Polymer Journal (2014) 46, 373–380

www.nature.com/pj

FOCUS REVIEW

Development of HRP-mediated enzymatic polymerization under heterogeneous conditions for the preparation of functional particles

Michinari Kohri

Enzymatic polymerization is a powerful tool for the synthesis of functional polymeric materials. Horseradish peroxidase (HRP)-mediated vinyl polymerization in the presence of hydrogen peroxide (H_2O_2) and β-diketone has been successfully applied to the synthesis of functional polymer particles as follows: (i) monodisperse polymer particles were prepared by enzymatic miniemulsion polymerization using surfmer (surfactant + monomer). (ii) Polymer particles stabilized by β-diketone moieties were prepared by enzymatic emulsifier-free emulsion polymerization. (iii) A novel method of surface-initiated enzymatic graft polymerization was used to synthesize core-shell particles. These methods could offer a practical approach for the synthesis of functional polymer particles because the polymerizations can be conducted at room temperature using a mild enzymatic process.
Polymer Journal (2014) **46,** 373–380; doi:10.1038/pj.2014.13; published online 26 March 2014

Keywords: emulsifier-free emulsion polymerization; enzymatic polymerization; horseradish peroxidase; miniemulsion polymerization; polymer particle; surface-initiated graft polymerization

INTRODUCTION

In 1951, Parravano reported the first example of an enzyme-mediated polymerization reaction through the demonstration of the oxidase-initiated polymerization of methyl methacrylate.[1] Enzyme-mediated polymerization, however, was not investigated further until the 1990s, when Derango *et al.*[2] reported the polymerization of methyl methacrylate and acrylamide (AAm) using isolated horseradish peroxidase (HRP) as a catalyst. Since then, the HRP-mediated polymerization of vinyl monomers has experienced a steadily growing popularity.[3] This type of reaction, the *in vitro* synthesis of polymers through enzymatic catalysis, or so-called enzymatic polymerization, has been extensively developed. Enzymatic polymerization possesses several advantages: (i) catalysis under mild reaction conditions with regard to temperature, pressure and pH; (ii) high enantio-, regio- and chemo-selectivities and high regulation of stereochemistry; and (iii) the use of a nontoxic natural catalyst with 'green' appeal for commercial benefits and ecological requirements.[4] Currently, lipase-catalyzed polyester synthesis and glycosyl hydrolase- or glucosyl phosphorylase-catalyzed polysaccharide synthesis are widely developed, and detailed enumerations of the polymer characteristics have been described in some excellent reviews.[5–8] Oxidase-catalyzed polyphenol derivative synthesis has also been widely investigated using peroxidase, laccase, xanthin oxidase and alcohol oxidase as a catalyst.[9] Large variety of monomers was used and led to the formation of new advantageous polymeric materials. For instance, Kobayashi *et al.* reported that laccase efficiently catalyzed the crosslinking of urushiol and laccol to produce the film having high gloss and good physical properties: artificial urushi.[10]

HRP is an important heme-containing enzyme that has been studied for more than a century[11] and is used for the oxidative polymerization of aromatic compounds such as polyphenol derivatives and the free-radical polymerization of vinyl monomers. Although the mechanism of the HRP-mediated redox reaction has not yet been fully elucidated, β-diketone radicals generated by the HRP-catalyzed oxidation of β-diketone (a representative β-diketone being acetylacetone (ACAC)) with hydrogen peroxide (H_2O_2) might initiate the radical polymerization of vinyl monomers.[12,13] A reasonable hypothesis for the catalytic mechanism is presented in Figure 1. As shown in Figure 1, HRP is oxidized by H_2O_2. It then passes from its native state through two catalytically active forms: HRP(*Ei*) and HRP(*Eii*). Each of these active forms oxidizes β-diketone, and then the enzyme returns to its native form. Excess H_2O_2 causes an inactive form, HRP(*Eiii*), which spontaneously reverts to the native form of the enzyme.[14–16] Therefore, the H_2O_2 concentration is a key factor in controlling the HRP-mediated polymerization reaction.[15] Many monomers, for example, methyl methacrylate,[17] styrene,[18] 4-methyl styrene,[18] 2-vinyl naphthalene[18] and AAm,[15,16] have been polymerized using the HRP/H_2O_2/ACAC system. Most studies that have used HRP as a catalyst have been investigated under homogeneous conditions, that is, solution and

Division of Applied Chemistry and Biotechnology, Graduate School of Engineering, Chiba University, Chiba, Japan
Correspondence: Dr M Kohri, Division of Applied Chemistry and Biotechnology, Graduate School of Engineering, Chiba University, Chiba 2638522, Japan.
E-mail: kohri@faculty.chiba-u.jp
Received 27 January 2014; revised 11 February 2014; accepted 14 February 2014; published online 26 March 2014

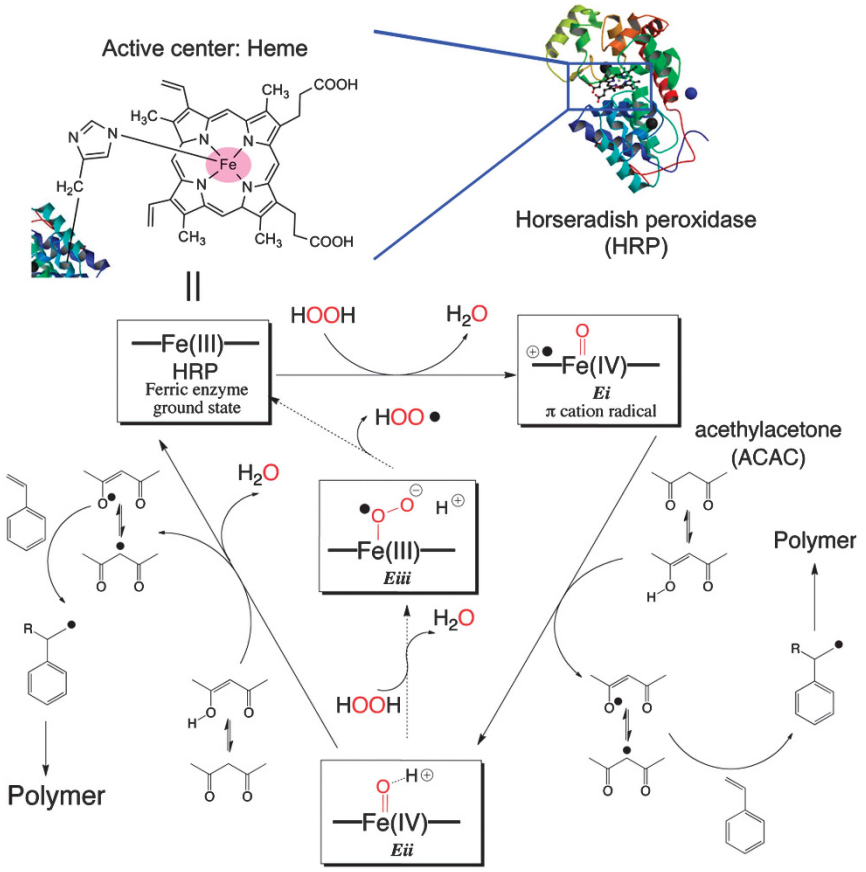

Figure 1 Proposed mechanism of the horseradish peroxidase (HRP)-mediated radical polymerization of vinyl monomers.

bulk polymerization. Great efforts are being directed at the implementation of enzymatic polymerization in various aqueous heterogeneous systems, which has been motivated by specific applications, industrial requirements for commercialization and environmental concerns, because heterogeneous polymerization processes are applicable to a much wider range of monomers. In fact, aqueous heterogeneous radical polymerization technique is one of the most important industrial processes to produce synthetic polymeric materials, and numerous applications exist for polymers prepared in aqueous heterogeneous systems. Their number will increase in the future owing to environmental regulations aiming at limiting the use of organic compounds. Thus, development of enzyme-mediated polymerization in aqueous heterogeneous system is an important issue as enzymatic polymerization is an environmental friendly alternative route. However, few reports have actually examined the HRP-mediated enzymatic polymerization under heterogeneous conditions.[19,20]

In this focus review, I provide an overview of the research performed by my research group and collaborators of HRP-mediated enzymatic polymerization under aqueous heterogeneous conditions for preparing functional particles.

ENZYMATIC MINIEMULSION POLYMERIZATION

Miniemulsion polymerization is a particular type of heterogeneous polymerization that allows the formation of functional polymer particles.[21] Miniemulsions are produced by high-energy homogenization, and they usually yield stable and narrow size monomer droplets. The polymerization of these miniemulsions gives rise to polymer particles with a diameter of 50–500 nm. The stability of oil-in-water miniemulsions is attained by the use of a surfactant and a highly water-insoluble molecule (hydrophobe). We have previously reported on HRP-mediated enzymatic miniemulsion polymerization.[22,23] The formation of radicals through the HRP-mediated enzymatic reaction takes place in the water phase. The entry of the resulting radicals into monomer droplets is stabilized by surfactants, and then polymerization can proceed (Figure 2a).

The selection of surfactants is a critical factor in controlling the polymerization efficiency during enzymatic miniemulsion polymerization because some surfactants act as denaturing agents for enzymes. We previously investigated the influence of the surfactant type on the enzymatic miniemulsion polymerization efficiency.[23] Styrene was used as a monomer, and ACAC was used as the β-diketone. Comparisons of four surfactants, the anionic surfactant SDS, the cationic surfactant cetyltrimethylammonium bromide (CTAB), the nonionic surfactant polyoxyethylene lauryl ether (Emulgen 109 P) and the cationic surfmer (that is, a surfactant + a monomer) *N*,*N*-dimethyl-*N*-*n*-dodecyl-*N*-2-methacryloyloxyethyl-ammonium bromide (C_{12}DMAEMA), were conducted for enzymatic miniemulsion polymerization. First, the effects of the surfactant type on the particle diameters measured by dynamic light scattering were investigated. These experiments were performed using a surfactant concentration greater than the critical micelle concentration because commercial polymer particles are usually prepared with surfactant levels greater than the critical micelle concentration to impart shear stability and reduce coagulation.[24] When an ionic surfactant, that is, SDS, CTAB or

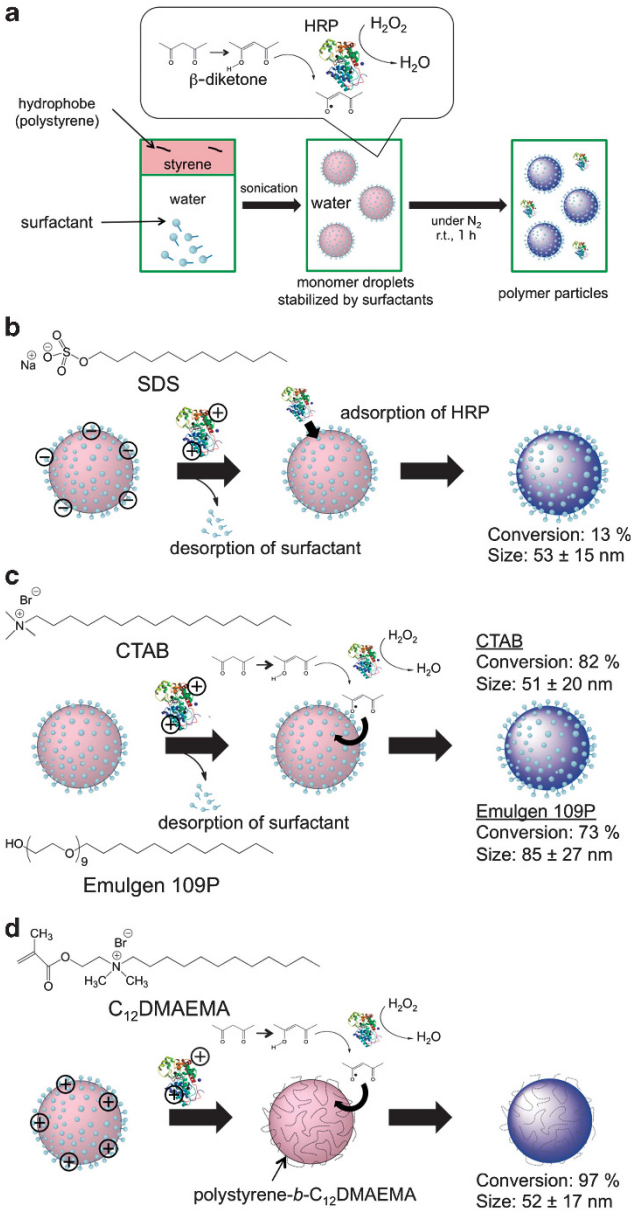

Figure 2 (**a**) Schematic representation of the enzymatic miniemulsion polymerization of styrene. The roles of (**b**) SDS, (**c**) cetyltrimethylammonium bromide (CTAB) or Emulgen 109 P and (**d**) *N,N*-dimethyl-*N*-*n*-dodecyl-*N*-2-methacryloyloxyethylammonium bromide (C_{12}DMAEMA) on enzymatic miniemulsion polymerization.

C_{12}DMAEMA, was used, polystyrene particles with average diameters of *ca* 50 nm were obtained. In contrast, switching to the nonionic surfactant Emulgen 109 P significantly increased the diameters (*ca* 85 nm). This phenomenon was most likely the result of the low stability of the monomer droplets or the resulting particles because Emulgen 109 P does not possess a charge, and it does not provide electrostatic stabilization to the miniemulsions and particles that subsequently form. To investigate the influence of surfactant type on HRP activity, the enzymatic activities in aqueous surfactant solutions were measured. HRP was incubated with the various surfactants at 25 °C for 30 min. The enzymatic activity of HRP was determined using a colorimetric test based on the HRP-catalyzed oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid[25] with H_2O_2 in the presence of SDS. For CTAB, Emulgen 109P or C_{12}DMAEMA, a colorimetric test based on 3,3′,5,5′-tetramethylbenzidine[26] was used, as these surfactants aggregated with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid and precipitated out of solution. HRP activity in the presence of SDS remained *ca* 85% compared with that in the absence of surfactant, but the monomer conversion was low in the presence of SDS. This phenomenon was likely due to the denaturation of HRP. Because HRP (isoelectric point: 7.2) is positively charged under the present polymerization conditions in deionized water, HRP will be adsorbed onto the negatively charged monomer droplets that are stabilized with SDS through electrostatic interactions. Therefore, HRP will be continually in contact with the styrene monomer, similar to an organic solvent, which causes the denaturation of HRP because natural enzymes are easily denatured and inactivated in the presence of organic solvents (Figure 2b). In contrast, it is difficult for the positively charged HRP to adsorb onto styrene monomer droplets that have been stabilized with a cationic surfactant (CTAB and C_{12}DMAEMA) or nonionic surfactant (Emulgen 109P), which prevents the denaturation of HRP from contact with the monomer. In fact, switching from SDS to CTAB or Emulgen 109P significantly increased the conversion of the monomer. When C_{12}DMAEMA was used, an essentially complete monomer conversion (*ca* 97%) was obtained. Incubation with CTAB, Emulgen 109P and C_{12}DMAEMA, however, decreased the HRP activity to *ca* 56%, 63% and 70%, respectively. This result suggested the partial inactivation of HRP in the presence of cationic and nonionic surfactants, in agreement with a report by Bhagwat and Motlekar,[27] as low-molecular-weight surfactants are released from the surface of the monomer droplets. Thus, in the case of CTAB and Emulgen 109P, the surfactant desorption caused the inactivation of HRP (Figure 2c).

Although the HRP activity decreased in the presence of C_{12}DMAEMA, this decrease in enzyme activity did not affect the polymerization efficiency (*vide supra*). To clarify this phenomenon, we performed gel permeation chromatography (GPC) measurements of the particles after different reaction times.[22] When the low-molecular-weight surfactant CTAB was used, a unimodal GPC trace was usually observed. In contrast, when the surfmer C_{12}DMAEMA was used, the resulting GPC trace was bimodal and contained two peaks, that is, peak I (retention time: 16 min) and peak II (retention time: 19 min; Figure 3a). The ratio of peak I/peak II increased with the reaction time. After 120 min, the conversion reached *ca* 100%, and the ratio stayed constant. It was noteworthy that the M_n of peak I increased with the reaction time to *ca* 90 000, whereas the M_n of peak II remained approximately *ca* 7000, indicating two different reaction loci during this polymerization. The reason for the bimodal peaks was likely due to the monomer ratio of the copolymers, as the system contained two monomers: styrene and C_{12}DMAEMA. The formation of a radical through the HRP-mediated reaction occurred in the water phase, followed by the resulting radical entry into the styrene monomer droplets stabilized by C_{12}DMAEMA. Thus, at the initial stage of polymerization, C_{12}DMAEMA near the surface of the monomer droplets was predominantly polymerized. These results suggested that peak II mainly represented the copolymers of C_{12}DMAEMA and styrene. As described above, the M_n of peak II remained approximately *ca* 7000, indicating the consumption of C_{12}DMAEMA during the early stage of polymerization. Thus, the desorption of C_{12}DMAEMA was suppressed, which caused increased monomer conversion and, as a result, a high-efficiency polymerization (Figure 2d). In contrast, peak I mainly represented the

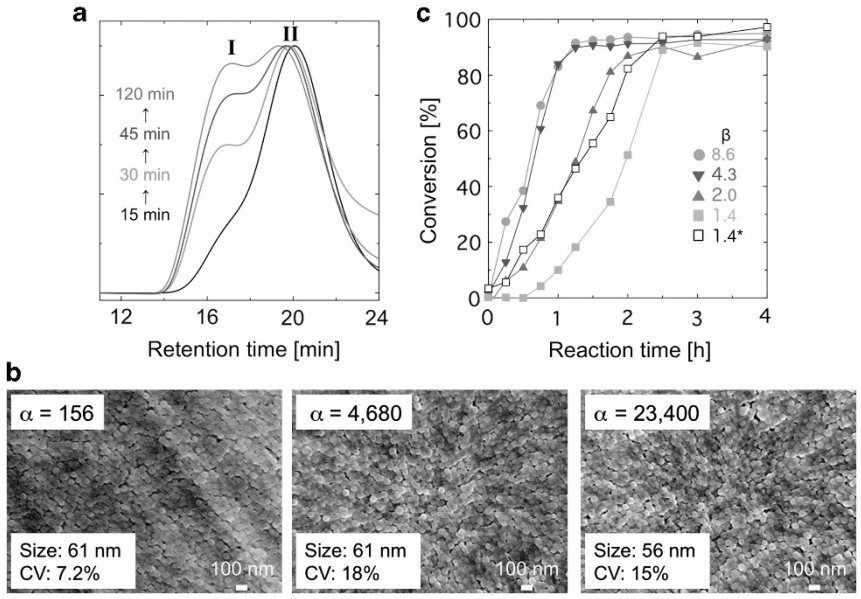

Figure 3 (**a**) GPC traces of polymer particles using *N,N*-dimethyl-*N*-*n*-dodecyl-*N*-2-methacryloyloxyethylammonium bromide with different reaction times. (**b**) Scanning electron microscopy images of polystyrene particles obtained from enzymatic miniemulsion polymerization. (**c**) Monomer conversion vs time profiles of the polymerization of styrene. *H_2O_2 was added five times to the mixture every 15 min with vigorous stirring. CV, coefficient of variation. A full color version of this figure is available at *Polymer Journal* online.

polystyrene homopolymer that grew by the polymerization of styrene in the interior of the monomer droplets. Infrared measurements and an elemental analysis of the polymers from the different reaction times also suggested two different polymerization loci in the polymerization system.[28] In addition, time-resolved small-angle neutron scattering provided direct evidence for the specific polymerization loci, which appeared during the early stage of polymerization using C_{12}DMAEMA on the surface of the droplet and which were the origins of the bimodal peaks that were observed in the GPC chromatogram.[28]

As described above, the HRP-mediated enzymatic reaction was not prevented, and polymer particles were obtained with a high-conversion rate using the cationic surfmer C_{12}DMAEMA system. The effects of various parameters of the enzymatic miniemulsion polymerization with C_{12}DMAEMA, including polymerization kinetics, were investigated.[22] It is important to investigate the influence of the H_2O_2 on the HRP initial concentration ratio (α) because H_2O_2 is both a substrate and an inhibitor of HRP.[15] Several experiments with α ranging from 10 to 23 400 were performed. The β-diketone (ACAC) to H_2O_2 initial molar ratio (β) was set at 2.0 because two ACAC molecules and one H_2O_2 molecules are consumed in the catalytic cycle of HRP. For values of α less than 77, the monomer conversion in 24 h was essentially 0%, and no particles were recoverable, indicating that lower limits existed for α. At low values of α, the initiation rate was too low to result in significant polymerization. Further increasing α from 156 to 23 400 caused an increase in the monomer conversion (*ca* 93–100%), and particle diameters measured by dynamic light scattering were approximately 60 nm. The particle diameters were controlled from *ca* 60 to 110 nm with C_{12}DMAEMA concentrations between 30 and 60 mM. Figure 3b shows scanning electron microscopy (SEM) images of the obtained particles ($\alpha = 156$, 4680 and 23 400). Relatively monodisperse particles were obtained with particle sizes of *ca* 60 nm. As their SEM images indicate, for $\alpha = 156$, 4680 and 23 400, the particle diameter distributions (coefficient of variation: CV) were, respectively, *ca* 7.2%, 18% and 15%, indicating that the CV increased with α. The obtained particles were stable in water, and no marked changes were observed after 3 months of storage. Upon the addition of excess H_2O_2 to the dispersion of particles, however, some particles coagulated and settled at the bottom of the glass cell, indicating poor stability of the obtained particles in the presence of excess H_2O_2. These results suggested the partial agglomeration of the miniemulsions or resulting particles in the presence of a high concentration of H_2O_2.

The effects of β (β-diketone to H_2O_2 initial molar ratio) on the polymerization kinetics of styrene were investigated because β-diketones act as polymerization initiators (Figure 3c).[22] Although the final conversions were essentially the same, a strong dependence on the polymerization time was observed. The theoretical value of β is 2.0 (*vide supra*). When β was lowered from 2.0 to 1.4, the monomer conversion proceeded slowly until 1 h. During the early stage of reaction, excess H_2O_2 likely caused an inactive form of HRP(*Eiii*), which is produced with the excessive exposure of protonated HRP(*Eii*) to H_2O_2 in a reaction that is partially mediated by a superoxide free radical.[14–16] To investigate this phenomenon, H_2O_2 ($\beta = 1.4$) was added a total of five times to the reaction mixture every 15 min, and the reaction proceeded smoothly. This result suggested that the formation of β-diketone radicals was inhibited by the presence of excess H_2O_2, which causes an inactive form, HRP(*Eiii*) (*vide supra*). When β was high ($\beta = 4.3$ and 8.5), the polymerization proceeded rapidly. Although many ACACs remained in the reaction medium, the presence of unreacted ACAC clearly did not affect the reaction efficiency.

Although some pioneering works have researched miniemulsion polymerization using functional surfmers to prepare polymer particles,[29–31] only one study on the post-functionalization of particle surfaces based on surfmers was found in the literature.[32] Froimowicz and Landfester reported on the preparation of particles by miniemulsion polymerization with a phosphonate surfmer, and the

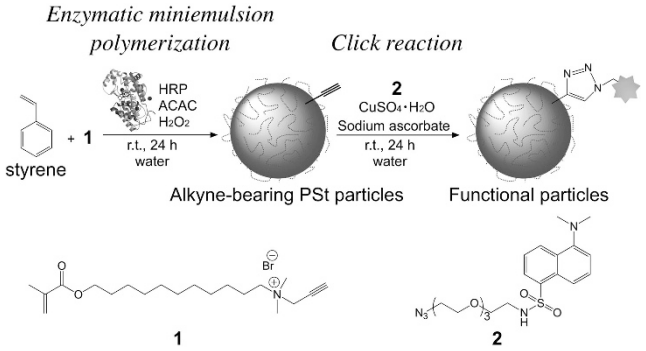

Figure 4 Schematic representation of the preparation of functional polymer particles through the combined use of enzymatic miniemulsion polymerization with clickable surfmer and click reaction. ACAC, acetylacetone; HRP, horseradish peroxidase; r.t., room temperature. A full color version of this figure is available at *Polymer Journal* online.

phosphonate-functionalized particles were used as functional scaffolds to bind calcium ions and subsequently mineralize hydroxyapatite on the surfaces of the particles. We synthesized alkyne-functionalized particles using enzymatic miniemulsion polymerization and then treated them with the click reaction to synthesize fluorescent particles (Figure 4).[33] The core polystyrene particles with alkyne moieties were first prepared using the clickable cationic surfmer *N*,*N*-dimethyl-propargyl-*N*-2-methacryloxy-11-undecyl ammonium bromide **1**, and spherical particles of *ca* 60 nm were obtained. The successful introduction of the alkyne moieties was determined by the appearance of the characteristic absorbance at 2120 cm^{-1} due to the C≡C stretching vibration band and at 1720 cm^{-1} due to the C = O stretching vibration band of **1** in the infrared spectrum. A click reaction was then performed using dansyl azide compound **2**, which contained polyethylene glycol (PEG) moieties to enhance the water solubility, with Cu(I) catalysis generated *in situ* by the reaction of $CuSO_4$ with sodium ascorbate for 24 h at room temperature. The introduction of **2** onto the particles was determined by the disappearance of the characteristic absorbance from both the alkyne (2120 cm^{-1}) and azide (2110 cm^{-1}) in the infrared spectrum. In addition, no fluorescence signals were obtained in the absence of $CuSO_4$ or sodium ascorbate, indicating that dansyl moieties were actually introduced onto the particles by the click reaction and not just physically adsorbed. Physically adsorbed **2** could be easily purified by washing with a methanol/water (1:4) solution. The fluorescence spectrum of the obtained particles exhibited a broad emission centered at *ca* 476 nm ($\lambda_{ex} = 340$). The fluorescence peak wavelength of the dansyl groups in the particles showed an extraordinary blue shift compared with **2** (*ca* 542 nm ($\lambda_{ex} = 325$)) in water, indicating that the microenvironment of the dansyl derivatives covalently bonded to the particle surface was less polar than the surrounding aqueous phase. It is well known that dansyl moieties exhibit polarity-dependent emission spectra and stokes shifts.[34] This method offered a practical and environmental friendly method for the preparation of surface-functional polymer particles because both the enzymatic miniemulsion polymerization and the click reaction were conducted at room temperature in water.

ENZYMATIC EMULSIFIER-FREE EMULSION POLYMERIZATION

Emulsifier-free emulsion polymerization is useful for fabricating polymer particles in the absence of surfactants.[35] The dispersion

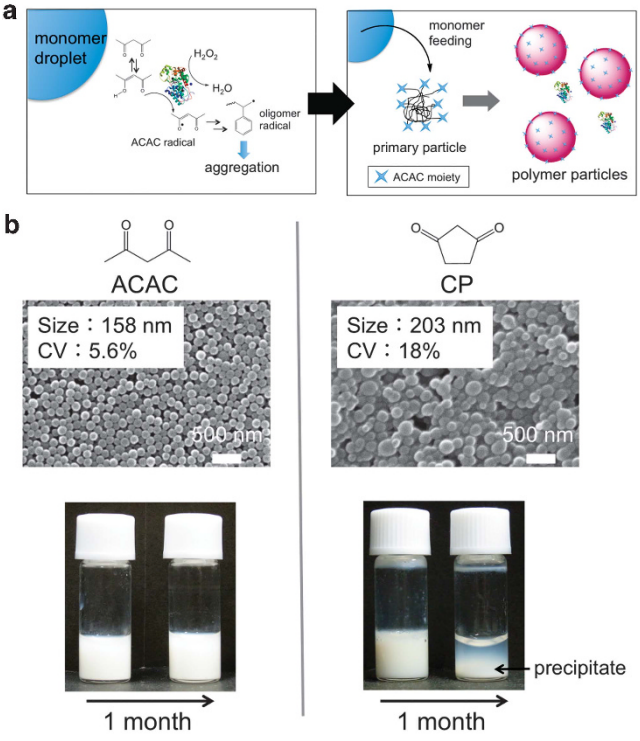

Figure 5 (**a**) Schematic representation of enzymatic emulsifier-free emulsion polymerization using the horseradish peroxidase (HRP)/H_2O_2/acetylacetone (ACAC) system. (**b**) Scanning electron microscopy (SEM) images of polystyrene particles from the enzymatic emulsifier-free emulsion polymerization prepared using ACAC and cyclopentanedione (CP) as the initiator. Photographs: dispersed solutions of particles.

stability of the obtained particles is enhanced by ionizable initiators or hydrophilic ionic co-monomers because the migration/desorption of ionic groups is avoided. Although many works have been reported on emulsifier-free emulsion polymerization using various types of initiators, there has been a lack of studies describing the use of enzyme-mediated reactions as initiating systems. Thus, we developed the enzymatic emulsifier-free emulsion polymerization using HRP as a catalyst (Figure 5a).[36] Because β-diketones act as initiators in the HRP-mediated reaction, the choice of the β-diketone was shown to affect the properties of the resultant particles. Two β-diketones were used to investigate the effects of the β-diketone structures on the enzymatic emulsifier-free emulsion polymerization. Two water-soluble β-diketones, ACAC and 1,3-cyclopentanedione (CP), generated polymer particles at a conversion rate of *ca* 25–50%. When ACAC was used as the β-diketone, the molecular weights of the obtained particles decreased with increasing amounts of ACAC, which is a general feature of radical polymerization. However, relatively similar molecular weights were obtained for the particles generated when CP was used as the β-diketone. This phenomenon was most likely due to the substrate specificity of HRP. Rodrigues *et al.* reported that HRP has a high specificity for ACAC, whereas cyclic β-diketones, for example, dimedon, showed less recognition.[37] Because it was difficult for CP, a cyclic β-diketone, to access the active center of HRP, very little CP was recognized by HRP. As a result, there was no difference between the molecular weights of the particles with CP. The particles were obtained with a moderate conversion using either ACAC or CP. This result was likely attributable to the mechanism of particle formation. With enzymatic emulsifier-free emulsion

polymerization, HRP is continually in contact with the styrene monomer, which results in the partial degradation of the HRP. Recently, we prepared PEGylated hematin, a hydroxylated protoporphyrin IX complex containing ferric ion (Fe^{3+}), and used it as a biomimetic catalyst for the fabrication of polyphenol derivatives.[38] PEGylated hematin exhibited good chemical stability toward styrene. Furthermore, it showed wide substrate specificity, as PEGylated hematin is a smaller molecule compound for HRP. In the future, more efficient processes will be developed using biomimetic catalysts, such as PEGylated hematin.

When ACAC was used as the initiator, the particle sizes measured by dynamic light scattering were *ca* 150 nm, and relatively monodisperse particles were obtained (ACAC particles). In contrast, when CP was used as the initiator, two peaks, one *ca* 300 nm and the other approximately 1200 nm, were observed, which were attributed to the individual particles and aggregates, respectively, as the particles obtained (CP particles) were polydisperse. As shown in Figure 5b, SEM images of the particles also indicated that the ACAC particles were spherical and monodisperse particles (CV = 5.6%), and the CP particles were polydisperse (CV = 18%). Furthermore, there were significant differences in their colloidal stabilities. Although ACAC particles showed no sign of aggregated precipitation over a 1-month duration, the CP particles partially aggregated and settled to the bottom of the sample vial after 1 month, indicating that the presence of the β-diketone moieties on the surface of the particles apparently affected the colloidal stability of the resultant particles (Figure 5b). No ionic surfactants or comonomers were used to stabilize the particles. Thus, the stability of the dispersion primarily resulted from the interparticle repulsion caused by the ACAC moieties located on the surfaces of the particles. Enzymatic emulsifier-free emulsion polymerization could offer a practical method for preparing polymer particles because polymerizations can be conducted at room temperature using a mild enzymatic process without surfactants.

SURFACE-INITIATED ENZYMATIC GRAFT POLYMERIZATION

Surface-initiated polymerization from initiators bound to material surfaces has been developed to be a powerful tool for preparing functional materials. So far, many groups, including our group,[39–42] have reported various types of surface-initiated polymerization techniques, for example, (living) radical, anionic, cationic and ring-opening polymerization.[43,44] However, HRP-mediated surface-initiated polymerization has never been reported. Thus, we developed surface-initiated enzymatic polymerization using HRP as a catalyst and used it to fabricate core-shell-type particles.[45]

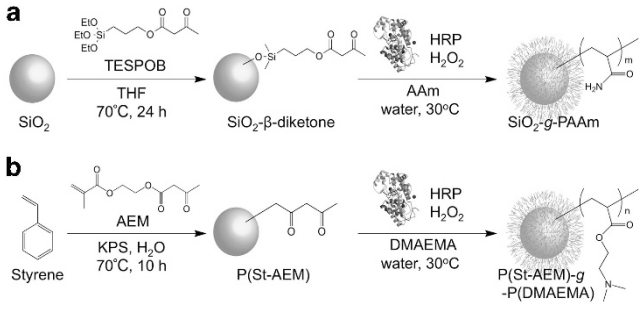

Figure 6 Schematic representation of surface-initiated enzymatic graft polymerization using (**a**) SiO_2 particles and (**b**) polystyrene particles as the core material. AAm, acrylamide; AEM, 2-(aceto-acetyloxy)ethyl methacrylate; DMAEMA, *N*,*N*-dimethyl-*N*-*n*-dodecyl-*N*-2-methacryloyloxyethylammonium bromide; HRP, horseradish peroxidase; PAAm, polyacrylamide; TESPOB, 3-(triethoxysilyl)propyl 3-oxobutanoate; THF, tetrahydrofuran. A full color version of this figure is available at *Polymer Journal* online.

First, polymer-grafted silica (SiO_2) particles were prepared by surface-initiated enzymatic polymerization (Figure 6a).[45] To introduce the initiating group onto SiO_2 particles, a β-diketone moiety-bearing silane coupling agent, 3-(triethoxysilyl)propyl 3-oxobutanoate (TESPOB), was synthesized. The modification of the initiating groups onto the SiO_2 particles was confirmed using infrared spectroscopy, as characteristic signals at *ca* 1735 and 1715 cm^{-1} corresponded to the C = O vibration mode of a TESPOB moiety. The surface initiator concentration was increased concomitantly with increasing TESPOB concentrations and was controlled to 0.58–3.29 initiator molecules per nm^2. SEM images confirmed that the dried SiO_2-β-diketone particles possessed a spherical surface. AAm was then polymerized onto the core SiO_2-β-diketone particles using surface-initiated HRP-mediated polymerization. A major peak at 1668 cm^{-1} corresponding to the C = O stretching vibration of the amide group ($-CONH_2$) confirmed the presence of polyacrylamide (PAAm) on the SiO_2-β-diketone particles. The spectra pattern closely resembled that of PAAm obtained by HRP using the free initiator ethyl acetoacetate. SEM analysis confirmed that the core-shell particles contained well-defined spherical SiO_2 cores with a narrow size distribution. The shell layer thickness of the resulting particles increased from 20 to 200 nm with increasing AAm concentration (0–500 mM), indicating that the shell layer thickness of the grafted polymer was controlled by the monomer concentration. The addition of AAm concentrations greater than 500 mM, however, caused coarse aggregation among the particles and in gelation, demonstrating that the excess addition of AAm increased the viscosity.

Polymers that are grafted onto a material's surface have an important role in many areas of science and technology, for example, colloidal stabilization, adhesion, lubrication, tribology and rheology. The conformations of grafted polymers in solvents dramatically change with graft density.[46] At low graft densities, they usually assume a 'mushroom' conformation with coiled dimensions. With increasing graft density, grafted polymers are obliged to stretch away from the material's surface, forming so-called 'polymer brushes.' Polymer brushes can be categorized into two groups that vary by graft density: semi-dilute and high-density polymer brushes. It is important to investigate the surface density of a grafted polymer because it is related directly to the material's properties. The surface density of the grafted PAAm (σPAAm) was determined from the weight loss calculated by thermogravimetric analysis and the molar mass of the grafted PAAm measured by GPC.[47] To investigate the σPAAm, the enzyme concentration was varied from 25 to 100 μM because the radical initiation is related directly to the HRP concentration (for these experiments, α was set at 1). When low concentrations of HRP were used (25 μM), σPAAm was *ca* 0.004 chains per nm^2, indicating a mushroom-type structure with coiled dimensions.[46] Although the shell layer thickness and M_n decreased as the amount of HRP was increased to 100 μM, σPAAm gradually increased to 0.012 chains per nm^2. These σ values described the semi-dilute brush regime, which is in agreement with the conventional surface-initiated free radical polymerization.[46] These results demonstrated that the grafting density of the brushes was controlled by the HRP amount.

Surface-initiated enzymatic polymerization has also been applied to the modification of the surfaces of polymer particles (Figure 6b).[48] Core polystyrene particles containing initiating groups, that is,

P(St-AEM), were prepared by the emulsifier-free emulsion polymerization of styrene and 2-(aceto-acetyloxy)ethyl methacrylate (AEM). The total β-diketone (initiator) concentration from ^{1}H NMR measurements (freeze-dried sample dissolved in $CDCl_3$) was comparable to the amount of AEM polymerized in the reaction (*ca* 5 mol%). During the early stage of emulsifier-free emulsion polymerization, a small amount of the styrene monomer molecules, which were dissolved in the aqueous phase, and the hydrophilic monomer AEM were polymerized. Oligomer radicals that formed in the aqueous phase grew via precipitation and aggregation, which led to the formation of primary particles. Polymerization of the styrene monomers continued within the swollen primary particles, which were supplied by diffusion from the monomer droplets. The resultant polymer particles were stabilized by the AEM moieties, which were located on the surfaces of the particles due to their hydrophilic nature. Although the particle size without the use of AEM was determined to be *ca* 500 nm, it monotonically decreased to *ca* 300 nm with an increase in the AEM feed concentration. The downward trend in the particle size by copolymerization with the hydrophilic monomers in the emulsifier-free emulsion polymerization of styrene was in agreement with previously reported results.[49] The surface-initiated enzymatic polymerization of DMAEMA was performed with DMAEMA concentrations from 50 to 500 mM in aqueous dispersions of P(St-AEM) particles. The particle size was easily controlled with the DMAEMA concentration and was able to reach *ca* 1100 nm. This methodology will be applied to a broad range of monomers to yield core-shell-type particles with tailored shell thicknesses.

CONCLUSIONS

In this focus review, novel research concerning HRP-mediated enzymatic polymerization under heterogeneous conditions, specifically, enzymatic miniemulsion polymerization, enzymatic emulsifier-free emulsion polymerization and surface-initiated enzymatic graft polymerization, was summarized. These methods could offer practical approaches for preparing functional polymer particles because enzymatic polymerization can be conducted at room temperature using a mild enzymatic process. Particles obtained by enzymatic miniemulsion polymerization will be expected to be applicable in some practical fields such as biotechnology and optoelectronics depending on their surface properties. β-Diketone-bearing particles by enzymatic emulsifier-free emulsion polymerization will be applied to hybrid particles because β-diketone group captures inorganic and metal particles, such as titania, zirconia and silica particles. Also core-shell-type particles prepared by surface-initiated enzymatic graft polymerization will be useful in developing adhesives, latex diagnosis and affinity chromatography. Recently, HRP[50,51] and its mimetic catalyst (either hematin[52] or hemin[53]) were used as the catalyst for living radical polymerization, that is, atom transfer radical polymerization. Studies using HRP or other relevant enzymes[54] are increasingly being conducted. The author hopes that these novel methods for the synthesis of polymer particles will contribute to the progress of environmental benign industrial processes.

ACKNOWLEDGEMENTS

I thank Professor Takayuki Nakahira, Professor Keiki Kishikawa and Professor Tatsuo Taniguchi and the students of the research group, particularly Ms Haruka Fukushima, Ms Ayaka Kobayashi, Mr Satoshi Uzawa and Ms. Yuri Nannichi. This work was partly supported by a grant from the Futaba Electronics Memorial Foundation, Oil and Fat Industry Kaikan Foundation and KAKENHI.

1 Parravano, G. Chain reactions induced by enzymic systems. *J. Am. Chem. Soc.* **73,** 183–184 (1951).
2 Derango, R. A., Chiang, L., Dowbenko, R. & Lasch, J. G. Enzyme-mediated polymerization of acrylic monomers. *Biotech. Lett.* **6,** 523–526 (1992).
3 Hollmann, F. in *Biocatalysis in Polymer Chemistry* (ed. Loos, K.) 143–163 (Wiley-VCH, Weinheim, 2011).
4 Kobayashi, S., Uyama, H. & Kimura, S. Enzymatic polymerization. *Chem. Rev.* **101,** 3793–3818 (2001).
5 Gross, R. A., Kumar, A. & Kalra, B. Polymer synthesis by *in vitro* enzyme catalysis. *Chem. Rev.* **101,** 2097–2124 (2001).
6 Kobayashi, S. & Makino, A. Enzymatic polymer synthesis: an opportunity for green polymer chemistry. *Chem. Rev.* **109,** 5288–5353 (2009).
7 Kadokawa, J. & Kobayashi, S. Polymer synthesis by enzymatic catalysis. *Curr. Opin. Chem. Biol.* **14,** 145–153 (2010).
8 Kadokawa, J. Precision polysaccharide synthesis catalyzed by enzymes. *Chem. Rev.* **111,** 4308–4345 (2011).
9 Uyama, H. & Kobayashi, S. Enzymatic synthesis of polyesters via polycondensation. *Adv. Polym. Sci.* **194,** 133–158 (2006).
10 Kobayashi, S., Uyama, H. & Ikeda, R. Artificial urushi. *Chem. Eur. J.* **7,** 4754–4760 (2001).
11 Veitch, N. C. Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry* **65,** 249–259 (2004).
12 Teixeira, D., Lalot, T., Brigodiot, M. & Marechal, E. Beta-diketones as key compounds in free-radical polymerization by enzyme-mediated initiation. *Macromolecules* **32,** 70–72 (1999).
13 Singh, A. & Kaplan, D. L. *In vitro* enzyme-induced vinyl polymerization. *Adv. Polym. Sci.* **194,** 211–224 (2006).
14 Singh, A., Ma, D. & Kaplan, D. L. Enzyme-mediated free radical polymerization of styrene. *Biomacromolecules* **1,** 592–596 (2000).
15 Durand, A., Lalot, T., Brigodiot, M. & Maréchal, E. Enzyme-mediated initiation of acrylamide polymerization: reaction mechanism. *Polymer (Guildf)* **41,** 8183–8192 (2000).
16 Kalra, B. & Gross, R. A. HRP-mediated polymerizations of acrylamide and sodium acrylate. *Green Chem.* **4,** 174–178 (2002).
17 Kalra, B. & Gross, R. A. Horseradish peroxidase mediated free radical polymerization of methyl methacrylate. *Biomacromolecules* **1,** 501–505 (2000).
18 Singh, A., Roy, S., Samuelson, L., Bruno, F., Nagarajan, R., Kumar, J., John, V. & Kaplan, D. L. Peroxidase, hematin, and pegylated-hematin catalyzed vinyl polymerizations in water. *J. Macromol. Sci. A Pure Appl. Chem.* **38,** 1219–1230 (2001).
19 Shan, J., Kitamura, Y. & Yoshizawa, H. Emulsion polymerization of styrene by horseradish peroxidase-mediated initiation. *Colloid Polym. Sci.* **284,** 108–111 (2005).
20 Qi, G., Jones, C. W. & Schork, F. J. Enzyme-initiated miniemulsion polymerization. *Biomacromolecules* **7,** 2927–2930 (2006).
21 Landfester, K. Miniemulsion polymerization and the structure of polymer and hybrid nanoparticles. *Angew. Chem. Int. Ed.* **48,** 4488–4507 (2009).
22 Kohri, M., Kobayashi, A., Fukushima, H., Kojima, T., Taniguchi, T., Saito, K. & Nakahira, T. Enzymatic miniemulsion polymerization of styrene with a polymerizable surfactant. *Polym. Chem.* **3,** 900–906 (2012).
23 Kohri, M., Kobayashi, A., Fukushima, H., Taniguchi, T. & Nakahira, T. Effect of surfactant type on enzymatic miniemulsion polymerization using horseradish peroxidase as a catalyst. *Chem. Lett.* **41,** 1131–1133 (2012).
24 Thames, S. F., Rawlins, J. W. & Mendon, S. K. in *Miniemulsion Polymerization Technology* (ed. Mittal, V.) 139–171 (Wiley-Scrivener, USA, 2010).
25 Wang, Q., Kromka, A., Houdkova, J., Babchenko, O., Rezek, B., Li, M., Boukherroub, R. & Szunerits, S. Nanomolar hydrogen peroxide detection using horseradish peroxidase covalently linked to undoped nanocrystalline diamond surfaces. *Langmuir* **28,** 587–592 (2012).
26 Gao, L., Wu, J. & Gao, D. Enzyme-controlled self-assembly and transformation of nanostructures in a tetramethylbenzidine/horseradish peroxidase/H_2O_2 system. *ACS Nano* **5,** 6736–6742 (2011).
27 Motlekar, N. A. & Bhagwat, S. S. Activity of horseradish peroxidase in aqueous and reverse micelles and back-extraction from reverse-micellar phases. *J. Chem. Technol. Biotechnol* **76,** 643–649 (2001).
28 Motokawa, R., Taniguchi, T., Sasaki, Y., Enomoto, Y., Murakami, F., Kasuya, M., Kohri, M. & Nakahira, T. Small-angle neutron scattering study on specific polymerization loci induced by a copolymerization of polymerizable surfactant and styrene in miniemulsion polymerization. *Macromolecules* **45,** 9435–9444 (2012).
29 Kitzmiller, E. L., Miller, C. M., Sudol, E. D. & El-Aasser, M. S. Miniemulsion polymerization: an approach to control copolymer composition. *Macromol. Symp.* **92,** 157–168 (1995).
30 Schoonbrood, H. A. S. & Asua, J. M. Reactive surfactants in heterophase polymerization. 9. Optimum surfmer behavior in emulsion polymerization. *Macromolecules* **30,** 6034–6041 (1997).
31 Sauer, R., Turshatov, A., Baluschev, S. & Landfester, K. One-pot production of fluorescent surface-labeled polymeric nanoparticles via miniemulsion polymerization with bodipy surfmers. *Macromolecules* **45,** 3787–3796 (2012).
32 Sauer, R., Froimowicz, P., Schöller, K., Cramer, J. M., Ritz, S., Mailänder, V. & Landfester, K. Design, synthesis, and miniemulsion polymerization of new phosphonate surfmers and application studies of the resulting nanoparticles as model systems for biomimetic mineralization and cellular uptake. *Chem. A Eur. J.* **18,** 5201–5212 (2012).

33 Kohri, M., Kobayashi, A., Nannichi, Y., Taniguchi, T. & Kishikawa, K. A green approach for the synthesis of fluorescent polymer particles by combined use of enzymatic miniemulsion polymerization with clickable surfmer and click reaction. *Trans. Mat. Res. Soc. Jpn* (in press).
34 Horie, K., Yamada, S., Machida, S., Takahashi, S., Isono, Y. & Kawaguchi, H. Dansyl fluorescence and local structure of dansyl-labeled core-shell and core-hair type microspheres in solution. *Macromol. Chem. Phys.* **204,** 131–138 (2003).
35 Ganachaud, F., Sauzedde, F., Elaïssari, A. & Pichot, C. Emulsifier-free emulsion copolymerization of styrene with two different amino-containing cationic monomers. I. Kinetic studies. *J. Appl. Polym. Sci.* **65,** 2315–2330 (1997).
36 Kohri, M., Uzawa, S., Kobayashi, A., Fukushima, H., Taniguchi, T. & Nakahira, T. Enzymatic emulsifier-free emulsion polymerization to prepare polystyrene particles using horseradish peroxidase as a catalyst. *Polym. J.* **45,** 354–358 (2013).
37 Rodrigues, A. P., da Fonseca, L. M., de Faria Oliveira, O. M., Brunetti, I. L. & Ximenes, V. F. Oxidation of acetylacetone catalyzed by horseradish peroxidase in the absence of hydrogen peroxide. *Biochim. Biophys. Acta* **1760,** 1755–1761 (2006).
38 Kohri, M., Fukushima, H., Taniguchi, T. & Nakahira, T. Synthesis of polyarbutin by oxidative polymerization using PEGylated hematin as a biomimetic catalyst. *Polym. J.* **42,** 952–955 (2010).
39 Kohri, M., Sato, M., Abo, F., Inada, T., Kasuya, M., Taniguchi, T. & Nakahira, T. Preparation and lectin binding specificity of polystyrene particles grafted with glycopolymers bearing S-linked carbohydrates. *Eur. Polym. J.* **47,** 2351–2360 (2011).
40 Kohri, M., Kohma, H., Shinoda, Y., Yamauchi, M., Yagai, S., Kojima, T., Taniguchi, T. & Kishikawa, K. A colorless functional polydopamine thin layer as a basis for polymer capsules. *Polym. Chem.* **4,** 2696–2702 (2013).
41 Kohri, M., Kohma, H., Shinoda, Y., Nannichi, Y., Yamauchi, M., Yagai, S., Kojima, T., Taniguchi, T. & Kishikawa, K. Facile synthesis of free-standing polymer brush films based on a colorless polydopamine thin layer. *Macromol. Rapid Commun.* **34,** 1220–1224 (2013).
42 Kohri, M., Abo, F., Miki, S., Fujii, T., Kasuya, M. & Taniguchi, T. Effects of graft shell thickness and compositions on lectin recognition of glycoparticles. *J. Colloid Sci. Biotechnol.* **2,** 45–52 (2013).
43 Edmondson, S., Osborne, V. L. & Huck, W. T. S. Polymer brushes via surface-initiated polymerizations. *Chem. Soc. Rev.* **33,** 14–22 (2004).
44 Barbey, R., Lavanant, L., Paripovic, D., Schüwer, N., Sugnaux, C., Tugulu, S. & Klok, H. A. Polymer brushes via surface-initiated controlled radical polymerization: synthesis, characterization, properties, and applications. *Chem. Rev.* **109,** 5437–5527 (2009).
45 Fukushima, H., Kohri, M., Kojima, T., Taniguchi, T., Saito, K. & Nakahira, T. Surface-initiated enzymatic vinyl polymerization: synthesis of polymer-grafted silica particles using horseradish peroxidase as catalyst. *Polym. Chem.* **3,** 1123–1125 (2012).
46 Tsujii, Y., Ohno, K., Yamamoto, S., Goto, A. & Fukuda, T. Structure and properties of high-density polymer brushes prepared by surface-initiated living radical polymerization. *Adv. Polym. Sci.* **197,** 1–45 (2006).
47 Inoubli, R., Dagréou, S., Delville, M. H., Lapp, A., Peyrelasse, J. & Billon, L. In situ thermo-dependant trapping of carbon radicals: a versatile route to well-defined polymer-grafted silica nanoparticles. *Soft Matter* **3,** 1014–1024 (2007).
48 Kohri, M. Development of environmentally-friendly preparation and surface-modification of polymer particles by enzymatic polymerization. *Kobunshi Ronbunsyu* **70,** 386–397 (2013).
49 Taniguchi, T., Murakami, F., Kasuya, M., Kojima, T., Kohri, M., Saito, K. & Nakahira, T. Preparation of titania hollow particles with independently controlled void size and shell thickness by catalytic templating core–shell polymer particles. *Colloid Polym. Sci.* **291,** 215–222 (2013).
50 Sigg, S. J., Seidi, F., Renggli, K., Silva, T. B., Kali, G. & Bruns, N. Horseradish peroxidase as a catalyst for atom transfer radical polymerization. *Macromol. Rapid Commun.* **32,** 1710–1715 (2011).
51 Ng, Y. H., di Lena, F. & Chai, C. L. PolyPEGA with predetermined molecular weights from enzyme-mediated radical polymerization in water. *Chem. Commun.* **47,** 6464–6466 (2011).
52 Yamashita, K., Yamamoto, K. & Kadokawa, J. Atom transfer radical polymerization of N-isopropylacrylamide by enzyme mimetic catalyst. *Polymer (Guildf)* **54,** 1775–1778 (2013).
53 Simakova, A., Mackenzie, M., Averick, S. E., Park, S. & Matyjaszewski, K. Bioinspired iron-based catalyst for atom transfer radical polymerization. *Angew. Chem. Int. Ed.* **52,** 12148–12151 (2013).
54 Silva, T. B., Spulber, M., Kocik, M. K., Seidi, F., Charan, H., Rother, M., Sigg, S. J., Renggli, K., Kali, G. & Bruns, N. Hemoglobin and red blood cells catalyze atom transfer radical polymerization. *Biomacromolecules* **14,** 2703–2712 (2013).

Michinari Kohri received his PhD from Tohoku University in 2007 under the supervision of Professor Shin-ichiro Shoda. He then moved to Tohoku University (the Professor Tokuji Miyashita laboratory) as a research assistant professor. From 2008, he worked as an assistant professor of Chiba University. His research interests focus on the development of functional materials based on the understanding of surfaces and interfaces. He received the Nanohana Venture Competition Award (Chiba University, 2011), Presentation Award (The Chemical Society of Japan, 2012), The Selected Lectures by Young Chemists (The Chemical Society of Japan, 2013), and the Award for Encouragement of Research in Polymer Science (The Society of Polymer Science, 2013).