Enzyme Catalysis

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Biocatalytic Synthesis of Poly(L-Lactide) by Native and Recombinant Forms of the Silicatein Enzymes**

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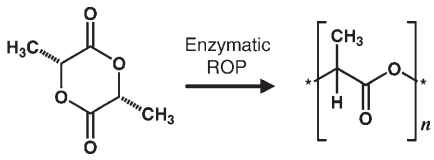
The synthesis and application of biocompatible and biodegradable polymers have recently emerged as areas of great interest. These materials are rapidly becoming viable alternatives to biologically intractable polymers for industrial uses such as packaging.[1] They are uniquely suited to specialist biomedical applications, such as the encapsulation and controlled release of bioactive molecules[2–5] and as scaffolds for tissue engineering.[6,7] Herein, we describe a novel enzymatic method for the low-temperature synthesis and surface deposition of one such polymer, poly(L-lactide) (PLA).

We have previously described the unusual utility of silicatein enzymes in the synthesis of inorganic materials. These proteins were first identified as filamentous aggregates occluded within the silica spicules of the Pacific demosponge *Tethya aurantia*. These filaments were subsequently found to consist of three individual protein species, which we have termed silicateins (silica protein) α, β, and γ according to their differential migration on an SDS-PAGE gel.[8] The filaments demonstrated unusual catalytic and templating activities, such that they were capable of biocatalytically directing the in vitro polycondensation of 1) polymeric silica and polysilsesquioxanes,[9] 2) nanocrystalline/amorphous titanium dioxide,[10] and 3) gallium oxohydroxide and spinel gallium oxide[11] from stable, water-soluble precursors under mild temperature and pH conditions.

Silicatein-α, which makes up 70% of the filaments by weight, has a surprisingly similar sequence identity to the family of papainlike cysteine proteases. Crucially, however, in silicatein-α, the active site cysteine is replaced with a serine. The corresponding decrease in the nucleophilicity of the catalytic triad considerably reduces the efficiency of silicatein-α as a protease.[9,12] We have suggested that this low catalytic activity is critical when the enzyme is applied to inorganic systems as the kinetically limited production of reactive-product species results in the slow growth of materials at the filament surface. This generates inorganic products with a nanoscale and/or microscale order that are otherwise unobtainable under ambient conditions.

We have recently also created a silicatein-based whole-cell biocatalyst through the recombinant expression of silicatein-α at the external surface of cells of the bacterium *Escherichia (E) coli*. This was achieved through the insertion of silicatein-α within the first extracellular loop of a recombinant form of the *E. coli* outer membrane protein A (OmpA).[13] We found that the advantages of silicatein-based synthesis are retained in this whole-cell system, and we are able to generate layered titanium phosphates at the outer membrane of silicatein-displaying cells at physiologically relevant temperature and pH.

Herein, we expand the range of products amenable to silicatein-based synthesis through the generation of PLA from the cyclic precursor L-lactide through a biocatalytically controlled ring-opening polymerization (ROP) (Scheme 1).



Scheme 1. Ring-opening polymerization (ROP) of poly(L-lactide).

Silicatein filaments and heat-denatured controls were reacted with L-lactide in an organic solvent and the results were investigated by using scanning electron microscopy (SEM). The filament surface is normally smooth, as is shown in Figure 1a. The heat-denatured filaments showed little change in surface morphology after incubation with L-lactide for 16 h (Figure 1b). In contrast, native filaments are seen to accumulate PLA at the filament surface (Figure 1c). Over longer timescales, coagulated structures were seen in which product formation was sufficient to join individual filaments (Figure 1d).

We also investigated the capacity of silicatein-displaying cells to synthesize PLA. Solubilized L-lactide was introduced to a suspension of silicatein-displaying cells in isotonic phosphate-buffered saline (PBS) solution. After overnight incubation (Figure 2a), polymeric products were apparent at the cell surface and were characterized by an increase in cell

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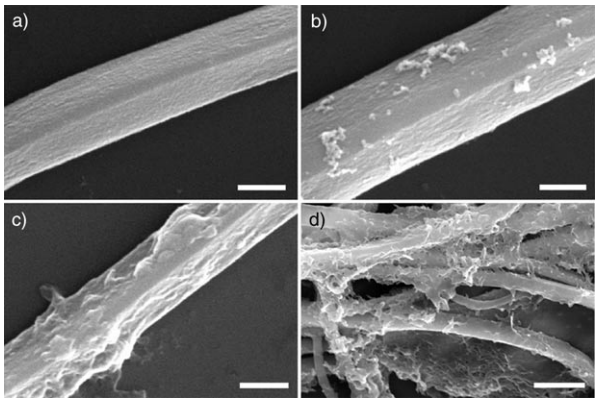

Figure 1. Silicatein filaments catalyze the synthesis of poly(L-lactide). a) Unreacted filament; b) denatured control, 16 h incubation with L-lactide; c) and d) filaments after incubation with L-lactide for 16 h and 90 h respectively. (a–c), Scale bar = 1 μm; d) scale bar = 4 μm.

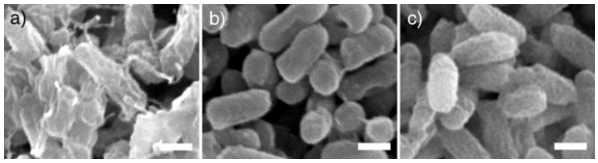

Figure 2. Cell-based synthesis of poly(L-lactide). *E. coli* cells displaying silicatein-α at the cell surface synthesize polymeric products a) apparent as thread-like structures between cells and as increased cell surface roughness. This product was significantly reduced in control cells that were preincubated with b) the protease inhibitor PMSF and c) in cells expressing recombinant OmpA only. Scale bar = 0.5 μm.

surface roughness and as "threads" between cells. These products were significantly reduced when cell suspensions were preincubated with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Figure 2b) and in cell preparations that expressed recombinant OmpA only (Figure 2c). Analysis by TEM further confirms the presence of surface products in filament-based and cell-based syntheses (see the Supporting Information).

As a result of the similarity between these data and our previous experiments with inorganic materials, we became interested to determine whether any crystalline organic-surface products might be observed. PLA has pseudo-orthorhombic[14] and hexagonal[15] phases that are typically formed by annealing from the molten state for several hours at $>130\,^{\circ}C$.[15–17] Crystals can also be grown from solutions in organic solvents at lower temperatures and over long-time periods.[15,18] However, selected-area electron diffraction patterns of both the filament and cell surfaces (see the Supporting Information) definitively show that only an amorphous polymer is formed during silicatein-based synthesis.

X-ray photoelectron spectroscopy (XPS) was used to analyze the PLA product on silicatein filaments. The XPS spectrum of PLA is relatively simple owing to the chemical simplicity of the repeat unit. The C1s orbital consists of approximately equivalent contributions from C–H, C–O, and C=O groups that occur at normalized binding energies of 285.0, 286.9, and 289.0 eV, respectively.[19–22] The O1s orbital is composed of two equivalent component peaks at normalized binding energies of 532.0 eV (C=O) and 533.4 eV (**O**–C=O).[22]

High-resolution scans of regions that correspond to the C1s, O1s, and N1s orbitals (Figure 3a–f) were recorded and

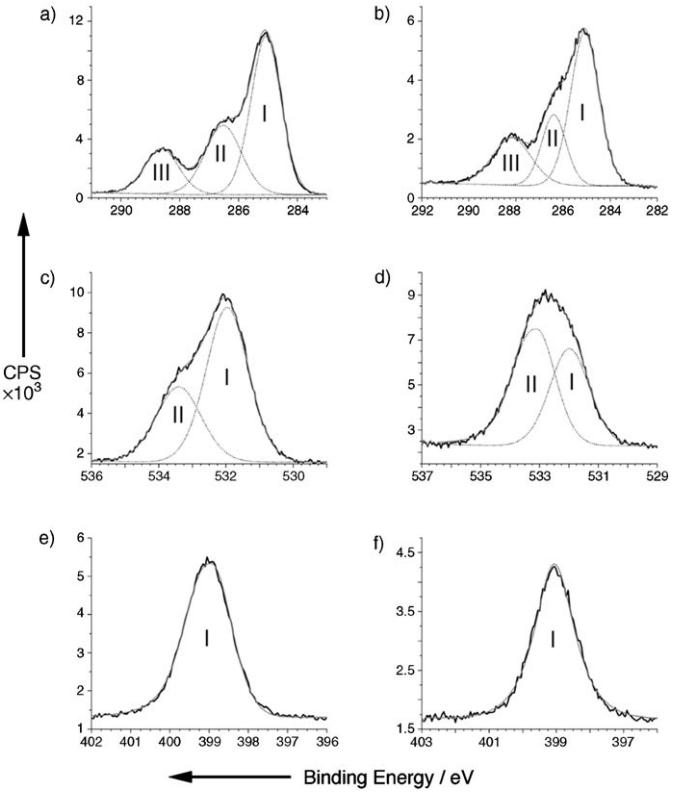

Figure 3. High-resolution XPS spectra of a), c), and e) filament controls and b), d), and f) filaments after reaction with L-lactide for 3 days. The synthetic component peaks of C1s (a and b), O1s (c and d), and N1s (e and f) orbitals are shown (see also Table 1). —— = experimental data; - - - - - = fitted components; —— = envelope of fitted data; CPS = counts per second

analyzed by curve fitting of synthetic component peaks. Native filaments that were reacted with L-lactide, demonstrated a relative increase in the C1s (Figure 3a,b) and O1s (Figure 3c,d) spectra at binding energies that are typically associated with ester groups ($\approx$288.5 eV and $\approx$533.2 eV, respectively). Quantitative values derived from these experiments are shown in Table 1. We concluded that the increase in these signals represents the formation of PLA at the filament surface.

The inherent gross surface roughness of the silicatein samples is nonideal for XPS analysis and results in peak broadening.[23] The individual orbital spectra of samples incubated with L-lactide are 0.5–1 eV broader than those in the control experiments; and full width at half maximum (FWHM) values of component peaks are >1.2. These considerations, combined with the complexities of the filament background signal (Figure 3a,c,e), obliged us to use a simplistic approach to peak fitting. Although this prevents a

Table 1: Position and quantification of component peaks from Figure 3.

Sample	Orbital	Peak	BE [eV][a]	FWHM [eV]	Composition [%]	Assignment
filament	C1s	I	285.07	1.20	53.73	**C**-C, **C**-H
		II	286.52	1.46	28.8	**C**-O, **C**-N
		III	288.61	1.36	17.47	**C**=O, O=**C**-O
	O1s	I	531.96	1.48	65.66	C=**O**, N-C=**O**
		II	533.41	1.59	34.34	C-**O**, **O**=C-**O**-C
	N1s	I	398.94	1.23	100	**N**-H, **N**-C, **N**-C=O
filament +lactide	C1s	I	285.08	1.42	55.02	**C**-C, **C**-H
		II	286.39	1.23	21.95	**C**-O, **C**-N
		III	288.17	1.93	23.03	**C**=O, O=**C**-O
	O1s	I	531.98	1.58	42.53	C=**O**, N-C=**O**
		II	533.09	1.52	57.47	C-**O**, **O**=C-**O**-C
	N1s	I	399.06	1.37	100	**N**-H, **N**-C, **N**-C=O

[a] BE = binding energy.

more detailed analysis of individual spectral components, it is sufficient for our purposes to confirm the synthesis of PLA.

Current limitations of this silicatein-based synthesis include the small quantities of enzyme available, the potential passivation of the catalytic surface, and competition from water. Furthermore, product isolation is complicated by the intimate contacts formed between the product and the reactive surfaces. As the enzyme is a catalyst rather than a ROP initiator, however, the product is expected to be adsorbed to the surfaces rather than covalently bound. Because of these inherent constraints, we have thus far been unable to generate the high product yields necessary to further characterize the PLA product by standard techniques such as NMR, IR, gel permeation chromatography and MS. We were also unable to determine the molecular-mass average of the product. Nevertheless, the observations reported here provide a first proof-of-principle that silicatein is capable of catalyzing the polymerization of the PLA precursor.

The controlled, surface-templated enzymatic synthesis of organic polymers at low temperature, shown here for the first time, has great promise as a nanotechnological tool. It has already been shown that polymer blends and organic/inorganic composites can be formed thorough enzymatic methods.[24,25] We therefore hope to extend the silicatein technology to the synthesis of a range of oriented PLA composites including materials with characteristics such as electrical conductivity[26] and those that incorporate bioactive glass[27] and hydroxyapatite.[28]

Experimental Section

Silicatein filaments were prepared from the silica spicules of *Tethya aurantia* by hydrofluoric acid demineralization according to standard methods.[8,9] For control experiments, protein denaturation was carried out at 100 °C for 2 h. As previously described, silicatein-α (Sil) is cloned into the first external loop of the *E. coli* OmpA resulting in the surface display of the recombinant fusion protein OmpA-Sil.[13] Briefly, the *E. coli* strain MC1061 was transformed with a derivative of pBAD33,[29] which contains the OmpA-Sil insert,[13,30] and grown in chloramphenicol-supplemented LB broth at 37 °C to a cell density of 0.9 at A_{600}. Protein expression was induced by the addition of L-(+)-arabinose to a final concentration of 0.2 % (w/v). At 6 h post induction, cultures were harvested in 50 mL aliquots by centrifugation at 3500 *g*, snap frozen and stored as pellets at −80 °C. When required, pellets ($\approx 5 \times 10^{10}$ cells) were washed with 50 mL of an isotonic PBS solution (8 M NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4, pH 7.4) and resuspended in PBS (10 mL).

L-Lactide (purchased from Sigma) was stored under argon at 4 °C and dissolved, as required, in chloroform/methanol/water, 1:1:0.08 (50 mM v/v) for filaments or in methanol ($\approx$ 1 M saturated solution) for cells. For the reaction with filaments, filament samples (1 mg) were pelleted at 6000 *g* for 15 min and resuspended in the lactide solution (400 μL) before being reacted at 16 °C and rotated on a mill for 16–90 h. For the cell-based reaction, lactide (0.5 mL of the 1 M solution) was introduced to the cells (10 mL) in PBS solution and reacted with gentle agitation for 16 h at 16 °C. The polymeric product was isolated from the cell solution by allowing it to settle under gravity. In control samples, OmpA-Sil cells were either incubated for 30 min with the protease inhibitor PMSF (0.1 mM) (purchased from Sigma) or cells that only express a recombinant OmpA were used.

For microscopic analysis, filament and cell samples were extracted from the aqueous phase by centrifugation and washed three times with milliQ water (1 mL). For SEM, samples were then dried directly onto glass cover slips (filaments) or lyophilyzed and transferred to cover slips with a plastic pipette tip (cells) prior to analysis with a Vega TS 5130 MM instrument (Tescan, Czech Republic) at an accelerating voltage of 20 kV. An energy dispersive spectrometer (EDS) attachment (IXRF Systems, Houston, TX) was used for elemental mapping of selected regions. For TEM, samples were dried onto holey-carbon copper grids (Ted Pella, Redding, CA) before imaging at 200 kV (FEI T-20, Hilllsboro, Oregon). Selected area electron diffraction patterns (SAEDs) were subsequently recorded from various surface regions. In all cases, representative micrographs were taken at random locations within the samples.

For XPS, filaments (5 mg) were reacted with L-lactide for three days as described above. Control reactions in which L-lactide was omitted were also performed. Samples were thoroughly washed in chloroform/methanol (1:1; v/v) and dried before being transferred to double-adhesive copper tape and analyzed with a Kratos Axis Ultra XPS instrument (Kratos Analytical, Chestnut Ridge, NY) by using a monochromatic $Al_{K\alpha}$ source (1486.6 eV) at 225 W. Spectra were acquired in the fixed analyzer transmission mode with vacuum maintained at 3.5×10^{-8} torr. Survey data were recorded between 0–600 eV at 160 eV pass energy and 0.5 eV/step and high-resolution spectra of the C 1s, O 1s and N 1s regions were recorded at 20 eV pass energy and 0.05 eV/step. Dwell times varied between 150–400 ms.

Data analysis and curve fitting was performed with the CasaXPS software. Regions of interest were fit to the lowest possible number of synthetic component peaks with a mixed Gaussian/Lorentzian line shape by using the background of Tougaard. All residuals (difference between experimental data and fitted envelope) were less than 1.5. Component quantification was performed by using appropriate sensitivity factors provided within the software. FWHM values were slightly larger than anticipated, which is attributed to the relative roughness of the sample surface. Peak assignments were referenced to literature data and to the C 1s aliphatic carbon peak at 285 eV.

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