Enzymatic Activities of Polycatalytic Complexes with Nonprocessive Cellulases Immobilized on the Surface of Magnetic Nanoparticles

Ranjan K. Kamat,‡ Yuting Zhang,∥ Murali Anuganti,‡ Wanfu Ma,∥ Iman Noshadi,‡ Hailin Fu,‡ Stephen Ekatan,‡ Richard Parnas,‡ Changchun Wang,∥∥ Challa V. Kumar,∥∥∥ and Yao Lin∥∥∥∥

†Polymer Program, Institute of Material Science, ‡Department of Chemistry, and ∥Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269, United States
∥State Key Laboratory of Molecular Engineering of Polymers and Department of Macromolecular Science, Fudan University, Shanghai 200433, China

Supporting Information

ABSTRACT: Polycatalytic enzyme complexes made by immobilization of industrial enzymes on polymer- or nanoparticle-based scaffolds are technologically attractive due to their recyclability and their improved substrate binding and catalytic activities. Herein, we report the synthesis of polycatalytic complexes by the immobilization of nonprocessive cellulases on the surface of colloidal polymers with a magnetic nanoparticle core and the study of their binding and catalytic activities. These polycatalytic cellulase complexes have increased binding affinity for the substrate. But due to their larger size, these complexes were unable to access to the internal surfaces of cellulose and have significantly lower binding capacity when compared to those of the corresponding free enzymes. Analysis of released soluble sugars indicated that the formation of complexes may promote the prospect of having consistent, multiple attacks on cellulose substrate. Once bound to the substrate, polycatalytic complexes tend to remain on the surface with very limited mobility due to their strong, multivalent binding to cellulose. Hence, the overall performance of polycatalytic complexes is limited by its substrate accessibility as well as mobility on the substrate surface.

INTRODUCTION

Enzymatic hydrolysis of cellulose is an important process for the conversion of biomass into fuels under mild conditions and for the modification of the cellulolic materials for desired properties.1−3 Although the chemical composition of cellulose is simple, the structure and morphology of native cellulose are not uniform, containing both crystalline and amorphous regions which differ in terms of their susceptibility to hydrolysis.4−7 Efficient hydrolysis of cellulose requires the cooperative actions of at least three types of cellulolytic enzymes: exoglucanases (also called cellobiohydrolases), endoglucanases, and β-glucosidase.8−10 Exoglucanases such as cellobiohydrolase I (CBHI) produced by the filamentous fungus Trichoderma reesei are processive enzymes, which bind at the ends of the cellulose chains in crystalline regions and “slide” along the chains progressively for continuing reactions.11 Cellobiose and glucose are released as the reaction products from the processive motions of exoglucanases.12,13 In contrast, endoglucanase such as EGII of T. reesei are thought to be nonprocessive and preferentially attack more disordered regions of cellulose by cleaving the bonds in the middle of the cellulose chains.14,15 Interestingly, significant amounts of cellobiose and glucose were still found in the hydrolysis of microcrystalline cellulose (e.g., Avicel) by EGII, but the proportion of the soluble sugar products varies considerably with the enzyme-substrate ratio and the reaction time.16 As the soluble sugars are not the expected products of a typical endoglucanase activity on a solid substrate, the sugar production pattern of EGII suggested that either multiple random attacks can happen on individual cellulose chains, resulting in small fragments that are further hydrolyzed in solution, or two-domain endoglucanases may possess some “pseudo-processivity” from their cellulose binding domain (CBD).17

We are interested in understanding whether the formation of artificial polycatalytic complexes from endoglucanase will further improve their capability in producing soluble sugars, as a consequence of both improved substrate binding and the proximity of multiple enzymes that may act on cellulose substrate in a concerted manner.18−20 Polycatalytic cellulase complexes made by immobilization of industrial cellulases on polymer or nanoparticle scaffolds are also technologically attractive due to the low cost and recyclability of these synthetic nanoscaffolds and the scale-up potential for biorefinery applications.21−27 However, the relatively large, polycatalytic
complexes could possess quite a different adsorption behavior and hydrolytic activity, in comparison to the corresponding free cellulases. First, the surface area of cellulose available for binding of the polycatalytic complexes may decrease considerably, as most of the internal surface area of pores may not be accessible to large complexes when the particle size is comparable to the average pore size. Second, once bound, the polycatalytic complexes may tend to stay on the surface of cellulose as a consequence of multivalent contacts between the polycatalytic complex and the cellulose substrate. Third, distinct reaction activities or product patterns may be observed in the polycatalytic complexes due to the proximity of enzymes present in these complexes and their collective actions. It is thus crucial to understand the benefits and limitations of polycatalytic complexes such that these multicatalytic particles could be rationally designed to achieve maximal hydrolytic efficiency.

Herein, we synthesized polycatalytic complexes by the immobilization of nonprocessive EGII on the surface of colloidal polymers with a magnetic nanoparticle (MNP) core, examined their adsorption and activities in the production of soluble sugars on Avicel substrate, and compared these attributes to those of EGII in its free state. The study shows that the formation of polycatalytic complexes significantly enhanced substrate binding to the integrated supramolecular structure and improved the prospect of having consistent, multiple attacks on cellulose chains to produce soluble sugars. The overall performance of polycatalytic complexes, however, is limited by the accessibility of complexes to the cellulose substrate surface.

**Experimental Section**

**Materials.** Cellulase mixture from *Trichoderma reesei* (Cellicast 1.5 L from Novozymes), microcrystalline cellulose (Avicel, PH101), and 4-methylumbelliferyl β-D-cellobioside were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and purchased from Fisher Scientific. EGII was purified from cellulase mixture using GE FPLC equipped with ion exchange columns. The purity of EGII and other individual enzymes was verified by SDS-PAGE and LC/MS and by the measurement of specific activity against 4-methylumbelliferyl β-D-cellobioside with that of stock solution with a predetermined amount of EGII. The suspension of unbound enzyme complexes was pelleted by centrifugation at 14,000 rpm for 5 min, and the supernatant was withdrawn. The concentration of soluble sugars in the solution by using HPLC. All experiments were done in triplicate.

**Adsorption and Activity Experiments for EGII.** 20 mg of Avicel was mixed with 1 mL of EGII solution (e.g., 0.025 and 0.1 µM) in 50 mM sodium acetate at pH 5 in a centrifuge tube and incubated at 37 °C on a mixer. At the given incubation time, the Avicel was pelleted by centrifugation at 14,000 rpm for 5 min, and the supernatant was withdrawn. The concentration of unbound EGII in the supernatants was determined by measuring the specific activity against 4-methylumbelliferyl β-D-cellobioside. The supernatant was used to measure the concentration of soluble sugars by using HPLC. All experiments were done in triplicate.

**Analysis of the Adsorption Isotherms.** For adsorption isotherms, increasing concentrations of EGII and EGII/MNP-PMAA complexes were mixed with 20 mg/mL of Avicel and incubated at 37 °C on a mixer. The unbound enzyme concentration was measured after 40 min using Amax and by comparing the specific activity against 4-methylumbelliferyl β-D-cellobioside with that of stock solution with a predetermined amount of EGII. The EGII/MNP-PMAA complex concentrations were determined by dividing the molarity of complexed EGII with the estimated number of EGII per particle. The Langmuir model was used to analyze the adsorption isotherms with good correlation. The equation for the Langmuir model is as follows:

\[
[B] = \frac{A_{\text{max}}[F]}{K_A^{-1} + [F]}
\]

where [B] and [F] are equilibrium concentration of bound and unbound enzymes or enzyme complexes, \(A_{\text{max}}\) is the maximum binding capacity, and \(K_A\) is the association constant for binding.

**Study on the Desorption and Motion of EGII Complexes on Avicel by Confocal Microscopy.** Laser-scanning fluorescence confocal microscopy (LFCM) was used to follow the adsorption and motions of individual EGII/MNP-PMAA complexes on Avicel surface. EGII/MNP-PMAA complexes were labeled with fluorescein isothiocyanate (FITC) by incubating FITC dye and the complexes (FITC:EGII = 20:1 in molecular ratio) for 2 h and then washing repeatedly by buffer solution to remove unbounded dyes. FITC-labeled EGII complexes were introduced into 50 mM sodium acetate buffer for five times to remove unbound proteins. The concentration of EGII bound to the MNP-PMAA particles was estimated by comparing the specific activity against 4-methylumbelliferyl β-D-cellobioside in 50 mM sodium acetate at 30 °C with that of EGII solutions with known concentration (Figure S2).

### Experimental Section

**Materials.** Cellulase mixture from *Trichoderma reesei* (Cellicast 1.5 L from Novozymes), microcrystalline cellulose (Avicel, PH101), and 4-methylumbelliferyl β-D-cellobioside were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and purchased from Fisher Scientific. EGII was purified from cellulase mixture using GE FPLC equipped with ion exchange columns. The purity of EGII and other individual enzymes was verified by their molecular weights estimated by comparing the specific activity against 4-methylumbelliferyl β-D-cellobioside with that of stock solution with a predetermined amount of EGII. The suspension of unbound enzyme complexes was pelleted by centrifugation at 14,000 rpm for 5 min, and the supernatant was withdrawn. The concentration of soluble sugars in the solution by using HPLC. All experiments were done in triplicate.

**Adsorption and Activity Experiments for EGII.** 20 mg of Avicel was mixed with 1 mL of EGII solution (e.g., 0.025 and 0.1 µM) in 50 mM sodium acetate at pH 5 in a centrifuge tube and incubated at 37 °C on a mixer. At the given incubation time, the Avicel was pelleted by centrifugation at 14,000 rpm for 5 min, and the supernatant was withdrawn. The concentration of unbound EGII in the supernatants was determined by measuring Amax and the specific activity against 4-methylumbelliferyl β-D-cellobioside. The supernatant was used to measure the concentration of soluble sugars by using HPLC. All experiments were done in triplicate.

**Analysis of the Adsorption Isotherms.** For adsorption isotherms, increasing concentrations of EGII and EGII/MNP-PMAA complexes were mixed with 20 mg/mL of Avicel and incubated at 37 °C on a mixer. The unbound enzyme concentration was measured after 40 min using Amax and by comparing the specific activity against 4-methylumbelliferyl β-D-cellobioside with that of stock solution with a predetermined amount of EGII. The EGII/MNP-PMAA complex concentrations were determined by dividing the molarity of complexed EGII with the estimated number of EGII per particle. The Langmuir model was used to analyze the adsorption isotherms with good correlation. The equation for the Langmuir model is as follows:

\[
[B] = \frac{A_{\text{max}}[F]}{K_A^{-1} + [F]}
\]

where [B] and [F] are equilibrium concentration of bound and unbound enzymes or enzyme complexes, \(A_{\text{max}}\) is the maximum binding capacity, and \(K_A\) is the association constant for binding.

**Study on the Desorption and Motion of EGII Complexes on Avicel by Confocal Microscopy.** Laser-scanning fluorescence confocal microscopy (LFCM) was used to follow the adsorption and motions of individual EGII/MNP-PMAA complexes on Avicel surface. EGII/MNP-PMAA complexes were labeled with fluorescein isothiocyanate (FITC) by incubating FITC dye and the complexes (FITC:EGII = 20:1 in molecular ratio) for 2 h and then washing repeatedly by buffer solution to remove unbounded dyes. FITC-labeled EGII complexes were introduced into 50 mM sodium acetate buffer solution containing Avicel. Complexes were allowed to adsorb on Avicel surface for 30 min by incubating at 37 °C on a mixer. After 30 min, Avicel was removed from the solution, washed, and resuspended in fresh buffer. Dilute Avicel suspension containing surface-bound EGII/MNP-PMAA complexes (∼50 µL) was placed between a glass slide and coverslip. Surface desorption and mobility of EGII complexes were followed by Nikon A1R spectral confocal microscopy. An environmental chamber was used to maintain the samples at a temperature of 37 ± 3 °C, and images were taken at an interval of 10 min. NIH ImageJ software was used in the image analysis.
Surface Plasmon Resonance (SPR) Study on the Adsorption of EGII/MNP-PMAA polycatalytic complexes on Cellulose Thin Films. The SPR chips (Reichert (catalog #13206060)) were cleaned by piranha solution (70:30 v/v H₂SO₄:H₂O₂) for 15 min followed by soaking in a sodium dodecyl sulfate solution for 20 min and rinsing with a large excess of DI water. Cellulose film (100−200 nm) was prepared by spin-coating of 2.5 wt % cellulose solution in 1-ethyl-3-methylimidazolium acetate on a precleaned SPR chips and washing the coated film in DI water and subsequently dried at 80 °C in the oven for 15 min. SPR signals were traced on an SR7000DC dual channel flow SPR spectrometer from Reichert Analytical Instruments (Depew, NY) with a semiautomatic injection setup with a 500 mL PEEK injection loop and Harvard Apparatus flow pump. The SPR data were collected at 37 °C in 50 mM sodium acetate, pH 5.

Figure 1. Adsorption isotherms of (A) EGII/MNP-PMAA polycatalytic complexes and (B) EGII on Avicel. Avicel concentration was kept constant (20 mg/mL), at increasing complex or enzyme concentrations.

Figure 2. Hydrolysis kinetics of Avicel by EGII/MNP-PMAA polycatalytic complex (A, B) and by EGII in the free state (C, D). Avicel concentration was kept constant at 20 mg/mL, and the concentrations of total enzymes are as indicated. Incubation was at 37 °C in 50 mM sodium acetate, pH 5.
and the concentration of particles (Figure S2). EGII/MNP-Avicel, which has a diameter of 160 nm, is practically irreversible. From the isotherm, the binding interaction and its dissociation is expected to be extremely slow, or binding interactions between the polycatalytic particle and the cellulose, 0.0001 μmol (pores of 1–10 nm in sizes) can be up to 2 orders of magnitude higher than the external area. From the isotherm, the binding interaction and its dissociation is expected to be extremely slow, or binding interactions between the polycatalytic particle and the cellulose, 0.0001 μmol (pores of 1–10 nm in sizes) can be up to 2 orders of magnitude higher than the external area.39

The Langmuir model was used to analyze the adsorption isotherms of both free EGII and EGII/MNP-PMAA complexes.38 Using a standard magnetic separation rack, the EGII/MNP-PMAA complexes were readily separated from unbound EGII and washed repeatedly. The complexes are thus free of interference from unbound enzymes. The surface densities of EGII on MNP-PMAA were determined from the amount of bounded EGII (e.g., measured by an enzymatic assay using 4-methylumbelliferyl β-D-cellobioside as the substrate) and the concentration of particles (Figure S2).38 EGII/MNP-PMAA containing ~200 enzymes per particle were used in the study.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of EGII/MNP-PMAA Conjugates.** EGII was purified to homogeneity from industrial cellulases (Novozymes Celluclast) by using ion-exchange columns and identified based on its molecular weight and its specific activity (see the Supporting Information, Figure S1).36 Following a previously reported method,36 we prepared core–shell particles (MNP-PMAA) with a magnetic nanoparticle (MNP) core of 100 nm in size and a poly(methacrylic acid) (PMAA) shell of about 30 nm. PMAA was cross-linked at about 10% cross-linking degree to provide optimized mechanical properties as the scaffold. Standard carbodiimide coupling (EDC) chemistry were used to conjugate EGII onto the activated carboxyl groups of the PMAA to obtain EGII/MNP-PMAA complexes.37 Using a standard magnetic separation rack, the EGII/MNP-PMAA complexes were readily separated from unbound EGII and washed repeatedly. The complexes are thus free of interference from unbound enzymes. The surface densities of EGII on MNP-PMAA were determined from the amount of bounded EGII (e.g., measured by an enzymatic assay using 4-methylumbelliferyl β-D-cellobioside as the substrate) and the concentration of particles (Figure S2).38 EGII/MNP-PMAA containing ~200 enzymes per particle were used in the study.

**Adsorption and Desorption Behavior of EGII/MNP-PMAA Conjugates.** At varying loadings, adsorption of EGII/MNP-PMAA and EGII to 20 mg/mL Avicel in 50 mM sodium acetate (pH 5) at 37 °C was followed. The Langmuir model was used to analyze the adsorption isotherms of both free EGII and EGII/MNP-PMAA complex (Figure 1). The apparent association constant of EGII/MNP-PMAA to Avicel was determined to be (1.3 ± 0.2) × 10^10 M^-1, almost 6 orders of magnitude higher than that of EGII, which is (9.2 ± 1.1) × 10^4 M^-1. Such tight binding could be attributed to the multivalent interactions between the polycatalytic particle and the cellulose, and its dissociation is expected to be extremely slow, or binding is practically irreversible. From the isotherm, the binding capacity for EGII/MNP-PMAA was determined to be 0.0012 ± 0.0001 μmol EGII/g Avicel. This is more than 1000 times lower than that of EGII, which is measured to be 1.16 ± 0.06 μmol EGII/g Avicel. The Avicel particles are known to have both external and internal surfaces, and the internal surface area (pores of 1–10 nm in sizes) can be up to 2 orders of magnitude higher than the external area.39–41 Obviously, a large fraction of Avicel’s internal surface is not accessible to EGII/MNP-PMAA, which has a diameter of 160 ± 20 nm (larger than the narrow pores of Avicel). The diminished accessibility to this internal surface may become a limiting factor to the overall performance of polycatalytic complexes.

**Hydrolytic Activity of EGII/MNP-PMAA Conjugates.** Enzymatic activities of EGII/MNP-PMAA and EGII on Avicel are compared in Figure 2, as obtained by monitoring the amount of cellobiose and glucose released from Avicel. The total enzyme concentrations were kept identical in the comparison. We found, at higher enzyme-to-cellulose ratio, EGII is more efficient in the production of soluble sugars than the polycatalytic EGII/MNP-PMAA. However, at the low enzyme-to-cellulose ratios, EGII/MNP-PMAA showed greater effectiveness. The control experiments with MNP-PMAA lacking EGII prove that the colloidal particles themselves do not have any hydrolytic activity. At the concentrations of enzymes examined in the experiments (0.025 and 0.1 μM EGII incubated with 20 mg/mL Avicel), almost 100% of the EGII adsorbed on the cellulose surface. In contrast, the adsorption of EGII/MNP-PMAA complexes on Avicel surface saturates quickly due to the low binding capacity of these complexes. To compare the inherent activity of soluble sugar production between EGII and EGII/MNP-PMAA complex, we calculated their reaction productivity by normalizing the amount of soluble sugar released at 24 h, based on the actual fraction of enzyme or enzyme complexes bound on the cellulose (Figure S3). Figure S4 indicates EGII/MNP-PMAA has a significantly higher productivity than EGII. The enhanced substrate interaction and succeeding attacks from multiple enzymes in the EGII/MNP-PMAA complexes seem to improve their capability in producing greater amounts of soluble sugars. The overall performance of polycatalytic complexes, however, is largely limited by their low binding capability to cellulose as they cannot access to the internal surface of Avicel.

The sugar production patterns were considerably different between polycatalytic complex and EGII. The proportion of the produced sugars (e.g., cellobiose vs glucose) for EGII/MNP-PMAA was rather independent of both the enzyme-to-substrate ratios and reaction time (Figure 2A,B), where glucose was the major product. In contrast, EGII produced more cellobiose than glucose at low enzyme-to-substrate ratios (Figure 2C,D). Only at much higher enzyme loadings (2 μM), glucose became the dominant product (Figure S5). This latter result was also reported in prior studies.42–46 The exact mechanism for the production of soluble sugars by EGII, an endoglucanase, is still not clear. But our result suggests that the formation of polycatalytic complexes that anchor on the cellulose substrate and possess many enzymes in the close proximity may enhance the prospect of having continuing, multiple attacks on cellulose chains and further digesting cello-oligosaccharides to glucose.
The collective actions of multiple enzymes in the polycatalytic complexes should facilitate the distinct reaction activities and product patterns.

**In-Situ Monitoring of Mobility of EGII/MNP-PMAA Conjugates on Cellulose Surface.** To confirm our findings derived from the experiments based on ensemble behavior, we used laser-scanning fluorescence confocal microscopy (LFCM) to follow the motions of individual EGII/MNP-PMAA complexes on Avicel. EGII/MNP-PMAA complexes were labeled with fluorescein isothiocyanate (FITC) for fluorescence imaging, and introduced into the buffer solution containing Avicel particles. The adsorption and desorption of individual complexes onto Avicel were followed by using time-lapse imaging (Figure 3 and Figure S6). Our study shows that once bound, the EGII/MNP-PMAA complexes rarely desorbed on the time scales of the experiments (~1 h). This is in agreement with the very high binding affinity we measured for the polycatalytic complexes. The coverage of EGII/MNP-PMAA complexes on Avicel was further revealed by field emission scanning electron microscopy (FESEM) (Figure 4). Interestingly, the hydrolytic reaction of EGII/MNP-PMAA complexes generated cavities at their anchoring sites, which can be visualized after merely 2 h of incubation. It also suggests that the complexes, as a whole, do not possess much lateral mobility on the surface of the cellulose substrate.

**Adsorption of Polycatalytic EGII/MNP-PMAA on Cellulose Film.** Lastly, we use regenerated cellulose thin films to confirm that the multivalent interaction of EGII/MNP-PMAA complexes can also play essential roles in their binding to cellulose other than Avicel. Cellulose thin films were made on SPR sensor chips by spin-coating cellulose dissolved in ionic liquids. Then the suspensions of EGII/MNP complexes in acetate buffer were flowed over the chips while monitoring the change of SPR signal to follow the adsorption of the complexes on the cellulose (Figure 5). After the adsorption reaches equilibrium, we washed the surface with buffer to elute the bound particles (the time when we replaced the solution with buffer is indicated by the dashed line in Figure 5). The apparent lack of dissociation of EGII/MNP complex from the cellulose surface suggested an extremely high binding constant. Because of the relatively large size and metal core of the complexes, the SPR signal from the adsorption and desorption of EGII/MNP-PMAA are much more significant than decrease in the signal due to the release of sugars from the cellulose film by EGII-mediated hydrolysis. Therefore, a simple association model based on a pseudo-first-order kinetics can be used to analyze the kinetic data (Figure 5). From the best fits to the kinetic data, the apparent association constants for EGII/MNP-PMAA complex were found to be in the range of 4 × 10^{12}–9 × 10^{13} M^{-1}. Again, this practically irreversible binding of the complexes to the cellulose film should result from the unique multivalent interaction of the polycatalytic complexes with the substrate.

**CONCLUSION**

Artificial polycatalytic complexes were developed by immobilizing EGII, a nonprocessive cellulase, on nanoparticles, and their adsorption and enzymatic activity were compared with those of the free enzymes. The formation of polycatalytic cellulase complexes enhanced the substrate binding of the integrated supramolecular structure as well as the prospect of having consistent, multiple attacks on cellulose substrate to release soluble sugars. The binding of the particles to cellulose is almost irreversible. However, the polycatalytic complexes are not accessible to the internal surface area of porous cellulose, and they tend to stay on the surface of cellulose with limited mobility due to multivalent binding to the surface. Therefore, the overall performance of polycatalytic complex is also limited by their restricted mobility on the cellulose substrate due to lack of dissociation. These limitations may be overcome by pretreatment of cellulose to form larger porous structures and by the use of smaller sized nanoparticles as scaffolds for the polycatalytic complexes.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.6b02573.
FPLC chromatography, SDS-PAGE gel and activity assays of cellulases; adsorption behaviors of EGI and EGII complexes, hydrolytic activity of EGII at different enzyme concentrations (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**
*E-mail ccwang@fudan.edu.cn (C.W.).
E-mail challa.kumar@uconn.edu (C.V.K.).
E-mail yao.lin@uconn.edu (Y.L.).

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by the US Department of Energy, Office of Basic Energy Sciences, Division of Materials Sciences and Engineering (DE-SC0005039) and Department of Education (GAANN P200A105330). Y.L. acknowledges a senior visiting scholarship provided by Fudan University and the University of Connecticut Confocal Microscopy Facility and Carol Norris for training, advice, and use of instrumentation. C.W. acknowledges the support from National Science and Technology Key Project of China (Grant No. 2012AA0202204). C.V.K. acknowledges the NSF EAGER grant (DMR-1401879).

**REFERENCES**


(30) Arantes, V.; Saddler, J. Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis


