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Electrospun Regenerated Cellulose Nanofibrous Membranes Surface-Grafted with Polymer Chains/Brushes via the Atom Transfer Radical Polymerization Method for Catalase Immobilization

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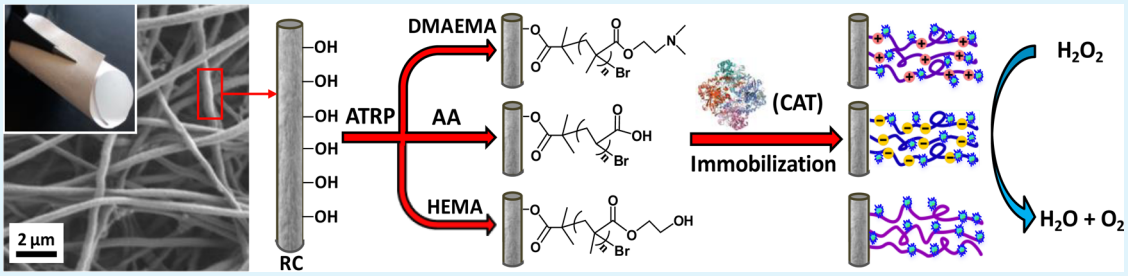
Supporting Information



ABSTRACT: In this study, an electrospun regenerated cellulose (RC) nanofibrous membrane with fiber diameters of ~200–400 nm was prepared first; subsequently, 2-hydroxyethyl methacrylate (HEMA), 2-dimethylaminoethyl methacrylate (DMAEMA), and acrylic acid (AA) were selected as the monomers for surface grafting of polymer chains/brushes via the atom transfer radical polymerization (ATRP) method. Thereafter, four nanofibrous membranes (i.e., RC, RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA)) were explored as innovative supports for immobilization of an enzyme of bovine liver catalase (CAT). The amount/capacity, activity, stability, and reusability of immobilized catalase were evaluated, and the kinetic parameters (V_{max} and K_m) for immobilized and free catalase were determined. The results indicated that the respective amounts/capacities of immobilized catalase on RC-poly(HEMA) and RC-poly(DMAEMA) nanofibrous membranes reached 78 ± 3.5 and 67 ± 2.7 mg g^{-1}, which were considerably higher than the previously reported values. Meanwhile, compared to that of free CAT (i.e., 18 days), the half-life periods of RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT were 49, 58, 56, and 60 days, respectively, indicating that the storage stability of immobilized catalase was also significantly improved. Furthermore, the immobilized catalase exhibited substantially higher resistance to temperature variation (tested from 5 to 70 °C) and lower degree of sensitivity to pH value (tested from 4.0 and 10.0) than the free catalase. In particular, according to the kinetic parameters of V_{max} and K_m, the nanofibrous membranes of RC-poly(HEMA) (i.e., 5102 μmol mg^{-1} min^{-1} and 44.89 mM) and RC-poly(DMAEMA) (i.e., 4651 μmol mg^{-1} min^{-1} and 46.98 mM) had the most satisfactory biocompatibility with immobilized catalase. It was therefore concluded that the electrospun RC nanofibrous membranes surface-grafted with 3-dimensional nanolayers of polymer chains/brushes would be suitable/ideal as efficient supports for high-density and reusable enzyme immobilization.

KEYWORDS: *ATRP, electrospinning, enzyme immobilization, nanofibrous membranes*

1. INTRODUCTION

Enzymes are protein-based biocatalysts with high efficiency, superior selectivity, and mild reaction conditions. A catalase of hydrogen peroxide (H_2O_2) oxidoreductase is a common enzyme for decomposition of H_2O_2 into oxygen and water, and it has been widely utilized in food, textile, and many other industries.[1,2] However, the practical applications of an enzyme (e.g., catalase) still encounter various difficulties/challenges. For example, the recovery of catalase from reaction systems is usually inconvenient due to its high water solubility; note that catalase has to be applied within a specific temperature range and chemical environment for achieving the optimal catalysis performance.[3] Over the past decade, numerous research endeavors have been devoted to overcome/mitigate those difficulties/challenges, and the immobilization of enzymes is a promising approach, in which enzyme molecules are adsorbed/bound onto water-insoluble supports via physical and/or chemical interactions.[4−6] Among different methods for enzyme immobilization, the physical adsorption method might be the most appealing one because the immobilized enzyme molecules

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would have relatively low degree of conformational variations on their 3-dimensional structures; in consequence, high catalytic activities could be retained. To date, many supports including beads of chitosan and its composite with poly(vinyl alcohol),[7,8] surface-modified fibers of collagen,[9] glass beads with high porosity,[10] submicron-sized spheres of titania,[11] and membranes of cellulose and its derivatives[12] have been studied for catalase immobilization; however, these supports have different limitations. For example, the enzyme loading amounts/capacities of conventional fibers (i.e., fibers with diameters being several microns or larger) are low due to small surface-to-mass ratios, and thus the immobilized enzyme molecules are easy to detach from these supports during practical applications. As another example, even though the enzyme loading amounts/capacities of microporous beads/spheres are quite high, the immobilized enzyme often cannot effectively contact the substrate due to the large curvature radii of microporous beads/spheres; in other words, the spatial/steric hindrances are usually serious.

It is well-known that atom transfer radical polymerization (ATRP) is a controlled/living polymerization method. The ATRP method has often been adopted for making linear polymer chains/brushes with controlled molecular lengths/weights since the propagation centers do not undergo chain termination and/or chain transfer during polymerization, and thus the molecular weights/lengths increase linearly with the conversion of monomers.[13,14] Therefore, this method provides a convenient approach for surface modification of supports, and the length and density of grafting polymer chains can be readily tailored.[13,14] The ATRP method is particularly suitable for grafting different polymer chains on the surface of cellulose since the hydroxyl groups in its repeating unit (i.e., glucose unit) can easily undergo chemical reactions with initiators, resulting in the formation of active species for propagation of polymer chains/brushes.[15] Several studies have demonstrated that micron-scaled fibers of regenerated cellulose (RC),[16] cellulose paper,[17] and bacteria-produced cellulose[18] can be surface-modified via the ATRP method, and the resulting materials/supports with grafted polymer chains/brushes have been explored for the immobilizations of nucleic acids and proteins/enzymes.[17,19−23]

The materials-processing technique of electrospinning utilizes the electric force to drive the spinning process and to produce the fibers with diameters typically in the range from tens to hundreds of nanometers (commonly known as "electrospun nanofibers"), and the electrospun nanofibers are usually collected as an overlaid mat/membrane.[24] In the recent years, electrospun nanofibrous mats/membranes have attracted growing interest for various applications such as filtration/separation, tissue engineering, drug delivery, sensor/detector, composite, and enzyme immobilization.[24−30] Cellulose is a natural polymer of particular interest for enzyme immobilization because of its abundance, biodegradability, compatibility with biological systems, and low nonspecific binding to proteins/enzymes.[31,32] Nevertheless, the processing of cellulose is restricted by its limited solubility in common solvents and its inability to melt. In our previously reported studies, electrospun cellulose acetate (CA) nanofibrous membranes were prepared first, and CA was then converted into RC through hydrolysis/deacetylation with the morphological structure of the nanofibrous membrane well-retained.[31,32] Subsequently, the electrospun RC nanofibrous membranes were further surface-functionalized with 3-dimensional nanolayers having varied thicknesses via the ATRP method, and the resulting materials/membranes exhibited excellent performance on bioseparation of proteins.[31] However, no report has been found in the literature on electrospun cellulose nanofibrous membranes surface-grafted with polymer chains/brushes for enzyme immobilization, while such membranes would be highly suitable for enzyme immobilization owing to their large surface-to-mass ratios and abundance of interaction sites.

In this study, the electrospun RC nanofibrous membrane was prepared first; subsequently, 2-hydroxyethyl methacrylate (HEMA), 2-dimethylaminoethyl methacrylate (DMAEMA), and acrylic acid (AA) were selected as the monomers for surface-grafting of polymer chains onto RC nanofibers via the ATRP method. Note that the optimal polymerization times were determined to control the length/thickness of grafted chains/brushes of poly(HEMA), poly(DMAEMA), and poly(AA). Thereafter, four electrospun nanofibrous membranes (i.e., RC, RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA)) were studied as innovative supports for high-density immobilization of bovine liver catalase (CAT). The amount/capacity, activity, stability, and reusability of the immobilized catalases were evaluated, and the kinetic parameters of both immobilized and free enzyme molecules were investigated. This study suggested that electrospun RC nanofibrous membranes (particularly those surface-grafted with 3-dimensional nanolayers of polymer chains/brushes) might be promising as efficient supports for high-density enzyme immobilization.

2. EXPERIMENTAL SECTION

2.1. Materials. Bovine liver catalase (enzyme commission number: 1.11.1.6), cellulose acetate (CA, Mn ∼ 30 000 g mol^{-1}, 39.8 wt % acetyl content), H_2O_2 (30 wt % in H_2O), KCl, KH_2PO_4, K_2HPO_4, CuCl, NaOH, NaCl, HCl, CH_3COONH_4, H_3BO_3, 2-propanol, Coomassie Brilliant Blue (G250), chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), diethyl amino ethyl chloride, tetrahydrofuran (THF), 2-bromoisobutyryl bromide (2-BIB), triethylamine (TEA), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA), 2-hydroxyethyl methacrylate (HEMA), 2-dimethylaminoethyl methacrylate (DMAEMA), acrylic acid (AA), and 1,4,8,11-tetraazacyclotetradecane were purchased from Sigma-Aldrich. The chemicals and materials were used without further purifications.

2.2. Preparation of Electrospun RC Nanofibrous Membranes. *2.2.1. Electrospinning.* Prior to electrospinning, a solution of 20 wt % CA in 1:1 (wt./wt.) THF/DMSO with 0.1 wt % diethylamino ethyl chloride was first prepared at room temperature. The solution was then filled in a 30 mL BD Luer-Lok tip plastic syringe having an 18-gauge 90° blunt end stainless-steel needle. The electrospinning setup included an ES30P high voltage power supply, purchased from the Gamma High Voltage Research, Inc. (Ormond Beach, FL), and a laboratory-produced roller with the diameter of 25 cm. During electrospinning, a positive high voltage of 15 kV was applied to the needle, and the flow rate of 1.0 mL h^{-1} was maintained using a KDS200 positive displacement syringe pump purchased from KD Scientific Inc. (Holliston, MA). The CA nanofibers were collected as an overlaid membrane on the electrically grounded aluminum foil that covered the roller. The rotational speed of this roller/collector during electrospinning was set at 100 rpm. A heating lamp was used to dry the nanofibrous membrane during electrospinning, while the membrane was further dried in a vacuum oven (∼27 mmHg) at 80 °C for 12 h after electrospinning. The collected CA nanofibrous membrane had a thickness of ∼200 μm and a mass per unit area of ∼50 g m^{-2}.

2.2.2. Hydrolysis/Deacetylation. An as-electrospun CA nanofibrous membrane was hydrolyzed/deacetylated upon immersion into 0.05 M NaOH aqueous solution for 24 h. The resulting RC nanofibrous

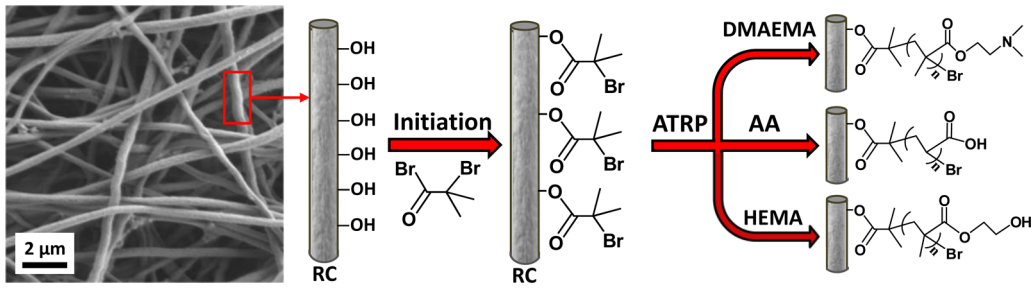

Figure 1. Schematic showing the grafting of poly(DMAEMA), poly(AA), and poly(HEMA) on the surface of an electrospun RC nanofibrous membrane via the ATRP method.

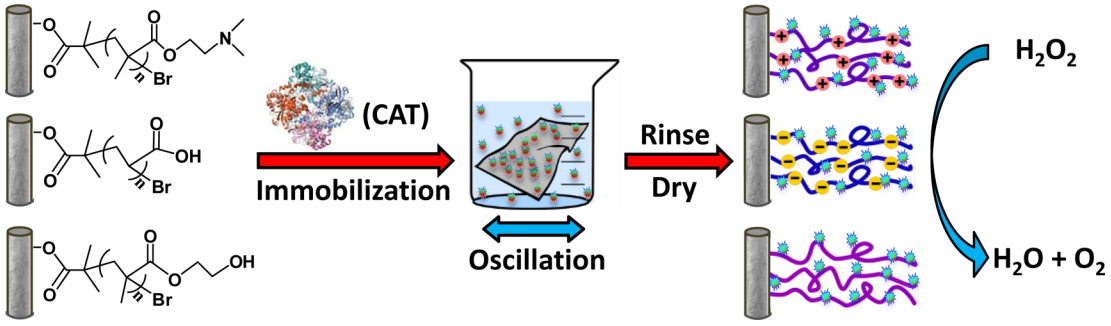

Figure 2. Schematic depicting the immobilization of catalase molecules on different RC nanofibrous membranes surface-modified with poly(DMAEMA), poly(AA), and poly(HEMA), respectively.

membrane was then rinsed with distilled water three times and dried at room temperature.

2.3. Surface Modification of the Electrospun RC Nanofibrous Membrane. *2.3.1. Initiation.* Prior to initiation, an electrospun RC nanofibrous membrane was first immersed in 20 mL of THF for 10 min. The membrane was then placed into a mixture of 63 μL of 10 mM 2-BIB, 70 μL of 10 mM TEA, and 50 mL of THF at 35 °C for 3 h. As depicted in Figure 1, the initiation reagent (i.e., 2-BIB) would react with hydroxyl (−OH) groups on the surface of electrospun RC nanofibers, while TEA would neutralize the byproduct of HBr. Thereafter, the acquired membrane was rinsed thoroughly with THF and deionized (DI) water.

2.3.2. Surface-Grafting of Polymer Chains/Brushes. After the above initiation, the acquired membrane was, respectively, surface-grafted with three types of polymer chains/brushes as schematically shown in Figure 1; the predetermined polymerization time was adopted to control the length/thickness of polymer chains/brushes.

(a) Surface-grafted with poly(HEMA): First, 24 mL of HEMA and a mixture of 400 μL of HMTETA and 24 mL of DMF were deoxygenated through three freeze−pump−thaw cycles; 100 mg of CuCl was then added into the HMTETA/DMF mixture inside an oxygen-free glovebox followed by being magnetically stirred for 2 h. Subsequently, the initiated RC nanofibrous membrane and HEMA were placed into the mixture of HMTETA/DMF/CuCl, and the system was kept in the glovebox at 25 °C for a predetermined time to complete the ATRP reaction of HEMA. Finally, the poly(HEMA)-modified RC nanofibrous membrane was rinsed with ethanol and dried in air.

(b) Surface-grafted with poly(DMAEMA): First, 20.24 mL of DMAEMA and a mixture of 300 μL of HMTETA and 39.76 mL of 2-propanol were deoxygenated through three freeze−pump−thaw cycles; 50 mg of CuCl was then added into the HMTETA/2-propanol mixture inside an oxygen-free glovebox followed by being magnetically stirred for 2 h. After that, the initiated RC nanofibrous membrane and DMAEMA were placed into the reaction system, and the system was kept in the glovebox at 40 °C for a predetermined time to complete the ATRP reaction of DMAEMA. Finally, the poly(DMAEMA)-modified RC nanofibrous membrane was rinsed with ethanol and DI water followed by being dried in air.

(c) Surface-grafted with poly(AA): First, 3.286 mL of AA, 3.65 g of NaOH, 11.78 g of NaCl, and 10.3 mg of 1,4,8,11-tetraazacyclotetradecane were added into 40 mL of DI water and bubbled with N_2 gas for 20 min to remove the O_2 gas, and the mixture solution was then transferred into an oxygen-free glovebox. Subsequently, 10 mg of CuCl was added into the mixture inside the glovebox, and the entire reaction system (with initiated RC nanofibrous membrane) was kept in the glovebox at 25 °C for a predetermined time to complete the ATRP reaction of AA. Thereafter, the poly(AA)-modified RC nanofibrous membrane was first rinsed with DI water at 25 °C for 30 min, then rinsed with DI water again at 60 °C for 60 min. followed by extra DI water rinsing at 25 °C for 30 min. Finally, the poly(AA)-modified RC nanofibrous membrane was dried in a vacuum oven at 45 °C.

2.4. Characterization of Electrospun Nanofibrous Membranes. A Zeiss Supra 40VP field-emission SEM was employed to examine morphological structures of the prepared nanofibrous membranes. Prior to SEM examination, the specimens were sputter-coated with gold to avoid charge accumulation, and the average nanofiber diameter of each sample was obtained through measuring 50 randomly selected fibers using the software of Image Pro-Plus 3.0. FT-IR spectra of different nanofibrous membranes (i.e., RC, RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA)) were obtained from a Bruker Tensor-27 FT-IR spectrometer equipped with a liquid-nitrogen-cooled mercury−cadmium−telluride (MCT) detector. The FT-IR specimens were prepared by pressing the ground samples with anhydrous KBr, and the FT-IR spectra were acquired by scanning the specimens 64 times in the wavenumber range from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.5. Immobilization of Catalase on Surface-Modified RC Nanofibrous Membranes. A solution with the catalase concentration of 1.0 mg mL^{-1} was first prepared by dissolving bovine liver catalase in 50 mM phosphate buffer solution (PBS, pH = 7.0). For immobilization of catalase (as schematically shown in Figure 2), the three surface-modified (as well as the unmodified) electrospun RC nanofibrous membranes (5 mg per sample) were immersed in 10 mL of catalase solution for 4 h at 20 °C under the shaking conditions. Thereafter, the membranes were rinsed with the same PBS until no catalase could be detected in the rinsing PBS solution; finally, the

Bradford assay was adopted to determine the amount of immobilized catalase,[33] which was calculated by using the following equation

$$Q_e = \frac{(C_0 - C_e)V_0 - C_rV_r}{M_d} \quad (1)$$

where Q_e is the amount of immobilized enzyme on unit mass of the nanofibrous membrane (mg g^{-1}); C_0 and C_e are the initial and equilibrium enzyme concentrations in the solution (mg mL^{-1}), respectively; C_r is the enzyme concentration in the rinsing PBS (mg mL^{-1}); V_o is the volume of enzyme solution; V_r is the volume of rinsing PBS solution; and M_d is the dry mass (g) of the nanofibrous membrane.

2.6. Activity Assays of Free and Immobilized Catalase. To test the respective activities of catalase under the free and immobilized conditions, 0.1 mL of catalase solution in PBS (1.0 mg mL^{-1}), as well as 5 mg of each nanofibrous membrane with immobilized catalase (i.e., RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT), were mixed with 50 mL of 50 mM H_2O_2 solution (pH = 7.0). The systems were then kept for 2 min at 35 °C (three replicates for each membrane). The activities of free and immobilized catalase were determined spectrophotometrically by measuring the decrease of absorbance at 240 nm as a consequence of H_2O_2 consumption. The specific activity of catalase was then calculated by using the following equation[34]

$$v = \frac{(A_0 - A) \times V}{T \times K \times E_w} \quad (2)$$

where v is the specific activity of free or immobilized catalase (μmol mg^{-1} min^{-1}); A_0 and A are the respective initial and final absorbance of solution at 240 nm; V is the volume of H_2O_2 solution (mL); T is the reaction time (min); K is the molar extinction coefficient of H_2O_2 at 240 nm (K = 0.0372 L $mmol^{-1}$ cm^{-1}); and E_w is the amount of catalase (mg).

2.7. Optimization of Immobilization Conditions. Various ATRP reaction times were studied to control the length/thickness of grafted polymer chains/brushes on the electrospun RC nanofibrous membrane. To determine the optimal reaction times, the amounts of immobilized catalase on the resulting RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT (with different ATRP times) were measured according to Section 2.5. Furthermore, to investigate the pH effect on immobilization, a series of 1.0 mg mL^{-1} catalase solutions with different pH values were prepared by using 50 mM acetate buffer solutions (pH values at 4.0, 5.0, and 5.5), 50 mM phosphate buffer solutions (pH values at 6.0, 6.5, 7.0, 7.5, and 8.0), and 50 mM borate buffer solution (pH value at 9.0). Subsequently, the RC-poly(HEMA), RC-poly(DMAEMA), RC-poly(AA), and (unmodified) RC nanofibrous membranes were immersed into the above catalase solutions (with varied pH values in the range from 4.0 to 9.0) for 4 h at 20 °C under the shaking condition, and the amounts/capacities of immobilized catalase (under different pH values) were then determined. Finally, the activities of immobilized catalase (immobilized at pH values from 4.0 to 9.0) were determined at 35 °C (pH = 7.0).

2.8. Evaluation of Immobilized Catalase. *2.8.1. Thermal and Storage Stability of Immobilized Catalase.* Thermal stability was investigated by incubating free and immobilized catalase at varied temperatures (i.e., 30, 35, 40, 45, 50, 55, and 60 °C) in 50 mM PBS (pH = 7.0) for 5 h; thereafter, the residual activities of free and immobilized catalase were determined at 35 °C. To test the storage stability, the free and immobilized catalase were stored in 50 mM PBS (pH = 7.0) at 4 °C for 40 days, and the half-lives of free and immobilized catalase were then calculated by using the following equation[35]

$$t_{1/2} = \frac{t \times \ln(2)}{\ln(E_0/E_t)} \quad (3)$$

where $t_{1/2}$ is the half-life of catalase (day) and E_0 and E_{+t} are the initial and residual activities of catalase (μmol mg^{-1} min^{-1}), respectively.

2.8.2. Temperature and pH Dependence of Free and Immobilized Catalase. To evaluate the temperature dependence, free and immobilized catalase were, respectively, mixed with 50 mL of 50 mM H_2O_2 solution (pH = 7.0) first. The activities were then measured in the temperature range from 5 to 70 °C. To investigate the pH dependence, the activities of free and immobilized catalase were determined at different pH values (from 4.0 to 10.0) for 2 min at 35 °C.

2.8.3. Kinetic Analysis of Free and Immobilized Catalase. To acquire the kinetic parameters of free and immobilized catalase, 0.1 mL of catalase solution (1.0 mg mL^{-1}) and 5 mg of each nanofibrous membrane with immobilized catalase were first mixed with 100 mL of H_2O_2 solution at the optimal pH values (according to Section 2.8.2), while the concentrations of H_2O_2 varied from 20 to 180 mM; the initial activity was then determined (for 2 min) at 35 °C. The kinetic parameters (i.e., V_{max} and K_m) of catalase under the free and immobilized conditions were calculated from a Lineweaver–Burk plot of the Michaelis–Menten equation (the detailed procedures are in the Supporting Information).[36]

$$\frac{1}{V_i} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (4)$$

where V_i and V_{max} are the respective initial and maximal rates of the enzyme-catalyzed reaction (μmol mg^{-1} min^{-1}); K_m is the Michaelis constant (mM); and [S] is the concentration of H_2O_2.

2.8.4. Reusability of Immobilized Catalase. To examine the reusability of immobilized catalase, the activity of each nanofibrous membrane with immobilized catalase was tested 10 times within 1 day. Before each test, the nanofibrous membrane with immobilized catalase was rinsed with PBS (pH = 7.0) to remove any residual substrate, and the activity of immobilized catalase was then tested in a fresh reaction medium.

3. RESULTS AND DISCUSSION

3.1. Morphological Structure of Different Nanofibrous Membranes. An electrospun RC nanofibrous membrane consisted of overlaid nanofibers with diameters of ~200–400 nm (Figure 3A), and the nanofibers were relatively uniform without identifiable beads and/or beaded nanofibers. After surface modifications via the ATRP method, the resulting RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA) nano-

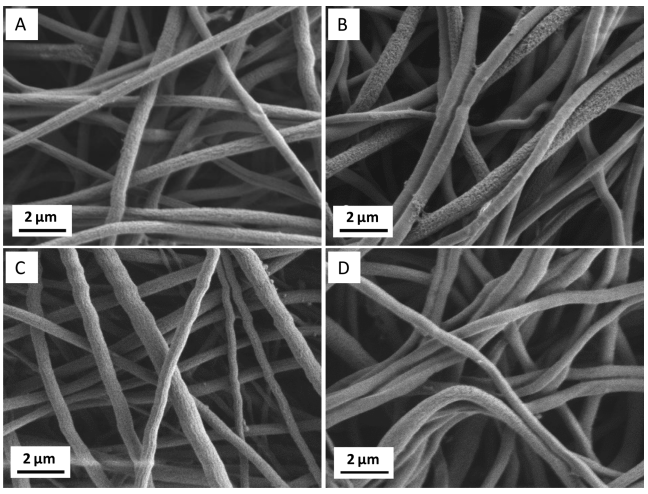

Figure 3. SEM images showing the morphological structures of (A) RC nanofibrous membrane, (B) RC-poly(HEMA) nanofibrous membrane after the ATRP reaction of HEMA for 40 min, (C) RC-poly(DMAEMA) nanofibrous membrane after the ATRP reaction of DMAEMA for 8 h, and (D) RC-poly(AA) nanofibrous membrane after the ATRP reaction of AA for 22 h.

fibrous membranes (as shown in Figure 3B, 3C, and 3D, respectively) exhibited no significant discrepancies. This indicated that the morphological structure of the nanofibrous membrane could be well-retained after the ATRP reactions with HEMA, DMAEMA, and AA. As compared to those of RC nanofibers, the diameters of RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA) nanofibers increased by approximately 20%, 20%, and 10%, respectively, and this was due to the surface-grafted polymer chains/brushes. The pore size among nanofibers would also be ideal for convective transport of substrate to the immobilized enzyme molecules, thus improving overall conversion efficiencies relative to porous beads and microfiber supports. As shown in Figure S1 (in Supporting Information), all of the nanofibrous membranes (i.e., CA, RC, and three types of surface-modified RC) had excellent mechanical flexibility.

3.2. FT-IR Analysis of Different Nanofibrous Membranes. FT-IR was employed to study chemical differences among the RC, RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA) nanofibrous membranes. The spectrum A in Figure 4 had a broad band around 3300−3500 cm^{-1}, an

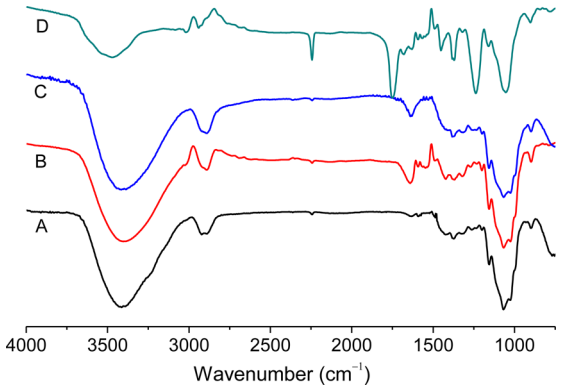

Figure 4. FT-IR spectra acquired from different nanofibrous membranes of (A) RC, (B) RC-poly(AA), (C) RC-poly(HEMA), and (D) RC-poly(DMAEMA).

indication of hydroxyl groups in RC. The two FT-IR bands centered at 1551 and 1428 cm^{-1} in the spectrum B were attributed to the asymmetric vibration and symmetric stretching motions of carboxylate ions in the surface-grafted poly(AA) chains/brushes.[31,37] In spectra B and C, the stretching vibration motion of carbonyl groups at 1641−1674 cm^{-1} was clearly identifiable, indicating the presence of ester and carboxyl groups after the RC membrane was, respectively, surface-grafted with AA and HEMA. Additionally, the spectrum D acquired from the RC-poly(DMAEMA) membrane had a band centered at 1749 cm^{-1}; this band could be attributed to the stretching motion of carbonyl groups in DMAEMA, indicating that poly(DMAEMA) chains/brushes were generated/grafted on the surface of RC nanofibers.[38]

3.3. Optimization of Immobilization Conditions. Initially, the amount of immobilized catalase generally increased with the increase of ATRP reaction time; after the amount reached a maximum value, it would then start to decrease gradually when further prolonging the reaction time. Upon the basis of the acquired results (Figure 5), the optimal reaction times of ATRP for poly(HEMA), poly(DMAEMA), and poly(AA) were determined at 40 min, 8 h, and 22 h, respectively; note that the corresponding immobilization amounts/capacities were 78 ± 3.5, 67 ± 2.7, and 34 ± 2.3 mg g^{-1}, representing the respective improvements of 178%, 139%, and 21% as compared to the value of the unmodified RC nanofibrous membrane (28 ± 1.8 mg g^{-1}). It is noteworthy that the immobilization amounts/capacities acquired from RC-poly(HEMA) and RC-poly(DMAEMA) nanofibrous membranes were substantially higher than the previously reported values acquired from other supports such as Zr(IV)-modified collagen fibers (45.4 mg g^{-1}),[9] porous glass beads surface-modified with 3-aminopropyltriethoxysilane (6.9 mg g^{-1}),[10] chitosan-*g*-poly(itaconic acid)-Fe(III) membrane (37.8 mg g^{-1}),[39] poly(styrene-D-glycidyl methacrylate)-tetraethyldiethylenetriamine microbeads (40.8 mg g^{-1}),[40] and polyacrylonitrile-glycopolymer nanofibrous membrane (46.5 mg g^{-1}).[41] Upon the ATRP reactions, the resulting nanofibrous membranes possessed 3-dimensional nanolayers, providing extremely large amounts of functional groups for enzyme immobilization; however, further prolonging the polymerization time would make the nanolayers thicker/denser, and thus some binding sites (i.e., functional groups) in poly(HEMA), poly(DMAEMA), and poly(AA) chains/brushes could not be readily accessed due to steric hindrance. Moreover, electrostatic interaction is an important factor affecting the immobilization amount/capacity. When pH = 7.0, the RC-poly(DMAEMA) nanofibrous membrane (a weak anion-exchange membrane; pK_a = 9.2−10) is positively charged,[38] while the bovine liver catalase is negatively charged (pI = 5.4).[42] Hence, the electrostatic attraction would provide additional binding/immobilization interaction between catalase molecules and the RC-poly(DMAEMA) nanofibrous membrane. For the RC-poly(HEMA) nanofibrous membrane, each repeat unit of poly(HEMA) chains/brushes has a hydroxyl group, and the catalase molecules would be adsorbed/bound onto the RC-poly(HEMA) nanofibrous membrane via hydrogen bonds and/or van der Waals forces; thus the RC-poly(HEMA) membrane also exhibited excellent performance on the immobilization of catalase.

The optimal pH values for immobilization of catalase onto nanofibrous membranes were investigated in the pH ranging from 4.0 to 9.0 at 35 °C. As shown in Figure 6, the maximum amounts/capacities for catalase immobilization on RC, RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA) nanofibrous membranes were determined at the pH values of 6.5, 6.5, 6, and 5, respectively. The electrostatic interaction played a crucial role in affecting the immobilization force between catalase molecules and nanofibrous membranes. Catalase molecules (pI = 5.4) are positively charged in the pH range of 4.0−5.0; note that the pK_a values of poly(DMAEMA) and poly(AA) are 9.2−10 and 4.5, respectively.[37] Therefore, at different pH values, the electrostatic interaction would vary the immobilization force between the charged catalase molecules and the nanofibrous membranes. Although it was speculated that the maximum amount/capacity for immobilization of catalase might be around the isoelectric point of catalase, a flocculent precipitate of catalase was observed at the pH value of 5.4 in this study. For the RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT membranes (with catalase immobilized at varied pH values in the range of 4.0−9.0), the specific activities were determined at 35 °C with pH value of 7.0. Note that among the four supports (i.e., RC, RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA)) the highest activities of immobilized catalase were acquired at the pH value of 7.0, indicating that the catalase

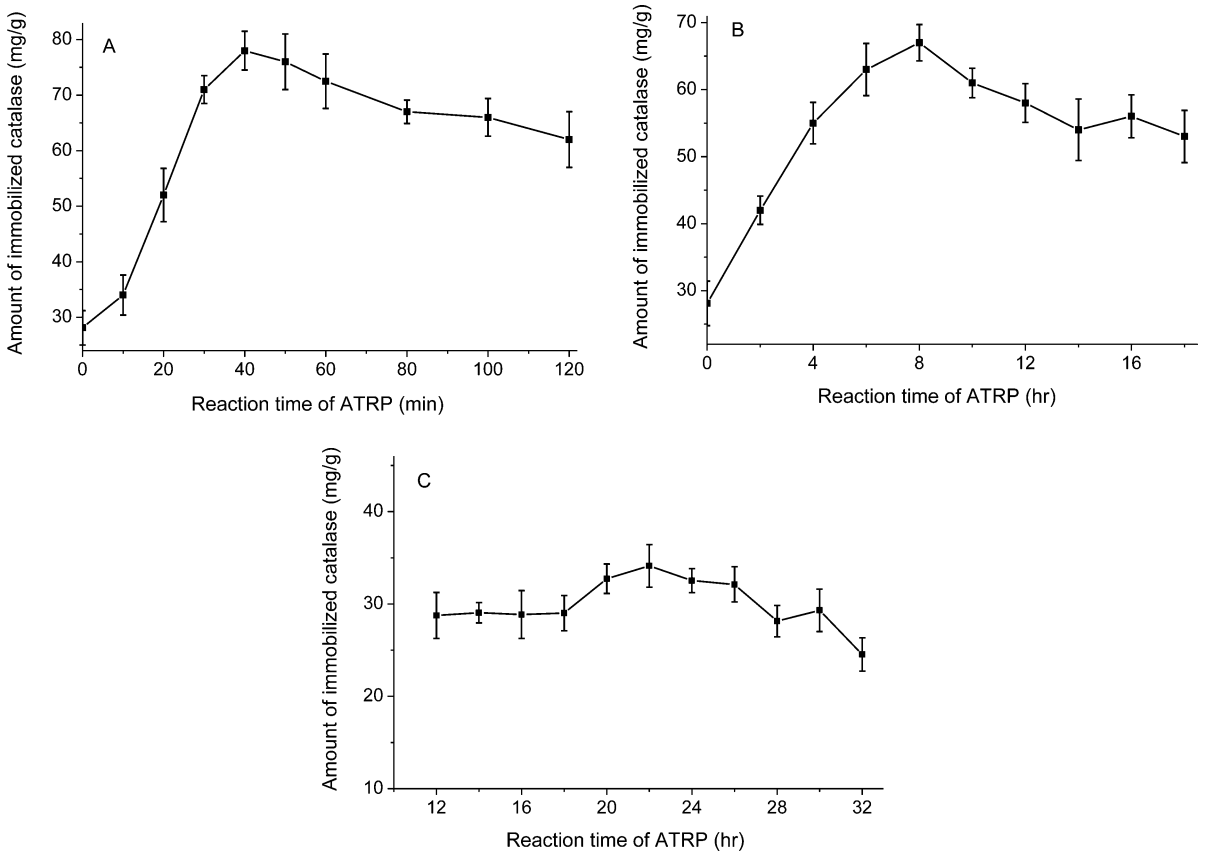

Figure 5. Amount of immobilized catalase versus reaction time of ATRP for (A) RC-poly(HEMA), (B) RC-poly(DMAEMA), and (C) RC-poly(AA) nanofibrous membranes. Error bars represent one standard deviation (n = 3).

molecules would possess the highly active conformation at the pH value of 7.0. Hence, a neutral condition (i.e., pH = 7) was selected for catalase immobilization in the following studies.

3.4. Thermal Stability and Storage Stability of Immobilized Catalase. Thermal stability of immobilized catalase is an important parameter in consideration of practical applications. Figure 7A shows the thermal stability variations of free and immobilized catalase incubated at different temperatures (i.e., 30, 35, 40, 45. 50, 55, and 60 °C) in 50 mM PBS (pH = 7.0) for 5 h; note that the activities of free and immobilized catalase incubated at 30 °C were set as 100%. In general, the activities of both free and immobilized catalase were reduced with the increase of incubation temperature. The immobilized catalase (i.e., RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT) retained more than half of the initial activity after incubation at 60 °C for 5 h, whereas the activity of free catalase was reduced for nearly 90% under the same conditions. The higher residual activity and thermal stability of immobilized catalase could be attributed to spatial/steric restrictions on molecular movements, which would limit/mitigate conformational changes of immobilized catalase molecules, thus resulting in the improved stability against inactivation/denaturalization.[43]

The relative activities of free CAT, RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT after being stored at 4 °C in PBS (50 mM, pH = 7.0) for 40 days were (22 ± 2.9)%, (57 ± 4.1)%, (62 ± 5.2)%, (61 ± 3.7)%, and (63 ± 4.1)%, respectively, when the corresponding initial activities were set as 100%, and their respective half-lives were 18, 49, 58, 56, and 60 days upon calculation by eq 3. The reduction in enzyme activity is a time-dependent natural phenomenon; however, the degree of enzyme activity reduction could be mitigated considerably through immobilization. The immobilized enzyme molecules could better retain their conformational morphology/structure; therefore, the inactivation/denaturation upon long-term storage would be prevented/mitigated, thus ameliorating the storage stability of immobilized catalase.[44]

3.5. Temperature and pH Effects on the Activity of Immobilized and Free Catalase. The effect of temperature on relative activity of free and immobilized catalase is depicted in Figure 8A. It was evident that the relative activity of catalase would be higher with the increase of reaction temperature initially, and it would then become lower when the temperature was further increased. The highest relative activities of catalase under both immobilized and free conditions were observed at 35−40 °C; furthermore, the immobilized catalase generally had higher activities than the free catalase in the entire temperature range from 5 to 70 °C. The higher relative activity would primarily be attributed to the increased morphological/structural stability of immobilized catalase molecules, while the multipoint interactions between catalase molecules and 3-dimensional nanolayers on the supports might provide the further protection against inactivation/denaturation at an elevated temperature.[9] The pH effect on relative activity of free and immobilized catalase is shown in Figure 8B. The optimal pH values for free CAT, RC-CAT, and RC-poly(HEMA)-CAT were identified at ~7.0, while the optimal pH values of RC-poly(DMAEMA)-CAT and RC-poly(AA)-CAT slightly shifted to 6.5 and 7.5, respectively. These results

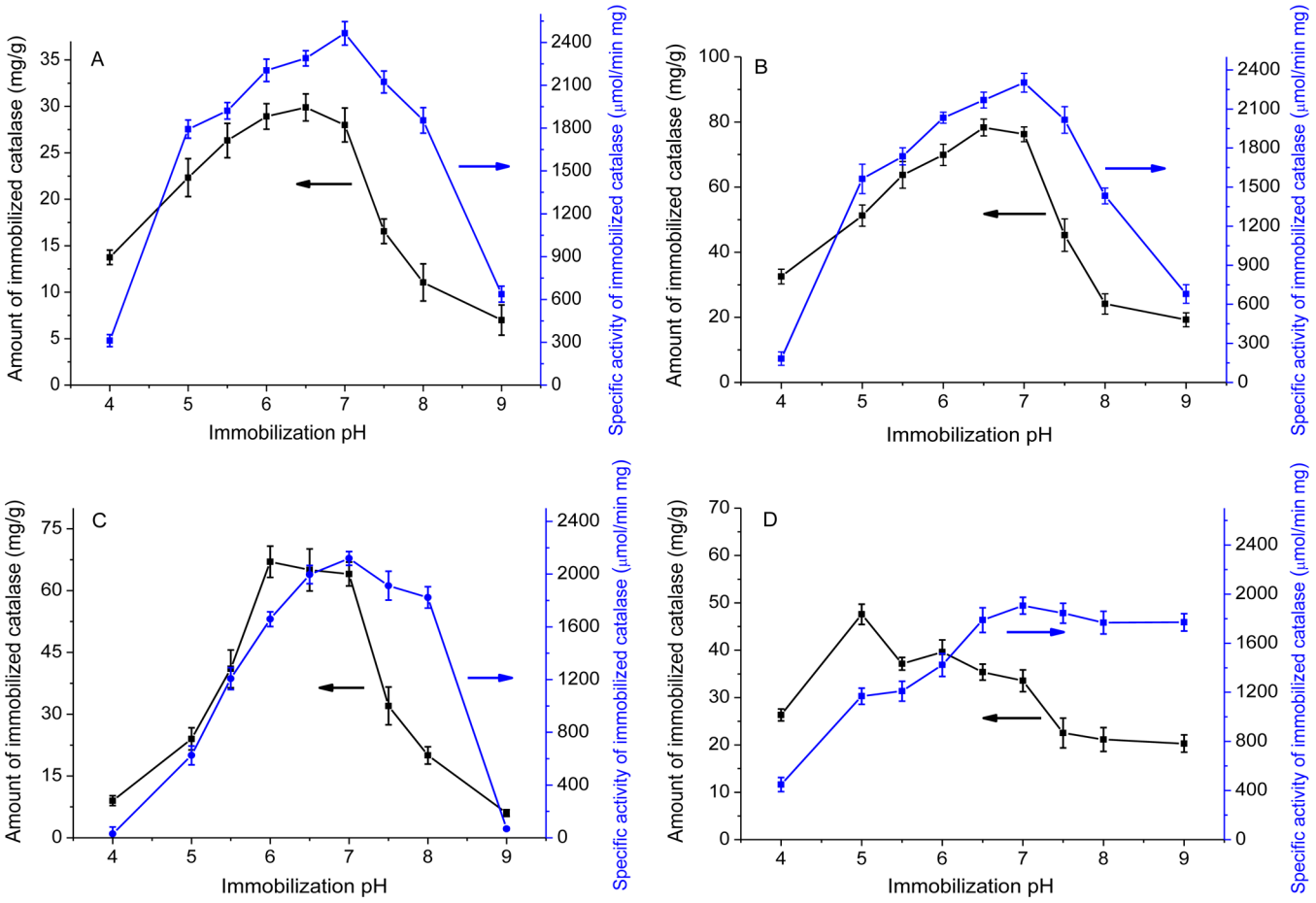

Figure 6. pH effect on the amount and activity of immobilized catalase. The catalase was immobilized on different nanofibrous membranes of (A) RC, (B) RC-poly(HEMA), (C) RC-poly(DMAEMA), and (D) RC-poly(AA) at 35 °C in the pH range of 4.0−9.0, while the specific activity for immobilized catalase was tested at 35 °C with pH value of 7.0. Error bars represent one standard deviation (n = 3).

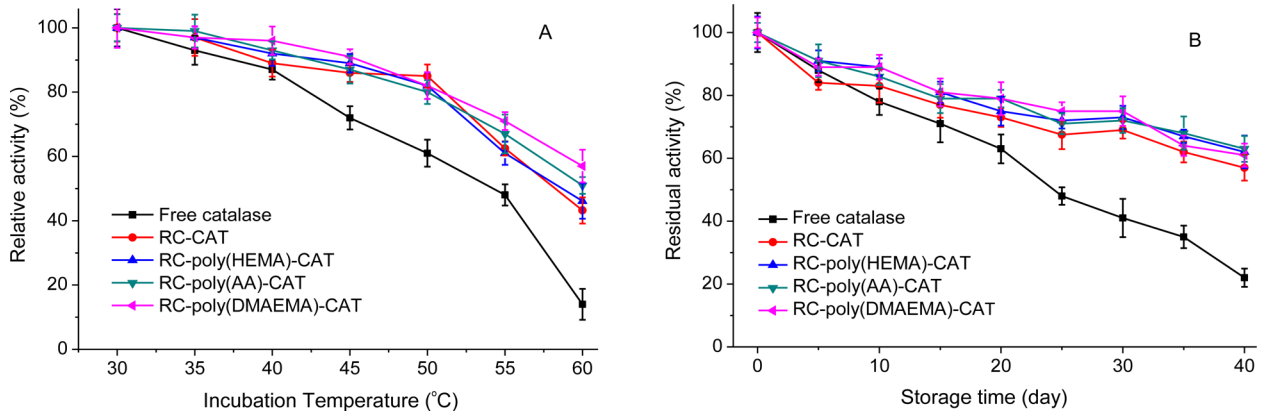

Figure 7. Thermal stability (A) and storage stability (B) of immobilized and free catalase. The residual activity was tested at 35 °C. Error bars show one standard deviation (n = 3).

suggested that the relative activities of immobilized catalase would be affected by the microenvironment (e.g., the pH value in the region close to the surface of nanofibrous membranes). For the RC-poly(DMAEMA) nanofibrous membrane (a weak anion-exchange membrane, pK_a = 9.2−10), each repeat unit of poly(DMAEMA) has a dimethylamino group, which is positively charged in neutral solution, and the hydroxide ions would be accumulated near the surface of the RC-poly(DMAEMA) nanofibrous membrane. As a result, to reach the same pH value (i.e., pH = 7.0) as free CAT, RC-CAT, and RC-poly(HEMA)-CAT in the microenvironment, the optimal pH value in the solution system might need to be slightly lower than 7. Similarly, each repeat unit of poly(AA) has a carboxyl group; thus the RC-poly(AA) nanofibrous membrane (i.e., a weak cation-exchange membrane, pK_a ∼ 4.5) is negatively charged in neutral solution, and the optimal pH value for immobilization of catalase on the RC-poly(AA) nanofibrous membrane in the solution system would increase to 7.5. Note

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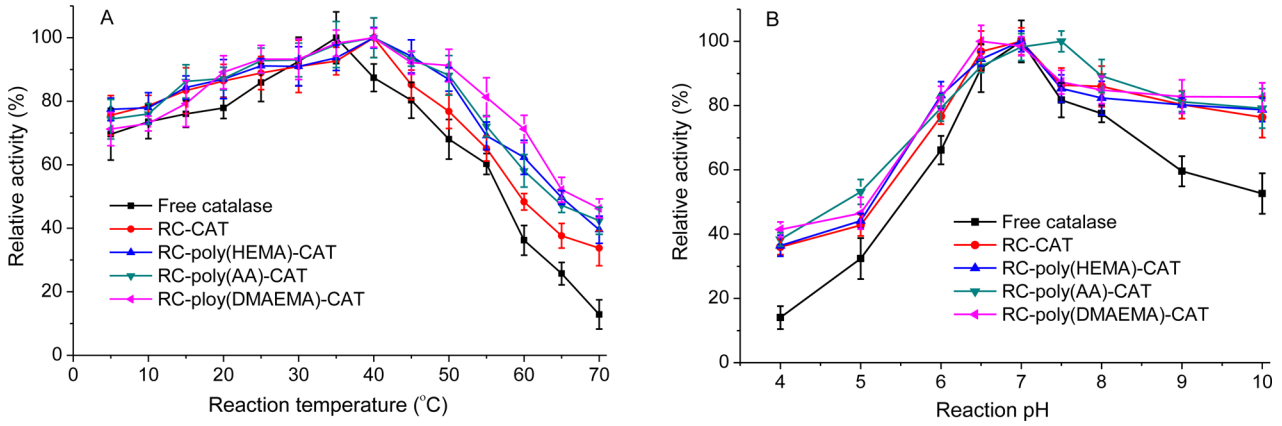

Figure 8. Effects of reaction temperature (A) and pH value (B) on relative activities of immobilized and free catalase. Error bars represent one standard deviation (n = 3).

Table 1. Specific Activity and Kinetic Parameters of Catalase under the Immobilized and Free Conditions[a]

	specific activity (μmol mg^{-1} min^{-1})	K_m (mM)	V_{max} (μmol mg^{-1} min^{-1})
free catalase	4202 ± 61	38.14	8474
RC-CAT	2464 ± 82	44.02	5434
RC-poly(HEMA)-CAT	2302 ± 72	44.89	5102
RC-poly(DMAEMA)-CAT	2210 ± 63	46.98	4651
RC-poly(AA)-CAT	1926 ± 48	65.07	4366

[a]Note: According to the optimal pH values described in Section 2.5, the initial activities of free catalase, RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT were determined at pH values of 7.0, 7.0, 7.0, 6.5, and 7.5, respectively.

that in the entire testing pH range from 4.0 and 10.0 the immobilized catalase (i.e., RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT) exhibited lower degrees of sensitivity to pH value, and the corresponding residual activities of immobilized catalase were generally higher than that of free catalase. Presumably, this was due to the reason that the generation of oxygen gas, formation of foams, and limitation of diffusion would occur on the surface of nanofibrous membranes.[45]

3.6. Kinetic Parameters of Immobilized and Free Catalase. The specific activities, K_m value (Michaelis−Menten constant), and V_{max} value (maximum reaction rate) are summarized in Table 1. Free catalase exhibited the highest specific activity at 4202 ± 61 μmol mg^{-1} min^{-1}, while the specific activities of RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT were 2464 ± 82, 2302 ± 72, 2210 ± 63, and 1926 ± 48 μmol mg^{-1} min^{-1}, respectively. The immobilization of catalase molecules on nanofibrous membranes would hinder the diffusion (i.e., decrease the accessibility) of H_2O_2 molecules to the active sites of immobilized enzyme molecules[46,47] and thus reduce the specific activities of immobilized catalase. As a result, for free catalase, the V_{max} value was higher and the K_m value lower than those of immobilized catalase. Note that the V_{max} is defined as the highest reaction rate when an enzyme is saturated with substrate, which reflects the intrinsic characteristics of enzyme, while the K_m is defined as the H_2O_2 concentration at the reaction rate of 1/2 V_{max}, which reflects the affinity of enzyme to substrate.[48−50] According to the kinetic parameters of V_{max} and K_m, the nanofibrous membranes of RC-poly(HEMA) and RC-poly(DMAEMA) had the most satisfactory biocompatibility with immobilized catalase.[51,52] Compared to that of free catalase, the V_{max} values of immobilized catalase from RC, RC-poly(HEMA), and RC-poly(DMAEMA) membranes remained at 64.1%, 60.2%, and 56.9%, respectively. These V_{max} values were significantly higher than those reported previously such as from Zr(IV)-modified collagen fibers (37.1%)[9] and porous glass beads surface-modified with 3-aminopropyltriethoxysilane (14.0%).[10]

3.7. Reusability of Immobilized Catalase. Unlike free catalase, immobilized catalase could be readily recovered/reused; note that the reusability of an enzyme is among the major factors/concerns for many practical applications. As shown in Figure 9, upon reuse for 10 times (the nanofibrous membrane with immobilized catalase was rinsed with PBS after each time), the residual activities of RC-CAT, RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT remained at (28 ± 2.6)%, (32 ± 6.4)%, (66 ± 3.3)%, and (39 ± 5.6)% of their initial activities, respectively. It

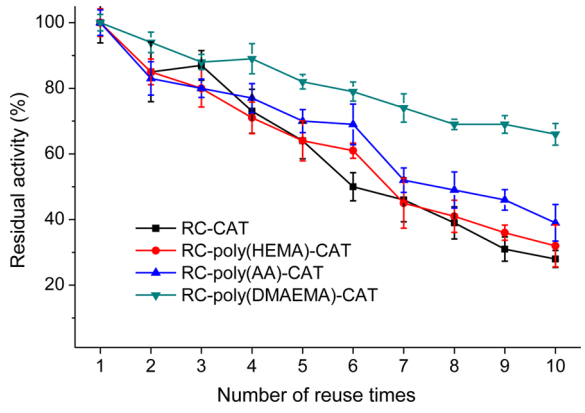

Figure 9. Residual activity (i.e., reusability) of the immobilized catalase (upon being tested 10 times within 1 day). Error bars represent one standard deviation (n = 3).

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is important to indicate that the RC-poly(DMAEMA)-CAT exhibited the best performance on reusability. As described before, the RC-poly(DMAEMA) nanofibrous membrane would be positively charged at the pH value of 7.0, while the catalase molecules would be negatively charged. Therefore, the electrostatic attraction/interaction could make the immobilization (between catalase molecules and the RC-poly(DMAEMA) nanofibrous membrane) quite stable. For the RC-poly(HEMA)-CAT nanofibrous membrane, each repeat unit of poly(HEMA) chains/brushes has a hydroxyl group, and the catalase molecules would be adsorbed/bound onto the RC-poly(HEMA) via hydrogen bonds and/or van der Waals forces. Thus, RC-poly(HEMA)-CAT exhibited reasonably high performance on the reusability, although it was relatively lower than that of RC-poly(DMAEMA)-CAT.

4. CONCLUSIONS

Herein, the electrospun RC nanofibrous membrane (with fiber diameters of ~200−400 nm) was surface-grafted with a 3-dimensional nanolayer of different polymer chains/brushes via the ATRP reaction for high-density immobilization of an enzyme of bovine liver catalase. The optimal ATRP reaction times for poly(HEMA), poly(DMAEMA), and poly(AA) were determined at 40 min, 8 h, and 22 h, respectively, while the corresponding immobilization amounts/capacities were 78 ± 3.5, 67 ± 2.7, and 34 ± 2.3 mg g^{-1}, representing the improvements of 178%, 139%, and 21% as compared to that of the unmodified RC nanofibrous membrane (28 ± 1.8 mg g^{-1}). In addition, compared to free catalase (18 days), the half-lives of RC-poly(HEMA)-CAT, RC-poly(DMAEMA)-CAT, and RC-poly(AA)-CAT were prolonged to 58, 56, and 60 days, indicating excellent storage stability of these surface-modified RC nanofibrous membranes. Moreover, all of the immobilized catalase exhibited substantially higher resistance to the variations of pH and temperature than the free catalase. More intriguingly, the activity and kinetic parameters (V_{max} and K_m) of RC-poly(HEMA)-CAT and RC-poly(DMAEMA)-CAT demonstrated superior affinity between immobilized catalase molecules and membrane supports, while the residual activity of RC-poly(DMAEMA)-CAT was retained at (66 ± 3.3)% of its initial activity upon reuse 10 times within 1 day. This study suggested that electrospun RC nanofibrous membranes (particularly those surface-grafted with polymer chains/brushes via the ATRP method) would be highly promising for efficient and reusable enzyme immobilization.

■ ASSOCIATED CONTENT

Supporting Information

Additional experiments and results are provided, including the images depicting excellent mechanical flexibility of the nanofibrous membranes of CA, RC, RC-poly(HEMA), RC-poly(DMAEMA), and RC-poly(AA); the determination of kinetic parameters (i.e., V_{max} and K_m) of free and immobilized catalase; the acquirement of the Lineweaver−Burk plot from the Michaelis−Menten equation; and the linear fitting results of the Michaelis−Menten equation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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