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Simultaneously and separately immobilizing incompatible dual-enzymes on polymer substrate *via* visible light induced graft polymerization

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ABSTRACT

Developing facile and mild strategy to construct multi-enzymes immobilization system has attracted considerable attentions in recent years. Here a simple immobilization strategy called visible light induced graft polymerization that can simultaneously and separately encapsulate two kinds of enzymes on one polymer film was proposed. Two incompatible enzymes, trypsin and transglutaminase (TGase) were selected as model dual-enzymes system and simultaneously immobilized on two sides of low-density polyethylene (LDPE) film. After immobilization, it was found that more than 90% of the enzymes can be embedded into dual-enzymes loaded film without leakage. And the activities of both separately immobilized enzymes were higher than the activities of mixed co-immobilized enzymes or the sequential immobilized ones. This dual-enzymes loaded film (DEL film) showed excellent recyclability and can retain >87% activities of both enzymes after 4 cycles of utilization. As an example, this DEL film was used to conjugate a prodrug of cytarabine with a target peptide. The successful preparation of expected product demonstrated that the separately immobilized two enzymes can worked well together to catalyze a two-step reaction.

1. Introduction

Immobilization of enzymes on or within a support is of great importance both scientifically and industrially [1–7]. Their moderate reaction conditions, high regio-selectivity, simple operation and reusability have made them potential in many sustainable applications [8–12]. In previous researches, many works focused on single enzyme immobilization system [13,14]. However, in many complex reactions, multi-enzymes working together are needed [15,16]. It is still very challenging to efficiently immobilize multi-enzymes in an organized way on one substrate.

One convenient method to fix several enzymes on one substrate is called co-immobilization, which usually immobilizes the mixture of enzymes like a single enzyme [17–20]. For example, Liang et al. [21] mixed glucose oxidase (GOx) and horseradish peroxidase (HRP) together and encapsulated them into a hydrogel fiber made from Zn^{2+} and adenosine monophosphate. It was found that both of the immobilized enzymes retained more than 70% of their initial activities after 15 days storage, and they were even more stable against the external environment than free enzymes. Although it is very simple, co-immobilization is only applicable to the enzymes that do not interfere with each other. Therefore, it is significant to explore alternative immobilization strategy toward organizing multi-enzymes in different area of substrate to avoid mutual interference. Recently, a great number of efforts have been devoted to constructing separated immobilization systems [22–25]. Inspiring by mitochondria, Shi et al. [26] prepared hybrid double membrane microcapsules. One kind of enzyme was embedded inside the microcapsules, while the other kind of enzyme was fixed in the inter membrane space. Comparing to the mixing co-immobilization method, this separated immobilization method

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significantly improved the selectivity, activity and stability of enzymes. Zhang et al. [27] sequentially absorbed organophosphate hydrolase (OPH) and acetylcholine esterase (AChE) *via* layer-by-layer strategy on polyethyleneimine/DNA modified multi-walled carbon nanotubes. This dual-enzymes immobilization system was used to discriminate between paraoxon and carbaryl pesticides, and it also exhibited good stability, sensitivity and reproducibility. Despite the obvious benefits of these contributions, the complicated process to localize each enzyme greatly hindered their practical application and the enzymes immobilized in the outer layers may block the activities of enzymes which immobilized in the inner layers. Moreover, some harsh conditions such as organic solvents, UV irradiation, acid or alkali may have to be used to realize the fabrication of the elaborately designed partition structure, but these factors may affect the activities of the immobilized enzymes [28,29]. Last but not least, when the incompatible enzymes system was involved, the enzymes at the interface of different part could still affect each other [30]. Hence, it is still a significant and challenging task to develop simple and mild approach to separately immobilize multi-enzymes for practical application.

In this study, an extremely simple and mild technique, called visible light induced graft polymerization [31–33], was used to immobilize two enzymes on polymer substrate separately and simultaneously. The mechanism of this technique composed of two chemical steps (Scheme 1). Firstly, isopropyl thioxanthone (ITX) was coupled to both side of low-density polyethylene (LDPE) film under UV irradiation (Scheme 1a). Secondly, the formed ITX semipinacol (ITXSP) dormant groups were photolyzed under visible light to generate surface carbon radical. And then the surface radical could initiate graft crosslinking polymerization of poly(ethylene glycol) diacrylate (PEGDA) (Scheme S1). When the enzymes/PEGDA solutions were cast onto each side of LDPE-ITXSP and irradiated by visible light (Scheme 1b), photografting reaction occurred on two sides and two enzymes could be encapsulated into different PEG networks isolated by the LDPE film. With the help of a photomask, two micropatterns loaded with different enzymes grafted on LDPE could be easily obtained. Compared with other separated immobilization methods, this strategy showed several advantages: 1) the immobilization of enzymes was performed under visible light and room temperature, which is mild enough to preserve the activity of enzymes; 2) the swelling of the PEG molecular network was markedly restricted by LDPE film thus is favorable to prevent the leakage of enzymes [31]; 3) it can immobilize two enzymes *via* a one-step reaction and greatly simplified the immobilization process; 4) two enzymes were completely separated by the film, eliminating the possibility of mutual interference.

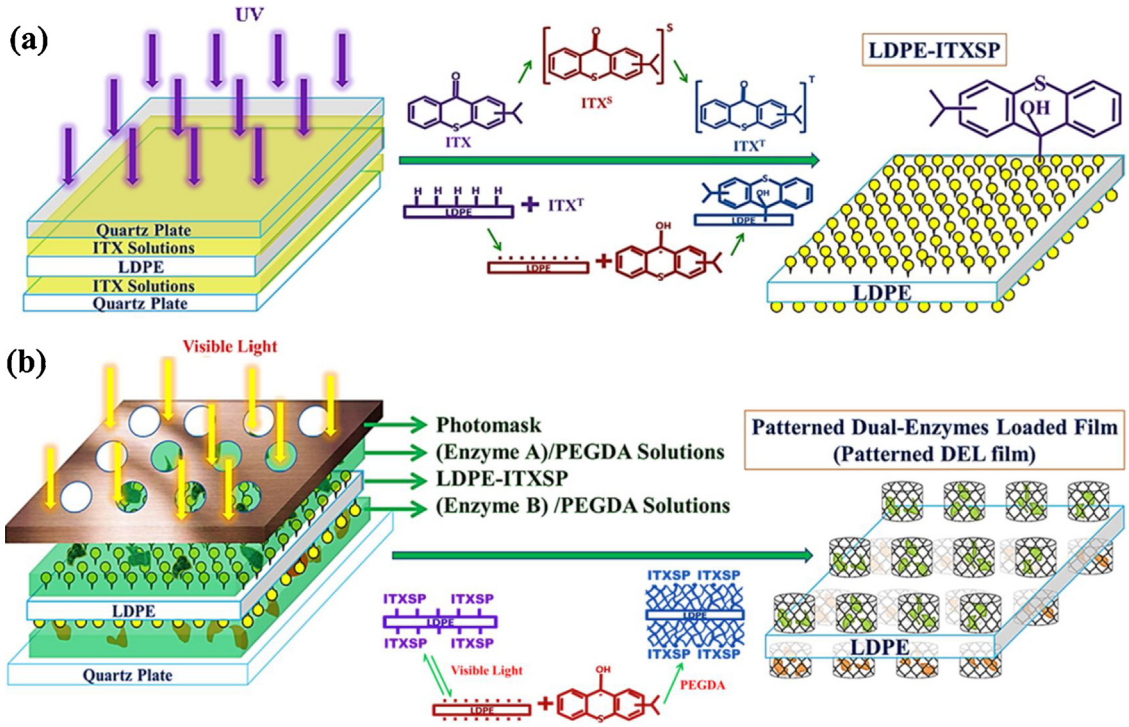
Herein, trypsin and transglutaminase (TGase) were chosen as model enzymes to fabricate the dual-enzymes loaded film (DEL film). TGase is one enzyme which can incorporate chemicals containing primary amines (*e.g.* lysine) into the protein substrate which contains γ-glutaminyl residues [34,35]. Trypsin is one specific digestive enzyme which can cut arginine or lysine on the carboxyl end of proteins or peptides [36–38]. When the two enzymes were mixed together, TGase may crosslink the trypsin and at the same time trypsin may hydrolyze TGase [39]. Therefore, completely separate immobilizing them on one substrate can not only retain their activities but also allow them to catalyze multi-steps reaction effectively.

To the best of our knowledge, this is the first time to provide a simple method that can immobilize two enzymes on one support separately and simultaneously. And we believe that this strategy is also applicable to immobilize other biomacromolecules.

2. Experimental

2.1. Grafting ITXSP on LDPE

Firstly, ITX solution (acetone as the solvent, 0.1 mL, 5 mmol mL^{-1}) was dropped onto a quartz plate, and then a 4 cm × 4 cm LDPE film was placed onto the quartz plate to spread out the solution uniformly. Secondly, ITX solution (acetone as the solvent, 0.1 mL, 5 mmol mL^{-1}) was dropped onto the front



Scheme 1. Schematic Route of (a) Planting the ITXSP on Both Side of LDPE Film under UV Irradiation; (b) Preparation of Patterned DEL Film *via* Visible Light-Induced Graft Polymerization.