Rapid Communication

Size-modulated synergy of cellulase clustering for enhanced cellulose hydrolysis

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Immobilization of enzymes onto nanoparticles for enhanced biocatalytic activity via enzyme clustering is a growing field. In this paper, the effect of nanoparticle size on the hydrolytic activity of artificial cellulosomes was investigated. A simple method based on metal affinity coordination was employed to directly conjugate two enzymes, an endoglucanase CelA and an exoglucanase CelE, onto CdSe–ZnS core–shell quantum dots (QDs) without the use of any chemical modification or linker molecules such as streptavidin. Artificial cellulosomes were created by clustering the enzymes onto two different QDs (5 and 10 nm) to systematically study the influence of particle size and QD to enzyme ratio on the enhancement in cellulose hydrolysis. Our results indicate that enzyme proximity is the most important factor for activity enhancement while the influence of particle size is relatively modest. This detailed understanding will provide insights for the design of other artificial cellulosomes based on nanoclustering of multiple catalytic domains with significantly enhanced activities, and may be applicable for designing improved nanobiocatalysts for biofuel production, bioremediation, and drug design.

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1 Introduction

Enzyme-clustering is a promising method in improving the efficiencies of sequential enzymatic reactions and has been successfully demonstrated for a wide range of applications including biodegradation and biofuel production [1–3]. The substantial improvement in catalytic efficiency is a result of the higher surface area to volume ratio as well as the enhanced substrate channeling between enzymes.

In nature, many micro-organisms also employ enzyme clustering as a method to control biological functions [4]. One of the most remarkable examples is the multi-enzyme complex displayed on the surface of many anaerobic micro-organisms called the cellulosome [5]. The main feature of the cellulosome is a structural scaffoldin consisting of at least one cellulose-binding module (CBM) and several repeating cohesin domains, which are docked individually with a different cellulase tagged with the 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. It has been demonstrated that the use of the ternary cellulose-enzyme-microbe cellulosome complex yields much higher rates of cellulose hydrolysis via both microbial adhesion onto the cellulose and the synergistic degradation by sequential hydrolysis [6].

Unfortunately, the use of native cellulosomes for large-scale biomass processing has not been economically viable because full-length cellulosomes cannot be efficiently recovered [7]. Several approaches have emerged in recent years to immobilize cellulases onto non-cellulosomal scaffolds to potentially improve the stabilities, stor-

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Abbreviations: CBM, cellulose-binding module; **PASC**, phosphoric-acid-swollen cellulose; **QD**, quantum dot

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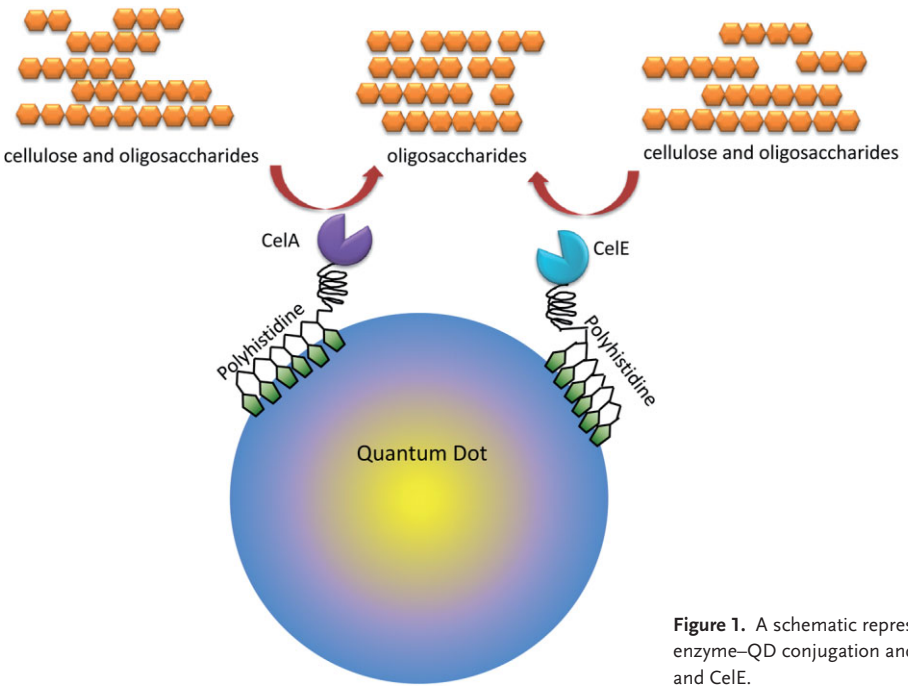


Figure 1. A schematic representation of the polyhistidine-mediated enzyme–QD conjugation and the synergistic cellulose hydrolysis by CelA and CelE.

age properties, and enzyme synergies [8–11]. Among them, nanoparticles, which are known for their minimum diffusional limitation, maximum specific surface area, and effective enzyme loading, are ideal supports for cellulase immobilization. For example, Kim and his colleagues reported the immobilization of endoglucanases and CBMs onto gold nanoparticles via the high-affinity biotin and streptavidin interaction for enhanced cellulose hydrolysis [11]. Although increased activity toward cellulose hydrolysis was demonstrated, the use of the bulkier biotin-streptavidin clustering method required not only an extra step for biotinylation, but also made it difficult to study the real size-effect on enzyme activity, especially for nanoparticles less than 10 nm.

To understand the effects of nanoparticle size on biocatalytic behaviors of artificial cellulosomes and eventually learn to modulate the enzyme activity, we used a simple and versatile approach for the conjugation of luminescent CdSe–ZnS core–shell QDs to cellulases through an engineered C-terminal polyhistidine tag [12] as depicted in Figure 1. By using this purely biological and direct conjugation method, we created artificial cellulosomes using QDs of two different sizes (5 and 10 nm). This detailed understanding at the nanoscale level is likely to provide the much needed insights for the design of artificial cellulosomes with enhanced hydrolytic efficiencies.

2 Materials and methods

2.1 Quantum dots preparation

Two different CdSe–ZnS core–shell QDs with emission maxima at 545 and 605 nm (Invitrogen Nanocrystal Technologies) were used in this study because of desirable size range (5 and 10 nm in diameter, respectively). These QDs were made water soluble via DHLA (dihydrolipoic acid, Sigma) cap exchanging as described elsewhere [13].

2.2 Protein preparation

Escherichia coli strains expressing the endoglucanase (CelA) from *Clostridium thermocellum* and the exglucanase (CelE) from *Clostridium cellulolyticum* were created early in our lab as described elsewhere [14]. The engineered cells were precultured overnight at 37°C in LB medium supplemented with appropriate antibiotics. The precultures were subinoculated into 200 mL LB medium supplemented with 1.5% glycerol and appropriate antibiotics at an initial OD of 0.01 and incubated at 37°C until the OD reached 1.5. The cultures were then cooled to 20°C, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 200 μM. After 1–3 h, cells were harvested by centrifugation (3000 g, 10 min) at 4°C, resuspended in Tris-HCl (50 mM Tris-HCl pH 8.0), and lysed with a sonicator. The cellulases were purified with a His-binding resin (Novagen) at 4°C. The purified enzymes were then dialyzed against PBS to remove the imidazole.

2.3 Quantum dots and proteins conjugation

QD–enzyme bioconjugates were prepared by incubating stock solutions of 1 μM QD with appropriate ratios of purified cellulases in 10 mM Hepes buffer pH 8 for 2 h. The formation of QD–enzyme bioconjugates were confirmed by the electrophoretic mobility shift assay using a 0.5% agarose gel. For the analysis of unbound proteins conjugation, cellulases were first labeled with FITC (Invitrogen) using the primary amine groups as described by the manufacturer (pH 9). After overnight reaction, unlabeled FITC was eliminated by spin dialysis (Centricon, Millipore). After conjugation with QDs, 20 μL of each assembled mixtures was analyzed by a 4–20% gradient SDS-PAGE gel.

2.4 Enzyme activity assay

The resulting QD assemblies were transferred into a reaction solution (1 g/L of phosphoric-acid-swollen cellulose (PASC) in 10 mM Hepes buffer pH 8) immediately prior to use. The total concentration of the enzymes used in the reaction solution was adjusted to 100 nM. Reactions were performed at 30°C with shaking, and samples were taken periodically. Reducing sugars were measured by the DNS method. Samples were collected periodically and mixed immediately with equal amount of DNS reagents (10 g/L dinitrosalicylic acid, 10 g/L sodium hydroxide, 2 g/L phenol, 0.5 g/L sodium sulfite) and incubated for 10 min at 95°C. A small amount of Rochelle salts was added to fix the color before the samples were cooled on ice. The absorbance at 575 nm was measured.

3 Results and discussion

3.1 Attachment of cellulases to CdSe–ZnS core–shell QDs via the polyhistidine tag

Numerous methods have been reported to attach biomolecules to QDs, including biotinylation and chemical conjugation. However, these methods require chemical modifications, which could be complex and resulted in a reduction in enzyme activities. In this work, a method based on metal-affinity coordination with an engineered polyhistidine tag (his tag) was chosen for enzyme conjugation to the surface of QDs as described by Goldman et al. [12].

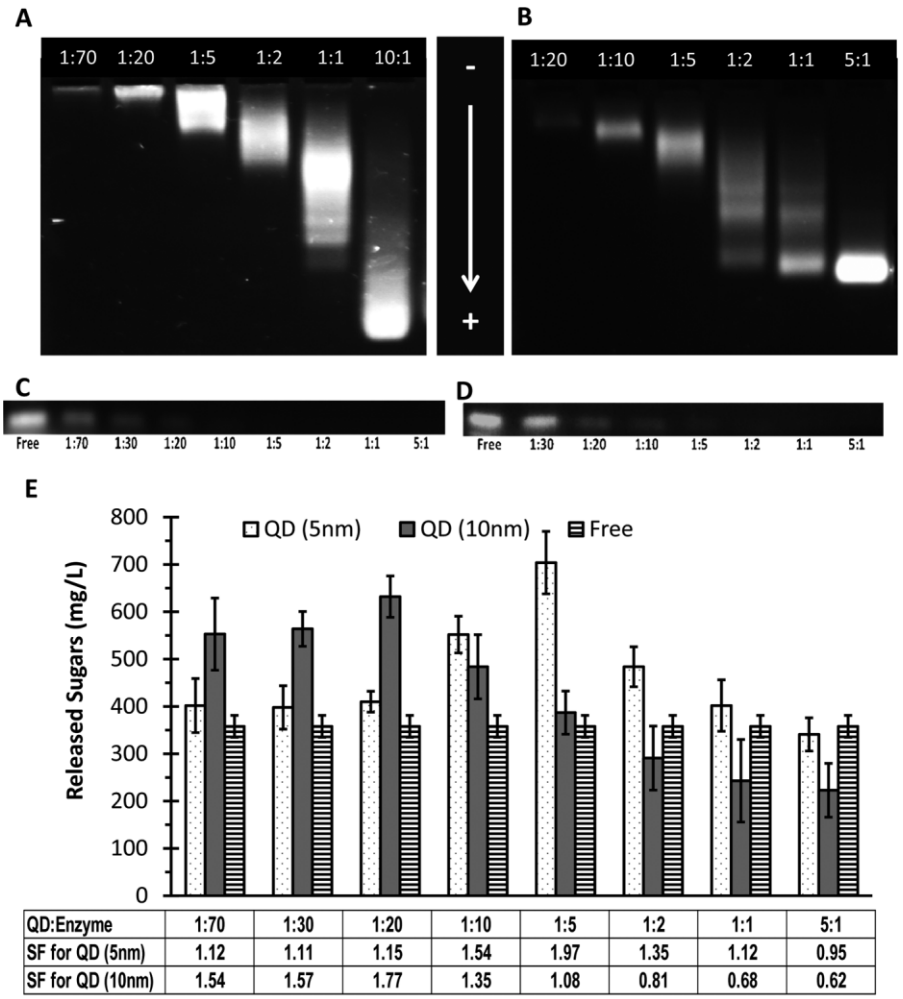
An endoglucanase (CelA), containing a C-terminus his tag, was used to construct the initial nanoscale artificial cellulosome. Two different QDs of 5 or 10 nm diameter were used as the nanoscaffolds. To investigate the loading capacity of the 10 nm QD, conjugation was conducted using different QD to CelA ratios from 10:1 to 1:70. To verify the level of conjugation, agarose gel electrophoresis was performed. As dihydrolipoic acid (DHLA)-functionalized QDs are strongly negatively charged due to the carboxyl group on the surface, the migration of different QD assemblies can be clearly visualized under UV light due to a shift in the electrophoretic mobility. Figure 2A demonstrated the successfully coupling of CelA to the QD (Lane 1–5) as the conjugates migrated differently from the unconjugated QDs (Lane 6). The smeary nature of the bands is likely a consequence of the varying degree of enzyme conjugation with the QDs. However, a clear reduction in the mobility was detected as the QD to enzyme ratio was decreased with the maximum shift in mobility occurred around a ratio of 1:20. A similar migration pattern for the 5 nm QD was observed; however, the maximum reduction in mobility was detected at a lower CelA loading, consistent with the roughly four times smaller available surface area ($\propto R^2$) for enzyme conjugation (Fig. 2B).

To further quantify the level of CelA conjugation, CelA was first labeled with FITC and the amount of unconjugated CelA was quantified using a 4–20% gradient SDS-PAGE gel (Fig. 2C, D). While unconjugated CelA was detected for the 10 nm QD at a QD–CelA ratio below 1:20, unconjugated CelA was only visible for the 5 nm QD at a QD to CelA ratio below 1:5. These results confirm that free enzymes are present below these respective conjugation ratios and could potentially reduce the overall nanoclustering effect.

3.2 Effects of QD size on enzyme synergy and cellulose hydrolysis

To analyze the hydrolytic activity of the different QD assemblies, the hydrolysis of PASC was examined (Fig. 2E). The same amount of CelA was used for the different QD–CelA ratios in order to evaluate only the effect of enzyme clustering on the overall enhancement. Regardless of the QD size, the hydrolytic activity of the QD–enzyme conjugates increased initially with an increasing CelA–QD ratio before reaching a maximum. The maximum activity was observed at an enzyme loading for each QD–CelA conjugate where no unconjugated CelA was detected. Since the maximum enzyme proximity and the highest fraction of CelA conjugated to QD were obtained at this ratio, this result suggests that enzyme proximity is indeed the more crucial in controlling the level of enzyme synergy rather than particle size.

Interestingly, while the immobilization of CelA onto the 10 nm QD showed a negative effect on the enzyme activity when the QD–enzyme ratio was below 1:5, a monotonic enhancement in the enzyme activity was observed when CelA was immobilized onto the 5 nm QD. At the lower enzyme loading, the steric effect caused by the bulky particle makes it more difficult for the immobilized CelA to interact with the larger cellulose substrate. It should be noted that the level of enzyme synergy appears to be higher using the smaller 5 nm QD, suggest-



QD:Enzyme	1:70	1:30	1:20	1:10	1:5	1:2	1:1	5:1
SF for QD (5nm)	1.12	1.11	1.15	1.54	1.97	1.35	1.12	0.95
SF for QD (10nm)	1.54	1.57	1.77	1.35	1.08	0.81	0.68	0.62

Figure 2. Characterization of enzyme–QD conjugates. (**A**) Characterization of CelA conjugation with the 10 nm QD at different QD–CelA ratios using a 0.5% agarose gel. (**B**) Characterization of CelA conjugation with the 5 nm QD at different QD–CelA ratios using a 0.5% agarose gel. (**C**) Detection of unconjugated CelA with the 10 nm QD using a 4–20% gradient SDS-PAGE gel. (**D**) Detection of unconjugated CelA with the 5 nm QD using a 4–20% gradient SDS-PAGE gel. Enzymes were labeled with FITC before conjugation with the (**C**) 10 nm QD or (**D**) the 5 nm QD. (**E**) Hydrolysis of 1 g/L of PASC by the different QD conjugates. Samples were taken after 48 h and the amount of reducing sugars released from cellulose hydrolysis was determined. The ratios of QD to enzyme are based on molarity. The synergy factor (SF) is defined as the amount of sugar released from the QD–enzyme conjugates (10 or 5 nm) over the amount of sugar released from free enzymes (Free). Data represent the mean ± standard deviation of three independent experiments.

ing that the mobility of the enzyme-QD conjugates is another important factor in controlling activity. This result is consistent with the enhanced binding effect using the smaller nanoparticles based on a diffusion-collision theory [15].

3.3 Synergy effect of CelA and CelE cluster on QDs

In a native cellulosome system, the synergistic cellulose hydrolysis is attributed to both the proximity effect from the scaffoldin and the substrate-targeting effect from the CBM [5]. On the scaffoldin, several different types of enzymes are brought together via the high-affinity dockerin–cohesin interaction and bound onto the substrate via the CBM, resulting in the substrate channeling between different enzymes.

To mimic this complex structure, we investigated the size effect of QD conjugates using a two-enzyme system. In addition to CelA, an exoglucanase CelE was chosen as this enzyme contains a catalytic domain and a CBM. Since the maximum enzyme synergy was observed at the saturating enzyme loading of each QD, the same enzyme loading was chosen for the dual-enzyme system. To maintain the 1:1 ratio of CelA and CelE, an equal molar ratio of CelA and CelE were mixed with the two QDs. The amount of sugars released from PASC hydrolysis was monitored periodically (Fig. 3). Regardless of the particle size, the hydrolysis rate was improved by about 2-fold when both cellulases were conjugated to the QDs. This two-fold improvement is similar to that observed when both CelA and CelE were assembled into a mini-cellulosome structure on the yeast surface [14]. Although the two enzymes were randomly attached onto the QDs, the nanometer dimension of the QDs likely provides a higher probability of two enzymes being immobilized next to each other, resulting in the improved substrate channeling. This is fully supported by the substantially higher initial hydrolysis rates by the QD–enzyme conjugates (461.1 μM/h for the 5 nm QD–enzyme conjugates and 533.3 μM/h for the 10 nm QD–enzyme conjugates), which are 4.9- and 5.6-

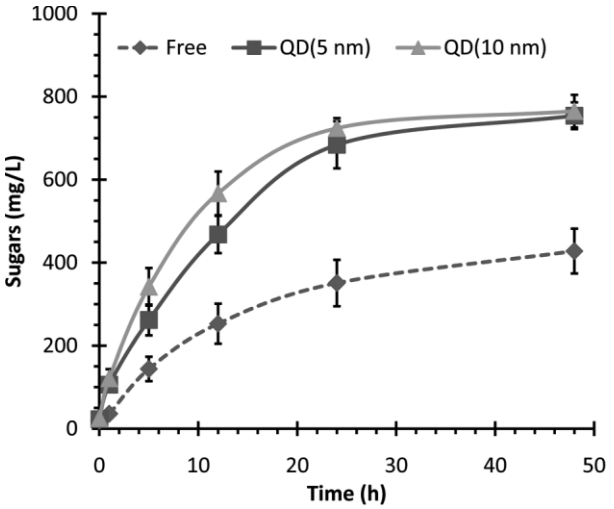


Figure 3. The time course of sugar released from 1 g/L of PASC using the two-enzyme QD conjugates. An equal amount of CelA and CelE was mixed thoroughly before conjugating with the QDs at the optimal ratio. Data represent the mean ± standard deviation of three independent experiments.

fold better than using free enzymes (94.4 μM/h). The fact that no substantial difference in the hydrolysis rate was observed between the two different QDs again suggests that enzyme proximity is more important than particle size in dictating the overall improvement in cellulose hydrolysis.

4 Concluding Remarks

This work demonstrated a systematic approach to discuss the size effect of nanoparticles on immobilized enzyme activity. To investigate the effects of nanoparticles smaller than 10 nm, conjugation onto QDs was based on coordination with a flanking his tag on the cellulases. For both the one-enzyme or two-enzyme setups, our results indicated that enzyme proximity is the most important in controlling the overall enhancement in enzyme activity rather than the particle size. As the integrative field of nanobiotechnology is gaining more interest, this information may be useful in designing improved nanobiocatalysts for biofuel production, bioremediation, and drug design.

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The authors declare no conflict of interest.

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