

## SPECIFIC INTERACTION ACTING AT A CELLULOSE-BINDING DOMAIN/CELLULOSE INTERFACE FOR PAPERMAKING APPLICATION

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Specific and strong cellulose-binding characteristics were utilized for promoting retention of additives in contaminated papermaking systems. Cellulose-binding domain (CBD) of cellulase derived from *Trichoderma viride* was separated by digestion with papain, and then introduced into anionic polyacrylamide (A-PAM) through a condensation reaction using water-soluble carbodiimide. The CBD-modified A-PAM (CBD-A-PAM) showed good retention on pulp fibers, resulting in high tensile strength paper sheets. The effect remained almost unchanged in the presence of model interfering substances such as ligninsulfonate and  $\text{Ca}^{2+}$  ions, whereas commercial cationic paper-strengthening polymer became ineffective. The cellulose-binding force of CBD was quantitatively determined by atomic force microscopy (AFM) in the liquid state. Histidine-tagged CBD protein was obtained using *Escherichia coli* via an expression of CBD derived from *Cellulomonas fimi*, and immobilized on a gold-coated AFM probe. A strong attractive force was detected only at a CBD/cellulose interface, even when  $\text{Ca}^{2+}$  ions were present in high concentration. Direct estimation of CBD affinity for cellulose substrate by AFM would provide significant information on the interfacial interactions useful for the functional design of papermaking additives.

*Keywords:* Cellulose-binding domain; Retention; Papermaking additive; Interfering substances; Atomic force microscopy; Force curve measurement

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### INTRODUCTION

Fibrous cellulosic materials consisting of plant cell walls have long been used extensively as raw materials for paper products. In industrial production, functional paper materials are commonly designed by simply adding a variety of papermaking additives to an aqueous fiber suspension in a part of the process called the wet-end. The wet-end system has attracted much attention as an effective and environmentally-friendly process for large-scale manufacture of cellulose fiber-based materials. However, that process has been considerably contaminated by use of recycled fibers of low quality and of recycled water in a closed water circulation system (Pelton et al. 1981). Charged contaminants, particularly in the form of dissolved and suspended colloidal substances, greatly interfere with the adsorption of papermaking additives on the surface of cellulosic pulp fibers. That type of interference arises because current wet-end systems generally

depend on an electrostatic interaction at a pulp fiber/additive interface in water (Zhang 1999).

Various polymeric additives, for example polyacrylamide (PAM), polyamideamine-epichlorohydrin (PAE), and cationic starch, have frequently been utilized in the papermaking process. Unfortunately, those (mostly ionic) water-soluble polymers are sensitive to anionic dissolved and colloidal substances (“anionic trash”) and inorganic ions accumulated in the paper furnish. Nonionic polymer systems, using polyethylene oxides and phenol formaldehyde resins, have been used to improve retention/drainage efficiency under industrial conditions (Pelton et al. 1980; Lindström and Glad-Nordmark 1984). However, those systems have limited applications and have not provided a fundamental solution. Consequently, there is a need for a novel concept in wet-end interactions, differing from conventional electrostatic interactions, for an additive retention system.

Novel wet-end interactions that could be used for a contaminated process should at least be able to clearly differentiate between cellulose and other organic trash, both with negative charges, and to give efficient retention even at high salt concentration. Cellulase is a ubiquitous enzyme in natural environments, and it can selectively hydrolyze cellulose even in the presence of many kinds and large amounts of other organic substances. Most cellulases have a modular structure composed of a catalytic domain (CD) linked to a cellulose-binding domain (CBD). Binding of cellulase to the surface of solid cellulose is initiated by CBDs, whereas CDs contribute to glycohydrolysis (Linder and Terri 1997). It has been reported that isolated CBDs were quasi-irreversibly adsorbed on the crystalline surface of cellulose with no catalytic activity (Din et al. 1991).

In a previous report (Kitaoka and Tanaka 2001), it was proposed for the first time that a CBD function was effective for enhancing the retention and performance of papermaking additives. CBD-conjugated anionic PAM (CBD-A-PAM) performed well under the wet-end condition with a high conductivity, while the commercial PAE became relatively ineffective. However, only meager quantitative information was obtained for the retention of CBD-A-PAM to cellulosic pulp fibers. Consequently, the cellulose-binding power of CBD molecules to cellulose substrate is unknown.

In the present study, the retention behavior has been compared for CBD-A-PAM and conventional papermaking polymer, in a contaminated process containing model anionic trash or inorganic ions. Furthermore, the direct interaction force between CBD and cellulose surface has been quantitatively assessed by atomic force microscopy (AFM), which is very useful for structural and functional studies of many biological samples (Ellis et al. 2006; Ueno et al. 2007; Yokota et al. 2007a, 2007b). AFM offers ultra-structural imaging with atomic-scale resolution, as well as force-distance measurements with femto-Newton force resolution (Ellis et al. 2006). In the present work we prepared pure CBD proteins via heterologous expression using *Escherichia coli* (*E. coli*), and quantified the cellulose-binding force using AFM force-distance measurements with a CBD-modified AFM probe and a flat cellulose model substrate.

## EXPERIMENTAL

### Preparation of CBD-A-PAM

Cellulase (EC 3.2.1.4, 1,4- $\beta$ -D-glucan 4-glucanohydrolase, Wako) produced by the fungus *Trichoderma viride* (*T. viride*), was partially purified by anion-exchange. Cellulase and papain (EC 3.4.22.2, Sigma) were pre-activated at 37 °C for 30 min in 50 mM phosphate buffer (pH 6.5). A portion of the activated papain solution (1 mg/mL) was added to 150 mL of cellulase solution (10 mg/mL) to give a cellulase/papain ratio of 30:1 (w/w), and the mixture was incubated at 37 °C for 30 min (Van Tilbeurgh et al. 1986). Affinity purification of CBDs was carried out by shaking the mixed enzyme solution with CB<sub>IND</sub><sup>TM</sup> 100 resin (Novagen) at 30 °C for 30 min, followed by sequential centrifugations (11,000 rpm, 12 min) using binding buffer (20 mM Tris-HCl, pH 7.5), washing buffer (20 mM Tris-HCl, pH 7.5, 0.8 M NaCl), and ethylene glycol (EG) solution, according to the protocol. The separation of CBD was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Subsequently, 70 mL of CBD/EG solution (ca. 3 mg/mL) and 3 mL of 10 % (w/w) aqueous 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) solution were gradually added to 150 mL of 0.1 % (w/w) aqueous A-PAM (HH-351, with molecular weight: ca.  $4 \times 10^6$  g/mol, and charge density: 0.83 meq./g; Kurita) with pH 4.75, with stirring at room temperature for 3 h (Kitaoka and Tanaka 2001). The CBD-A-PAM obtained was partially purified by three-times centrifugations using 2-propanol before handsheet preparation.

### Handsheet-making and Physical Testing

The desired amounts of CBD-A-PAM, or A-PAM or PAE (Japan PMC) were added (0-0.8 % based on dry weight of pulp) to a 0.15 % pulp suspension prepared from a commercial bleached hardwood kraft pulp (Canadian Standard Freeness: 450 mL). The cationic demand of the pulp suspension was adjusted to 20-100  $\mu$ eq./L by addition of a sodium ligninsulfonate as a model of anionic trash, before addition of polymer. The conductivity of the suspension was adjusted to 0.5-4.0 mS/cm by addition of calcium chloride (CaCl<sub>2</sub>). Handsheets with a basis weight of 60 g/m were prepared according to the TAPPI test method (T205 sp-95), by dewatering, wet-pressing, and drying in an oven at 105 °C for 10 min. After conditioning at 23 °C and 50 % relative humidity for 24 h, the handsheets were subjected tensile strength testing in the dry condition according to the TAPPI test method (T494 om-88). Additive retention was determined by combustion analysis for nitrogen.

### Expression of Designed CBD Protein with *E. coli*

The pET-38b(+) vector that contains the CBD<sub>cex</sub> from *Cellulomonas fimi* (*C. fimi*) and histidine (His) tags (Novagen) was transferred to *E. coli* BL21 (DE3) pLysS (TaKaRa). The transformant was grown in LB broth containing kanamycin (15  $\mu$ g/mL) and chloramphenicol (34  $\mu$ g/mL) with shaking (120 rpm) at 37 °C, until an OD<sub>600</sub> of 0.4-0.5 was reached. Then isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, followed by incubation with shaking for 5 h. The cells were harvested by centrifugation (5,000 rpm, 4 °C, 10 min) and then re-

suspended in Lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT)), followed by freezing overnight at -80 °C. After re-melting at 4 °C, the cell suspension was incubated with 2 mg/mL lysozyme, 0.1 mg/mL DNase I, and 10 µM phenylmethyl sulfonylfluoride at 30 °C for 60 min. The cell lysate obtained by sonication was centrifuged (9,500 rpm, 4 °C, 30 min), then rinsed three times with washing buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM DTT). For inclusion bodies, pellets were solubilized in 10 mL of Lysis buffer (8 M urea, 50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 50 mM DTT) in reduced light at 25 °C for 2 h, then centrifuged (15,000 rpm, 4 °C, 10 min). The supernatant fluid, mixed with CB<sub>IND</sub><sup>TM</sup> 100 resin, was dialyzed against TBS buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 250 mM NaCl) at 4 °C for 36 h, then CBD protein was collected according to the protocol referred to previously.

### Force Measurement at a CBD/cellulose Interface

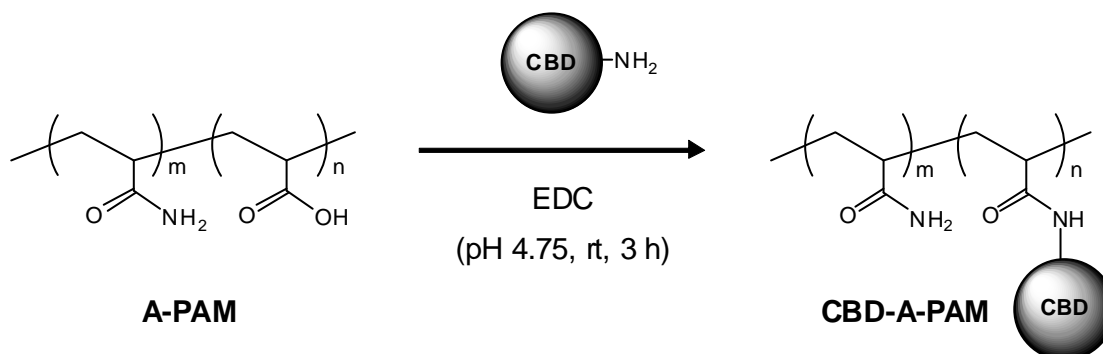
His-tagged CBD protein was immobilized on a gold-coated Si<sub>3</sub>N<sub>4</sub> probe (cantilever length: 200 µm, spring constant: 0.06 N/m, Veeco Instruments) via Ni<sup>2+</sup>-mediated complexation using 3,3'-dithiobis(*N*-(5-amino-5-carboxypentyl)propionamide-*N'*,*N'*-diacetic acid) dihydrochloride (dithiobis(C<sub>2</sub>-NTA)), as reported previously (Murata et al. 2001). The self-assembled cellulose layer was prepared on an Au-sputtered Si substrate using cellulose thiosemicarbazone (degree of polymerization: ca. 200) according to our previous report (Yokota et al. 2007c). Force-distance measurement with CBD-modified AFM probe and cellulose substrate was carried out in pure water, using a NanoScope IIIa atomic force microscope (Veeco Instruments) in contact mode with a J-type scanner. The force curves obtained were analyzed using a Scanning Probe Image Processor (SPIP Version 3.1.0.2, Image Metrology).

## RESULTS AND DISCUSSION

### Preparation and Characteristics of CBD-A-PAM

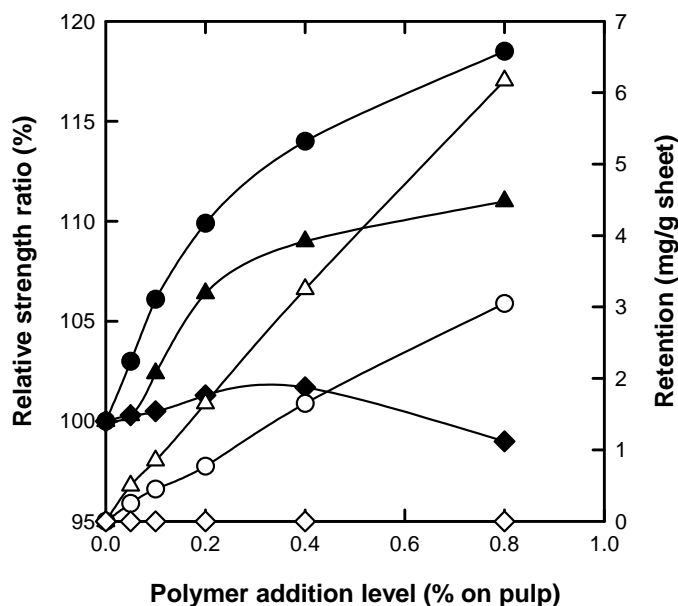
Figure 1 shows schematically the introduction of the isolated CBD fractions into A-PAM via condensation using a water-soluble carbodiimide, EDC. Matrix-assisted laser desorption/ionization time-of-flight mass analysis confirmed that limited proteolysis by papain provided a small fragment (ca. 9 kDa) possibly originating from *T. viride* CBD. The degree of CBD-substitution (DS) of the carboxyl group of A-PAM was estimated by elementary analysis as approximately 0.0259, indicating that one of every 39 carboxyl groups were conjugated with the CBD proteins. Taking into account the presence of acrylamide units on A-PAM chains, only one or two CBDs might have been introduced per 1000 structural units of an A-PAM chain. The N-termini of the isolated CBDs were covalently linked to the carboxyl groups of A-PAM (acrylamide + acrylic acid). SDS-PAGE analysis (data not shown) indicated that self-condensation of the CBDs by EDC-mediated reaction occurred to a negligible extent, because the amount of carboxyl groups of A-PAM was more than 40 times that of CBDs added in the reaction system (see Experimental section). Moreover, the carbohydrates located in the neighborhood of the

C-termini of the CBDs would have suppressed self-condensation, which is not desirable, and facilitated formation of CBD-A-PAM.



**Fig. 1.** Schematic representation of the preparation of CBD-A-PAM from isolated CBD and A-PAM via EDC-mediated condensation reaction.

Figure 2 shows the tensile strength and additive retention of the handsheets prepared with CBD-A-PAM or PAE or A-PAM under the same papermaking conditions. Increasing levels of CBD-A-PAM and PAE increased additive retention, resulting in strength enhancement of the paper sheets. In the case of the CBD-free A-PAM, neither A-PAM retention nor paper strength improvement was found, because both A-PAM and pulp fibers are negatively charged at the wet-end, leading to electrostatic repulsion.

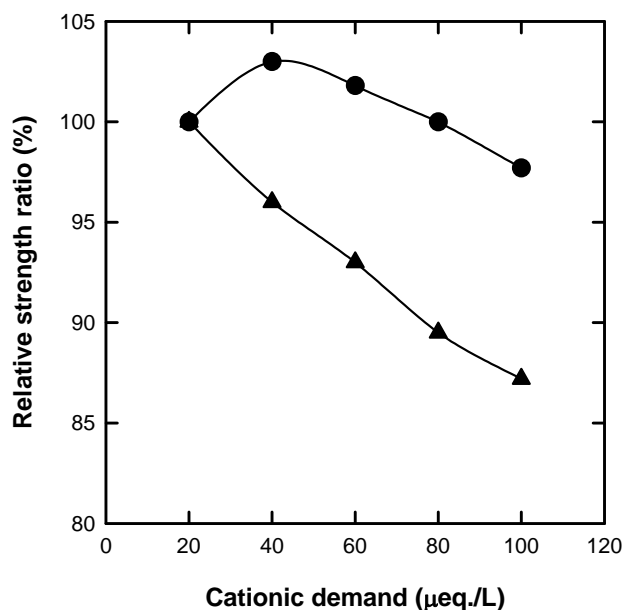


**Fig. 2.** Effects of additives on paper strength (closed) and retention (open): CBD-A-PAM (circles), PAE (triangles) and A-PAM (diamonds).

Although the amount of retained CBD-A-PAM was only about half that of PAE, CBD-A-PAM had a more beneficial effect than PAE on the physical properties of paper sheet; this implies favorable performance of CBD-A-PAM as a paper strength additive. Presumably the CBD segments of the CBD-A-PAM chains have an attractive interaction with cellulose fibers, and AM units form hydrogen bonds with cellulose fibers. Non-hydrolytic disruption of cellulose fibers with CBDs as previously reported (Wang et al. 2008) was sufficiently small as to be neglected in this study, possibly because of the slight amount of CBD introduced. Direct introduction of cellulase to A-PAM may be possible as another approach; however, it is presumed that the EDC-mediated condensation reaction must be less efficient due to high molecular weight of cellulase, and the complete inactivation of CD portions is indispensable for avoiding the hydrolytic degradation of cellulose fibers. Consequently, overall, the CBD-A-PAM composed of CBD moieties and A-PAM demonstrated wet-end functions as both a cellulose anchor and a paper strength enhancer.

### Additive Performance of CBD-A-PAM in the Contaminated Wet-End

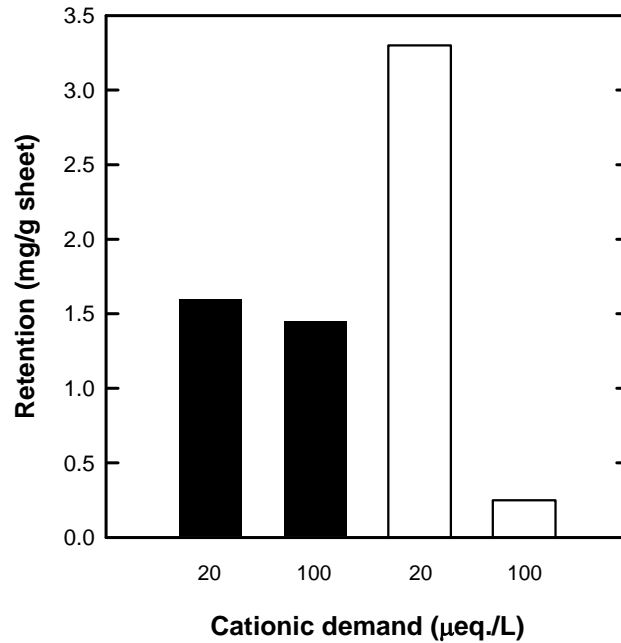
Figure 3 shows the influence of anionic trash on the tensile strength of handsheets prepared with addition of 0.4 % (based on dry pulp) CBD-A-PAM or PAE. In the model wet-end condition with cationic demand ranging from 20 to 100  $\mu\text{eq./L}$ , the tensile strength of paper to which PAE had been added was remarkably reduced, compared with the tensile strength of paper prepared with tap water (cationic demand: 20  $\mu\text{eq./L}$ ).



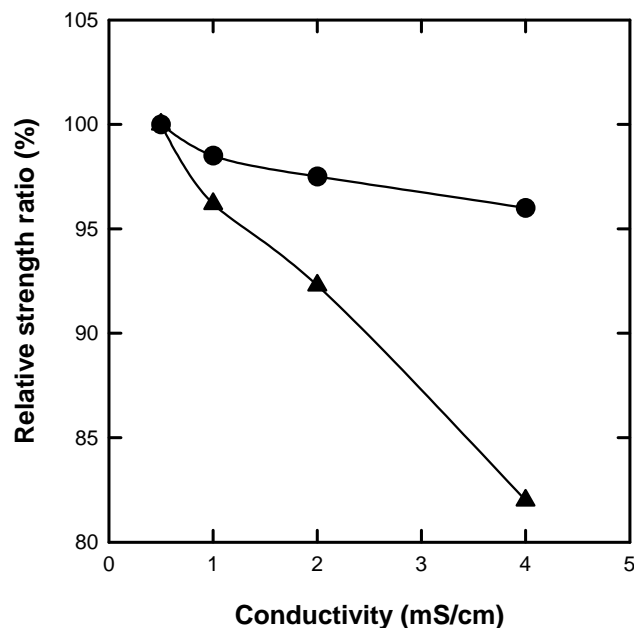
**Fig. 3.** Influence of anionic trash on the tensile strength of paper sheets prepared with CBD-A-PAM (circles) and PAE (triangles).

As shown in Fig. 4, retention of PAE was quite low in the high cationic demand condition (100  $\mu\text{eq./L}$ ), strongly suggesting that anionic ligninsulfonate interfered significantly with the electrostatic interaction between cationic polyelectrolytes and anionic pulp fibers at the wet-end. On the other hand, both the retention of CBD-A-PAM

and the tensile strength of CBD-A-PAM-treated paper sheets remained almost unchanged in contaminated paper-making conditions (Figs. 3, 4). Consequently, CBD-A-PAM was selectively retained on the pulp fibers even in the presence of negatively-charged interfering trash.

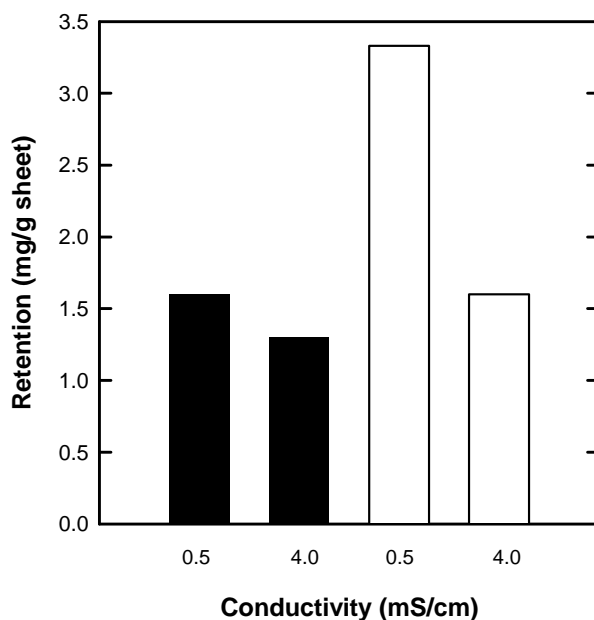


**Fig. 4.** Influence of anionic trash on the retention of CBD-A-PAM (filled bars) and PAE (open bars).



**Fig. 5.** Influence of paper stock conductivity on the tensile strength of paper sheets prepared with CBD-A-PAM (circles) and PAE (triangles).

The influence of multivalent ions on the tensile strength and retention contents were also investigated. Whereas the higher conductivity of pulp stock containing interfering inorganic salts ( $\text{CaCl}_2$ ) drastically reduced the performance of PAE, the strength and additive retention of the handsheets prepared with CBD-A-PAM remained almost unchanged (Figs. 5, 6). It is well known that divalent cations such as  $\text{Ca}^{2+}$  ions in the fiber stock greatly disturb PAE retention on paper sheets due to weakening of the anionic charges of the pulp fibers, resulting in fatal decrease in paper strength (Espy and Rave 1988). At a stock conductivity of 4.0 mS/cm, a larger amount of PAE was retained in the paper sheet than that at cationic demand of 100  $\mu\text{eq./L}$  (Figs. 4, 6), although PAE-added paper demonstrated higher relative strength at less PAE retention (Figs. 3, 5). In the case of anionic trash, PAE was simply consumed by its preferential adsorption to the trash. On the other hand, some molecular conformation of PAE might be affected at a high salt concentration in addition to the electrostatic interfering, and thus it can possibly be presumed that the retained PAE could not effectively perform as a paper strength additive.



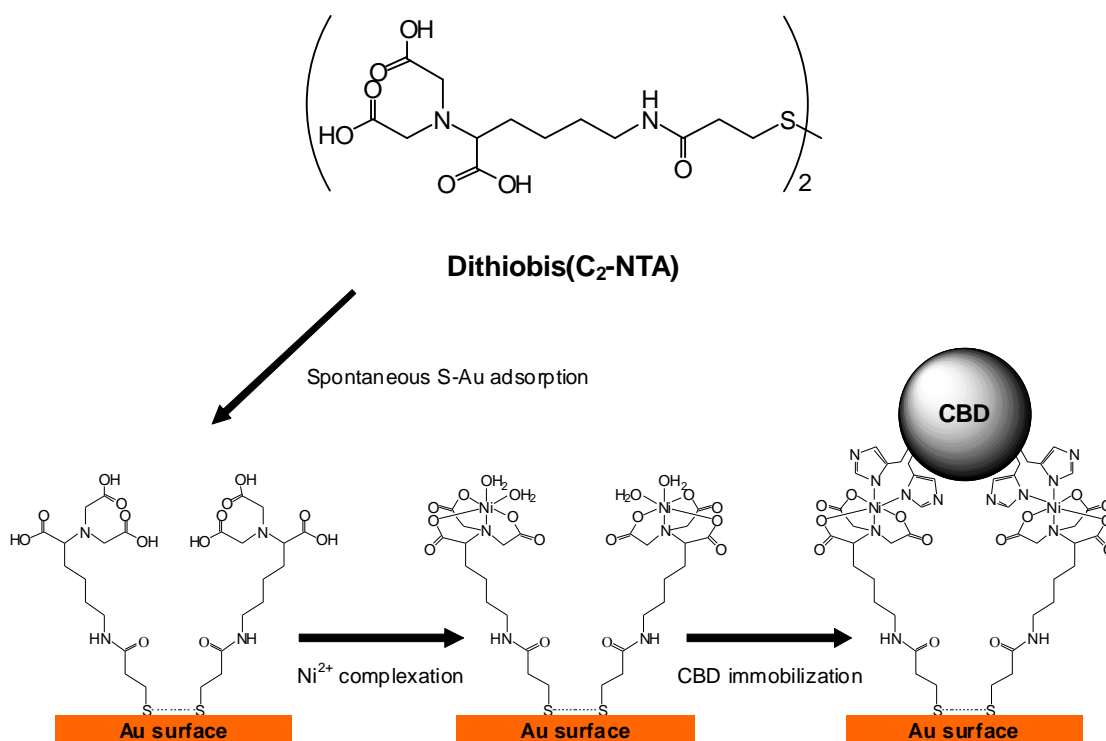
**Fig. 6.** Influence of paper stock conductivity on the retention of CBD-A-PAM (filled bars) and PAE (open bars).

These results strongly suggest that additive adsorption systems governed by ionic interactions are likely to be ineffective in papermaking, because their performance deteriorates as interfering substances accumulate. On the other hand, both organic trash with negative charges and inorganic cations had negligible influence on the function of CBD-A-PAM (Figs. 3-6). This suggests that CBD-A-PAM ‘recognized’ cellulose fibers via a CBD-mediated interaction, even under highly contaminated wet-end conditions. Thus CBD-conjugated polymers are promising wet-end additives that are not significantly affected by sudden changes in the ionic environment of papermaking systems. At this stage, CBD-polymers may not possess a good cost-effectiveness in

industrial processes due to high-priced cellulase, but they would have potential advantages for production of functional papers, e.g. electrolyte-free papers for insulating products.

### Direct Measurement of CBD Affinity for Cellulose Substrate

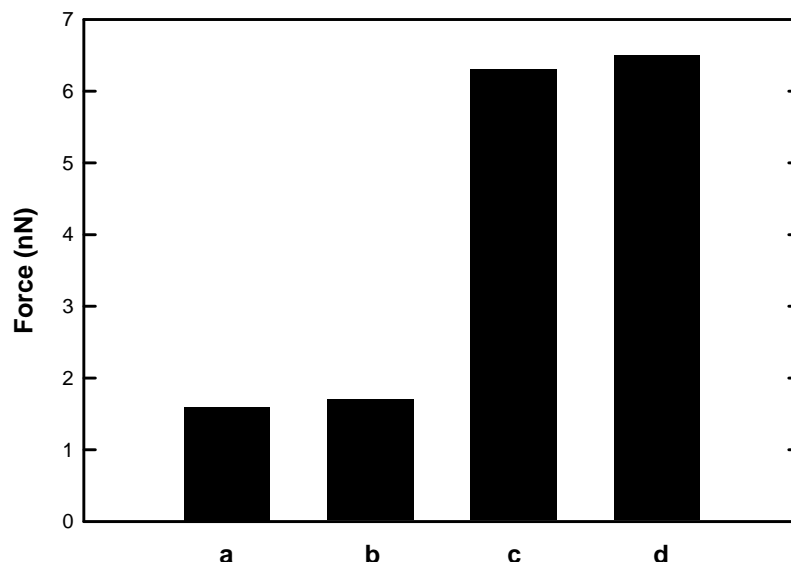
CBD-A-PAM served as an effective paper strength agent at the wet-end, and its interaction with pulp fibers differed greatly from the conventional wet-end interaction based on electrostatic attraction. To further elucidate the binding features of CBD to cellulose, atomic force microscopy (AFM) analysis was used to investigate the detailed interactions between the CBD moieties and a cellulose substrate. AFM provides quantitative physical information through probe/sample interactions, and the force curve profiles measured by the deflection of the cantilever give pico- to nano-Newton-level sensitivity even in liquid media (Ellis et al. 2006). In the present work, AFM force-distance measurements were carried out using a CBD-modified AFM probe and a flat cellulose model surface, in aqueous conditions. The Au-coated AFM probe was modified with pure CBD protein via  $\text{Ni}^{2+}$  complexation (Fig. 7). As shown in a previous section, fungal CBD from *T. viride* was used for the paper experiments; however, heterogeneous expression of fungal proteins generally involves great difficulties. Consequently, bacterial CBD<sub>ceX</sub>, a commercial product, from *C. fimi* containing His-tag was expressed by well-established protocol using *E. coli* for AFM analysis. SDS-PAGE analysis of the



**Fig. 7.** Chemical structure of dithiobis(C<sub>2</sub>-NTA) and schematic illustration of the modification of Au-coated Si<sub>3</sub>N<sub>4</sub> probe with CBD molecule.

protein obtained from the protein collection system, using CB<sub>IND</sub><sup>TM</sup> 100 resin, presented a clear single band at approximately 17 kDa according to the molecular weight of CBD<sub>ceX</sub> (data not shown), indicating that the CBD protein with cellulose-binding property was successfully isolated.

In general, detailed investigation of interfacial phenomena is very difficult to achieve for native cellulose such as pulp fibers because of their bulky, fibrous shapes, the presence of co-existing substances (hemicellulose and lignin), their rough and porous morphology, and various crystalline states. The problem can be overcome by using a flat cellulose nanolayer with native (cellulose I) crystal structure, (Yokota et al. 2007c), and that cellulose substrate was utilized in the AFM analysis in the present study. Figure 8 compares the attractive forces between each probe and substrate. It was confirmed that only a small attractive force (ca. 1.7 nN) was detected at an unmodified probe/Si wafer or a CBD-modified probe/Si wafer interface (Fig. 8), indicating that the AFM probes with or without CBDs interacted weakly with a Si substrate. By contrast, the attractive force between CBD-modified probe and the cellulose substrate in deionized water was relatively strong (6.3 nN), suggesting that CBD immobilized onto the AFM probe may attach selectively to a crystalline cellulose surface. It was found that a probe that had not been modified with CBD showed negligible adhesion to the cellulose substrate. Consequently, the strong attraction of the CBD-modified probe to the substrate is attributed to the CBD function.



**Fig. 8.** Attractive force between AFM probe and substrate. a) Au-coated Si<sub>3</sub>N<sub>4</sub> probe/Si wafer, b) CBD-probe/Si wafer, and c, d) CBD-probe/cellulose. AFM measurements were performed in deionized water (a-c) and 6 mM CaCl<sub>2</sub> solution (d).

Although the number of CBD molecules on the AFM probe is not known, the greater attractive force of CBD-modified probe to cellulose substrate was involved in the retention behavior of CBD-A-PAM at the wet-end. Moreover, the binding power remained almost unchanged (ca. 6.5 nN) in an aqueous salt solution, suggesting that inorganic salts do not inhibit binding of CBD<sub>ceX</sub> to cellulose. This observation is in

accordance with the additive performance of CBD-A-PAM at high salt concentration. The differences in the affinities for cellulose substrate between fungal and bacterial CBDs must be investigated in a future work. Nevertheless, the quantitative information provided by AFM, on the binding power of the CBD/cellulose attractive interaction, will assist design of functional additives composed of sugar-binding domains, for paper-making applications.

## CONCLUSIONS

1. Cellulose-binding domain (CBD) from *T. viride* was successfully introduced to A-PAM, which has no binding ability to cellulose fibers, via EDC-mediated condensation. The CBD-A-PAM product demonstrated significant retention, and enhanced performance as a paper strength additive, by comparison with commercial PAE.
2. Both additive retention and tensile strength of paper sheet prepared with CBD-A-PAM remains almost unchanged at the wet-end contaminated with anionic trash or multivalent inorganic cations, while the performance of PAE is inadequate.
3. Pure CBD protein with His-tags from *C. fimi* obtained by heterologous expression using *E. coli*, gives successful estimation of the attractive force of CBD to a cellulose model surface, and elucidation of the unchanged binding power of CBD to cellulose at high salt concentration.

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