The immobilization of penicillin G acylase on modified TiO_2 with various micro-environments

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Abstract:

Immobilization of penicillin G acylase(PGA) on carriers was an effective strategy for running away from the drawbacks of free PGA. In this work, modified TiO_2 was employed as a multifunctional carrier for the immobilization of PGA. Firstly, TiO_2 was used as a nucleus and modified by a one-step modification approach and a two-step modification approach to construct and regulate the micro-environment of carrier by changing the type of functional immobilization groups, the flexibility of arms and arm-length of immobilization sites. In the one-step modification approach, TiO_2 was altered by glutaraldehyde, 3-glycidoxypropyltrimethoxysilane (3-GCDPTMS) and 3-aminopropyltriethoxysilane (3-APTMS), separately; while in the two-step modification

approach, primary TiO_2 was firstly altered with a small amount of glutaraldehyde or 3-GCDPTMS, then followed by a secondary modification process using glutaraldehyde, 3-GCDPTMS and 3-APTMS, respectively. Secondly, the influence of the micro-environments of the surface of carrier on the catalytic performance of immobilized PGA, such as enzyme loading capacity (ELC), enzyme activity (*EA*) and activity retention ratio (*EAR*) was investigated. In the one-step modification approach PGA immobilized on TiO_2 modified by 3-GCDPTMS, meanwhile in the two-step modification approach, PGA on carrier that sequently altered by glutaraldehyde and 3-GCDPTMS had higher *EA* and *EAR*. Those results suggested that not only the interaction between polar groups of PGA and hydrated functional groups on carrier surface, but also the properties of immobilization site, including the flexibility of arms and the length of arm, had a critical influence on the performance of PGA. Among those impact factors, the physical attract interaction was most essential for stabilizing the conformation of PGA and improving its catalytic activity; secondly, the higher the flexibility of arms and the longer the arm-length of immobilization site, the better the accessibility of PGA to substrate, and the better the catalytic performance of immobilized PGA.

Keywords: Modified TiO_2; Penicillin G acylase; Micro-environments; Catalytic performance

1. Introduction

Penicillin G acylase (PGA) was an important industrial biocatalyst and widely existed in about 40 microorganisms (including bacterial fungi and yeast) [1]，being extensively used for enzymatic production of 6-amimopenicillanic acid(6-APA) [2, 3]and 7-aminodeacetoxyceph-alosporanic acid (7-ADCA)[4], which were the major pharmaceutical intermediates for producing semisynthetic β-lactam antibiotics[5, 6]. However, there was still a big challenge to employ free PGA in large scale application in industrial fields for its lack of structural stability in aqueous solution, being unable to reuse and the remaining difficulties of separation products from residual substrate[7,

8]. Immobilization of PGA on carriers to prepare non-soluble catalyst in reaction system provided a meaningful approach to overcome some of the weakness of free PGA, such as poor stability and reusability, and weak tolerability in reaction media. Studies of immobilization PGA on kinds of materials, such as encapsulating PGA in microsphere, entrapping PGA by covalent bonds on inorganic supports and physical adsorption of PGA on resins had been reported[9-11]. After a screening of the overall performance of immobilized PGA on supporting carrier, it revealed that the micro-environment of carrier surface not only determined the conformation and mobility of itself, but also has a critical effect on the diffusion rate and the accessibility to enzyme of substrate[12], hence, which in turn determined the *EA* and stability of the enzyme in catalytic reaction. For PGA dissolved in aqueous phase, that was uniformly distributed, and could fully align and self-assemble to the reactive sites of target molecules according to their shape and external structure, thus possessing the highest *EA* and catalytic efficiency in reaction. Whilst for PGA that was immobilized on solid carriers, which exhibited as a catalyst in fixed state with a certain conformation and some combined water molecules, therefore it was relatively difficult to change the spatial orientation of targeting immobilization sites according to the size and structure of enzyme, thus resulting in poor uniformity and limited overall performance of immobilized enzyme. Therefore, how to combine the merits of PGA that existed in solvation state and that immobilized on carrier in a catalytic system to run away from the disadvantages of the two cases when they come alone is a challenging question deserving further study.

TiO_2 was a polycrystalline semiconductor material not only had strong ultraviolet adsorption, physical and chemical stability, but had merits of low cost and having multiple polyhydroxy groups on its surface[13, 14]. In addition, that can be modified by various compounds to introduce extra functional groups, such as amino group, aldehyde group and carboxyl group to make it as extensible multipurpose carrier[15, 16]. Herein, a novel soft carrier was designed and prepared by hunting TiO_2 nanoparticle as a solid nucleus and grafting liner molecules as hair-likely arranged long arms on the surface of TiO_2 to immobilize PGA. The TiO_2 nucleus was responsible for carrying out the immobilized enzyme as sediment and the long arms were counting on

make the soft carrier with different microenvironment for conducting the catalyzation reaction in quasi liquid environment. Firstly, TiO_2 nanoparticle was used as the insoluble part to act as solid nucleus, and bifunctional coupling agent with various chain length, such as glutaraldehyde, 3-GCDPTMS and 3-APTMS, were applied as intermediate connection part to act as arms for grabbing PGA. Secondly, in order to investigate the influence of arms length to the catalytic performance of immobilized PGA, TiO_2 was modified by two approaches, i.e., the one-step modification approach and two-step modification approach. In the one-step modification approach, TiO_2 was altered by glutaraldehyde, 3-GCDPTMS and 3-APTMS, separately; while in the two-step modification approach, primary TiO_2 was firstly altered with a small amount of glutaraldehyde or 3-GCDPTMS, then followed by a secondary modification process using glutaraldehyde, 3-GCDPTMS and 3-APTMS, respectively. Last but not the least, the interaction force between carrier and PGA had a marvelous effect on the catalytic performance of immobilized PGA. Therefore, the functional groups of carrier, including aldehyde group, epoxy group and amino group were screened to regulate the microenvironment of carrier and alter the interaction force between carrier and immobilized PGA as well. The influence of arm length and microenvironment of the as-prepared soft carrier on the catalytic performance of immobilized PGA, including *ELC*, *EA* and *EAR* were studied in detail. Moreover, the reusability of immobilized PGA was also studied.

2. Experimental section

2.1 Materials and reagents

Titanium dioxide (TiO_2) (rutile type) was obtained from Tianxing new material Co. Ltd., Nanjing, China. Penicillin acylase (PGA, E. coli) (16900U/g), 6-aminopenicillanic acid (6-APA) and penicillin G potassium (PG) were purchased from Lantian Pharmaceutical Co, Ltd., Hubei, China. Potassium dihydrogen phosphate (KH_2PO_4, AR) was supported by Tianjin Beichen Founder Reagent Factory Co., Ltd. Ethanol (C_2H_5OH, AR) and phosphoric acid (H_3PO_4, AR) were purchased from Tianjin Fu Yu Fine Chemical Co., Ltd. Ammonium molybdate were supplied by Kaida Chemical Plant, Fourth Chemical Reagent Plant of Tianjin. Glutaraldehyde was supplied by Tianjin Damao Chemical Reagent Factory Co., Ltd and ascorbic acid were

from Tianjin Basf Chemical Co., Ltd., respectively. Hydroxylamine hydrochloride ($HONH_3Cl$, AR), dipotassium phosphate ($K_2HPO_4 \cdot 3H_2O$), methyl red, boracic acid, bromocresol green, 3-GCDPTMS and 3-APTMS were all purchased from Shanghai Macklin Biochemical Technology Co., Ltd. All the chemicals were used as received except those detailedly processed was introduced in the paper.

2.2 Activation of TiO_2

The activation method of TiO_2 was referred to previous literature [17].

2.3 Modification methods of TiO_2

The modification conditions of TiO_2 were as follows, particle size(d), d = 3.3 μm, active temperature (T_a), T_a = 120 °C, reaction temperature (T_m), T_m = 45 °C, pH of reaction media solution, pH = 6.0.

2.3.1 Modification of TiO_2 by Glutaraldehyde

5.00 g activated TiO_2 was added in a 150 mL Erlenmeyer flask, then mixture of 50 % (v/v) aqueous solution of glutaraldehyde solution and 70 mL absolute ethanol was added. The conical flask was placed in water bath at 45°C 24 h. Thereafter, the reaction system was filtrated to exclude the sediment from the solution media, and the obtained solution was diluted to measure the absorbance of glutaraldehyde dioxime for the purpose of calculation the graft ratio of Glutaraldehyde immobilized on titanium dioxide[11]. The formula was listed in below:

$$G = \frac{(C_0 - C_1)V}{m} \times 100\% \quad (1)$$

Where, G (%) represented the graft ratio of glutaraldehyde and/or silane coupling agent on the surface of TiO_2. C_0 (mg/L) and C_1 (mg/L) were the initial concentration of glutaraldehyde and/or silane coupling agent in the stock solution and that left in the filtrate after the reaction conducted, V (mL, $\times 10^{-3}$) was the volume of the stock solution and m (g) stood for the mass of TiO_2 involved in the reaction.

Next, the modified TiO_2 was collected and washed by distilled water several times until the absorbance of glutaraldehyde dioxime in the washing media was lower than 0.003. The obtained TiO_2 was vacuum dried at 40 °C to a constant weight, stored for further use.

2.3.2 Modification of TiO_2 by 3-GCDPTMS

Several pieces of 1.50 g activated TiO_2 were added in 150 mL Erlenmeyer flasks separately, then a series of 100 mL 3-GCDPTMS with varied concentration were introduced. After ultrasonic dispersion, the flasks were sealed and placed in water bath at 45°C for 24 h. Thereafter, the obtained mixture in flasks were filtrated and 3.00 mL of filtration solution was pumped out to test the concentration of 3-GCDPTMS by silicon molybdenum blue spectrophotometry [18], then calculating the graft ratio of 3-GCDPTMS according to equation (1). The modified samples of TiO_2 were rinsed several times with distilled water and the solution collected from the last rinsing time was adopted to measure the solution adsorption until the absorbance below 0.003. Finally, the modified TiO_2 samples were collected and dried in vacuum oven at 40 °C to a constant weight, stored in desiccator.

2.3.3 Modification of TiO_2 by 3-APTMS

The modification procedures of TiO_2 by 3-APTMS were similar as that of 3-GCDPTMS. The content of nitrogen in each sample was measured by Kjeldahl determination method [19] and the concentration of 3-APTMS in solution was calculated by equation (2). Then the graft ratio of 3-APTMS on TiO_2 was calculating using equation (1).

$$C = \frac{(V_1 - V_2) \times 0.1085 \times 0.014}{m \times \frac{10}{100}} \times 15.8 \times 100 \quad (2)$$

C (g/100g) was the concentration of 3-APTMS in the filtrate. V_1 (mL) and V_2 (mL) were the volume of standard hydrochloric acid solution (0.1085 mol/L) consumed by the testing sample and the controlling solution, respectively, m (g) was the mass of sample used in the test, 15.8 was the coefficient of converting the mass of nitrogen to that of 3-APTMS.

2.3.4 Modification of TiO_2 by glutaraldehyde and 3-GCDPTMS

A series of modified TiO_2 of 1.50 g with glutaraldehyde graft ratio of 8%, 14%, 20%, 25%, 29%, 35% and 42% were added in 150 mL Erlenmeyer flasks respectively. Then a series of 3-GCDPTMS standard solution (5.0 g/L) with varied volume were introduced into the above flasks to graft 3-GCDPTMS according to the method

illustrated in section 2.3.2. Finally, the obtained samples were applied for the immobilization of PGA.

2.3.5 Modification of TiO_2 by glutaraldehyde and 3-APTMS

A series of modified TiO_2 of 1.50g with glutaraldehyde graft ratio of 8%, 14%, 20%, 25%, 29%, 35% and 42% were added in 150 mL Erlenmeyer flasks respectively. Then a series of 3-APTMS standard solution (5.0 g/L) with varied volume were introduced into the above flasks to graft 3-APTMS according to the method illustrated in section 2.3.3. Finally, the obtained samples were applied for the immobilization of PGA.

2.3.6 Modification of TiO_2 by 3-GCDPTMS and glutaraldehyde

A series of modified TiO_2 of 1.50g with 3-GCDPTMS graft ratio of 10%, 15%, 18%, 25%, 28% and 33% were added in 150 mL Erlenmeyer flasks respectively. Then a series of glutaraldehyde standard solution (5.0 g/L) with varied volume were introduced into the above flasks to graft glutaraldehyde according to the method illustrated in section 2.3.1. Finally, the obtained samples were applied for the immobilization of PGA.

2.4 Determination of the enzyme activity of free PGA

The *EA* of free PGA was measured on the basis of its catalytic activity towards PG, and the amount of reaction product was determined by PDAB chromogenic method using PBS buffer solution as the reference[2, 20]. The details were as follow: 0.10 mL of free PGA with concentration of 2.50% (v/v) was added into a test tube that had been loaded with 5.00 mL 50.000 g/L PG, which was fixed in water-bath oscillator with 37 °C constant temperature for reaction for 5 min. Then 0.10 mL of the reaction mixture was pipetted out and diluted to 70 volume times with pH = 7.8 phosphate buffer solution(PBS), and 0.50 mL of the diluted solution was pipetted out and injected into a cuvette that had been loaded with 3.5 mL of 40.000g/L PDAB coloring solution to react for 5 min, then the absorbance of which was determined at absorption wavelength of 420 nm[21]. The *EA* of free PGA was calculated using Equation (3):

$$EA_{\mathrm{v}} = \frac{C \times V}{V_0 \times \mathrm{t}} \tag{3}$$

Where EA_v (U/mL) was the *EA* of free PGA per unit volume; *C* (mmol/L) and *V* (mL) were the concentration of 6-APA in the reaction system and the volume of the system, respectively. V_0 (mL) was the volume of free PGA introduced into the catalytic reaction and *t* (min) was the reaction time.

2.5 Immobilization of PGA on modified TiO_2

The immobilization conditions of immobilizing PGA on modified TiO_2 were investigated in detail previously, which included the pH of solution, PGA concentration, immobilization temperature and reaction time [22]. Herein, the immobilization procedure was illustrated below: 0.2000 g modified TiO_2 (ultrasonically dispersed in PBS and centrifugally separated prior to use) and 10.00 mL PGA solution with concentration of 2.50% (v/v) was added in a test tube, which was fixed in a water bath of 35°C and oscillated for 24 h. Then the reaction mixture was transferred to a centrifuge tube and centrifuged at 10000 r/s for 3 min. Afterwards, the activity of residual free PGA in supernatant fraction was tested similar as that in Section 2.4, and results was employed to determine the loading capacity of PGA on modified TiO_2. Finally, the obtained immobilized PGA was transferred to a bottle that had been fitted with certain volume by PBS to wash away free PGA and repeated this procedure for several times until the absorbance of washing solution was less than 0.003. Measuring the activity of immobilized PGA, drying it in vacuum oven at 35 °C for 24h till constant weight, and then storing it at desiccator for further usage. The loading capacity of immobilized PGA were calculated using Equation (4):

$$ELC = (EA_{v_0} - EA_{vr}) \times V \tag{4}$$

Where *ELC* was the loading capacity of immobilized PGA (U), EA_{v0} (U/mL) and EA_{vr} (U/mL) were the enzyme activity of free PGA in original solution and the remaining activity in residual solution respectively. *V* was the volume of reaction system (mL).

2.6 Evaluation of the catalytic performance of immobilized PGA

0.2000 g immobilized PGA and 10.00 mL penicillin G potassium solution with concentration of 50.000 g/L were added in a test tube, which was placed in a water bath

of 37 °C for 5 min. Afterwards, the *EA* of residual free PGA in supernatant fraction was tested similar as that in Section 2.4. The *EAR* of PGA was established by measuring the remaining activity of free PGA in residual solution and the activity of the immobilized PGA. The *EA* and *EAR* of immobilized PAG were calculated according to Equation (5) and Equation (6), respectively.

$$EA_{\mathrm{g}} = \frac{C \times V}{m \times \mathrm{t}} \tag{5}$$

$$EAR = \frac{EA_g \times m}{ELC} \times 100\% \tag{6}$$

Where, EA_g (U/g) was the *EA* of immobilized PGA per unit mass; *C* (mmol/L) and *V* (mL) were the concentration of 6-APA in the reaction system and the volume of the system, respectively. And *m* (g) was the mass of PGA that had been immobilized on carrier and *t* was the reaction time (min). *EAR* (%) was the EAR of immobilized PAG.

2.7 Reusability

The reusability of immobilized PGA was carried out for evaluating the stability of PGA in catalytic process. The immobilized PGA was recovered through centrifuging in each cycle and rinsed with PBS solution (pH = 8.0) until the absorbance of supernatant was lower than 0.003, then dried to constant weight at 35 °C and the *EA* of immobilized PGA in each cycle was measured similar as described in 2.6, and calculated according to Equation (5).

3. Results and discussion

3.1 The catalytic performance of PGA immobilized on TiO_2 with a single modifier

Fig 1 showed the influence of graft ratio of modifier on *ELC, EA* and *EAR* of immobilized PGA. The graft ratio of modifiers on TiO_2 followed the sequence of glutaraldehyde, 3-GCDPTMS, and then 3-APTMS. Seen from Fig 1 a), in general, the *ELC* firstly increased with the increasing of graft ratio of modifier and then kept constant when the graft ratio of modifier further increased. Detailedly, the growth rate of *ELC* of each modifier was different as the graft ratio increased. The *ELC* of PGA on 3-GCDPTMS had the biggest growth rate, then came with that on glutaraldehyde, that on 3-APTMS had the least growth rate. In particular, 3-GCDPTMS had the biggest *ELC*

of about 11000 U at graft ratio of 33%, glutaraldehyde had the second *ELC* of about 8800 U at graft ratio of 46% and 3-APTMS reached to its biggest value at graft ratio of 27% with *ELC* of about 3500 U. The influence of modifier graft ratio on *ELC* can be ascribed to the influence of increased number of reactive functional groups (the increased immobilization site provided by different modifier) on the grafting reaction involved in. In terms of glutaraldehyde, as the graft ratio of it increased, the immobilization site of enzyme on carrier would increase and which led to the increasing of *ELC* consequently. When the graft ratio of glutaraldehyde reached at 40%, the *ELC* increased to the maximum amount because of the reaction between aldehyde groups and PGA molecules attempted to achieve the dynamic equilibrium state due to the steric hindrance effect. The immobilized PGA molecules side-around arranged compactly on the surface of TiO_2 and no more PAG molecules could be attached to the carrier, hence the loading capacity become constant.

Seen from Fig 1 b), *EA* of modified TiO_2 also increased with the increasing of modifier graft ratio. Generally speaking, all of the prepared carriers had an excellent maximum *EA* of about 15000 U/g, which is irrelevant to the *ELC* of PGA on each modifier. Detailedly, TiO_2 modified by 3-APTMS had a relatively higher *EA* of 12300 U/g even if at low graft ratio of 4%, and which achieved its maximum of 14938 U/g at the graft ratio 27%. TiO_2 modified by 3-GCDPTMS had the biggest *EA* of 15024 U/g at graft ratio 33%, and that modified by glutaraldehyde had the maximum *EA* of 14872 U/g at the graft ratio 46%. Therefore, the catalytic efficiency of PGA that immobilized on TiO_2 with 3-APTMS as modifier had the biggest catalytic efficiency towards PG, that modified by 3-GCDPTMS was in the second place, and that modified by glutaraldehyde was the least.

The influence of modifier graft ratio on the *EA* of immobilized PGA can be ascribed to the deformation of active center of enzyme. Naturally, the deformation degree of active center of PGA would also change with the *ELC* of PGA on carrier. At lower *ELC*, the deformation degree of active center was notable, which was caused by the multi-point immobilization effect of PGA by aldehyde groups, and/or hydroxyl groups derived from silane coupling agent, then resulted in lower *EA* of enzyme. For

example, at low graft ratio of glutaraldehyde, the *EA* of PGA on carrier was the smallest when compared with that of PGA on carriers modified by 3-GCDPTMS and 3-APTMS. A glutaraldehyde molecule could only react with only one hydroxyl group on the surface of TiO_2 particle, while a 3-GCDPTMS or 3-APTMS molecule would react with three hydroxyl groups on surface of TiO_2, therefore the amount of residual hydroxyl groups on carrier that modified by glutaraldehyde was the largest, and the residual hydroxyl groups would interact with PGA to form multipoint immobilization site, which greatly influenced the conformation of active center of PGA. Moreover, the flexible chain of 3-GCDPTMS had three more carbon atoms than other chains, which made the accessibility between immobilized PGA and substrate become more easier, thus resulting in higher *EA* at lower modifier graft ratio. When the *ELC* gradually increased, the multi-point immobilization trend of PGA would decrease and the deformation degree of active center would reduce, therefore the *EA* of enzyme increased and which would reach its maximum value along with the loading capacity of PGA.

Seen from Fig 1 c), the *EAR* exhibited similar increasing trends as that of *ELC*. The relationship between *EA* retention ratio and modifier graft ratio can be deduced from two aspects: the influence of modifier graft ratio on *ELC* and the influence of *ELC* on the deformation degree of the active center of PGA. At low modifier graft ratio, *ELC* was small and the multi-point immobilization effect of PGA was prominent[6, 23] because of the reaction between the surplus functional groups on PGA and TiO_2, which gave rise to higher deformation degree of the active center of enzyme immobilized. With the increment of modifier graft ratio, *ELC* increased, the multi-point immobilization effect decreased and the deformation degree of enzyme active center waked, therefore the *EAR* increased. When *ELC* on carrier reached to its maximum, the deformation degree of the active center of PGA was minute because of the multi-point immobilization effect of PGA was negligible and all the immobilized PGA molecules were closely arranged on the surface of modified TiO_2, thus the *EAR* of PGA achieved to its maximum. In addition, when comparing the catalytic performance of PGA immobilized on different carrier (PGA on carrier modified by 3-GCDPTMS and by

glutaraldehyde, respectively), we could realize that the carriers with longer arm length and higher arm flexibility was profitable for improve the catalytic performance of immobilized PGA.

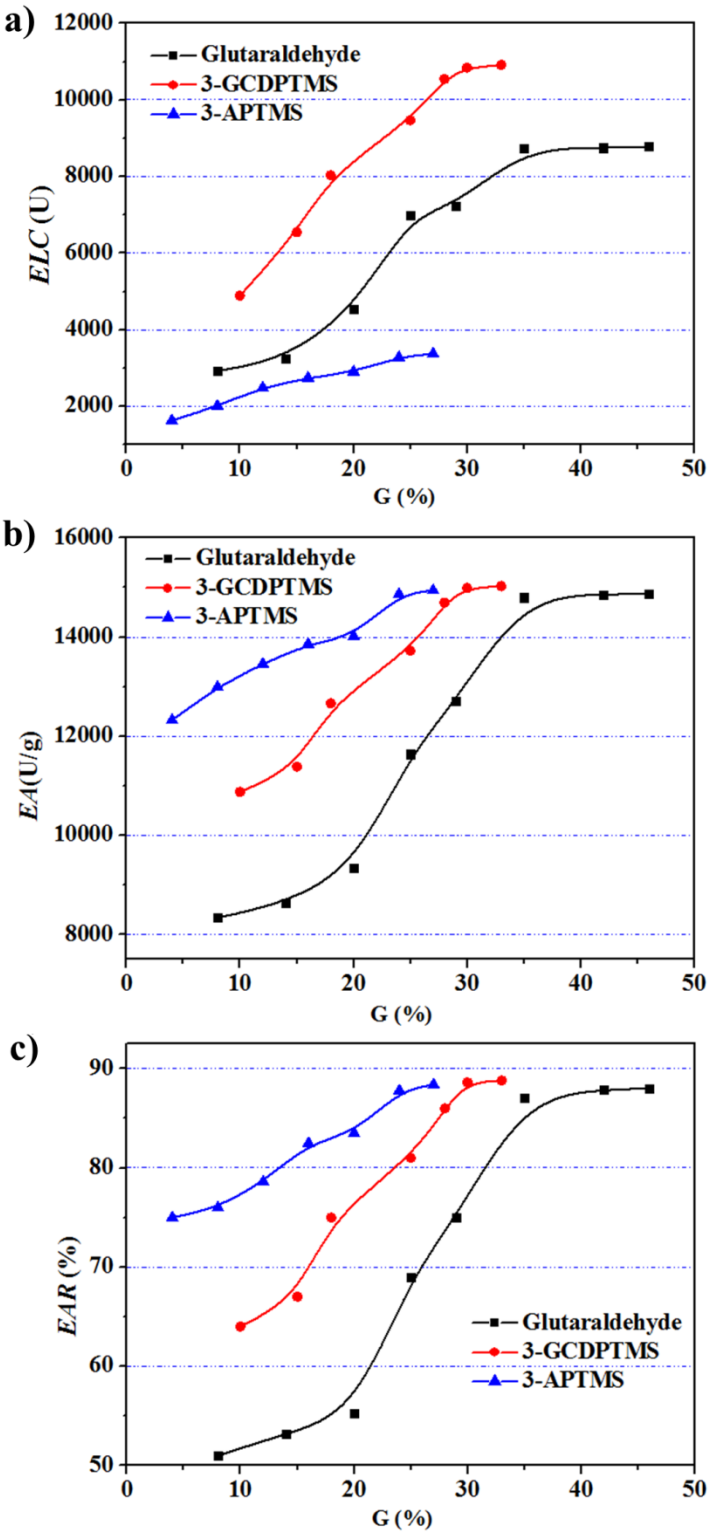

Fig.1 The influence of graft ratio of modifier on a) the *ELC*, b) the *EA* and c) the *EAR*

of immobilized PGA

3.2 The catalytic performance of PGA immobilized on TiO_2 with two component modifier

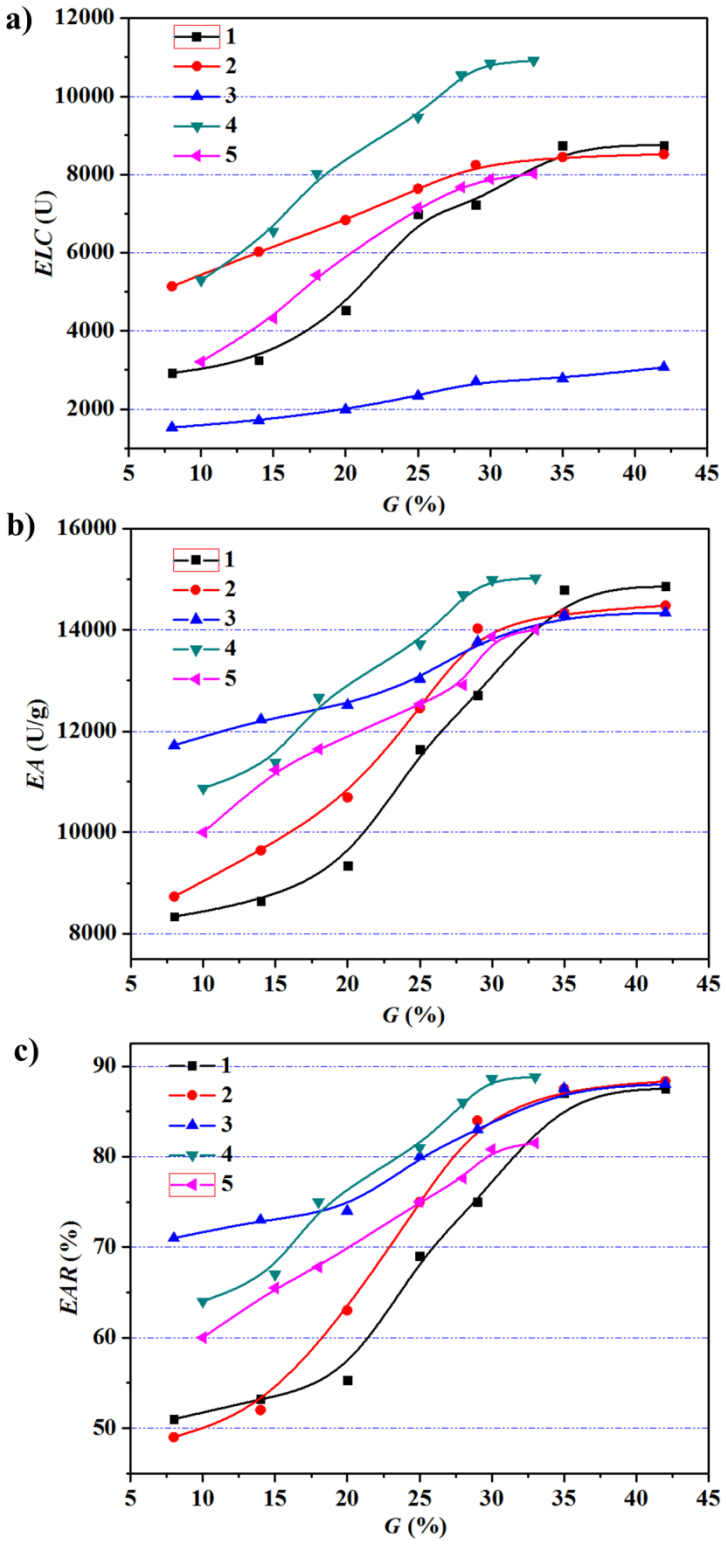

Fig.2 The influence of graft ratio of modifier on a) the *ELC*, b) the *EA* and c) the

EAR of immobilized PGA (Line 1, the modifier was glutaraldehyde, Line 2, the modifiers were glutaraldehyde and 3-GCDPTMS, Line 3, the modifiers were glutaraldehyde and 3-APTMS, Line 4 was 3-GCDPTMS and Line 5 the modifiers were 3-GCDPTMS and glutaraldehyde)

The amount of modifier preserved on TiO_2 in the two step modification method was listed in Table 1, Table 2 and Table 3, the catalytic performance of PGA immobilized on TiO_2 with two component modifier was illustrated in Fig 2.

A second modifier 3-GCDPTMS was introduced to modify TiO_2 on the basis of modification TiO_2 by glutaraldehyde, the amount of glutaraldehyde consumed and that of 3-GCDPTMS increased in the second graft process on TiO_2 was shown in Table 1. The amount of target immobilization sites preserved on TiO_2 reduced considerably as the graft ratio of 3-GCDPTMS increased, which indicated that the reaction between a silanol group and several aldehyde groups occurred and that came to be the main reaction along with the increment of 3-GCDPTMS amount. Seen from Fig 2 a), *ELC* of carrier that modified with two component modifier was higher than that of carrier modified with glutaraldehyde only when the graft ratio of glutaraldehyde was less than 35%, and which turn to a reverse trend after the graft ratio of glutaraldehyde was higher than 35% (demonstrated on Line 1 and Line 3 in Fig 2 a)). This phenomenon can be illustrated by the change of the micro-environment of carrier. At lower graft ratio of glutaraldehyde, there were many hydroxyl groups leftover on the surface of original TiO_2, which could react with the silanol group of 3-GCDPTMS in the second graft process, and the reaction between silanol group and aldehyde group was not notable, therefore the amount of immobilization site greatly increased as the introduced 3-GCDPTMS could produce extra silanol groups that derived from the hydrolysis of alkoxy silicone groups when compared with carrier that just grafting glutaraldehyde on TiO_2. What's more, the micro-environment of immobilization site on carrier after modified by 3-GCDPTMS was getting more close to a solid-liquid interface rather than a solid surface for immobilization of enzyme, so that the grafting of enzyme on carrier became easier, so that the *ELC* increased. Along with the increment of the graft ratio of glutaraldehyde, the amount of residual hydroxyl groups of TiO_2 decreased and the

reaction between the residual hydroxyl groups and silanol group of 3-GCDPTMS became minute, while the reaction between aldehyde groups and silanol group became notable, therefore the total amount of immobilization sites decreased, which further leading to the decrement of *ELC*.

Seen from Fig 2b), *EA* of PGA on carrier that modified with two component modifier was higher than that of carrier modified with glutaraldehyde only when the graft ratio of glutaraldehyde was less than 35%, and which turn to a reverse trend after the graft ratio of glutaraldehyde was over than 35% (demonstrated on Line 1 and Line 3 in Fig 2b)). When immobilization of PGA by carrier with a secondary modification process, the reactivity of carrier was decreased as the hydroxyl group on TiO_2 with higher activity could react with the alkoxy silicone group of 3-GCDPTMS in the second graft process, therefore the functional groups preserved on carrier were epoxy groups and aldehyde groups, which had a relatively lower reactivity towards PGA, so that the multi-point immobilization effect of PGA decreased and the deformation degree of enzyme active center waked, thus resulting in higher *EA*. Meanwhile, the immobilization of PGA on carrier with a secondary modification process had higher reactivity, could be ascribed to two factors, i.e., the extended arm-length of immobilization site and the mimetic liquid micro-environment of immobilization site.

In the two-step modification process, the arm length of carrier was longer and the flexibility of it was also improved, therefore the mimetic micro-environment of immobilization site was similar as a solid-liquid interface, so that the catalytic performance of immobilized PGA was the best as the micro-environment of which was more close as which in the aqueous solution. Along with the increase of glutaraldehyde graft ratio, the *EA* of PGA would increase because of the decrement of the deformation degree of carrier. As a result, PGA immobilized on carrier with a secondary modification process would have higher *EAR* as well, as shown in Fig 2 c). When the second modifier was changed to 3-APTMS, the amount of glutaraldehyde consumed and that of 3-APTMS increased in the second graft process on TiO_2 was shown in Table 2. The amount of aldehyde groups leftover on TiO_2 decreased greatly in the secondary modification procedure, which indicated that there was a reaction between the aldehyde

group on carrier and the amino group of 3-APTMS. Meanwhile, the increment of the amount of 3-APTMS was greater than the decrement of the amount of aldehyde group on carrier, indicating that there was a reaction between the alkoxy silicone group of 3-APTMS and the hydroxyl group on TiO_2 as well.

While for PGA immobilized on TiO_2 that grafted by 3-APTMS, PGA molecules were absorbed on carrier by physical interaction and there were not covalent bond existing between them, so that the PGA which immobilized on carrier would easily run away from carrier to get into the reaction media, thus leading to the decrease of *ELC* and *EA*.

Table 1 the amount of glutaraldehyde and 3-GCDPTMS on TiO_2 in the two step modification reaction

Item	Amount of modifier (moL/100g)						
Amount of glutaraldehyde on TiO_2	0.080	0.140	0.200	0.250	0.280	0.330	0.420
Amount of glutaraldehyde after grafting 3-GCDPTMS on TiO_2	0.060	0.070	0.090	0.120	0.130	0.140	0.170
Amount of glutaraldehyde consumed in the second graft process	0.020	0.070	0.110	0.120	0.150	0.190	0.250
Amount of 3-GCDPTMS on TiO_2	0.047	0.059	0.085	0.101	0.120	0.127	0.158

Table 2 the amount of glutaraldehyde and 3-APTMS on TiO_2 in the two step modification reaction

Item	Amount of modifier (moL/100g)						
Amount of glutaraldehyde on TiO_2	0.080	0.140	0.20	0.250	0.280	0.330	0.420
Amount of glutaraldehyde after grafting 3-APTMS on TiO_2	0.050	0.104	0.157	0.197	0.214	0.239	0.318
Amount of glutaraldehyde consumed in the second graft process	0.030	0.036	0.043	0.053	0.066	0.091	0.102
Amount of 3-APTMS on TiO_2	0.038	0.041	0.052	0.062	0.088	0.105	0.113

Table 3 the amount of 3-GCDPTMS and glutaraldehyde on TiO_2 in the two step modification reaction

Item	Amount of modifier (moL/100g)						
Amount of 3-GCDPTMS on TiO_2	0.025	0.036	0.054	0.072	0.091	0.109	0.122
Amount of 3-GCDPTMS consumed in the second graft process	0.004	0.008	0.008	0.010	0.005	0.013	0.011
Amount of glutaraldehyde on TiO_2	0.040	0.060	0.090	0.130	0.160	0.180	0.210

3.3 The reusability of immobilized PGA

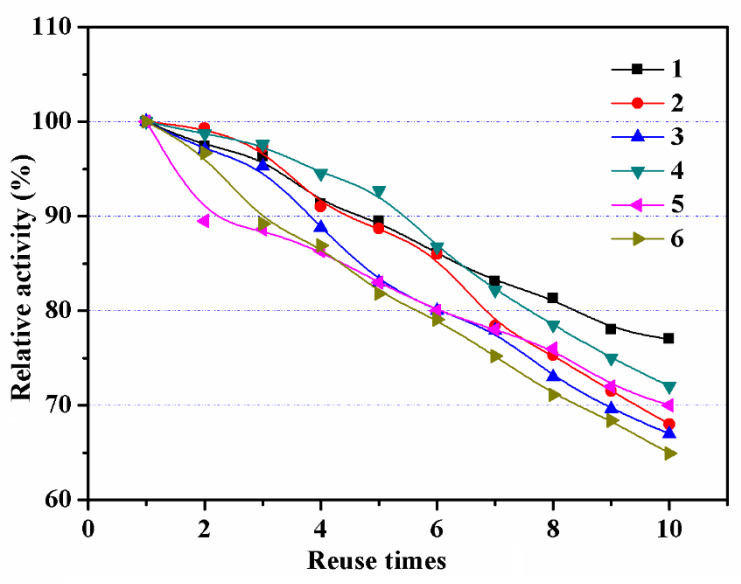

Fig.3 The reusability of immobilized PGA on different carrier (Line 1, the modifier was glutaraldehyde; Line 2, the modifiers were glutaraldehyde and 3-GCDPTMS; Line 3, the modifiers were glutaraldehyde and 3-APTMS; Line 4 was 3-GCDPTMS and Line 5 the modifiers were 3-GCDPTMS and glutaraldehyde; Line 6 was 3-APTMS)

Fig 3 illustrated the relationship between the relative activity of immobilized PGA and its reuse time. From the perspective of application, the reusability of immobilized PGA was a critical parameter for evaluation of its integrative performance. The reusability of immobilized PGA was closely related to the microenvironment of carrier. PGA immobilized on TiO_2 grafted by 3-GCDPTMS had the best reusability in the first six cycles, while that on carrier grafted by glutaraldehyde had the best performance in ten cycles and that grafted by 3-APTMS had the worst performance after ten cycle of usage. For PGA immobilized on TiO_2 that grafted by 3-GCDPTMS, PGA molecules were covalently bonded on the surface of carrier and that would not leach out to reaction media in the catalytic process. Whereas, phenyl acetic acid would be formed during the hydrolysis process of PGA, which in turn would lead to the hydrolysis of the covalent bonds between PGA molecules and epoxy groups, thus the loading capacity of PGA decreased as the reuse time increased, further led to the decrement of relative activity. While for PGA immobilized on TiO_2 that grafted by 3-APTMS, PGA molecules were absorbed on carrier by physical interaction and there were not covalent bond existing

between them, so that the immobilized PGA would easily run away from carrier to reaction media, leading to the decrease of *ELC* and *EA*. The relative activity of PGA immobilized on TiO_2 with glutaraldehyde as modifier was decreased along with the increment of reuse time, but had the highest activity after ten cycles of usage, which indicated that the Schiff base structure derived from the reaction between aldehyde group and PGA had excellent stability in weak acidic environment and not easy to decomposition.

4. Conclusions

In this work, TiO_2 was modified by two kinds of methods to prepare soft carriers with various micro-environment to immobilize PGA. The modification methods included a one-step modification approach of modifying TiO_2 by glutaraldehyde, 3-GCDPTMS and 3-APTMS, separately; and a two-step modification approach of altering primary TiO_2 with a small amount of glutaraldehyde or 3-GCDPTMS, then coming along with a secondary reaction by grafting glutaraldehyde, 3-GCDPTMS and 3-APTMS, respectively. The influence of the micro-environments of carrier on the catalytic performance, including *ELC, EA* and *EAR*, and reusability of immobilized PGA were investigated in detail. The results showed that both of the functional group and arm length of immobilization site of carrier had a great influence on the catalytic performance of immobilized PGA. PGA immobilized on carrier that modified just by 3-GCDPTMS, and modified by glutaraldehyde and 3-GCDPTMS sequently possessed higher *EA* and *EAR*, demonstrating that the physical attract interaction between polar groups of PGA and hydrated functional groups on carrier surface had high stabilizing effect on PGA. In addition, the carriers with longer arm length and higher arm flexibility was profitable to improve the catalytic performance of immobilized PGA.

Acknowledgments

This work is supported by National Natural Science Foundation of China (Grant No. 51563015).

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