Current Science
CS 5 (4), 3713-3724 (2025)
Received 19 April 2025 | **Revised 18 May** 2025 | **Accepted 20 June**. 2025 | **Online Available 27 July** 2025
https://doi.org/ 10.52845/CS/2025-5-4-32

Current Science
Current Science

OPEN ACCESS

ISSN(O)2795-8639

Original Article

Immobilization of Lipase on Zwitterionic Polymer-Modified Magnetic Chitosan Microspheres for Enhancing Enzyme Performance

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Abstract:
In this work, the novel zwitterionic polymer-modified magnetic chitosan microspheres (PMCTS) were fabricated by a simple one-pot synthesis for covalent immobilization of *Candida rugosa* lipase (CRL). The zwitterionic polymer used was a derivative of poly(isobutylene-*alt*-maleic anhydride) and *N*,*N*-dimethylethylenediamine, with some residual anhydrides. The immobilization conditions and the characterization of immobilized lipase were evaluated systematically. The resulting immobilized lipases (PMCTS-CRL) exhibited higher relative activity than CRL on the unmodified chitosan magnetic microspheres (MCTS-CRL), comparable to the activity of free counterpart under optimal conditions. Moreover, PMCTS-CRL showed enhanced thermal, pH and storage stabilities in comparison with free CRL and MCTS-CRL, and better reusability than MCTS-CRL. PMCTS-CRL exhibited a 21.3-fold and 5.49-fold higher half-life than free one and MCTS-CRL at 50 °C. These results demonstrated that zwitterionic polymer-modified chitosan magnetic microsphere is a potential support for enzyme immobilization.
Keywords: zwitterionic polymer; magnetic chitosan microspheres; lipase immobilization; stability

Introduction

Lipase (triacylglycerol acyl hydrolases, EC 3.1.1.3) is an enzyme with excellent chemical, regional and enantiomeric specificity in catalyzing hydrolysis, esterification, transesterification reactions [1, 2]. However, lipases as macromolecular proteins are prone to denaturation by environmental conditions including pH, temperature and organic solvents [3, 4]. Coupled with the difficulty of recycling and reuse, the process cost will increase, limiting their large-scale application in industry. Enzyme immobilization provides a direction to address these problems [5, 6]. Immobilization facilitates isolation of lipases form the reaction system, allowing them to be reused for a less costly process. Besides, the immobilized enzymes performed better operational stability, most notably thermostability and pH tolerance.

Several methods have been applied for enzyme immobilization including cross-linking of enzymes without a solid carrier, entrapment enzymes into a hydrogel or polymer matrix, or attachment them to an organic or inorganic carrier via physical adsorption or covalent binding [7]. Each method exhibits distinct merits and defects. Immobilization by physical adsorption and entrapment depends on weak forces, contributing to maintenance of enzyme activity, while immobilization via covalent interactions provides more strong and stable binding, promoting enzyme stability [8]. Additionally, the choice of supports is also crucial for enzyme immobilization, and the interaction between enzyme and supports has a significant effect on the stability and kinetics of the enzyme. In various organic or inorganic materials, Fe_3O_4 nanoparticles have received extensive attention

due to their superior properties, such as large surface area, low mass transfer resistance, and easy separation and recovery under external magnetic fields [9, 10]. However, Fe_3O_4 nanoparticles are prone to aggregation in aqueous solution due to high surface energy, and high chemical activity promotes their susceptibility to oxidation in the air, losing magnetism and dispersibility [11, 12]. This trouble can be solved by surface coating with appropriate polymers (polyethylene glycol, polydopamine, polyethyleneimine, chitosan, dextran, etc.) or inorganic metals and oxides (gold, silica, alumina, titania, etc.) [13, 14]. Of those, chitosan is an attractive cationic polysaccharide with abundant amino and hydroxyl groups, allowing it to react with other compounds. The integration of Fe_3O_4 nanoparticles into the chitosan network may enhance the dispersibility, physical stability and biocompatibility of the particles, making the resulting composites more suitable for enzyme immobilization [13, 15].

Even though good results were obtained in enzyme stability and reusability, the lower catalytic activities of immobilized enzymes than free one was reported due to the unsuitable conformation changes and mass-transfer limitations between enzyme and supports [16, 17]. That can be improved by adjusting the surface properties of the carrier material. Zwitterionic polymers with both anionic and cationic groups in polymer chains are promising materials for protein stability, antifouling coatings and drug delivery, owing to their strong hydration and charge balance [18-20]. It has been reported that zwitterionic polymer modified-surfaces could reduce non-specific adsorption and improve the stability and water-dispersibility of particles [18, 21]. In order to obtain better surface properties for enzyme immobilization, this work thus designed a novel zwitterionic polymer-modified magnetic chitosan microspheres by a simple one-pot synthesis. The zwitterionic polymer (pID) with residual anhydride groups was used for facile modification with chitosan, which was synthesized by controlling the reaction of poly (isobutylene-*alt*-maleic anhydride) (PIMA) with *N, N*-dimethylenediamine (Fig. S1) [22]. *Candida rugosa* lipase was then covalently immobilized on the prepared magnetic microspheres, and the immobilization conditions and the characterization of immobilized lipase were evaluated systematically. The results would provide a sound reference for further exploration.

2. Material and Methods

2.1. Materials

Candida rugosa lipase (CRL), poly(isobutene-*alt*-maleic anhydride) and *N,N*-dimethylethylenediamine (DMEA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N, N*-Dimethylformamide (HPLC grade), paraffin and bovine serum albumin were obtained from Macklin Biochemical Technology (Shanghai, China). Nano Fe_3O_4 (20 nm, sphere) and *p*-nitrophenyl acetate (*p*-NPA) were obtained from Aladdin (Shanghai, China). Chitosan was acquired from Shanghai Yuanye Bio-Technology (Shanghai, China). Acetic acid and petroleum ether were obtained from Fuyu Chemical (Tianjin, China). Span-80, disodium hydrogen phosphate, sodium dihydrogen phosphate, and other reagents were of analytical grade from Sinopharm (Beijing, China).

2.2. Synthesis of Zwitterionic Polymers

Through controlling the molar ratio of anhydride on polymer chains to amino groups of DMEA, zwitterionic polymer with 70% anhydride ring-opening of PIMA (denoted as pID 0.7) was synthesized following the reported method as shown in Fig. S1 [22]. The zwitterionization degree of the resultant polymer was demonstrated by ^{1}H NMR spectroscopy (Fig. S2). More details are provided in the **Supplementary Material**.

2.3. Preparation of Zwitterionic Polymer-Modified Magnetic Chitosan Microspheres

The zwitterionic polymer-modified magnetic chitosan microspheres were fabricated as described in the literature [23] with slight modification. As shown in Fig. 1, 250 mg of chitosan was dissolved in 10 mL of 5% acetic acid solution, followed by adding 50 mg of Fe_3O_4 nanoparticles. The mixture was poured dropwise into 40 mL of paraffin oil containing 2.5 mL of Span 80 as emulsifier in a 250 mL three-neck round-bottom flask under mechanical stirring at 1500 rpm for 30 min. Then, 5 mL of 5% pID-0.7 in deionized water was added to react with chitosan for 1 h with a mechanical stirrer (1500 rpm) at room temperature. Thereafter, the suspension was added with 10 mL of 6% glutaraldehyde (GA) and stabilized for another 1 h

at 500 r/min and 40 ℃. Subsequently, the mixture was adjusted to pH 9-10 with 1 M NaOH, and kept under stirring for a further 2 h at 70 ℃. The final product (PMCTS) was collected using a magnet and successively washed with petroleum ether, ethanol, and phosphate buffer solution (50 mM, pH 7.0). For comparison, the unmodified magnetic chitosan particles (MCTS) were also prepared following the similar procedure. The microspheres with different crosslinking intensities were prepared by varying GA concentrations.

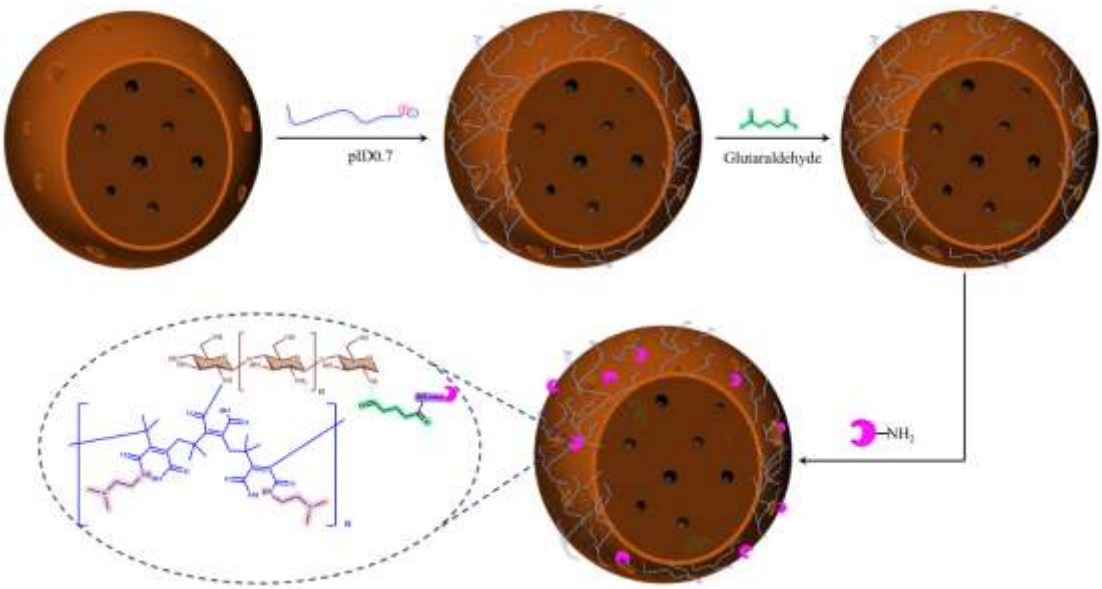

Figure 1. Schematic for the preparation of zwitterionic polymer-modified magnetic chitosan microspheres and lipase immobilization.

2.4. Characterization of Magnetic Microspheres

The particle size and size distribution of PMCTS and MCTS were determined using a laser particle size analyzer (Malvern MS-2000, Worcestershire, UK). Thermogravimetry analysis (TGA) was performed with a synchronous thermal analyzer (STA449F5, NETZSCH, Selbu, Germany) by heating the samples from 25 ℃ to 800 ℃ at 10 ℃/min under a nitrogen atmosphere. The surface morphologies of the dried particles were observed with scanning electron microscopy (SEM, Quanta 250 FEG, FEI, Columbia, US) with a magnification of 5000 X under an accelerated voltage of 800 kV. The magnetic properties of composites were characterized by a vibrating sample magnetometer (Squid-VSM, Quantum Design, San Diego, US) at room temperature with a magnetic field strength of -10 kOe~10 kOe.

2.5. Immobilization of Lipase

PMCTS and MCTS were washed several times with 50 mM phosphate buffer (pH 7.0) before immobilization. 100 mg of supports were dispersed into 10 mL of lipase solution (0.1 mg/mL) in 50 mM phosphate buffer (pH 7.0), and the mixture was incubated in a shaker at 25 ℃ and 130 rpm for 4 h. Then, the particles were collected with a magnet and washed three times with the same buffer, and the resulting immobilized enzymes were named as PMCTS - CRL and MCTS-CRL, respectively. The protein concentration was assayed by the Bradford method, and the protein loading was determined using the difference in protein content between the initial and final solutions.

Effects of immobilization conditions on immobilized CRL were investigated by using magnetic microspheres cross-linked with GA ranging from 5% to 10%, lipase concentration from 0.05 mg/mL to 1.00 mg/mL, and reaction time from 1 h to 10 h. The specific activity of free CRL at a certain condition was defined as 100% to calculate the relative activity (%).

2.6. Determination of Enzyme Activity

Lipase activity was determined using *p*-nitrophenyl acetate (*p*-NPA) as substrate based on the previous procedure [24] with minor

modifications, as described in more detail in the **Supplementary Material.**

2.7. Enzymatic Reaction Kinetics

The kinetic parameters were determined by measuring the lipase activity with *p*-NPA in different concentrations from 0 to 200 mM under the activity assay conditions. In the reactions, the amount of free CRL was equivalent to that of CRL loaded on MCTS and PMCTS, i.e. 0.83 ug/mL or 1.38×10^{-5} mM based on the molecular weight of CRL (60 kDa). The maximum reaction rate (V_{max}) and Michaelis constant (K_m) were determined by fitting the experimental data to the Michaelis-Menten equation.

2.8. Stability Investigation

The temperature endurance of free CRL, MCTS-CRL and PMCTS-CRL was investigated by keeping the enzyme samples at different temperatures varying from 25 ℃ to 60 ℃ for 60 min. The residual activity was measured as described above, and the original activity was defined as 100%.

To further evaluate the thermal stability, the three lipase samples were incubated at 50 ℃ in 50 mM phosphate buffer (pH 7.0) for 180 min. Aliquots of the enzyme samples were withdrawn every 30 min, followed by measuring the residual activities as described above.

pH tolerance was evaluated by assaying the residual activities of the free and immobilized CRLs after incubating at 25 ℃ in different buffers varying pH from 4.0 to 8.0 for 4 h. The storage stabilities were evaluated by keeping three enzyme samples in 50 mM phosphate buffer (pH 7.0) at 4 ℃. The residual activity was measured at regular intervals to compare to its initial activity defined as 100%.

2.9. Reusability

MCTS-CRL and PMCTS-CRL were used to catalyze the hydrolysis of *p*-NPA at 37 ℃ for 10 min. After magnetic separation, the immobilized lipases were rinsed twice with 50 mM phosphate buffer (pH 7.0) and then reintroduced into a fresh substrate solution for the next catalytic cycle. The enzyme activity in the first run was recorded as 100% to calculate the residual activity. The average values of three replicate experiments are reported with standard deviations.

3. Results and Discussion

3.1. Characteristics of MCTS and PMCTS

Fig. S3 presents TGA curves of MCTS and PMCTS from 25 ℃ to 800 ℃. Both supports showed a weight loss of about 5% at temperatures below 100 ℃, due to the removal of water molecules adsorbed physically on the support surfaces [15]. A mass loss of 32.8% was observed for PMCTS within the temperature range of 185-400 ℃, higher than 29.7% of MCTS. The extra mass loss was assigned to the thermal decomposition of pID modified on the chitosan chains.

Fig. S4 exhibits the particle size distribution of PMCTS and MCTS. The majority of MCTS and PMCTS present a wide size distribution of 30-150 μm with the mean size of 56.3±0.99 μm and 53.4±0.99 μm, respectively, while a second peak was observed on the distribution plots with smaller dimension between 1-10 μm for both supports, may due to local concentration differences caused by uneven stirring during the dripping process of chitosan solution.

SEM characterizations in Fig. S5 show that MCTS and PMCTS presented spherical morphologies with rough surfaces and heterogeneous diameters, attributed to the shrinkage of particles caused by water loss during drying.

The magnetization curves of the two supports are shown in Fig. S6. The saturation magnetization of MCTS and PMCTS-P7 were observed to be 0.041 emu/g and 0.038 emu/g, respectively. The slightly lower saturation magnetization of PMCTS may be explained by the modification of zwitterionic polymer resulting in the small proportion of Fe_3O_4. In addition, S-curves with no hysteresis were found for both microspheres, indicating good superparamagnetism [13].

3.2. Optimization Condition for Lipase Immobilization on MCTS and PMCTS

The performances of immobilized lipase were influenced by the immobilization procedure, and thus the immobilization conditions were optimized and the results were presented in Fig. 2. Fig. 2a shows the effect of glutaraldehyde concentration on the activity of immobilized lipase, in which lipase was immobilized on magnetic microspheres cross-linked with GA

concentrations in the range of 5%-10%. It can be seen that the activity of PMCTS-CRL first increased and then decreased with increasing GA concentration, and the maximum relative activity (98.1%) was obtained for CRL immobilized on PMCTS cross-linked with 6% GA. Though more free aldehyde groups on the support surface with increasing GA concentration resulted in more bound enzymes, the excessive interaction between enzymes and aldehyde groups would alter the enzyme conformation, causing damage to the active site and decreased activity [23, 25]. Therefore, 6% cross-linked PMCTS was considered the optimum for CRL immobilization. However, CRL bound on MCTS presented a decrease in activity with the increase of GA concentration (Fig. 2a) and reached the relative activity of 71.0% at 5% GA and 69.6% at 6% GA. Together with higher cross-linking and protein loading (data not shown) with higher GA, 6% of GA should be considered to be more suitable for the preparation of MCTS-CRL. In addition, it can be found in Fig. 2a that lipases immobilized on pID-modified chitosan carriers exhibited significantly higher catalytic activity than those on MCTS, which may be attributed to the stabilizing effect of zwitterionic polymers on enzyme proteins, maintaining the conformation and the structural stability of enzyme molecules, and ensuring functional activity of enzymes around polymers [22, 26]. Another reason may be the hydrophobic interaction of alkyl chains of pID with the hydrophobic peptide chains covering the active site of the lipase, leading to an active structure, allowing substrates more accessible to the active sites of immobilized lipase [27].

The effect of initial enzyme concentration on the activity of immobilized enzymes is shown in Fig. 2b. The increase in activity was found for both immobilized CRLs with the increase of initial lipase concentrations from 0.05 to 0.10 mg/mL, reaching the highest relative activities for PMCTS of 97.3% and MCTS of 73.3% at 0.10 mg/mL. The activities of MCTS-CRL and PMCTS-CRL began to decrease at the initial lipase concentrations higher than 0.10 mg/mL, in spite of the increase in protein loading. In the process of enzyme immobilization, increasing the initial enzyme concentration can promote more enzyme molecules to bind with the active groups of supports, whereas excessive enzyme piled up in MCTS and PMCTS may generate steric hindrance, astricting the substrate access to the active sites of the enzyme, leading to a decrease in enzyme activity [23, 28]. Therefore, a lipase concentration of 0.10 mg/mL was chosen for higher activities of MCTS-CRL and PMCTS-CRL. Fig. 2c shows the effect of reaction time on the activities of immobilized CRLs. The relative activity of MCTS-CRL and PMCTS-CRL appeared to increase first and then decline with prolonging the immobilization time, and the apparent peaks occurred at 4 h. The extending immobilization time is conducive to sufficient contact between enzyme and support, promoting more enzymes bound on supports, while for a long time, many enzyme molecules would squeeze and form the steric hindrance against each other, resulting in the substrate less access to the active sites, and lowering enzyme activity [29]. Thus, the optimal reaction time of 4 h was considered for immobilization of CRL onto MCTS and PMCTS, and the resulting PMCTS-CRL and MCTS-CRL presented the relative activity of 100.2±3.30% and 77.9±2.47% coupling with the protein loading of 3.96±1.11 mg/g carrier and 5.59±0.43 mg/g carrier, respectively.

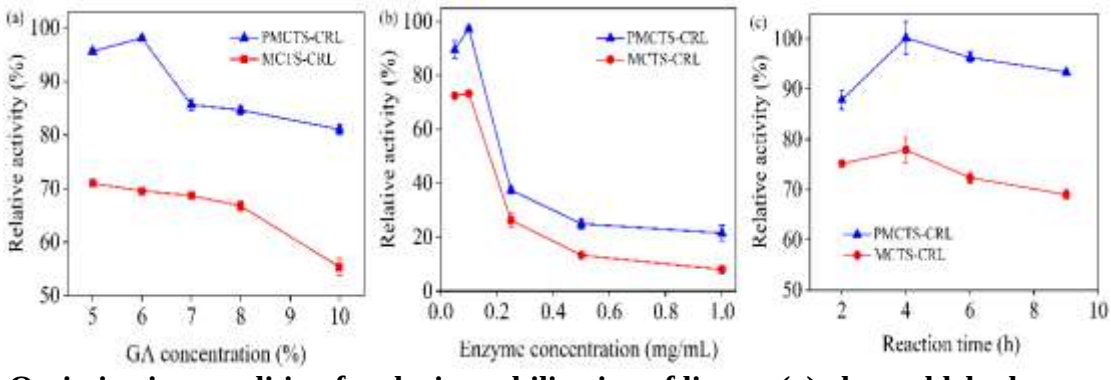

Figure 2. Optimization condition for the immobilization of lipase: (a) glutaraldehyde concentration, (b) initial lipase concentration, (c) reaction time.

3.3. Enzymatic Reaction Kinetics

The kinetic parameters of enzyme samples were determined by fitting the experimental data to the Michaelis-Menten equation (Fig. S7) and the results are presented in Table 1. The K_m values of both immobilized CRLs increased compared to the free ones, indicating a decrease in the affinity of the substrate to the enzyme after immobilization. This may be related to the loss of conformational flexibility of lipase coupled with supports and mass transfer resistance caused by the stereo-hindrance of the support after immobilization [28, 30]. Similar results have been reported in other studies [31, 32]. The k_{cat} value of CRL immobilized on PMCTS (14.75 s^{-1}) was reduced by 14.5% compared to that of free CRL (17.25 s^{-1}), which was 6.1-fold higher than MCTS-CRL (2.42 s^{-1}). Moreover, PMCTS-CRL presented a 3.9-fold higher k_{cat}/K_m than MCTS-CRL. These results indicated the enhancement of enzymatic reaction efficiency for PMCTS-CRL compared with MCTS-CRL, which is attributed to the reasonable hydrophilic/hydrophobic arrangement of the support surface introduced by the modification of pID with the hydrophobic alkyl side chains and hydrophilic charge groups (Fig. S1), allowing the lipase to be activated by the hydrophobic part and providing a hydrophilic microenvironment by the hydrophilic one [33, 34].

Table 1 Kinetic parameters of the free CRL, MCTS-CRL and PMCTS-CRL

Parameters	Free CRL	MCTS-CRL	PMCTS-CRL
$V_{max}\times10^5$ (mM s^{-1})	23.80±0.36	3.34±0.63	20.36±0.07
K_m (mM)	7.09±0.59	12.06±2.47	18.99±1.11
k_{cat} (s^{-1})	17.25±0.26	2.42±0.46	14.75±0.05
k_{cat}/K_m (s^{-1} mM^{-1})	2.43±0.04	0.20±0.04	0.78±0.01

3.4. Stability Properties

Fig. 3 shows the impact of temperature on the thermal stabilities of free and immobilized CRLs. The residual activities of free CRL, MCTS-CRL and PMCTS-CRL declined continuously with increasing temperature. Compared with free CRL, the two immobilized lipases displayed a slower rate of activity loss in the studied temperatures of 25-60 ℃, and PMCTS-CRL shows better thermal stability than MCTS-CRL. After heat treatment at 60 ℃ for 1 h, PMCTS-CRL maintained 75.6% of its original activity, while free CRL and MCTS-CRL retained 20.6% and 68.5%, respectively. In order to further confirm the thermostability of immobilized enzymes, the three enzyme preparations were kept at 50 ℃ for a longer time (180 min) and their residual activities were compared in Fig. 4. PMCTS-CRL and MCTS-CRL presented a moderate decrease in activity as the heating time increased, while free CRL showed a sharp loss in activity at the first 30 min, followed by a slow decrease over time. For example, free CRL lost almost 50% of its original activity at 50 ℃ for 30 min, as opposed to 5% for PMCTS-CRL and 20% for MCTS-CRL. After heating for 180 min, PMCTS-CRL still retained 76.1% of the initial activity, while free CRL and MCTS-CRL retained only 24.5% and 43.9%, respectively. Furthermore, a two-step series model was employed to described to thermal inactivation process of the three enzyme samples [35, 36],

$$E \xrightarrow{k_1} \overset{\alpha_1}{E_1} \xrightarrow{k_2} \overset{\alpha_2}{E_2} \quad (1)$$

where k_1 and k_2 are inactivation rate coefficients, E and E_i represent the initial and intermediate states of an enzyme, α_i represents the ratio of specific activity of E_i to E. By assuming that the content of E_1 and E_2 is zero at the beginning, the specific activity (a) at time t can be represented as:

$$a = (1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1})exp(-k_1 t) - (\frac{k_1}{k_2 - k_1})(\alpha_1 - \alpha_2)exp(-k_2 t) + \alpha_2 \quad (2)$$

As shown in Fig. 4, the scatters were well distributed on the calculated curves, indicating the validity of the selected model. The deactivation parameters are listed in Table 2, which were obtained by fitting the experimental data to the model via Matlab software. The decreased k_1 and k_2 values can be found after enzyme immobilization. Compared with MCTS-CRL, PMCTS-CRL exhibited the smaller inactivation rate constant, demonstrating that the polymer chains around the enzyme may inhibit the change rate of the protein structure at high temperature, which may be due to the super hydrophilicity of zwitterionic polymer (pID) triggering a favorable microenvironment around enzyme bound on supports, and stabilizing the native structure of the immobilized proteins [27, 37]. Moreover, the half-life ($t_{1/2}$) increased by 3.89-fold for MCTS-CRL and 21.3-fold for PMCTS-CRL, further indicating the stabilizing effect of zwitterionic polymers on protein structure for better thermostability of PMCTS-CRL.

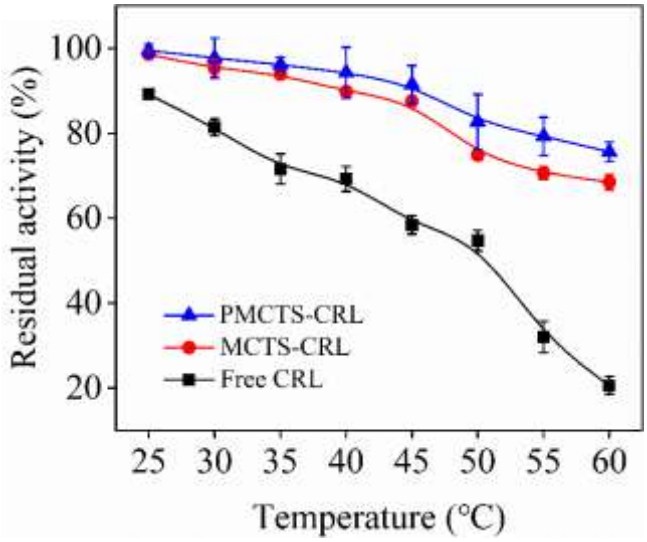

Figure 3. Thermal stabilities of free and immobilized CRLs as a function of temperature.

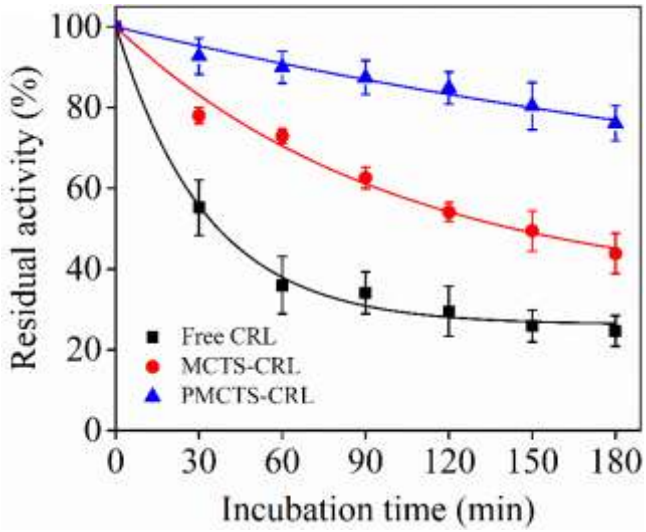

Figure 4 Thermal stabilities of lipase preparations in phosphate buffer (pH 7.0) at 50 °C. The solid lines are calculated from the kinetic deactivation schemes shown in formula 2.

Table 2 Lipase deactivation parameters at 50 °C obtained from the kinetic deactivation scheme.

Parameters	Free CRL	MCTS-CRL	PMCTS-CRL
k_1	1.811	0.697	0.228
k_2	1.840	0.585	0.218
α_1	0.249	0.439	0.567

α_2	0.262	0.338	0.455
$t_{1/2}$ (h)	0.614	2.387	13.14
$t_{1/2}/ t_{1/2\,0}$ [a]	1	3.888	21.34

[a] $t_{1/2\,0}$ represents the half-life of native CRL.

Fig. 5 shows the pH tolerance of three CRL preparations. The free and immobilized CRLs displayed reduced activity to a certain extent after 4 h incubation at pH 4.0-9.0, and obvious differences in pH tolerance were found under alkaline conditions along with more serious activity loss. Free CRL only retained 16.7% of its initial activity at pH 9.0. In contrast, both immobilized enzymes presented relative stability with approximately 80% of the residual activities at pHs of 4.0-9.0, albeit with slightly enhanced stability for PMCTS-CRL. Those can be explained by the greater structural rigidity for immobilized proteins and the buffering effect of the zwitterionic groups of pID on acid-base variables, reducing its susceptibility to conformational changes and denaturation.

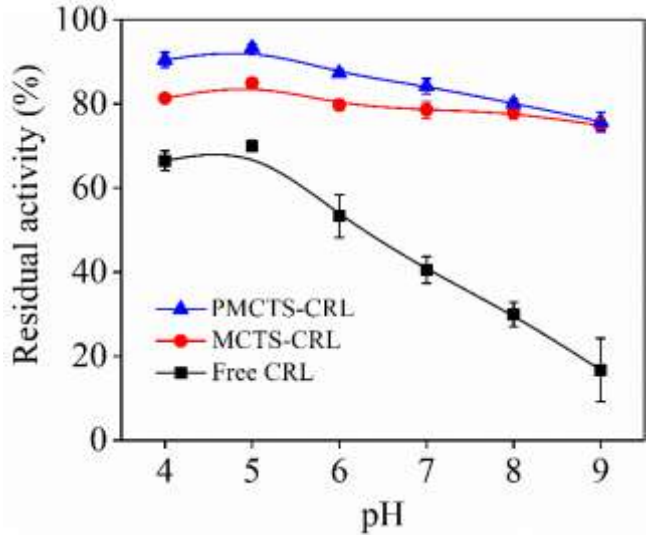

Figure 5 pH tolerances of free and immobilized CRLs.

The storage stability of free and immobilized CRLs at 4 ℃ was depicted in Fig. 6. The gradually declined activity was observed for the three enzyme samples with prolonged storage time. However, PMCTS-CRL exhibited less activity loss than free CRL and MCTS-CRL. After 30 days of storage, PMCTS-CRL maintained its initial activity at 67.5%, while free CRL and MCTS-CRL remained at 44.1% and 50% of their original activities, respectively. The better storage stability of PMCTS-CRL was ascribed to the zwitterionic nature of pID balancing the interactions between the enzyme and water molecules, allowing enzyme protein more stable in aqueous [27, 37].

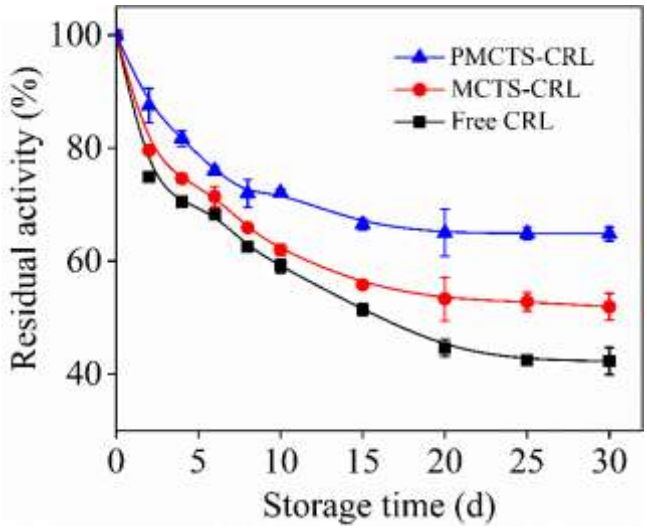

Figure 6 Storage stability of free and immobilized CRLs at 4 ℃.

3.5. Reusability

The reusabilities of MCTS-CRL and PMCTS-CRL are investigated by catalyzing hydrolysis of *p*-NPA. As shown in Fig. 7, compared to MCTS-CRL, PMCTS-CRL preserved relative stability in the first five cycles, with the residual activity of 87.7% for PMCTS-CRL versus 74.9% for MCTS-CRL after 5 cycles. Subsequently, the residual activities of both immobilized CRLs decreased quickly, but PMCTS-CRL consistently exhibited better reusability than MCTS-CRL. After 10 consecutive operations, the MCTS-CRL and PMCTS-CRL remained 24.6% and 27.5% of their original activities, respectively. The increasing activity loss can be attributed to probable enzyme inactivation and the loss of immobilized particles, during the frequent reaction, washing and recovery process [33, 38].

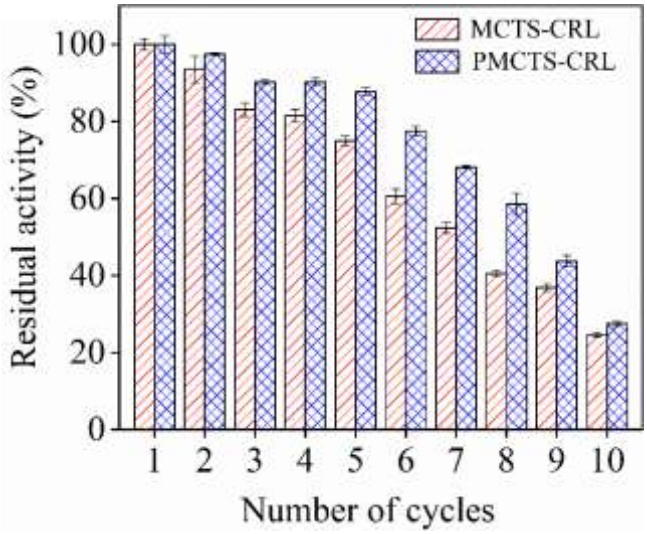

Figure 7. The reusabilities of MCTS-CRL and PMCTS-CRL

4. Conclusions

In this work, the innovative zwitterionic polymer-modified magnetic chitosan microspheres (PMCTS) were fabricated by a simple one-pot synthesis via the reaction of anhydride of pID0.7 with amino group of chitosan, and the MCTS was used for comparison. CRL was immobilized onto PMCTS and MCTS via covalent attachment, and the optimization of GA concentration, enzyme amount and immobilization time for lipase immobilization were conducted to enhance the performance of immobilized CRLs. PMCTS-CRL and MCTS-CRL presented the relative activity of 100.2% and 77.9% at the optimal conditions, respectively. The higher k_{cat} and k_{cat}/K_m were found for PMCTS-CRL compared with MCTS-CRL, indicating that the higher catalytic

efficiency for PMCTS-CRL. Moreover, PMCTS-CRL showed enhanced thermal, pH and storage stabilities in comparison with free CRL and MCTS-CRL. The half-life ($t_{1/2}$) increased by 3.89-fold for MCTS-CRL and 21.3-fold for PMCTS-CRL at 50 ℃. Furthermore, PMCTS-CRL displayed better reusability than MCTS-CRL for hydrolysis of *p*-NPA. These results indicated that immobilized enzymes onto zwitterionic polymer-modified chitosan magnetic carriers could be versatile candidates with great potential for industrial exploitation.

CRediT Authorship Contribution Statement

Shaokang Fang: Methodology, Investigation, Data curation, Writing - original draft. Hongyang Qi: Investigation, Validation, Formal analysis, Writing - original draft. Xianxiu Li: Writing - review & editing. Xinyi Jiang: Investigation, Validation. Chunyu Zhang: Writing - review & editing, Supervision, Funding acquisition. Shaokang Fang and Hongyang Qi should be considered joint first author.

Declaration of Interest Statement

The authors declare no competing interests.

Acknowledgments

This work was funded by the Natural Science Foundation of Shandong Province (No. ZR2021QC206) and the Doctoral Scientific Research Starting Foundation of Yantai University (SM20B85).

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/xxxxxx

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