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ARTICLE

Laccase-catalyzed Controlled Radical Polymerization of *N*-Vinylimidazole

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Laccase from *Trametes versicolor* is a multi-copper-containing oxidoreductase which was found to catalyze the polymerization of *N*-vinylimidazole under conditions of atom transfer radical polymerization (ATRP) in aqueous solution (pH 4, 100 mM acetate buffer) at ambient temperature by using sodium ascorbate as reducing agent. The reaction followed first order kinetics and resulted in polymers with controlled number-average molecular weights (between 1660 and 9970 g mol^{-1}) and relatively narrow, monomodal molecular weight distributions (*Đ* between 1.27 and 1.56) according to gel premeation chromatography. Purified polymers were also analyzed by mass spectrometry which revelead a *Đ* of 1.07. The enzyme could be separated quantitatively from the polymer, lowering the metal content of the purified polymers below the detection limit of ICP-OES of 9 ppb. The enzyme retained its polymerization activity for more than eight hours, but formed electrostatic complexes with the polymer and underwent conformational changes at the beginning of the reaction. Biocatalytic controlled radical polymerization allows to synthesize poly(*N*-vinylimidazole) with well-defined molecular weight. Such polymers will be useful building blocks in many applications, such as drug- and gene-delivery, fuel cell membranes and polyionic liquids.

Introduction

Derivatives of the heterocyclic aromatic compound imidazole have an extraordinary importance in nature, since the aromatic ring is a structural element of the amino acid histidine, the hormone histamine, and other naturally occurring molecules. Moreover, the imidazole moiety is also present in many biologically active pharmaceuticals and agrochemical compounds. Polymers featuring imidazole rings, such as poly(*N*-vinylimidazole) (PNVIm), poly(2-vinylimidazole), poly(4-vinylimidazole) and polymers of *N*-vinylimidazolium salts, have attracted great interest not only in material science, but also in biomedical applications.[1-3] Poly(vinylimidazoles) and their quarternized salts show potential in applications that range from non-toxic gene delivery vectors,[4, 5] to fuel cells,[6-10] lithium ion batteries,[11] membranes for metal ion complexation[9, 12-15] and as molecularly imprinted matrices.[3, 16] Moreover these polymers have been explored as carbon dioxide absorbing membranes,[17] anticorrosion coatings,[18] catalysts and catalyst supports,[19-23] for drug-delivery[24] and as polymerized ionic liquids.[3] The most widely used monomer that features an imidazole side chain is *N*-vinylimidazole (NVIm). Homo- and copolymers of this monomer are used as washing formulation ingredients,[25] in cosmetic and hair care products,[26, 27] and as filtration materials to remove metal ions from alcoholic beverages like wine.[28] Many of these applications would benefit from PVIm with a predetermined molecular weight and a narrowly dispersed molecular weight distribution. Such well-defined polymers allow to conclusively study their structure-property relationships and consequently to tailor their properties. For example, the transfection efficiency and cytotoxicity of polycationic gene delivery vectors strongly depends on the molecular weight of the polymers.[29, 30] Moreover, advanced polymer architectures such as block, graft and star polymers are crucial for the conception of high-tech materials in many fields of application.[1-3] However, vinylimidazoles are commonly polymerized by free radical polymerization,[1, 31-35] which yields broadly distributed polymers with limited control over the molecular weight and architecture of the produced polymers due to the occurrence of the termination reactions.

Reversible-deactivation radical polymerizations (controlled radical polymerizations (CRP)) limit termination reactions through a dynamic equilibrium between propagating (active) and non-propagating (dormant) polymer chains. Thus, methods like atom transfer radical polymerization (ATRP),[36-38] nitroxide-mediated polymerization (NMP)[39] and reversible addition-fragmentation chain transfer (RAFT)[40] polymerization enable the synthesis of well-defined functional polymers with predetermined molecular weights, a low dispersity (*Đ*), and defined functional groups at the chain ends. Moreover, these methods allow synthesizing block- and other copolymers with advanced architectures.

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While the isomers of NVIm, i.e. 4-vinylimidazole, and quarternized *N*-vinylimidazolium salts have been polymerized by RAFT,[24, 41-44] NMP[1] and cobalt-mediated controlled radical polymerization,[45] NVIm itself is much more difficult to polymerize in a controlled way.[1] Recently, Long and coworkers even stated that "*controlled radical polymerization of N-vinylimidazole is not achievable".*[41] The propagating radicals are highly reactive because they are not stabilized by resonance and therefore tend to undergo chain termination and chain transfer reactions. Only RAFT polymerization[46, 47] and photo-polymerization in the presence of an organotellurium chain transfer agent[48] have been used with limited success. All reported attempts to polymerize NVIm with ATRP in a controlled way failed. In some cases, only small or no conversion was observed.[47] In other cases, the polymers were grown from surfaces and were not further characterized with respect to their molecular weight, their chain end functionalization and their molecular structure.[49] The difficulty encountered to polymerize imidazoles by ATRP is that the monomers and the resulting polymers tightly complex metal ions.[1, 12, 13, 15] This strips the transition metal ions from the complexes that are conventionally used as ATRP catalysts. Even if ATRP would yield well-defined PVIms, the catalyst would be difficult to remove quantitatively from the polymers due to the polymer's capability to bind metal ions. Residual metal traces, however, can cause unwanted coloration of the polymer product, inhibit its use in biomedical applications due to the toxicity of transition metal ions and deteriorate the material properties when used in electronic, energy, or filtration applications.

Our group has recently discovered that some metallo-proteins, such as horseradish peroxidase (HRP) and hemoglobin (Hb) are able to catalyze ATRP of acrylate and acrylamide monomers.[50-53] In parallel, di Lena and coworkers found that catalase, laccase, and peroxidases catalyze similar polymerizations.[54, 55] Moreover, these "*ATRPases*" were also used in surface-initiated ATRP of *N*-isopropylacrylamide ,[56] and for the initiation of RAFT polymerizations.[57, 58]

Here we report that laccase from the fungus *Trametes versicolor* (LacTv) (benzenediol oxygen oxidoreductase, EC 1.10.3.2) can catalyze the controlled radical polymerization of NVIm (Scheme 1a). The enzyme circumvents the major problem of conventional ATRP catalysts in the polymerization of vinylimidazoles, because laccases bind their copper ions tightly within their three-dimensional structure.[59] Polymerization of NVIm under biocatalytic activators regenerated by electron transfer (ARGET) ATRP conditions allowed to synthesize well-defined PNVIm with narrow molecular weight distribution. Moreover, the produced polymer is free of metal ions after purification from the enzyme.

Laccases are multi-copper oxidoreductase that usually catalyze oxidation reactions utilizing molecular oxygen.[60-64] Moreover, laccases are well-established biocatalysts for the free radical polymerization of various aromatic and phenolic compounds as well as vinyl monomers.[65-69] LacTv is known to have its optimum pH in acidic conditions between pH 3 and pH 5.[70] It has a molecular mass of about 66 kDa and contains three different types of copper ions (Scheme 1b). Type 1 copper is located near the protein surface and is the primary oxidation site for substrates. Three additional copper ions (one Type 2 and two Type 3 coppers) are arranged in a trinuclear cluster within the protein, at which the reduction of molecular oxygen takes place.[71]

Experimental

Materials. *N*-Vinylimidazole (NVIm, ≥99%) purchased from Sigma-Aldrich was distilled under reduced pressure at 72oC and kept under argon until used. 2,2'-Azobis(2-methylpropionitrile) (AIBN, 98%) from Sigma-Aldrich was freshly recrystallized from methanol and kept under nitrogen until used. Laccase from *Trametes versicolor* (LacTv; 10 U·mg^{-1}, 1 U corresponds to the amount of enzyme which converts 1 µmol catechol per minute at pH 4.5 and 25°C), horseradish peroxidase (HRP; 250 U mg^{-1} lyophilized powder), hemoglobin from bovin blood (Hb; lyophilized powder), N,N-dimethyl formamide (DMF; Chromasolv plus), lithium bromide (LiBr, ≥99%), nitric acid (ACS reagent, 70%), 2-hydroxyethyl-2-bromoisobutyrate (HEBIB; 95%), (+)-sodium L-ascorbate (NaAsc; crystalline, ≥98%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; ≥98%), 1,4-dioxane (anhydrous, ≥99.8%), sodium acetate (anhydrous, ≥99%), acetic acid (puriss), sodium phosphate mono and dibasic (≥99.0%), and sodium hydroxide (anhydrous pellets, ≥98%) were purchased from Sigma-Aldrich, and used as received. Deuterium oxide (D, 99.9%) was obtained from Cambridge Isotope Laboratories. Argon (99.998%, Carbagas) was used with further purification by using an Oxiclear disposable gas purifier (LabClear, USA).

Synthetic procedures

Laccase-catalyzed polymerization of *N*-Vinylimidazole. The following paragraph describes the protocol for laccase-catalyzed polymerization reaction under ATRP conditions with a molar ratio of reagents of 1:50:0.2:0.005 (HEBIB:NVIm:NaAsc:Lac). For other reaction mixtures, the amount of reagents was adjusted accordingly. HEBIB (59.1 mg, 0.28 mmol) and NVIm (1.32 g, 14.0 mmol) were weighed in a round-bottom flask and dissolved in 6.1 mL sodium acetate buffer (100 mM, pH 4.0) in argon atmosphere. Solutions of Lac and NaAsc were prepared by dissolving Lac (184.8 mg, 2.8 µmol) in 1.0 mL acetate buffer and NaAsc (22.2 mg, 0.11 mmol) in 1.0 mL acetate buffer, respectively. All solutions were deoxygenated by bubbling argon for ca. 30 min. 0.5 mL of the enzyme solution was transferred into the initiator/monomer flask using an argon purged syringe. The polymerization was started by adding 0.5 mL of the reducing agent solution to the reaction mixture with an argon-purged syringe. The reaction was stirred at room temperature. 1 mL samples were removed from the polymerization mixture for analytical purposes at periodic time intervals. The samples were exposed to air, diluted with 1.5 mL non-deoxygenated D_2O and filtered over a small plug of neutral aluminium oxide. The filtrate was analyzed by ^{1}H NMR to determine the conversion. The NMR sample was

recovered and prepared for GPC measurements by evaporating the solvents of the polymer sample in vacuum to yield partially solid consistency with a low amount of residual solvent. The sample was redissolved in the GPC eluent (DMF containing 0.05 M LiBr). The residual water was removed by using activated Molecular Sieve UOP Type 3A beads (Sigma- Aldrich). The sample was filtered through a PTFE syringe filter (Chromafil O-20/15 MS, Macherey-Nagel; pore size: 0.20 µm, filter diameter: 15 mm) and analyzed by GPC.

Purified PNVIm samples for ^{1}H-NMR, MALDI-ToF MS, ICP-OES, isothermal dehydrobromination and UV-*vis* analysis were prepared by stopping a reaction as described above after a predetermined time. The sample was filtered over a small plug of neutral aluminium oxide. The filtrate was centrifuged to remove any remaining alumina particles. From the supernatant the solvent was evaporated in vacuum using a rotary evaporator to yield a white-yellowish solid. The product was dissolved in methanol and the dissolved polymer was purified by precipitation in acetone and dried under vacuum, yielded with pale-yellowish powder.

Free radical polymerization (FRP) of *N*-vinylimidazole. In a typical free radical polymerization reaction, a 100 mL round bottom flask was charged with NVIm (5 mL, 55.2 mmol) and 19 mL 1,4-dioxane. Stock solutions of initiator were prepared by dissolving AIBN (0.1 g, 0.61 mmol) in 2 mL 1,4-dioxane. The reaction mixture and the stock solution were then deoxygenated by bubbling argon for about 30 min. After adding the AIBN stock solution to the reaction mixture, the solution was further deoxygenated under vigorous stirring for another 10 min. The reaction mixture was kept in an oil bath at 70 °C for 5 hours. A light yellow polymer formed. It was dissolved in methanol and precipitated in acetone two times. The precipitated powder was filtered and dried under vacuum at 60 °C. The yield was 81%. The purified PNVIm was characterized by GPC and NMR (M_n(GPC) = 2.52 x 10^3 g mol^{-1}, M_w(GPC) = 5.55 x 10^3 g mol^{-1}, $Đ$ = 2.20; ^{1}H NMR (200MHz, D_2O): δ 1.72-2.34 (2H, CH_2 backbone), 2.48-3.86 (1H, CH backbone for syndio-, hetero- and isotactic triads), 6.50-7.23 (3H, CH groups of imidazole ring)).

Recovery of enzyme after a polymerization. It was attempted to remove the polycationic PNVIm from LacTv at the end of typical polymerizations (as described above) by washing with and exchanging the buffer to 100 mM sodium phosphate buffer (pH 7). To this end, the polymerization mixture was centrifuged in Amicon Ultra-0.5 mL spin centrifugal filter units (Ultracel-3K and Ultracel-30K, regenerated cellulose 3000 g mol^{-1} and 30000 g mol^{-1} NMWL, respectively), which removed first the acetate buffer and small molecules like unreacted monomers and ascorbate, followed by removing polymer from the enzyme by using the higher molecular weight cut-off (Ultracel-30K) filter unit.

Instruments and measurements

Nuclear magnetic resonance (NMR). NMR spectra were recorded at room temperature on a Bruker Advance III 300 MHz spectrometer using deuterated solvents. Chemical shifts (δ) are reported in ppm, whereas the chemical shifts are calibrated to the solvent residual peaks. The chemical composition and the purity of the compounds were determined using D_2O as solvent. Conversion of NVIm was calculated from phase and baseline-corrected spectra using the integrals of the signal at 7.77 ppm, which corresponds to one proton of the residual monomer, and the signals between 2.30 and 1.70 ppm, which correspond to the backbone protons of the polymer. The number average molecular weight (M_n) was calculated from the integrals of initiator peak (corresponds to 6H, around 1.8 ppm) and the signals (corresponds to 2H, between 1.70 and 2.30 ppm) of the polymer backbone.

Gel permeation chromatography (GPC). The molecular weight and the dispersity ($Đ$) of polymers were determined by GPC using PSS GRAM columns (for PNVIm produced by LacTv: 1 guard column and 2 mixed bed PSS GRAM analytical linear 10 µm (300 mm x 8.0 mm), separation range from 500 to 10^6 g mol^{-1}; for the polymer synthesized by free radical polymerization: 1 guard column, a 30 Å column and two 1000 Å columns, all 10 µm) thermostatted to 60°C in DMF containing LiBr (LacTV-synthesized PNVIm: 50 mM LiBr; free radical PNVIm: 20 mM LiBr) with a flow rate of 1.0 mL min^{-1} by using a refractive index detector. The molecular weight determination of the PNVIm samples were based on narrow molecular weight distribution poly(methyl methacrylate) (PMMA) standards in the range of 800 to 1.6·10^6 g mol^{-1}.

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS). Mass analysis and detection of the chemical nature of the end groups of the polymers was carried out on a Bruker Utraflextreme MALDI-ToF mass spectrometer. The instrument was working in positive ionization method in reflecting mode for all samples. Samples for MALDI-ToF MS were prepared using α-cyano-4-hydroxycinnamic acid (HCCA) as matrix and potassium- and sodium chloride salts. Data was processed with Bruker Daltonicsflex Analysis software. Number average molecular weight and weight average molecular weight were calculated from $\boldsymbol{Mn} = \frac{\sum_i N_i M_i}{\sum_i N_i}$ and, $\boldsymbol{Mw} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i}$ where M_i is the molecular weight of the chain and N_i is the number of chains of that molecular weight. The data were analyzed using Polymerix software (Sierra Analytics, Modesto, CA, USA).

Isothermal dehydrobromination. 76.6 mg (2 h sample) and 160.5 mg (24 h sample) PNVIm were dissolved in 20 mL 1,2,4-trichlorobenzene, respectively. In thermo-oxidative experiments the evolution of free hydrogen bromide (HBr) from these samples was monitored with a Metrohm PVC Thermomat 763 instrument. The degradation temperature was 200 °C and the oxygen flow rate was constant at 3.8 L·h^{-1}. The formed HBr was purged with the oxygen, quantitatively dissolved in 60 mL distilled water and then detected with a conductometer. A calibration curve was measured without oxygen flow, with various concentrations of acid solutions. The degree of bromine termination of the polymers was calculated from the

quantitative isothermal dehydrobromination results and the M_n of the polymers as determined by GPC.

Inductively coupled plasma atomic absorption spectrometry (ICP-OES). A Perkin Elmer Optima 7000 ICP-OES was used to measure the concentration of the residual Cu-ions at 224.700 nm. For the calibration a 1000 mg·L^{-1} aqueous stock solution of $Cu(NO_3)_2$ was used to prepare a dilution series containing from $0.91 \cdot 10^{-5}$ to $3.63 \cdot 10^{-4}$ mg·L^{-1} copper ions in the same aqueous media. 1 mL of each calibration solution was diluted with 11 mL nitric acid and measured. To determine the copper content of samples (polymerization reaction mixture; 1 mg·mL^{-1} purified PNVIm, respectively) 1 mL of each sample solution was diluted with 11 mL nitric acid and measured. The background emission of pure water was compensated by the preparation of the sample and calibration solutions in the same media. Three parallel measurements were carried out and the mean value is reported as result.

UV-*visible* spectroscopy (UV-*vis*). UV-*vis* spectroscopy measurements were conducted using an Analytik Jena Specord 50 PLUS spectrometer from 190 nm to 700 nm, using quartz cuvettes with a path length of 10 mm.

Fluorescence spectroscopy. The measurements were carried out using a Jasco FP-6200 fluorimeter. For the tryptophan fluorescence analysis the supernatant of the diluted, centrifuged reaction samples (with 0.1 nmol·mL^{-1} enzyme concentration) were used. Emission spectra from 300 to 500 nm were recorded with an excitation at 295 nm using a 1 nm bandwidth in both excitation and emission paths.

Activity assay. The oxidase activity of laccase was measured by following the oxidation of ABTS at 420 nm (absorption band of the oxidized derivative) (ε_{420} = 36000 mol·L^{-1}·cm^{-1}) in a UV-*vis* spectrometer. The reaction mixture was prepared by mixing 100 μL of 30 mM ABTS solution with 700 μL of 100 mM acetate buffer (pH 4.0) in a cuvette. The reaction was started by adding and short mixing of 200 μL 20x diluted reaction mixture and LacTv solution, respectively. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol product per minute at room temperature.

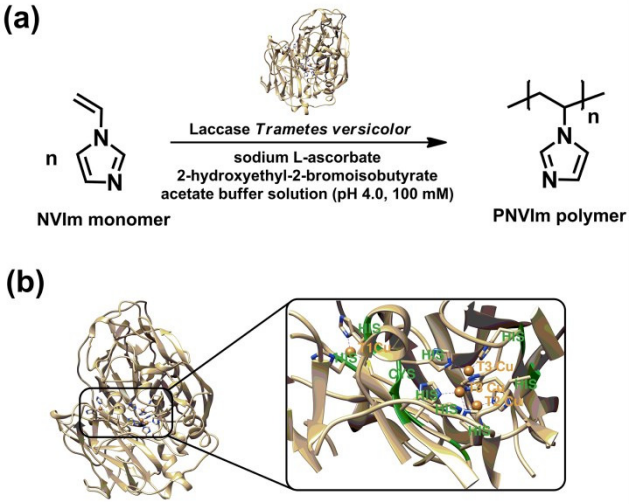
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Gel electrophoresis was performed with Mini-PROTEAN® TGX™ precast polyacrylamide gels in a Bio-Rad Miniprotean tetra cell setup at 120 V for 40 min. Broad range Bio-Rad prestained SDS-PAGE standard was used as molecular weight marker. The gels were stained with Coomassie brilliant blue G-250 (Bio-Safe Coomassie Stain, Bio-Rad) according to the manufacturer's instructions.

Results and discussion

In order to find a suitable biocatalyst for the controlled radical polymerization of NVIm, three enzymes that have been previously used for ATRP, namely LacTv, HRP and bovine Hb, were tested for their activity to polymerize NVIm in aqueous solution. In a typical polymerization experiment, the initiator 2-hydroxyethyl-2-bromoisobutyrate (HEBIB) and the monomer NVIm were dissolved in aqueous buffer under argon bubbling. Then, deoxygenated buffered solutions of enzyme and of the reducing agent sodium ascorbate (NaAsc) were added to the polymerization mixture to start the reaction, which was stirred at room temperature. While LacTv yielded PNVIm with approx. 70% conversion within 24 h reaction time, as proven by ^{1}H NMR (Table S1, entry 4-10; see also Figure S1), HRP and bovine Hb resulted in very low conversions (Table S1, entry 1-3). The reaction mixture containing LacTv retained its light yellow-brownish color during the reaction. In contrast, the HRP solution changed from rose-tint to greenish within 8 h of polymerization, indicating drastic changes in the coordination sphere of the peroxidase's heme group and/or a change in its oxidation state. Taking together, these results show that out of the three tested enzymes, LacTv is the most promising one to catalyze the polymerization of NVIm (Scheme 1). Therefore, its polymerization activity was investigated in detail.

Scheme 1. Controlled radical polymerization of NVIm catalyzed by LacTv. (a) Reaction scheme of the LacTV-catalyzed controlled radical polymerization and (b) three-dimensional structure of LacTv, showing the arrangement of the four copper atoms (Protein Data Bank ID: 1GYC).[71]

Several reaction parameters, i.e. the initiator-to-monomer



ratio, the concentration of LacTv, the reaction temperature and the pH, were varied in order to find conditions that allow for a good control over the polymerization (Table S1). Reactions carried out with reagent ratios of HEBIB:NVIm:NaAsc:LacTv 1:50:0.2:0.005 and 1:100:0.2:0.005 at room temperature and

pH 4.0 resulted in the lowest dispersities (*Đ*). Under these conditions, the LacTv-catalyzed polymerization of NVIm proceeded with a first order kinetic and a linear increase in molecular weight with conversion (for initiator:monomer ratio of 1:50 see Figure 1 and Table S1, entry 4-10; for initiator:monomer ratio of 1:100 see Figure S2 and Table S1, entry 11-17). The *Đ* of the polymers ranged from 1.27 at the beginning of the reaction to 1.57 after 24 h reaction time, as determined by GPC. The GPC traces were monomodal and clearly shifted towards higher molecular weight while the polymerization proceeded. The resulting polymers had higher molecular weights than predicted by the conversion and the initiator-to-monomer ratio, probably because of early termination of some chains. The fact that the dispersity increased during the reaction indicates that the enzyme's efficiency in controlling the polymerization was not optimal. Nevertheless, these results clearly demonstrate that the enzymatic polymerization of NVIm was a controlled radical polymerization.

Biocatalytically produced PNVIm was purified from a reaction (HEBIB:NVIm:NaAsc:LacTV 1:50:0.2:0.005 at pH 4.0) after 7 h for further characterization. To this end, the solution was filtered over a plug of neutral alumina. The filtrate was centrifuged to remove alumina particles and the water was evaporated from the supernatant in vacuum. The product was redissolved in methanol and purified by precipitation in acetone, followed by drying under vacuum. The obtained polymer was analyzed by MALDI-ToF MS to obtain precise information about its molecular weight and molecular weight distribution. The MALDI-ToF MS spectrum (Figure 1d, see also Figure S3) shows a series of peaks between 1000 to 8000 m/z, with a main peak separation Δ(*m/z*) of 94.2. This value characteristic for the NVIm repeating unit with a molecular weight of 94.1 g mol^{-1} and confirms that the reaction yielded PNVIm. The molecular weight of the sample was M_n = 4300 g mol^{-1} and M_w = 4610 g mol^{-1}, as calculated from the mass spectrum. This corresponds to a *Đ* of 1.07. The very low molecular weight distribution recorded by MALDI-ToF MS is a further evidence that the enzyme controlled the polymerization of NVIm. The molecular weights are similar to those measured by NMR and GPC (M_n(NMR) = 3340 g mol^{-1}, M_n(GPC) = 3730 g mol^{-1}, M_w(GPC) = 5840 g mol^{-1}, *Đ* = 1.57) but the dispersity is lower than determined by GPC. This difference could be caused by the different measurement methods. In this context it should be noted that the GPC measurements were calibrated with poly(methyl methacrylate) standards.

A closer analysis of the peaks in the mass spectrum reveals several repeating peaks (Figure S3) which correspond to PVIm chains bearing a hydroxyethyl isobutyrate residue from the initiator and H, ene and OH chain ends. Signals of bromine-terminated chains, which would be indicative of an atom transfer mechanism, are very weak. However, MALDI-ToF MS of ATRP-derived polymers often fails to detect bromine chain ends, as the halogen is easily cleaved of during the ionization of the molecules.[72, 73] In order to investigate if a fraction of the

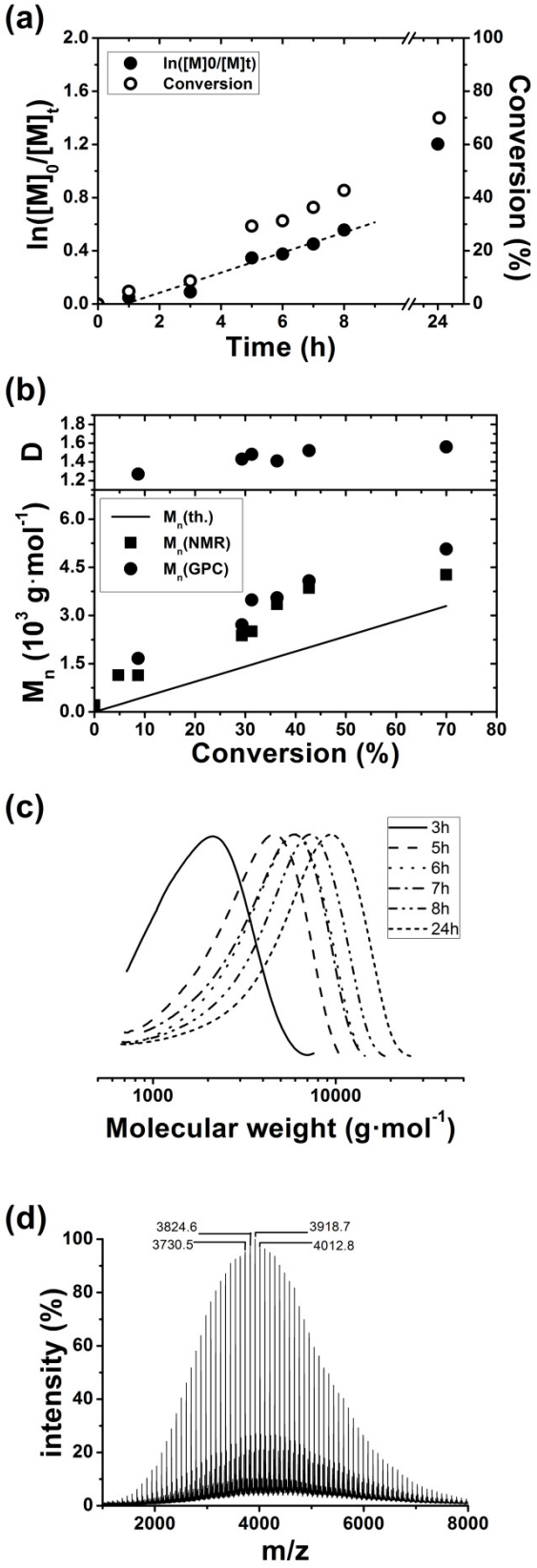


Figure 1. Kinetic investigations of polymerizations catalyzed by LacTv under conditions of ATRP. (a) First order kinetic plot, (b) evolution of molecular weight and dispersity with monomer conversion, (c) GPC traces and (d) MALDI-ToF MS reflective spectrum of the purified PNVIm polymer, prepared by LacTv-catalyzed polymerization. (Experimental conditions: ratio of HEBIB:NVIm:NaAsc:LacTV 1:50:0.2:0.005 at pH 4.0).

PVIm chains carry bromine atoms, isothermal dehydrobromination of PVIm samples was carried out.
This characterization method revealed that bromine is present in the samples, but the degree of bromine termination is low (around 11.7% at 2 h reaction time and approx. 2.8% for a 24 h sample). The results allow to conclude that atom transfer plays a minor role in the biocatalytic reaction and that the observed control of the PVIm polymerization by the enzyme is mainly due to a different mechanism. Detailed mechanistic studies will follow, but the interplay of ATRP, organometallic-mediated radical polymerization and/or catalytic chain transfer has been previously reported for other transition metal catalysts.[37, 74]

In fungal laccases the copper ions are embedded within the three-dimensional structure of the enzyme. Therefore, it can be expected that the metal ions can be completely removed from biocatalytically synthesized PNVIm if the enzyme is separated from the polymer. To test this hypothesis, a polymerization (ratio of HEBIB:NVIm:NaAsc:LacTv 1:50:0.2:0.005 at pH 4.0) was run for 24 h and the polymer was purified as described above. The sample was analyzed for its metal content by ICP-OES before and after removal of the enzyme. The reaction mixture had a copper content of 203 ppm which corresponds to a copper concentration of 167 nmol mL^{-1} LacTv, as each laccase hosts four copper ions. This measured concentration is in agreement with the weighted LacTv concentration of 170 nmol mL^{-1} that was used in the reaction. The copper ion content of the purified polymer powder was below the ICP-OES detection limit, i.e. ≤ 9.1 ppb in the dry mass of PNVIm. Moreover, Zn and Fe ions, which could be contaminants of Lac preparations, were also not detectable. Thus, a few simple purifying steps (filtration over alumina, redissolving in methanol and precipitation in acetone) are sufficient to remove the protein and its metal ions from the strongly metal-binding polymer and to obtain virtually metal-free, purified PNVIm.

The UV-*vis* spectra of the pure enzyme, of a purified biocatalytically-produced polymer and, for reference, of PNVIm synthesized by free radical polymerization are shown in Figure 2. LacTv absorbs at 280 nm with a shoulder at 320 nm but shows no significant absorbance at 610 nm. The enzyme's spectrum is typical for "*yellow*" copper oxidases in which the paramagnetic Type 1 copper (that gives most laccases a characteristic blue color by absorbing around 610 nm) is complexed in a non-absorbing state.[61, 75] PNVIm has an absorption band with maxima at 205 and 211 nm because of the heteroaromatic rings in PNVIm. The absorption spectra of the biocatalytically synthesized PNVIm is similar to the one produced by free radical polymerization. It does not show major traces of LacTv, which proves that the enzyme could be removed from the polymer and corroborates the results of ICP-OES that the enzymatically produced PNVIm is free of copper ions.

In order to assess the stability of the enzyme during the polymerization, samples were withdrawn during regular time intervals from a typical polymerization and the residual oxidase activity of the enzyme was measured colorimetrically by the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Figure 3a). Native LacTv had an ABTS activity of 12.3 U mg^{-1}. The activity of the enzyme decreased by 20 % to 9.8 U mg^{-1} at the beginning of the reaction. One reason could be the purging with argon during the preparation of the enzyme solution, because proteins can denature at the interface of gas bubbles.[76] During the first hour of the reaction the activity decreased further to 50 % (6.1 U mg^{-1}). Thereafter, it remained constant until at least 8 h. These results indicate that, after an initial adaption to the reaction conditions, the enzyme remained stable during the crucial first eight hours of the polymerization (c.f. Figure 1a). The decrease in oxidase activity in the first hour of the reaction might be due conformational rearrangements around the active site or changes in reduction state of the metal centers. When assayed after 24 h of exposure to the polymerization conditions, the activity dropped to 0.1 U·mg^{-1}, indicating that at prolonged exposure to the polymerization conditions the enzyme had lost most of its oxidase activity. Nevertheless, it should be noted that the protein did not precipitate from solution, indicating that it did not unfold.

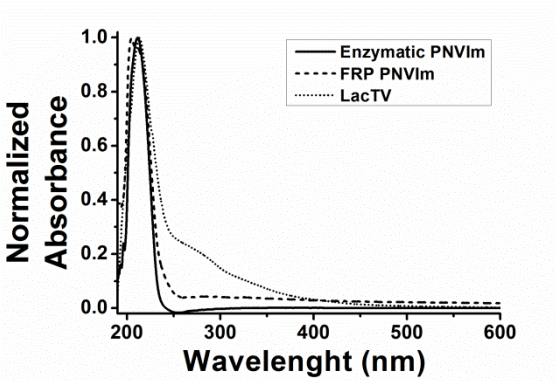
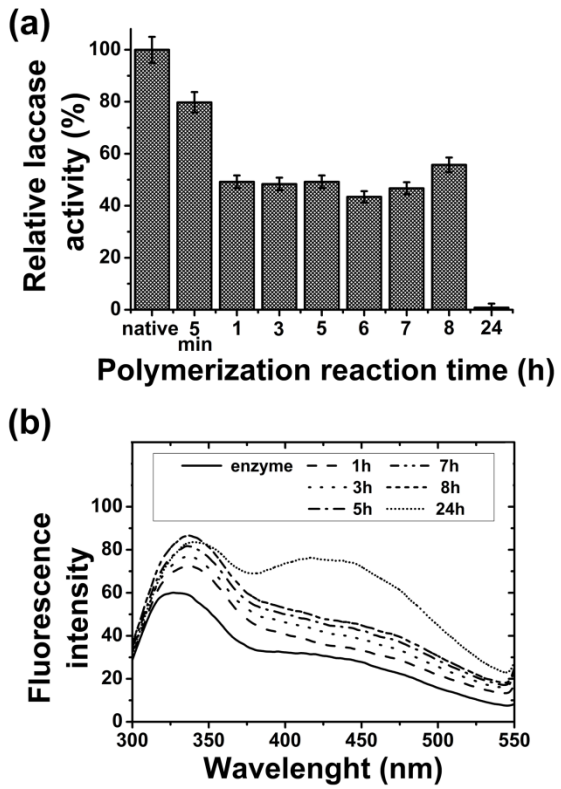


Figure 2. UV-vis spectra of PNVIm synthesized by LacTv-catalyzed polymerization (Enzymatic PNVIm: ratio of HEBIB:NVIm:NaAsc:LacTv 1:50:0.2:0.005 at pH 4.0, purified after 24h of reaction time; FRP PNVIm: prepared by free radical polymerization) and of the enzyme LacTv.

LacTV has several tryptophan residues (Trp) close at its surface (Figure S4). Conformational changes of the protein can be investigated by measuring the fluorescence of Trp, as its fluorescence emission depends on the polarity of the direct environment of the Trp residues.[77, 78]. Therefore, samples were drawn at regular time-intervals from a typical polymerization and their fluorescence emission spectrum was recorded (Figure 3b). For comparison the spectrum of LacTv at pH 4 in the absence of any polymerization reagents was also measured. The latter exhibits a maximum at 327 nm, which is in accordance with previously reported Trp-fluorescence spectra of this enzyme.[79] After one hour of polymerization, the maximum of the Trp fluorescence was red-shifted by 10 nm to 337 nm. The position and shape of the emission band remained constant during the next seven hours. These observations indicate that the enzyme underwent some conformational changes during deoxygenation of the enzyme solution and the subsequent exposure of the laccase to the reaction conditions. Then, the enzyme kept this conformation for several hours.

Figure 3. Characterization of LacTv before, during and after polymerization of NVIm under conditions of ATRP (ratio of HEBIB:NVIm:NaAsc:LacTv 1:50:0.2:0.005 at pH 4.0).



(a) Residual activity of LacTv, as measured with the ABTS activity assay, (b) fluorescence spectra of the enzyme.

The red-shift of the fluorescence of Trp is indicative of an increased exposure of the enzyme´s Trp residues to the aqueous medium and the polycations therein.[77, 80] The fluorescence emission intensity of the laccase increased during the reaction. This may be attributed to a reduction of the enzyme´s copper, as described previously for the reduction of the Type 1 copper of a laccase with ascorbic acid under oxygen-free conditions.[81] After 24 h reaction time the emission peak red-shifted to 345 nm, which indicates that the enzyme is partially unfolded (fully unfolded LacTv is reported to have its emission maximum at 352 nm[77]). Moreover, another maximum at 420 nm appeared, which could be due to energy transfer between tryptophan as donor and the surrounding PNVIm as acceptor.[82-84] Taken together, the fluorescence measurements correlate well with the ABTS activity data and show that during the first eight hours a stable, but non-native conformation of LacTv catalyzes the reaction. Circular dichroism spectroscopy could give further insight into the structure of the enzyme. Unfortunately, it could not be measured, because monomer and polymer absorb in the deep UV and therefore interfere with the measurements.

Attempts to purify the enzyme from the reaction mixture after a polymerization by size exclusion spin centrifugation combined with buffer exchange were not successful, as residual polymer was observed in sodium dodecyl sulfate polyacrylamide gel electrophoresis of recovered enzyme solutions. The remaining PNVIm polymers smeared on the gels and resulted in a strong Comassie staining (Figure S5). Most likely the protonated, cationic polymer formed electrostatic complexes with the anionic enzyme (pI approx. 3.5[71]) that prevented the purification of the enzyme. In contrast, a LacTv solution that lacked the polymer gave a strong band at around 66 kDa which corresponds to the molecular mass of the laccase.

Conclusions

In conclusion, the results of this study show that LacTv is able to catalyze the controlled radical polymerization of NVIm in aqueous media. Although the degree of control is not perfect, laccase-catalysis is currently the only way to produce PNVIm with predetermined molecular weight and relatively narrow molecular weight distributions. The polymerization followed first order kinetics and the molecular weight of the polymer increased linearly with conversion, thus showing the controlled character of the reaction. The enzyme could be quantitatively removed from the polymer by simple purification steps, yielding metal-free PNVIm. Conformational changes of the enzyme, denaturation at prolonged exposure to the reaction conditions, a reduction in its oxygenase activity and electrostatic complexation of the polymer to the protein were observed. Nevertheless, this did not impede the ability of LacTv to catalyze the polymerization of NVIm.

Metal-free PNVIm with defined molecular weight can unlock the full potential of PNVIm as gene-delivery nanocarrier, as building block in metal ion-imprinted nanostructures and in fuel cell membranes. Moreover, biocatalytically synthesized PNVIm could be easily transformed into polyionic liquids with its myriad of applications.

Research is underway to elucidate mechanistic details of the polymerization, to identify reaction parameters that enhance the performance of laccases in the polymerization of vinyl monomers with *N*-heterocyclic aromatic functional groups and to further explore the scope of biocatalytic controlled radical polymerizations.

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Notes and references

1. M. D. Green, M. H. Allen Jr, J. M. Dennis, D. S.-d. l. Cruz, R. Gao, K. I. Winey and T. E. Long, *Eur. Polym. J.*, 2011, **47**, 486-496.

2. K. Nakabayashi and H. Mori, *Eur. Polym. J.*, 2013, **49**, 2808-2838.
3. S. Yuan, Q. Deng, G. Fang, J. Wu, W. Li and S. Wang, *J. Chromatogr. B*, 2014, **960**, 239-246.
4. S. Asayama, T. Hakamatani and H. Kawakami, *Bioconjugate Chem.* 2010, **21**, 646-652.
5. J. E. Ihm, K.-O. Han, C. S. Hwang, J. H. Kang, K.-D. Ahn, I.-K. Han, D. K. Han, J. A. Hubbell and C.-S. Cho, *Acta Biomateri.*, 2005, **1**, 165-172.
6. Y. Guan, H. Pu, H. Pan, Z. Chang and M. Jin, *Polymer*, 2010, **51**, 5473-5481.
7. Y. S. Guan, H. T. Pu, M. Jin, Z. H. Chang and D. C. Wan, *Fuel Cells*, 2010, **10**, 973-982.
8. A. H. Tian, J.-Y. Kim, J. Y. Shi and K. Kim, *J. Power Sources*, 2008, **183**, 1-7.
9. J. Tu, J. Zhou, C.-F. Wang, Q. Zhang and S. Chen, J. Polym. Sci., Part A: Polym. Chem., 2010, **48**, 4005-4012.
10. H. I. Unal, O. Erol and O. Y. Gumus, *Colloids Surf A Physicochem Eng Asp.*, 2014, **442**, 132-138.
11. K. Yin, Z. Zhang, L. Yang and S.-I. Hirano, *Journal of Power Sources*, 2014, **258**, 150-154.
12. Z. Ajji and A. M. Ali, *J. Hazard. Mater.*, 2010, **173**, 71-74.
13. H. Bessbousse, T. Rhlalou, J. F. Verchère and L. Lebrun, *Chem. Eng. J.*, 2010, **164**, 37-48.
14. H. El-Hamshary, M. M. G. Fouda, M. Moydeen and S. S. Al-Deyab, *Int. J. Biol. Macromolec.,* 2014, **66**, 289-294.
15. F. Özmen, P. A. Kavaklı and O. Güven, *J. Appl. Polym. Sci.*, 2011, **119**, 613-619.
16. M. Llorina Rañada, M. Akbulut, L. Abad and O. Güven, *Radiat. Phys. Chem.,* 2014, **94**, 93-97.
17. K. Yao, Z. Wang, J. Wang and S. Wang, *Chem Commun (Camb).* 2012, **48**, 1766-1768.
18. S. Yuan, S. O. Pehkonen, B. Liang, Y. P. Ting, K. G. Neoh and E. T. Kang, *Corros. Sci.*, 2010, **52**, 1958-1968.
19. I. P. Beletskaya, A. V. Selivanova, V. S. Tyurin, V. V. Matveev and A. R. Khokhlov, *Russ. J. Org. Chem.* 2010, **46**, 157-161.
20. Y. Han, X. Yuan, M. Zhu, S. Li, M. J. Whitcombe and S. A. Piletsky, *Adv. Funct. Mater.*, 2014, **24**, 4996-5001.
21. B. Ö. Öztürk, B. Sariaslan, N. P. Bayramgil and S. K. Şehitoğlu, *Appl. Catal., A.*, 2014, **483**, 19-24.
22. A. V. Selivanova, V. S. Tyurin and I. P. Beletskaya, *ChemPlusChem*, 2014, **79**, 1278-1283.
23. Y. Zhou, M. Zhu and S. Li, *J. Mater. Chem.,* 2014, **2**, 6834-6839.
24. B. M. Blunden, A. Rawal, H. Lu and M. H. Stenzel, *Macromolecules*, 2014, **47**, 1646-1655.
25. J. Detering, C. Schade, W. Trieselt and J. Tropsch, US 5846924 A, 1998.
26. B. Biatry and E. Lheureux, US2003/133889 A1, 2003.
27. J. Wood, A. Meuser and J. Schneider, US2013/251662 A1, 2013.
28. M. Schubert and M. A. Glomb, *J. Agric. Food Chem.*, 2010, **58**, 8300-8304.
29. J. M. Layman, S. M. Ramirez, M. D. Green and T. E. Long, *Biomacromolecules*, 2009, **10**, 1244-1252.
30. A. C. Rinkenauer, S. Schubert, A. Traeger and U. S. Schubert, *J. Mater. Chem. B*, 2015, **3**, 7477-7493.
31. C. G. Overberger and N. Vorchheimer, *J. Am. Chem. Soc.*, 1963, **85**, 951-955.
32. C. G. Overberger, R. Corett, J. C. Salamone and S. Yaroslavsky, *Macromolecules*, 1968, **1**, 331-334.
33. C. G. Overberger and C.-M. Shen, *Bioorg. Chem.*, 1971, **1**, 1-12.
34. C. G. Overberger, R. C. Glowaky and P. H. Vandewyer, *J. Am. Chem. Soc.*, 1973, **95**, 6008-6013.
35. S. Santanakrishnan and R. A. Hutchinson, *Macromol. Chem. Phys.*, 2013, **214**, 1140-1146.
36. N. V. Tsarevsky and K. Matyjaszewski, *Chem. Rev.*, 2007, **107**, 2270-2299.
37. F. di Lena and K. Matyjaszewski, *Prog. Polym. Sci.*, 2010, **35**, 959-1021.
38. K. Matyjaszewski, *Macromolecules*, 2012, **45**, 4015-4039.
39. D. Bertin, D. Gigmes, S. R. A. Marque and P. Tordo, *Chem. Soc. Rev.*, 2011, **40**, 2189-2198.
40. D. J. Keddie, *Chem. Soc. Rev.*, 2014, **43**, 496-505.
41. M. H. Allen, S. T. Hemp, A. E. Smith and T. E. Long, *Macromolecules*, 2012, **45**, 3669-3676.
42. H. Mori, M. Yahagi and T. Endo, *Macromolecules*, 2009, **42**, 8082-8092.
43. R. Skouta, S. Wei and R. Breslow, *J. Am. Chem. Soc.*, 2009, **131**, 15604-15605.
44. Y. Yang, Y. Yu, Y. Zhang, C. Liu, W. Shi and Q. Li, *Process Biochem.*, 2011, **46**, 1900-1908.
45. J. Detering, C. Schade, W. Trieselt and J. Tropsch, *US 5846924 A*, 1998.
46. Z. Ge, D. Xie, D. Chen, X. Jiang, Y. Zhang, H. Liu and S. Liu, *Macromolecules*, 2007, **40**, 3538-3546.
47. S. Jana, V. A. Vasantha, L. P. Stubbs, A. Parthiban and J. G. Vancso, *J. Polym. Sci. A Polym. Chem.*, 2013, **51**, 3260-3273.
48. S. Yamago, *Chem. Rev.*, 2009, **109**, 5051-5068.
49. J. Li, H. Han, Q. Wang, X. Liu and S. Jiang, *J. Sep. Sci.*, 2010, **33**, 2804-2810.
50. M. V. Dinu, M. Spulber, K. Renggli, D. Wu, C. A. Monnier, A. Petri-Fink and N. Bruns, *Macromol. Rapid Commun.*, 2015, **36**, 507-514.
51. G. Kali, T. B. Silva, S. J. Sigg, F. Seidi, K. Renggli and N. Bruns, in *Progress in Controlled Radical Polymerization: Mechanisms and Techniques*, American Chemical Society, 2012, vol. 1100, ch. 11, pp. 171-181.
52. S. J. Sigg, F. Seidi, K. Renggli, T. B. Silva, G. Kali and N. Bruns, *Macromol. Rapid Commun.*, 2011, **32**, 1710-1715.
53. T. B. Silva, M. Spulber, M. K. Kocik, F. Seidi, H. Charan, M. Rother, S. J. Sigg, K. Renggli, G. Kali and N. Bruns, *Biomacromolecules*, 2013, **14**, 2703-2712.
54. Y.-H. Ng, F. di Lena and C. L. L. Chai, *Chem. Commun.*, 2011, **47**, 6464-6466.
55. Y.-H. Ng, F. di Lena and C. L. L. Chai, *Polym. Chem.*, 2011, **2**, 589-594.
56. G. Gao, M. A. Karaaslan, J. F. Kadla and F. Ko, *Green Chem.*, 2014, **16**, 3890-3898.
57. B. Zhang, X. Wang, A. Zhu, K. Ma, Y. Lv, X. Wang and Z. An, *Macromolecules*, 2015, **48**, 7792-7802.
58. A. P. Danielson, D. B. Van Kuren, M. E. Lucius, K. Makaroff, C. Williams, R. C. Page, J. A. Berberich and D. Konkolewicz, *Macromol. Rapid Commun.*, 2016, **37**, 362-367.
59. R. Malkin, B. G. Malmström and T. Vänngård, *European J. Biochem.*, 1969, **7**, 253-259.
60. I. Goncalves, C. Silva and A. Cavaco-Paulo, *Green Chem.*, 2015, **17**, 1362-1374.
61. V. Madhavi and S. Lele, *BioResources*, 2009, **4**, 1694-1717.
62. D. M. Mate and M. Alcalde, *Biotechnol. Adv.*, 2015, **33**, 25-40.
63. S. Riva, *Trends Biotech.*, 2006, **24**, 219-226.

64. S. Rodríguez Couto, A. Rodríguez, R. R. M. Paterson, N. Lima and J. A. Teixeira, *Lett. Appl. Microbiol.*, 2006, **42**, 612-616.
65. I. Gitsov, L. Wang, N. Vladimirov, A. Simonyan, D. J. Kiemle and A. Schutz, *Biomacromolecules*, 2014, **15**, 4082-4095.
66. R. Ikeda, H. Tanaka, H. Uyama and S. Kobayashi, *Macromol. Rapid Commun.*, 1998, **19**, 423-425.
67. K. Junker, R. Kissner, B. Rakvin, Z. Guo, M. Willeke, S. Busato, T. Weber and P. Walde, *Enzyme Microb .Technol.*, 2014, **55**, 72-84.
68. N. Mita, S.-i. Tawaki, H. Uyama and S. Kobayashi, *Macromol. Biosci.*, 2003, **3**, 253-257.
69. S.-i. Shoda, H. Uyama, J.-i. Kadokawa, S. Kimura and S. Kobayashi, *Chem Rev*, 2016, **116**, 2307-2413.
70. J. Margot, C. Bennati-Granier, J. Maillard, P. Blánquez, D. A. Barry and C. Holliger, *AMB Express*, 2013, **3**, 1-14.
71. K. Piontek, M. Antorini and T. Choinowski, *J. Biol. Chem.*, 2002, **277**, 37663-37669.
72. C. Barner-Kowollik, T. P. Davis and M. H. Stenzel, *Polymer*, 2004, **45**, 7791-7805.
73. A. C. Crecelius, C. R. Becer, K. Knop and U. S. Schubert, *J. Polym. Sci. A Polym. Chem.*, 2010, **48**, 4375-4384.
74. R. Poli and M. P. Shaver, *Chem. Eur. J.*, 2014, **20**, 17530-17540.
75. A. A. Leontievsky, T. Vares, P. Lankinen, J. K. Shergill, N. N. Pozdnyakova, N. M. Myasoedova, N. Kalkkinen, L. A. Golovleva, R. Cammack, C. F. Thurston and A. Hatakka, *FEMS Microbiol. Lett.*, 1997, **156**, 9-14.
76. M. Caussette, A. Gaunand, H. Planche and B. Lindet, in *Progress in Biotechnology*, eds. F. J. Plou. J. L. Iborra. A. Ballesteros and P. J. Halling, Elsevier, 1998, vol. Volume 15, pp. 393-398.
77. X. Li, P. Yu, L. Yang, F. Wang and L. Mao, *Anal. Chem.*, 2012, **84**, 9416-9421.
78. M. Lucius, R. Falatach, C. McGlone, K. Makaroff, A. Danielson, C. Williams, J. C. Nix, D. Konkolewicz, R. C. Page and J. A. Berberich, *Biomacromolecules*, 2016, **17**, 1123-1134.
79. X. Li, P. Yu, L. Yang, F. Wang and L. Mao, *Anal. Chem.*, 2012, **84**, 9416-9421.
80. M. M. Andersson, R. Hatti-Kaul and W. Brown, *J. Phys. Chem. B*, 2000, **104**, 3660-3667.
81. M. Goldberg and I. Pecht, *Proc. Natl. Acad. Sci. U.S.A.*, 1974, **71**, 4684-4687.
82. G. N. Kumar and K. Srikumar, *Cellulose*, 2013, **20**, 115-125.
83. B. Wang, H.-J. Liu and Y. Chen, *RSC Advances*, 2016, **6**, 2141-2148.
84. Z.-H. Zhang, X.-F. Shi, X. Guo, C.-F. Wang and S. Chen, *Soft Matter*, 2013, **9**, 3809-3814.