

REVIEW

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Biomolecular assembly strategies to develop potential artificial cellulosomes

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Abstract

Cellulosic biomass is a sustainable source for fuels and value-added chemicals, and is available in large quantities. One of the key challenges in biomass processing is associated with the establishment of an efficient enzymatic degradation of plant cell wall. A multi-enzymatic complex, cellulosome, was identified as a highly efficient biocatalyst for the hydrolysis of cellulosic biomass in nature. Significant progress has been achieved on cellulosome production and application since its discovery, but there is still a gap for industrial use. Artificial systems are being developed by employing various pairs of proteins and scaffolds with the objective of reconstructing this natural multi-enzymatic complex for sustainable biotechnology application.

Keywords: Artificial cellulosome, Biocatalyst, Cellulosome, Lignocellulose, Multi-enzymatic system

Biomass degradation and the multi-enzymatic cellulosome system

Currently, there is an emergent need for the development of green and sustainable biotechnology. Since global warming and energy crises are examples of serious problems in our society, innovative and renewable substitutes for petroleum are extremely necessary. Lignocellulosic biomass is a sustainable source of natural carbohydrate polymers, which can be converted into fuels and is available in large quantities. Nevertheless, the conversion of lignocellulosic biomass to fermentable sugars still depends on challenges such as the development of highly efficient and cost-effective catalysts. Most of the present raw biomass conversion processes rely on chemical/physical pretreatment and high enzyme loading, resulting in expensive manufacturing and final product. To overcome technical and economical barriers on biomass conversion, innovative solutions are needed.

Multi-enzyme cellulosome systems were described as one of the most efficient natural biocatalyst for degradation of lignocellulosic biomass, and it is a potential alternative for reducing enzyme loading in industrial processes. The synergism among the multiple enzymes and their proximity to the target substrate are essential to the

degradation of cellulose [1]. With the advancement of molecular biology and conjugation technologies, artificial cellulosomes are being developed with the objective of reconstructing this natural multi-enzymatic system to enhance biomass degradation. Once native cellulosome system is not yet compatible with industry, the manufacturing of a resembling system using advanced technological tools is desired. This mini review will focus on the development of novel multi-enzymatic complexes, especially for lignocellulosic biomass degradation. We will highlight recent advances on artificial cellulosome, describing different methods to fabricate this innovative catalyst.

Artificial cellulosomes for cellulosic biomass hydrolysis

The general idea of developing an artificial cellulosome was based on the need to create an efficient enzymatic complex system that resembles the cellulolytic ecosystem, and is compatible with industry. The first type of artificial cellulosome proposed was so called designer cellulosome and was suggested by Bayer and co-workers in 1994 [2]. Designer cellulosomes are especially based on the interaction between cohesin and dockerin; and the modification of these key points can potentiate the interaction among enzymes from different species. Furthermore, it is possible to control the specific location of enzyme binding sites. Designer cellulosome was first constructed in 2001

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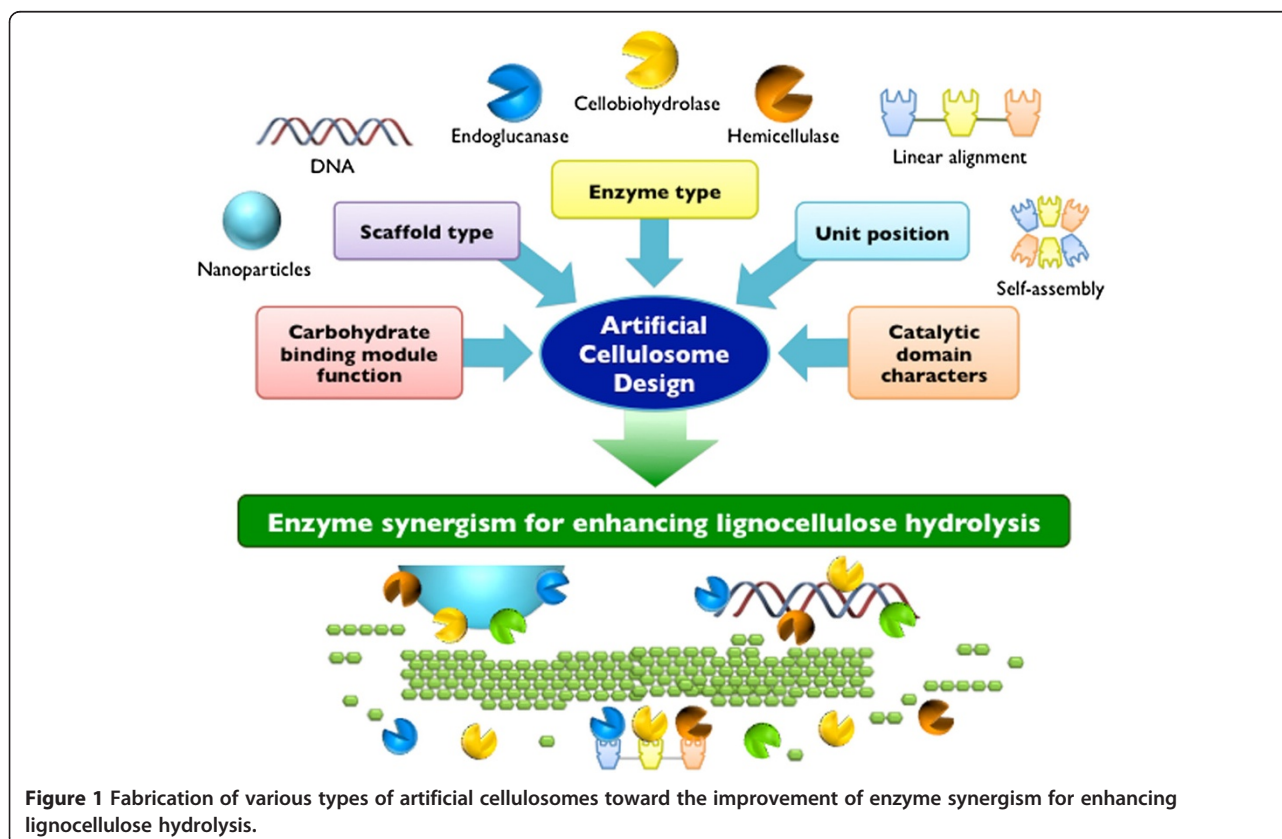
[3], and since that time significant development and advances have been done. No longer after the construction of the first designer cellulosome, a new model of artificial cellulosome was proposed using staphylococcal protein A, as an artificial scaffold, containing Ig-binding domains, as cohesin modules, and using engineered affibody, as dockerin modules. The protein A cellulosome-like complexes, combined to CBM modules, successfully bound to cellulose surface. However, no cellulolytic enzyme was analyzed in this study [4]. Recently, other types of artificial cellulosomes are being developed, based on different enzyme-scaffold conjugation methods, other than cohesin-dockerin interaction, seeking for alternatives for an efficient and profitable manufacturing process. For example, the use of polystyrene nanoparticles, DNA and streptavidin, have been successfully applied in the creation of artificial cellulosomes (Figure 1).

Artificial cellulosomes based on cohesin-dockerin interaction

Artificial cellulosome was first proposed based on the tight binding between cohesin and dockerin modules, and it was constructed *in vitro*, by combination of equimolar amounts of two enzymes and one scaffold [3]. With the creation of designer cellulosomes, the combination of enzymes from different organisms in one single

scaffold became possible, as well as the conversion of non-cellulosomal enzymes to cellulosome mode. The flexibility of building designer cellulosomes allowed researchers to probe more deeply into questions about enzyme synergism, such as enzyme position, catalytic domain mobility, and CBM function. For example, a study about cellulosome geometries, comparing different shapes of designer cellulosomes [5], has demonstrated that the intrinsic mobility of catalytic subunits and the use of a single CBM for substrate targeting are important characteristics of the native system that should be taken into consideration on the design of artificial forms. A comprehension of the individual modules of native cellulosomes is crucial for the development of artificial cellulosomes. The high-affinity of cohesin-dockerin and CBM-cellulose interactions are noticeable points in the structural function of cellulosomes. These features have raised interest in many research fields and have led to innovative applications in the industrial biotechnology (reviewed by Hyeon et al. [6]).

Minicellulosomes are smaller and simpler versions of designer cellulosomes, and are also used for enzyme synergy studies, in order to improve plant cell wall degradation. Minicellulosomes display the main advantage of requiring low levels of enzymes, which in turn facilitates their expression in microbial hosts. Recent study by Xu et al. [7], has



shown that the application of artificial multicatalytic cellulases on minicellulosomes increased cellulose hydrolysis, due to high intramolecular synergism. Overall, artificial (mini)cellulosomes are still less active than natural cellulosomes on crystalline cellulose and raw substrate. Nevertheless, the reproduction of a full size cellulosome from *Clostridium thermocellum* has been successfully achieved, with about 20% less activity than the native one in the hydrolysis of Avicel [8].

The development of artificial designer cellulosomes has boosted the understanding of cellulose hydrolysis in different aspects, but the comprehension of cellulosome structure-function is still not completely clarified. It was only recently, with the combination of advance techniques, such as X-ray crystallographic and cryo-electron microscopic, that high-resolution structural insights of cellulosomes were demonstrated (reviewed by Smith and Bayer [9]). These recent developments in the cellulosome field, related to the plasticity of the cohesin-dockerin interaction and cellulosome assembly, will bring significant impact in the development of novel forms of artificial cellulosomes.

Novel approaches to design artificial cellulosomes

Artificial cellulosomes, based on cohesin-dockerin interaction, have demonstrated to be efficient over soluble enzymes on the degradation of cellulosic materials. Nevertheless, with the advancement of protein engineering and bioconjugation technology, new clustered enzyme complexes also emerged (Table 1). Different strategies were described to organize multi-enzymes complexes, based on the scaffold material, enzyme type, and conjugation technique. Ring-shaped proteins, carboxysome

microcompartment, capsid protein, bacteriophage, RNA and DNA, are examples of synthetic scaffolds for the production of artificial multi-enzyme systems, with potential application for the development of artificial cellulosomes [10].

Nanoscale platforms of different materials are being used as analog to the cellulosomal scaffold. Nanospheres have proven to be a potential scaffold for cellulase immobilization, since they present high specific surface area and minimum diffusional limitation. Using streptavidin-biotin interaction, Kim and co-workers demonstrated the conjugation of catalytic domains and CBM modules onto inorganic nanoparticles [12]. Various ratios of CBMs and catalytic domains were combined using streptavidin or streptavidin-conjugated CdSe nanospheres as cellulosome scaffolds; the increase of CBM modules had positive effect on cellulose hydrolysis (Figure 2). Chemical conjugation technique was also used to immobilize cellulases onto polystyrene nanospheres, the cellulase-nanosphere-complexes were more efficient on crystalline cellulose and recalcitrance wood biomass than on soluble carboxymethyl cellulose [13]. More recently, using metal affinity, a new method of direct conjugation was proposed to cluster cellulases onto nanospheres, CdSe-ZnS core-shell quantum dots. As a consequence, more uniform nanoparticles were obtained, nanoparticle size and enzyme proximity were evaluated. In general, enzyme proximity seems to be more crucial in controlling enzyme synergism than particle size [14].

The fabrication of nucleic acid-protein conjugates became very attractive in the last decade, especially in the biomedical area. DNA brings the advantage of being easily manipulated and produced, even in high-length and

Table 1 Design of artificial cellulosome for lignocellulose hydrolysis

Year	Artificial cellulosome	Scaffold-base	Enzyme type	Conjugation technique	Reference
2001	First designer cellulosome	Engineered chimeric scaffoldin	<i>Clostridium cellulolyticum</i> , CelA, CelF	Cohesin-dockerin interaction	[3]
2003	Protein A- CBM – enzyme conjugates	Staphylococcal protein A	EGFP, <i>Fusarium solani pisi</i> lipase cutinase	IgG-affibody interaction	[4]
2009	Rosettazyme	Group II Chaperonin (Rosettazomes)	<i>Clostridium thermocellum</i> , Cel9B, Cel9K, Cel9R, Cel48S	Cohesin-dockerin interaction	[11]
2011	Nanocluster	Streptavidin-immobilized inorganic nanoparticles	<i>Aspergillus niger</i> , EglA	Avidin-biotin interaction	[12]
2012	Nanocluster	Polystyrene nanosphere	<i>Trichoderma viride</i> cellulase	EDC and Sulfo-NHS coupling chemistry	[13]
2013	Nanocluster	CdSe-ZnS core-shell quantum dots (QDs)	<i>Clostridium thermocellum</i> CelA, <i>Clostridium cellulolyticum</i> CelE	Metal affinity between core-shell QDs and polyhistidine tag	[14]
2013	Cellulase-ankyrin fusions	Ankyrin	<i>Clostridium thermocellum</i> CelA, <i>Thermotoga maritima</i> , Cel12A	Protein fusion	[15]
2013	DNA-cellulase covalent conjugates	Double strand DNA	<i>Thermobifida fusca</i> , Cel5A	MTG mediated cross-linking	[16]
2014	DNA-cellulase non-covalent conjugates	Double strand DNA	<i>Clostridium thermocellum</i> , CelA	Zinc-finger protein guided assembly	[17]

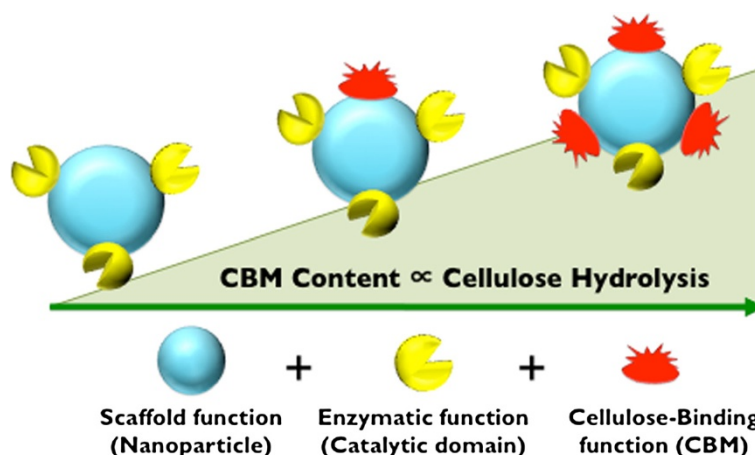


Figure 2 The importance of CBM module on cellulose hydrolysis in the case of nano-cluster artificial cellulosome design.

complex forms, it has great mechanical rigidity and high physicochemical stability. The use of DNA to produce a potent artificial cellulosome was demonstrated for the first time [16]. To prepare DNA-cellulase conjugates, a protein crosslinking enzyme, microbial transglutaminase, has been used. We found that there may be an optimal density of the conjugated enzyme, Cel5A endoglucanase from *Thermobifida fusca*, on the DNA scaffold. Enhancement of 5.7-fold in enzymatic saccharification was observed when comparing to free enzyme, in the hydrolysis of Avicel in the presence of free endoglucanase and β -glucosidase. No later, a different preparation form of DNA-enzyme conjugate was proposed using zinc-finger proteins, based on the ability of these proteins to recognize specific sequences of double-stranded DNA. The combination of catalytic domain and CBM modules were also tested in DNA scaffolds; 1.7-fold enhancement on cellulose hydrolysis was observed with the conjugation of one CelA and one CBM module onto DNA scaffold. The addition of an extra CBM module on the artificial DNA cellulosome increased only 15% in reducing sugar production [17].

Other synthetic cellulosomes were created, associating protein engineering and cohesin-dockerin interaction, as for example, the multi-enzyme rosetazyme [11]. A synthetic cellulosome was constructed based on the self-assembling protein complex, rosettasome, containing cohesin, combined with maximum four *C. thermocellum* cellulases. Interestingly, rosetazyme artificial cellulosome proved to be more efficient when combining two or more enzymes in the scaffold. The addition of a specific CBM module was also analyzed in the rosetazyme, but no improvement was observed in the cellulase activity. Recently, a study has demonstrated the use of ankyrin scaffold combined with endocellulase catalytic domains, as a potent strategy to fabricate artificial cellulosomes. Ankyrin protein proved to be a stable scaffold, increasing cellulase thermostability

and activity, especially because the cellulase domains were internal to the scaffolding protein [15].

Toward practical application of cellulosomes to industry, the integration of recombinant cellulosomes into natural systems has been demonstrated. To accomplish a consolidate process for biofuel production, where cellulase production, cellulose hydrolysis and sugar fermentation occurred in one step, the display of cellulosomes on yeast surface has been developed [18]. Natural cellulosomes are found on the cell wall of microorganisms, and this feature can be advantageous for saccharification process, once it enhances enzyme local concentration, and facilitates the consumption of cellobiose and celloextrin, decreasing product inhibition [19]. A bioenergetic model of celloextrin consumption for the *C. thermocellum* was previously described, demonstrating that assimilation of high lengths of glucose chain are usually associated with ATP saving [20]. Nevertheless, enzyme-microbe synergy is not explained only by the removal of inhibitory products, this complex can also affect the properties of the substrate in the region between cellulose and the adhered cellulolytic microorganism [21]. A mini-cellulosome displayed at *B. subtilis* cell surface, was more active than the free version of the same cellulosome on low-accessibility Avicel or high-accessibility regenerated amorphous cellulose [22]. Furthermore, to enhance lignocellulosic biomass degradation other strategies focused on the degradation of hemicellulose have been shown, such as the creation of xylanase-containing designer cellulosomes [23], artificial xylanosomes [24] or minihemicellulosomes [25].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Wrote the paper: GALG and NK. Designed the figures: YM and NK. All authors read and approved the final manuscript.

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