was obtained by fusing the glutathione S-transferases (GSTs) with a dodecapeptide isolated from a bacterial display library which specifically binds to polystyrene. Again, GSTs immobilized onto the sub-nanometer sized polystyrene beads showed 10-fold higher specific activity, likely due to an increased localized enzyme concentration and stability [22]. Although these binding peptides or proteins allow the site-specific immobilization of enzymes, the interactions between the enzymes and nanomaterials are reversible; enzymes can be eluted by either extensive washing or changes in solution conditions.

Another widely used binding peptide is the polyhistidine tag (his-tag), which is composed of six or nine histidines that interact specifically with many transition metal ions (Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+}) because of the interaction between the imidazole ring and metal ions [23, 24]. Like most affinity tags, his-tags are a versatile tool in the immobilization of enzymes, particularly to metallic nanoparticles. Aromatic hydrocarbons are highly toxic to enzymes because of the induced conformational changes in the protein structure. By simply immobilizing 1,2-dioxygenase (CatA) from *Corynebacterium glutamicum*, which cleaves the ring of catechol, onto Ni^{2+} modified magnetic nanoparticles through a hexa his-tag, a highly stable biocatalyst that functions even at a high concentration of aromatic hydrocarbons was created [25]. This stabilization effect is likely the result of enhanced enzyme rigidity after immobilization and may be used as a generalized strategy for many other biodegradation reactions. The reversibility of His-tag binding has been exploited

for the repeated immobilization of enzymes [26]. Carbon nanotubes functionalized with N_{α},N_{α} -bis(carboxymethyl)-L-lysine hydrates were used for immobilization of his-tagged NADH oxidase. The resulting nanobiocatalyst maintained 92 % of the original activity and retained over 86 % enzyme loading even after regeneration with EDTA.

4 Enzyme Assisted Covalent Immobilization of Biocatalysts

For some applications, a stronger covalent interaction between enzymes and nanomaterials is desirable for improved stability. This is usually achieved without the use of chemical conjugation by using an enzyme that catalyzes the formation of a covalent bond between the enzyme and a surface-immobilized capture ligand. For instance, the microbial transglutaminase (MTG) is an enzyme that catalyzes protein cross-linking between the ϵ -amino group of lysine and the γ -carboxy-amide group of glutamine [27]. Alkaline phosphatase genetically tagged with a peptide containing a lysine residue was covalently bound to agarose beads modified with β -casein containing multiple glutamine residues in the presence of MTG [28]. The DNA repair protein O^6 -alkylguanine-DNA alkyltransferase (hAGT) transfers an alkyl group from O^6 -alkylguanine-DNA to one of its cysteine residues. Glutathione S-transferase (GST) fused with hAGT was specifically and covalently coupled to O^6 -benzylguanine-functionalized agarose beads while retaining its activity [29]. Another example is through a dityrosine cross-link. Tyrosine residues, in the presence of oxidative enzymes or radical initiators, can be converted into free radicals and react with each other to form dityrosines. Short tyrosine-containing peptide (Y-tag) with only 5 amino acids was introduced to a hyperthermophilic alkaline phosphatase. The recombinant enzyme was immobilized onto water-in-oil-in-water type microcapsules through a radical polymerization reaction in the oil phase. The immobilized enzyme exhibited higher catalytic ability in repeated usages due to the irreversible covalent interaction [30].

5 Leucine Zippers

Leucine zippers are helical peptides that dimerized based on hydrophobic coiled-coil interactions. Each α -helix contains multiple leucine residues at ~ 7 -residue intervals, creating a hydrophobic region on one side of the coil for dimerization between two complementary leucine zippers. Because of the highly specific nature of the dimerization, leucine zipper pairs have been used to create fusion proteins with multiple functions. For example, an albumin binding protein (ABP) was fused to the N-terminal of a Jun leucine zipper [31], while an IgG-binding zz domain and an alkaline phosphatase (PhoA) were fused to opposite ends of the Fos leucine zipper. The resulting combination allowed PhoA to bind to several microtiter plates covered with either albumin, IgG, or even Ni^{2+} through the use of a his-tag on the ABP.

Leucine zipper pairs can be used to create multi-functional enzymes intracellularly (Fig. 3). Steinmann et al. [32] produced galactose oxidase (GOase) bound to PhaC inclusion bodies using two complementary leucine zippers. Although the bound GOase was 35 % less active than the unbound enzymes, it is noticeably easier to purify the complex since centrifugation is adequate in separating the heavy complex from the cell lysate. However, the selection of which inclusion body to use is important as the PhaC inclusion bodies do not show signs of massive aggregation in aqueous buffers, a property highly desirable for the subsequent applicability of the purified enzymes.

Leucine zippers can also be used to spontaneously create enzyme hydrogels (Fig. 3). Wheeldon et al. [33] showed that it is possible to attach α -helices to both ends of different proteins and enzymes to create self-assembling bio-active hydrogels. Although the α -helices were identical in sequence, a random coil is placed between the enzyme and the α -helix to promote physical separation and to increase the water content of the resulting hydrogel. The same group demonstrated this strategy with several proteins and

enzymes including organophosphate hydrolase (OPH) [34] and aldehyde dehydrogenase (AdhD) [33]. In the case of AdhD, it was reported that the hydrogel was not only enzymatically active, but the rigid 3D structure of the hydrogel also minimally affected activity. Hydrogel formation allows the use of enzymes in a 3D structure as most immobilization techniques are limited to flat surfaces. The proximity of enzymes can be controlled, creating a much more concentrated 3D structure than can be afforded in solution. The high water content of hydrogels may play a huge role in substrate transport, as a 3D enzyme structure would be impractical if substrate transport is limited.

6 Other Potential Tags for Biocatalysts: Unnatural Amino Acids

While the use of affinity tags reduces the risk of denaturation, the addition of such tags has been limited to the N and C terminals of enzymes. This limitation hinders our ability to control the precise orientation of the active sites and their accessibility. With the emergence of detailed crystal structures for many enzymes, we are now able to predict the specific location of each amino acid residue.

Based on structural insights, one possibility to provide better control over the active site accessibility is to attach the enzyme onto nanomaterials via conjugation at a specific amino acid residue. To this end, the incorporation of an unnatural amino acid onto the enzyme surface is particularly attractive as a unique functional group not available in other 20 natural amino acids can be exploited for site-specific conjugation. Chin et al. [35] were the first to demonstrate how to replace an amino acid with an unnatural one in a specific location in a protein expressed in *E. coli*. By replacing a carefully chosen codon with an amber codon (TAG), a *p*-azido-L-phenylalanine was incorporated in vivo with high fidelity and better yield.

Using this method, Seo et al. [36] replaced a specific residue in DrrA, a pathogenic protein that binds to human Rab1, to study the effectiveness of a general strategy for controlled and oriented protein immobilization. After incorporation of *p*-azido-L-phenylalanine, the azide group was coupled to biotin by click chemistry which was in turn used to immobilize the whole enzyme onto an avidin-modified surface. The authors reported that the unnatural amino acid incorporated DrrA had higher affinity and faster binding of Rab1 than that of randomly biotinylated DrrA. This served as evidence that the unnatural amino acid allows immobilization without any compromise to the binding site. Although the use of unnatural amino acids for immobilization has not been applied to enzymes, the substantially improved biological properties afforded by this method make it ideal for nanobiocatalyst preparation.

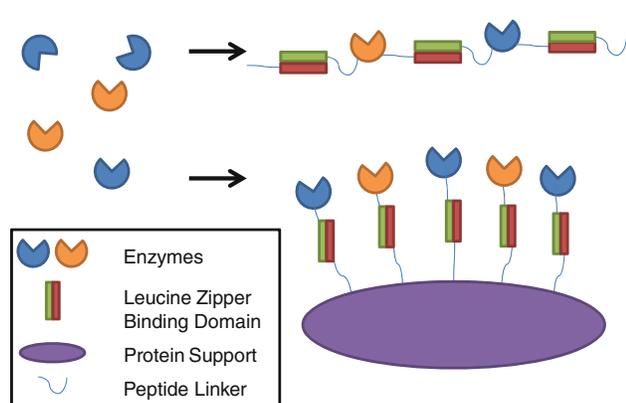


Fig. 3 Leucine zippers allow enzymes **a** to form hydrogels and **b** to other protein supports such as inclusion bodies

7 Protein Scaffoldin/Self-Assembly

In nature, many proteins have the inherent virtues of self-assembly. Examples included the formation of nanoscale assemblies with remarkable structural specificity through the coordination of multiple subunits, the organization of structural motifs into defined 2D or 3D structures, the packaging of virus coat proteins, etc. These protein nanostructures can serve as scaffolds for the self-assembly of complex biological structures displaying catalytic modules.

A naturally occurring system for nanoscale assembly is based on the crystalline S-layer protein found in the cell envelope of many bacteria and archaea [37]. The identical protein subunits can be self-organized into precisely defined 2D arrays of oblique, square or hexagonal symmetry with regularly spaced pores [37]. Glucose-1-phosphate thymidyltransferase, which is involved in the catalysis of activated sugar metabolites, was fused to the C-terminus of the S-layer protein from *Geobacillus stearothermophilus* and self-assembled in solution to form p2 symmetric monolayers on liposomes [38]. These novel biocatalysts assembled by S-layers showed improved storage stability and offer the possibility of recycling.

Similarly, the highly organized stress-related stable protein SP1 has been employed as a protein scaffold for biocatalysts self-assembly. SP1, first isolated from aspen plants, forms a ring shaped dodecamer composed of 12 subunits that are tightly bound to each other. When GOx was fused to SP1, 12 GOx monomers were assembled around one SP1 dodecamer [39]. Enzyme nanotube structures containing hundreds of GOx per tube were also formed by gathering dozens of dodecamers. The enzyme and protein scaffold was expressed as an insoluble aggregate and showed significantly improved stability.

Another interesting example is based on the polyhydroxyalkanoate (PHA) system. PHAs are granular biopolyesters of R-hydroxyalkanoic acid (HA) that are formed intracellularly for carbon and energy storage [40]. PHA synthase is the key enzyme for PHA biosynthesis and PHA granule formation. Enzyme fusions to PHA synthase can be created to guide the covalent attachment of enzymes onto the PHA granule surface [41]. β -galactosidase immobilized onto PHA granules this way showed improved stability and can be easily recycled without loss of activity.

The interaction between a peptide ligand and its corresponding binding domain has also been employed to build synthetic protein scaffolds with the desired enzyme stoichiometry to overcome the bottleneck of metabolic pathways and to optimize sequential catalytic reactions. The gene encoding for enzymes acetoacetyl-CoA thiolase (AtoB), hydroxyl-methylglutaryl-CoA synthase (HMGS) and hydroxyl-methylglutaryl-CoA reductase (HMGR) were introduced into *E. coli* cells as part of the steroid

biosynthesis. Three pairs of peptide ligands and interaction domains from metazoan cells were used to genetically link these enzymes onto a synthetic protein scaffold where the stoichiometry of each enzyme was tunable [42]. This scaffold-assembled multi-enzyme system was up to 60-fold more efficient due to the substrate channeling and activity fine-tuning.

8 Virus-Like Particles

Viruses contained highly organized protein capsids/coated proteins that are useful as scaffolds for building nanomaterials. These highly organized protein shells are nanoscale materials with robust chemical and physical properties that can be modified by both genetic and chemical methods. By combining the self-assembly and reproduction properties of viruses, a plant virus was used to construct a self-assembled catalytic system by genetically fusing an enzyme with its coat proteins [43]. Lipase B, an efficient biocatalyst for many chemical hydrolysis reactions, was introduced to the N-terminus of the capsid proteins of the potato virus X, resulting in a virus-anchored biocatalyst that was catalytically active and had the capability to be mass-produced by cell culture. The M13 bacteriophage, which contains 2,700 copies of the major coat protein and 5 copies each of the four minor proteins, was used to display multiple enzymes. By fusing the endo-1, 4-xylanase I from *Aspergillus niger* and endo-1, 4-xylanase A from *Bacillus subtilis* to the minor coat protein g3, phage particles displaying both enzymes were obtained and were shown to have hydrolytic activity towards an arabinoxylan substrate [44, 45].

Since viral assembly is mainly driven by the organization of protein capsids, one promising alternative to the use of whole viral particles is to design virus-like particles (VLPs) that are also organized and self-assembled from virus-derived structural proteins but lack the genetic material necessary for replication. Fiedler et al. [46] created a nanobiocatalyst based on virus-like particles. In this case, they took advantage of the nanocage structures formed using coat proteins of the bacteriophage Q β for enzyme packaging. Adapting the concept that these phage particles packed their single-stranded RNA genome by virtue of the high-affinity interaction between a hairpin structure and the interior-facing residues of the coat protein, two binding domains were introduced to the coat protein mRNA. Expression of the bifunctional mRNA in parallel with the production of an enzyme containing an arginine-rich peptide resulted in not only the synthesis of the coated proteins, but also packaging of the enzymes inside the resulting nanocages. This is made possible due to binding of the arginine-rich peptide on the enzyme to a corresponding RNA aptamer on one end of the mRNA and

the interaction with the interior of the coat protein using the hairpin structure on the other end. This method of packaging enzymes inside a protective protein shell has had several attributes that distinguish it from existing technologies, such as the active nature of the encapsulated enzymes and the ability of the capsid shell to stabilize the enzymes against thermal degradation, protease attack, and hydrophobic adsorption.

9 Cellulosomes

In nature, anaerobic microorganisms have developed an elaborate enzyme complex known as the cellulosome for efficient carbon turnover. This cell-bound multienzyme complex is responsible for the synergistic deconstruction of both cellulose and hemicellulose, two of the most abundant carbon-rich polymers in the world. The main feature of this nanomachine is a structural scaffoldin consisting of at least one cellulose-binding domain (CBD) and repeating cohesin domains, which are docked individually with a different cellulases tagged with a corresponding dockerin domain. This highly ordered structure allows the assembly of multiple enzymes in close proximity, mediated by the high-affinity protein–protein interaction ($>10^{-9}$ M) between the dockerin and cohesin modules, resulting in a high level of enzyme–substrate–microbe synergy.

Because of the modular nature of the cellulosomal subunits, artificial “designer” cellulosomes have been created [47, 48]. A trifunctional chimeric scaffoldin containing the cohesin domains from three different species was constructed and was shown to bind specifically to the corresponding dockerin-borne cellulolytic enzymes [48]. The resulting sixfold improvement in cellulose hydrolysis over free enzymes suggests that this “designer cellulosome” concept can be similarly exploited for whole-cell

hydrolysis of cellulose and ethanol production. Our group recently reported the functional assembly of this nanocatalyst on the yeast surface (Fig. 4) and demonstrated up-to-3-fold increase in cellulose hydrolysis and ethanol production from phosphoric acid-swollen cellulose (PASC) compared with free enzymes [49, 50]. As the self-assembly of this nanomachine is purely based on protein–protein interactions, similar ideas can also be adapted to other specific protein–protein interaction systems. For instance, Ito et al. [51] constructed a cell surface display system to assemble two cellulases based on the interaction between a cohesin and dockerin pair and the Z domain of protein A and the Fc domain of human immunoglobulin G.

Not surprisingly, several cellulosome-like nanostructures have been explored [52–58]. Two of the most popular are based on rosetazyme-derived and stable protein-derived nanoscaffolds. [59, 60]. Rosettasomes from the hyperthermoacidophilic archaeon *Sulfolobus shibatae* are thermostable, group II chaperonins [61], which in the presence of ATP/Mg²⁺ assemble into 18-subunit, double-ring structures. Another protein scaffold SP1, first isolated from aspen (*Populus tremula*) trees [62], is a ring-shaped, highly stable homododecamer protein with a diameter of 11 nm and an internal cavity of 3 nm, which can be potentially utilized to self-assemble different modules and enzymes in a specific and oriented manner. In both cases, they fused a cohesin module to a circular permutant of the self-assembled protein complex subunit, resulting in nanoscaffolds displaying functional cohesin domains. Again, binding of dockerin-tagged cellulases onto these cohesin-containing nanoscaffoldins increased cellulose-degrading activity compared to their activity in solution. We can easily envision that by exploring such protein scaffolds, one can potentially position selected nano-scale objects, such as metal particles, peptides, protein modules or even whole enzymes, in an oriented manner, thereby creating a nanobiocatalyst for desired chemical reactions.

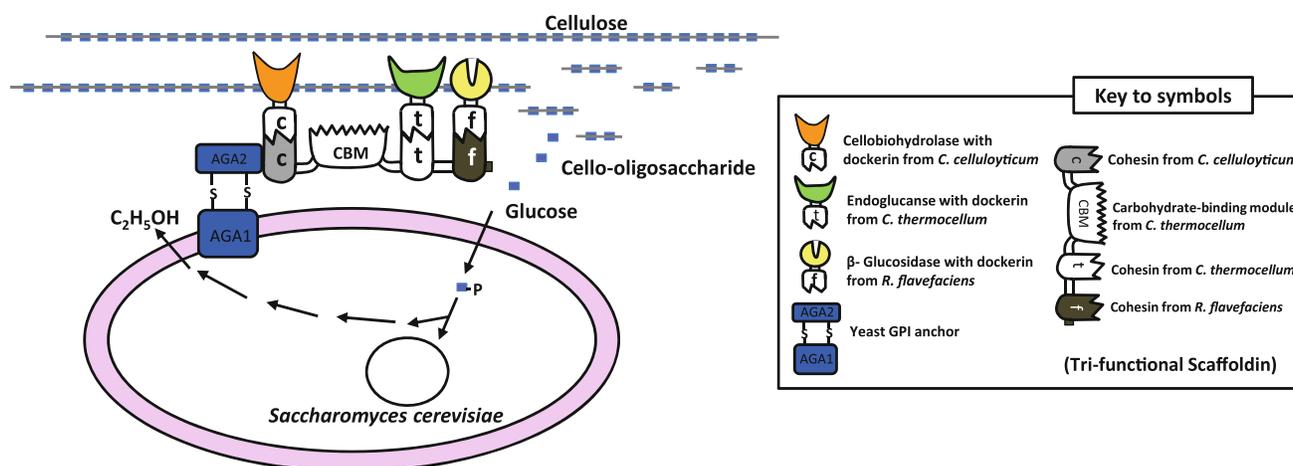


Fig. 4 Surface assembly of a functional mini-cellulosome

10 Conclusion

Interest in nanobiocatalysts has been growing rapidly because of their mild working conditions, renewability, low cost, and high substrate specificity. Biological methods are particularly attractive for nanobiocatalyst assembly because of the ability to preserve enzyme activity and proper orientation. In this paper, we discussed several biological assembly methods based on the use of either DNA scaffolds, synthetic protein scaffolds, or affinity tags. In all cases, the resulting nanoscale biocatalysts showed improved stability and activity when compared to the free enzymes systems. It should be noted that the methods described here comprise of only a small subset of approaches found in many natural systems, further development in this area will likely result in a plethora of tools for even more sophisticated applications.

Acknowledgments This work was supported by Grants from NSF (CBET 1129012) and DOE (EE0000988).

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