Cellulose Fiber−Enzyme Composites Fabricated through Layer-by-Layer Nanoassembly

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Cellulose microfibers were coated with enzymes, laccase and urease, through layer-by-layer assembly by alternate adsorption with oppositely charged polycations. The formation of organized polyelectrolyte and enzyme multilayer films of 15−20 nm thickness was demonstrated by quartz crystal microbalance, ζ-potential analysis, and confocal laser scanning microscopy. These biocomposites retained enzymatic catalytic activity, which was proportional to the number of coated enzyme layers. For laccase−fiber composites, around 50% of its initial activity was retained after 2 weeks of storage at 4 °C. The synthesis of calcium carbonate microparticles on urease−fiber composites confirmed urease functionality and demonstrated its possible applications. This strategy could be employed to fabricate fiber-based composites with novel biological functions.

Introduction

The use of nanotechnology in forest products has attracted much interest. Intensive research has been focused on the fundamental composite material, cellulose fibers.[1] Long, hollow cellulose fibers with diameters of around 20 μm can be obtained by pulping wood chips and delignified to contain less than 1% of the remaining lignin. These fibers are generally used to manufacture paper and paperboard products.[2] During manufacturing process, nano- and microparticles such as titania and calcium carbonate are used as additives for whiter and brighter paper.[3] New nanomaterial technologies provide the opportunity to develop higher value and higher performance composite products based on cellulose fibers. Magnetic particles and noble metal nanoparticles have been successfully synthesized in the presence of cellulose fibers.[4] These nanoparticle-containing cellulose fibers were used to make special paper with magnetic properties or high-performance catalysis for chemical reactions under mild conditions. Cellulose micro- and nanofibrils were extracted from fiber cell walls and used to prepare light cellulose composites of high strength.[5]

Layer-by-layer (LbL) self-assembly is a versatile method to form tailored multilayered thin films.[6] The basic idea of the LbL method is the alternate deposition of polycations and polyanions through electrostatic interactions. The assembly process involves resaturation of polycation and polyanion adsorption, which results in the reversal of the terminal surface charge after deposition of each layer. A great variety of substances including linear and branched polyelectrolytes, inorganic nanoparticles, and proteins have been employed to deposit on planar and spherical substrates.[7] Polyelectrolyte and nanoparticle multilayers created by LbL nanoassembly have been utilized to modify the surface of cellulose fibers.[8,9] These modified fibers obtained special functions without losing their basic structure and properties. It was reported that the sequential LbL coating of cellulose fibers with oppositely charged polyelectrolytes and the subsequent formation of paper from these fibers resulted in an enhanced tensile strength of paper.[8a,9a] Using the LbL method to deposit organized multilayers of nanoparticles on cellulose fibers, Lu et al. found an increase of brightness and porosity without changing the tensile strength index of the paper.[9b]

The LbL technique appeared to be an advantageous method to immobilize bioactive molecules such as enzymes adsorbed on top or inside LbL polyelectrolyte films, due to the possibility of maintaining the structure and functionality of the enzymes.[7d,10] In addition, as compared with other noncovalent binding methods of immobilizing enzymes on cellulose, LbL is more versatile without the assistance of carbohydrate-binding modules or cellulose-binding polysaccharides.[11] This work immobilized laccase and urease on cellulose fibers through electrostatic LbL nanoassembly to fabricate functional biocomposites; see Figure 1. Such enzyme modified composites could be used to decompose urea or lignin or synthesize inorganic nanoparticles or polyphenols. The enzyme nanocoating on cellulose fibers was systematically analyzed by quartz crystal microbalance (QCM), ζ-potential, and confocal laser scanning microscopy. The activity and storage ability of enzymes in these biocomposites were evaluated, and the biomineralization application of urease-coated fibers was demonstrated.

Materials and Methods

All chemicals and enzymes were purchased from Sigma-Aldrich and used without further purification. Poly(dimethyldiallyl ammonium chloride) (PDDA, M_w 100 000−200 000 Da) and sodium poly(styrene sulfonate) (PSS, M_w 70 000 Da) were used as polycation and polyanion, respectively. Laccase from *Trametes versicolor* has an activity of 22.6 U/mg, and urease type IX from *Jack beans* has an activity of 65.7 U/mg. Fluorescein isothiocynate (FITC) was used to label the enzyme for confocal imaging. Beaten bleached Kraft softwood fiber sheets, supplied by International Paper Company, were dispersed in water to obtain cellulose fibers.

To maximize the enzyme activity, the experiments were performed under optimum pH conditions. Both enzymes were negatively charged under the optimum pH conditions and could be assembled alternately with polycations. For the assembly of laccase, all chemicals were prepared at a concentration of 2 mg/mL in 0.05 M sodium acetate buffer at pH 4.5. Dry fibers (4.5 mg) were dispersed in deionized water. Then, a standard LbL assembly procedure[9a] was applied with an adsorption time of 10 min for polyelectrolytes and 20 min for enzymes. After

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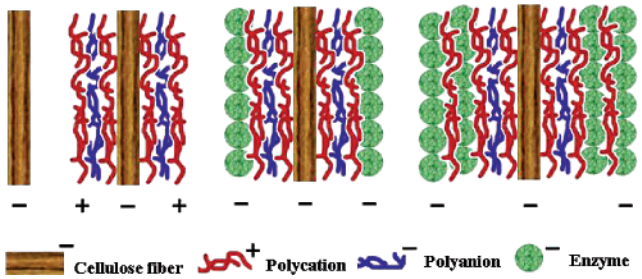

Figure 1. Illustration of the LbL assembly of polyions and enzymes on cellulose fibers.

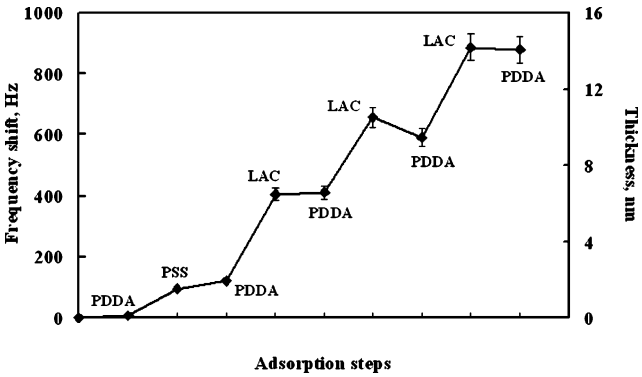

Figure 2. QCM monitoring (frequency and thickness change vs adsorption steps) of laccase(LAC)/PDDA assembly. The first three polyion layers (PDDA/PSS/PDDA) were precursor films.

three precursor polyelectrolyte layers, laccase and PDDA were alternately deposited with the enzyme layer as the outermost layer. For the assembly of urease, all chemicals were prepared at a concentration of 2 mg/mL in 0.02 M Tris-HCl buffer at pH 7.2.

The thickness of the coating was estimated with a quartz crystal microbalance (QCM, USI-system). Multilayer films were deposited on a silver QCM resonator in the same way as was done for coating on fibers. The frequency shift was monitored after each adsorption cycle and converted to thickness using the Sauerbrey equation: ΔD (nm) = $-0.017\Delta F$ (Hz). The surface potential variation was monitored using a Brookhaven Zeta Plus microelectrophoretic instrument. Scanning electron microscope (AMRAY, model 1830) was used to image dried cellulose fibers and $CaCO_3$ microparticles. Confocal laser scanning microscopy (Leica TCS SP2) was used to analyze the nanocoatings on cellulose fibers.

The activity of laccase was measured by monitoring the oxidation of ABTS.[12] Samples were mixed with 2.9 mL of 0.4 mM ABTS in 0.05 M sodium acetate buffer (pH 4.5). The UV absorbance data at 420 nm were continuously recorded for 20 min. For each sample, the same amount of enzyme-coated fibers was added to the test solution. An activity was calculated from the slope of the absorbance curve of each test. Urease activity was measured by a colorimetric assay based on the hydrolysis of urea, as reported in previous work.[13]

Results and Discussion

Assembly of Laccase Multilayers on QCM Electrodes. First, the assembly was elaborated on silver QCM resonators to determine the optimal conditions for the multilayer growth. After deposition of each layer, the change in frequency was recorded; this corresponded to the amount of mass deposited on the electrode. The assembly results are shown in Figure 2. A stepwise growth of a laccase/polycation (LAC/PDDA) multilayer on the QCM resonator was observed. The average frequency shift ($-\Delta F$) for the alternate LAC/PDDA adsorption cycle was 253 ± 63 Hz and was 276 ± 26 Hz for laccase adsorption and a small negative frequency change for PDDA.

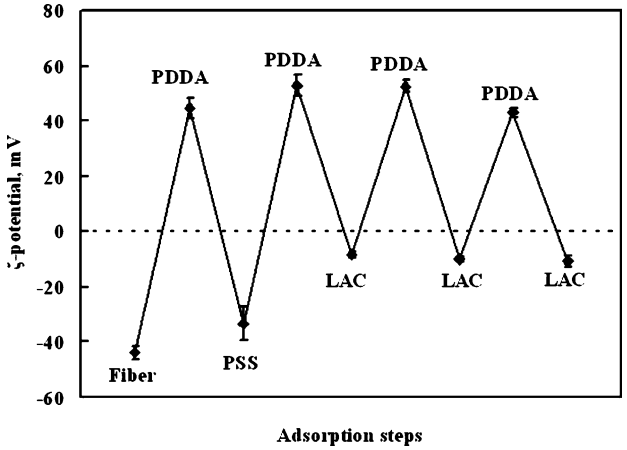

Figure 3. ζ-Potential of the coated short cellulose fiber vs the number of adsorption steps for multilayer film composition of {PDDA/PSS/(PDDA/laccase)$_3$}. Data are shown as means ± SD (n = 3).

This is a typical phenomenon that occurs in protein/polyelectrolyte LbL assembly.[7d] When PDDA was deposited on top of the protein layer, its flexible linear structure enabled PDDA to penetrate between protein molecules. At the same time, the strongly charged PDDA peeled off some of the weakly attached outermost proteins and recharged the surface. The enzyme layer thickness calculated from the Sauerbrey equation was 4.7 ± 0.4 nm. This value is consistent with the molecular dimension of laccase (6.5 nm × 5.5 nm × 4.5 nm),[14] suggesting a relatively uniform laccase monolayer formation.

Formation of Polyelectrolytes/Laccase Multilayers on Cellulose Fibers. One of the prerequisites for the sequential deposition of polyelectrolytes and enzymes onto cellulose fibers is the charge inversion at every deposition step. Figure 3 shows the ζ-potential changes for laccase layers alternating with PDDA during the assembly on cellulose fibers. The cellulose fibers have a negative potential of −43.8 ± 2.5 mV. The first three precursor polyelectrolyte layers provided even coating and showed a regular alternation of surface potential with values of +45 ± 3.7 mV for cationic PDDA and −33 ± 6.2 mV for anionic PSS. The isoelectric point (IP) of laccase from *Trametes versicolor* is around 4.0.[15] The charge of laccase is weakly negatively charged at pH 4.5. Therefore, when laccase formed the outermost layer, the surface had a small negative potential, −8.5 ± 1.2 mV. A deposition of the next PDDA layer recharged the surface and restored the positive potential of +52.6 ± 2.1 mV. Then, again values of −10.2 ± 1.0 mV for laccase and +43.1 ± 1.9 mV for PDDA were found. For each layer deposited, the underneath layer could have a different moleculer distribution and conformation, which may have resulted in the slight variations of the measured values.[16] Overall, alternate ζ-potential changes were observed for all laccase/PDDA multilayer films, depending on whether the polyelectrolyte or the enzyme formed the outermost layer. This result proves the LbL assembly of laccase and PDDA in organized multilayers.

Confocal laser scanning microscopy was employed to visualize the location of the laccase multilayer nanocoatings on cellulose fibers. For this purpose, laccase was labeled with FITC (green fluorescence) and assembled with PDDA as described previously. Figure 4 shows the uniform laccase coatings on the surface of the fibers. Our previous work has shown that only low molecular weight polyelectrolytes may penetrate into the fiber walls.[9a] Because of the resolution limitation of the microscope, it is impossible to accurately estimate how deep the polymer can penetrate. In this work, we deposited precursor PDDA/PSS/PDDA layers before we assembled the enzyme.

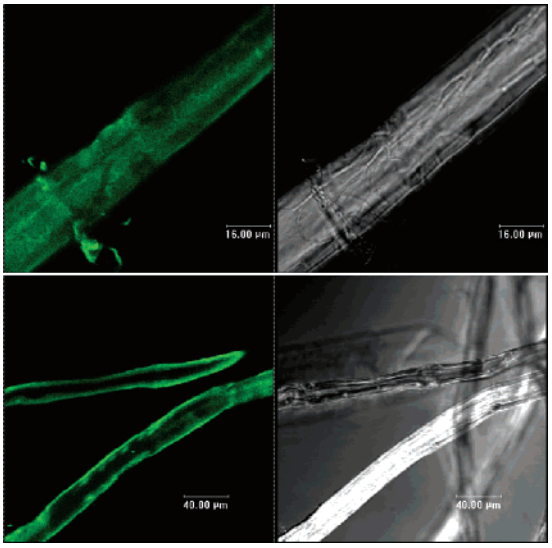

Figure 4. Laser scanning confocal microscope images of cellulose fibers coated with three bilayers of FITC-labeled laccase (green fluorescence) in fluorescent mode and transmission mode.

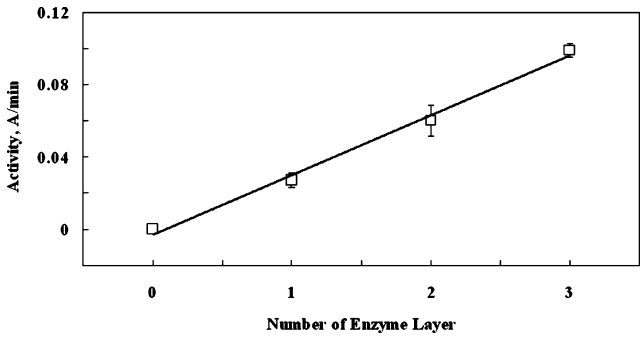

Figure 5. Catalytic activity of laccase−fiber biocomposites with one to three (PDDA/ laccase) bilayers on cellulose fibers. Data are shown as means ± SD ($n = 3$).

These polyelectrolytes formed a complex that blocked the small cell wall pores—the mean pore width of the cell wall voids ca. 5 nm.[17] In addition, the test of activity versus enzyme layer showed no difference from the result of enzymes adsorbed on a solid surface.[18] Therefore, it is unlikely that a major part of the enzyme molecules penetrated into the fiber cell wall in this case.

Enzymatic Activity of Biocomposites. Figure 5 shows the activity for cellulose fibers coated with one to three PDDA/ laccase bilayers. As expected, an increase in the enzymatic activity proportional to the enzyme layer number was detected. This demonstrates that the embedded first, second, and third enzyme layers remained accessible to the ABTS substrate. A linear proportionality with enzyme layers indicates a homogeneous laccase adsorption for each layer. It is established that protein/polymer LbL multilayers are permeable for small molecules, so ABTS substrate can rapidly penetrate into the films.[16] There are only three bilayers of PDDA/laccase on cellulose fibers (of ca. 14 nm thickness), so diffusion limitation did not play an important role in the enzyme activity test. One of the advantages for assembling enzyme thin films via the LbL method is that the enzymatic activity could be tuned by varying the number of enzyme layers. This has been demonstrated on flat substrates[19,20] and colloid particles.[16,18] Our work shows that this tunable feature is also valid on a hollow tubular substrate with a rough surface coated with a three layer polyelectrolyte precursor.

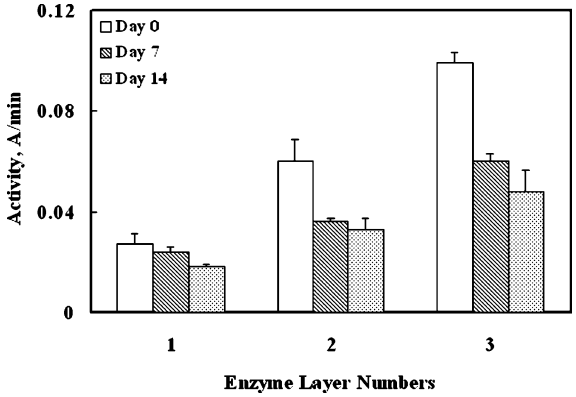

Figure 6. Storage ability of enzyme−fiber biocomposites with one to three (PDDA/laccase) layers at 4 °C. Data are shown as means ± SD ($n = 3$). For two and three bilayer samples, days 7 and 14 data points have no significant difference.

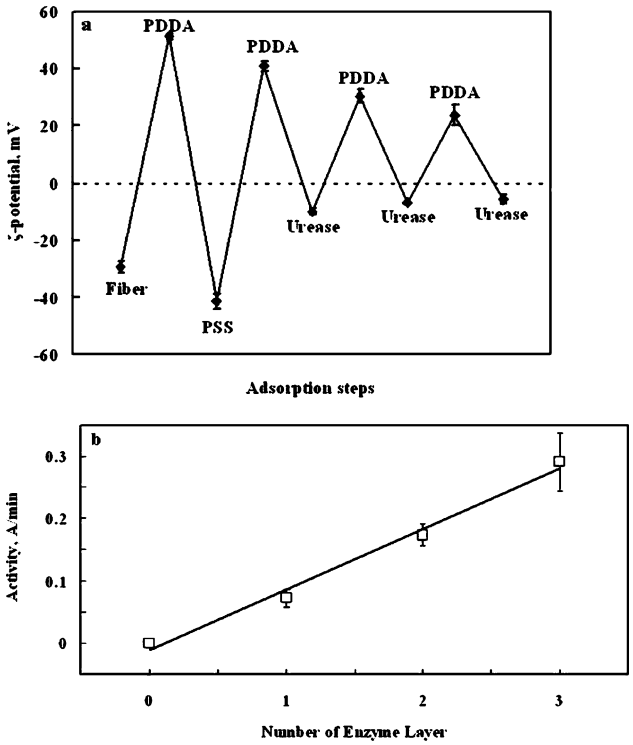

Figure 7. Characterization of urease−fiber biocomposites. (a) ζ-Potential during LbL assembly of PDDA and urease on fibers and (b) enzymatic activity for one to three (PDDA/urease) bilayers on cellulose fibers. Data are shown as means ± SD ($n = 3$).

To determine the stability of laccase immobilized in the biocomposites, samples with an architecture of one to three bilayers of PDDA/laccase were stored in water at 4 °C for up to 14 days. In all cases, laccase was the outermost layer. Figure 6 shows the biocomposite activity changes during the 14 days. All samples have declined enzymatic activities with elapsed time. Especially in the first 7 days, the activity decreased by about 40% for the $(PDDA/laccase)_2$ and $(PDDA/laccase)_3$ biocomposites. The rapid loss of activity could be attributed to the laccase desorption from the outermost enzyme layer. A similar protein desorption from polyelectrolyte multilayer films assembled on flat substrates or on colloids has also been reported.[7d,21] However, from 7 to 14 days, the biocomposite only lost ca. 10% of its activity and maintained around 50% of its initial enzymatic activity at day 14. Additionally, there was no significant difference between these two data points. During this period, the activity of biocomposites had reached a stable

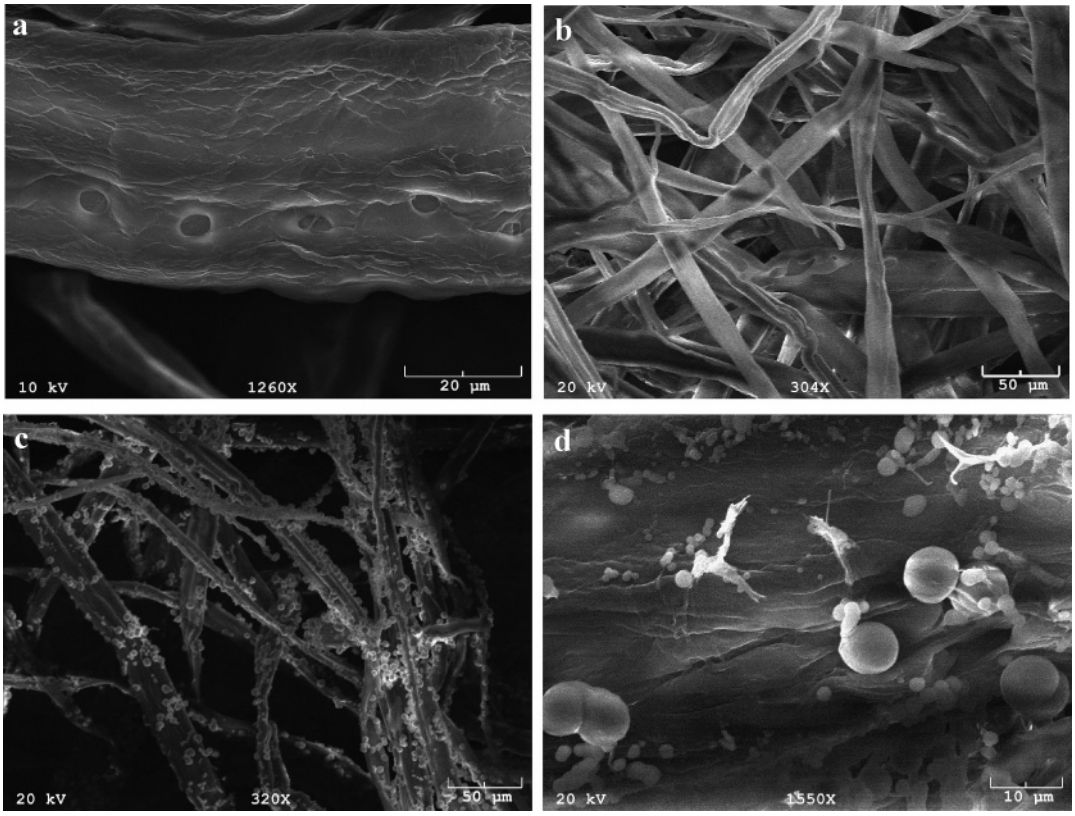

Figure 8. SEM images of enzyme−fiber biocomposites. (a) Biocomposites with three (PDDA/urease) layers; (b) negative contol: fibers with $(PDDA/PSS)_3$ coating reacted with Ca^{2+}—no microparticle formation observed; and (c and d) composites after biocatalysis reaction-$CaCO_3$ microparticle formed on fibers.

stage. A similar enzyme activity decrease has been observed in other LbL enzymatic research. After exponentially losing activity at the initial stage, immobilized enzymes maintained pretty stable activity for more than 30 days.[22] It is possible that the laccase−fiber biocomposites could maintain high activity for at least one month. Laccase is a phenol oxidase that catalyzes the oxidation of various aromatic compounds (particularly phenols, including lignin). It was used to fabricate biosensors to detect polyphenols in wastewater.[23a] In the paper making process, laccase was studied either as a bio-bleaching catalysis or as a fiber modification agent.[23b,c] The laccase−fiber biocomposites could find possible applications in these areas.

Formation of Polyelectrolytes/Urease Multilayers and Biomimetic Synthesis of Calcium Carbonate Microparticles. Urease was also successfully assembled with PDDA on cellulose fibers. The resulting biocomposites were characterized in the same methods. QCM results indicated the step-by-step urease thin film formation with ca. 6.9 ± 1.4 nm thickness. Figure 7 a shows the ζ-potential alternate change with the layer number for oppositely charged urease and PDDA. The surface potential of the urease layer decreased from −10 to −5 mV, indicating a decrease of surface charge density. Therefore, the subsequent PDDA adsorption became less, and the resulting surface potential also decreased from +40 to +24 mV. Obviously, the urease/PDDA layers assembled on fibers showed an increase in activity with layer number, as revealed by Figure 7b. After 7 days storage at 4 °C, the biocomposites activity also decreased by ca. 50% and remained stable. It was reported that enzymes embedded in multilayer polyelectrolyte films prevent desorption.[24] We also found that with two bilayers of PDDA/PSS on top of the outermost urease layer, the biocomposites could retain 70% activity after 7 days.

Urease has been widely used as a catalyst for biomineralization reactions. Biomimetic synthesis of inorganic composites is a developing research area lying at the nexus of chemistry, biology, medicine, and materials science. Moreover, biomineralization is one of the most promising methods to form hybrid inorganic/organic nanomaterials, which often display unique and desirable morphological, structural, and mechanical properties and represent informative models for the design of complex functional structures.[25] This process usually occurs at room temperature and mild conditions and requires the presence of organic nanotemplates like Langmuir monolayers, self-assembled monolayers, micelles, and emulsions.[26]

We demonstrated the application of urease−fiber biocomposites in biomineralization to produce hybrid inorganic/organic composites. Urease−fiber biocomposites were introduced into a mixture of 0.5 M urea and 1 M $CaCl_2$ for 10 min. Then, the biocomposites were washed in deionized water 3 times. The formation of $CaCO_3$ precipitates began immediately after urea decomposition into ammonia and CO_3^{2-} catalyzed by urease in the LbL multilayer. Urea decomposition and CO_3^{2-} formation occurred on the fiber surface, whereas calcium cations diffused from the surrounding solution. According to previous work,[27] to prevent undesirable formation of $CaCO_3$ in bulk solution, a higher concentration of Ca^{2+} is required, so that it consumes all CO_3^{2-} produced at the surface vicinity. Using a Ca^{2+} concentration below 0.1 M leads to the formation of $CaCO_3$ particles in the solution. Before initiation of calcium carbonate microparticle formation, urease-coated fibers looked similar to the uncoated fibers (no particles were detected). The surface was rough, and cell wall openings were still visible; see Figure 8a. After reaction with urea and $CaCl_2$, calcium carbonate microparticles were formed on the surface of the cellulose fibers,

as shown in Figure 8c. Most of these particles have a spherical shape with diameter of 1−7 μm. Formation of such composites may be useful for the paper making industry, where $CaCO_3$ microparticle loading is an important technique to improve paper brightness.[2b] A negative control proved that the addition of Ca^{2+} to polyelectrolyte-coated fibers would not cause reconstruction of films without biocatalysis (Figure 8b).

It is interesting to compare our approach with the work on immobilization of an enamel matrix derivate protein on polylysine/polyglutamic acid multilayer and its LbL assembly with this polyacid.[28] This assembly could be of great interest for biomineralization of hydroxyapatite crystals for dental implants, but no enzymatic activity of enamel formation was demonstrated yet.

Conclusion

The possibility of employing cellulose microfibers as a support to fabricate bioactive composites with organized enzyme multilayers was demonstrated. Through the LbL nanoassembly, laccase and urease were sequentially deposited with polycations, which acted as electrostatic glue between proteins. The biocomposites were enzymatically active, and this activity could be tuned by varying the number of enzyme layers in the coating. For laccase−fiber composites, around 50% of the initial enzyme activity was preserved after 14 days of storage in water. Urease−fiber composites were successfully applied for biomineralization to grow calcium carbonate microparticles needed for paper whitening. The strategy presented could be used for the creation of cellulose fiber-based biocomposites with various functions that can be precisely controlled by the film nanoarchitecture.

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