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Encapsulation of Biocatalysts (Cell/Enzyme) with High Retaining Activity

A thesis submitted in partial satisfaction of

the requirements for the degree

Master of Science in Chemical Engineering

by

Tao Liu

2015

ABSTRACT OF THE THESIS

Encapsulation of Biocatalysts (Cell/Enzyme) with High Retaining Activity

by

Tao Liu

Master of Science in Chemical Engineering
University of California, Los Angeles, 2015
Professor Yunfeng Lu, Chair

Enzymes are always considered as great gifts from nature since they are holding brilliant properties, including high activity, selectivity and specificity. Nowadays, a variety of enzymes have been applied to many industry processes. However, challenges are still needed to be addressed while applying enzymes. It is worth to point out that enzymes are sensitive to the change of ambient conditions. Most of enzymes are unstable and work under certain sort of temperature and pH conditions. Since enzymes could be denatured when subject to unnatural conditions, their work environment has to be controlled.

Researchers have been developed a variety of methods to improve the stability of bio-catalysts under various non-biological conditions. However, the immobilization process might harm the activity of enzymes. Therefore, even though immobilization approach has stabilized the stability of bio-catalysts, alternative strategies are still necessary to maintain the enzymes' activity during the encapsulation process.

In my research, two novel strategies were successfully developed to maintain enzymes' activity during encapsulation processes. Enzyme-based microgels and nanogels were successfully synthesized at cellular and enzymatic level for various applications, which are briefly outlined below:

Cellular level: An approach was envisioned in this section to improve biocatalyst stability, while maintaining their activities at the maximum during the encapsulation process. The new technology employs materials self-assembly to form a protective layer coating on cells surface. Enzymes are restricted within the cell all the time, without disturbing the structure during the encapsulation process. Therefore, this strategy maintains enzymes' activity at maximum. What's more, the protective polymer coating significantly increases biocatalyst viability in harsh thermal environment, different pH conditions, while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. Compared with the conventional enzymes' encapsulation method, such robust microgels exhibit significantly improved activity and catalytic stability. Meanwhile, such robust single cells make the immobilized whole cells much cheaper to use than an immobilized enzyme.

Enzymatic level: In this section, surface coating with zwitterionic polymers was studied at the single enzymatic level without disturbing the enzyme structure during the encapsulation process in order to maintain enzymes' activity. The protective polymer coating can significantly increase biocatalyst viability while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. Zwitterionic polymer shells have an efficient antibiofouling property which reduce the protein or cell adsorption, reduce immune response and prolong the circulation time of nanogels in blood circulation system.

Overall, my researches focus on maintaining enzymes' activity during the encapsulation process. Protective layers stabilize enzymes and create new surface functions. The encapsulation process is without disturbing enzymes' 3D structure. The resulted enzyme microgels or nanogels gain high activity and various new functionalities. With these technologies, we can envision a promising prospect in environmental, therapeutic and analytical applications of enzymes.

The thesis of Tao Liu is approved.

Selim Senkan

Yoram Cohen

Yunfeng Lu, Committee Chair

University of California, Los Angeles

2015

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Chapter 1. Application Scope of Proteins and Existing Challenges

Enzymes are always considered as great gifts from nature since they are holding brilliant properties, including high activity, selectivity and specificity. Enzymes are able to modulate the most complicated processes under benign conditions. In addition, enzymes get involved with almost every biological process, mediating different kinds of living organism functions, such as metabolism, neurotransmission, photosynthesis, and DNA synthesis.

According to the basic knowledge on structures and functions of enzymes accumulates, it has been proved valuable even beyond enzymes' intrinsic biological roles. The applications of enzymes might be back to several thousand years ago, when people started producing cheese, beer or wine using microorganisms and enzymes. Up to nowadays, the applications of enzymes are widened dramatically. In chemical industry, for instance, because of benign reaction conditions, high chemo- and stero-selectivities and specificity to substrate, enzymatic catalysis is treated as a promising field. In therapeutic applications, on the other hand, therapeutic enzymes are applied to treat different kinds of diseases, specially the diseases caused by enzyme deficiency. Moreover, enzymes are also important to bio-sensing, bio-remediation and so on.

Note that enzymes have been applied to bio-technology recently as well. Thanks to recombining DNA technologies, most of the products of enzymes are acceptable commercially [1-2]. However, challenges are still needed to be addressed while applying enzymes. It is worth to point out that enzymes are sensitive to environmental changes, including physical and chemical ambient conditions. Hence, they are easy to denature by small increment of temperature and changes of pH. Enzymes are also susceptible to poisons. Most of enzymes are unstable and working under

certain sort of temperature or pH conditions [3-4]. Since enzymes could be denatured when subject to unnatural conditions, their work environment has to be controlled. However, enzymes are required to be workable with chemicals such as organic solvents, under high temperature or at extreme pH conditions in some industrial processes, which tends to cause functional inactive or loss of them. So to speak, the deterioration of enzymes' viability renders the promising bio-catalysts improper in the applications of detection, protection and decontamination field.

Researchers have been dedicating to investigate the stabilization methods for bio-catalysts, such as porous supporting [5-6], multipoint immobilization [7], and multi-subunit immobilization [8-10] which is able to increase the stability of enzymes with different successes. Note that the high stabilization is attributed to the rigidification of the three-dimensional structures of enzymes [11-14]. However, the enzyme conformational changes are involved in enzyme inactivation. Thus, immobilization processes might disturb enzymes' structure and harm enzymes' activity. Therefore, even though immobilization approach has reached great success, alternative strategies are still necessary to maintain the activity of enzymes during the processes of encapsulation.

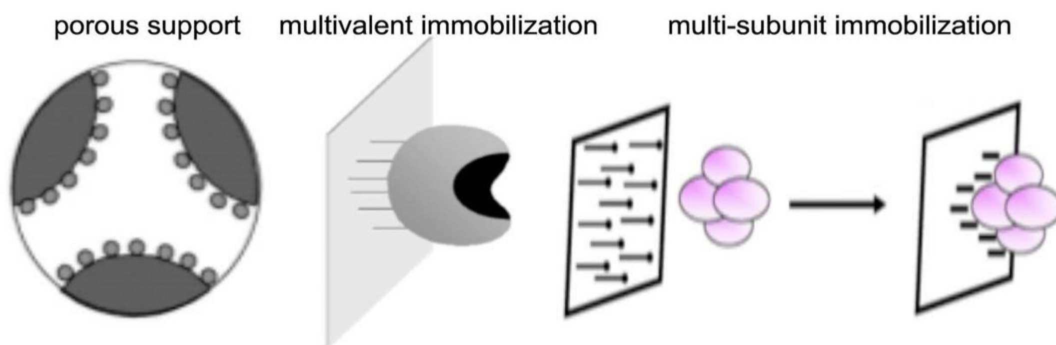


Figure 1 Immobilization strategies that can stabilize the enzymes

The other drawback is that foreign enzymes in blood circulation system are easily to be attacked by the macrophagocyte and cleared out by immune system [15-16]. Many methods were developed

to improve the enzyme autifouling property, avoiding being recognized by immune system. Traditional methods can encapsulate the enzyme effectively with autifouling polymers but this immobilization method is often achieved at the expense of the enzyme activity.

The charged monomers can attach to the enzyme surface thought the electrostatical interaction and encapsulate the enzyme with high retaining activity. But the charged polymers have the cytotoxicity and the enzyme with charged polymers is easily attacked by the macrophagocyte and cleared out by immune system. Neutral monomers, such as zwitterionic monomers, are biocompatible and nontoxic, but they are incapable of encapsulating the enzyme. If we want to use zwitterionic monomers to encapsulate the enzyme, we need to modify of the enzyme surface to give the enzyme reactive site and then conjugate polymers onto enzymes. This method can encapsulate the enzyme effectively; however, it easily denatures the enzyme during the encapsulation process. The contradiction between the encapsulation efficiency and the enzyme activity impedes applications of enzymes.

In this thesis, two novel strategies were successfully developed to maintain enzymes' activity during the encapsulation process and protective layers give the enzyme new properties. In my research, enzyme-based microgels and nanogels were successfully synthesized at cellular and enzymatic level for various applications.

Chapter 2. Cellular Level: Fabrication of Highly Robust Single Bacteria Biocatalysts

2.1. Introduction

Many intracellular microbial enzymes are produced in quantities large enough to be used in industrial processes. However, the cost for their isolation and purification is quite high. Therefore, it would be of interest to be able to encapsulate directly whole cells such as yeast or bacteria to avoid tedious separation and purification procedures. Bacteria could behave as a “bag of enzymes” and retain enzymes within their natural surroundings. The encapsulation of cells could preserve enzymes’ activity and avoid enzymes’ leaching during repetitive operations.

The number of papers describing the encapsulation of whole cells is very limited, but they show that the process is feasible [17-20]. Encapsulated yeast cells have also been used for environmental protection and metal recovery [21]. They are able to accumulate heavy metals (Hg^{2+}, Cd^{2+}...) from aqueous solutions. The porosity of the gel allows nutrients to reach the cell and by-products to escape [22-23].

A bacteria encapsulation approach was developed to maintain their activities during the encapsulation process, while improving biocatalyst stabilities in harsh environment. The new technology employs material self-assembly to form a protective layer of polymer coating on cell surface under non-denaturing, aqueous conditions. The protective polymer coating significantly increases biocatalyst viability in harsh thermal, different pH conditions, or chaotropic environment, while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. Compared with the conventional catalytic materials prepared by

encapsulating multiple enzyme aggregates within a polymer, such robust single cells exhibit significantly improved catalytic activity and stability. Meanwhile, such robust single cells make the immobilized whole cells much cheaper to use than an immobilized enzyme.

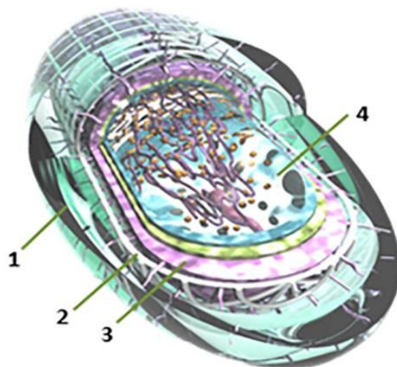


Figure 2 Single-cell microgel (1-microgel shell, 2-polymer interface, 3-cell wall, 4-cell)

In this research, we focus on encapsulating bacteria cells and present a method to fabricate enzymatic catalyst with high activity and enhanced stability directly from the microorganism. To demonstrate the concept, organophosphorus hydrolase (OPH) is selected as the sample enzyme. OPH is an organophosphotriester (OP)-hydrolyzing enzyme that can effectively hydrolyze a series of chemical warfare agents, such as sarin and soman [24-25]. The OPH shows great potential in detection and neutralization of these nerve agents. Protein engineering techniques, such as site-directed mutagenesis, have been used to afford OPH mutant with improved catalytic activity towards several nerve agents [26-27].

Moreover, although modern advances in DNA recombinant technology have paved the way for economic expression of functional proteins in microorganisms [28-29], enzymatic decontamination still has significantly higher cost compared with chemical decontamination agents, placing a hurdle for its application. Purification contributes to the majority of cost for recombinant protein.

Eliminating the purification steps without the compromise of activity will greatly reduce the production cost.

To the best of our knowledge, no studies have yet reported on the use of immobilized bacteria whole cells expressing OPH for hydrolysis of organophosphorus. In my work, recombinant E.coli whole cells harboring the OPH were immobilized with polymers coating using electrostatic adsorption, and then OPH activity, stability and hydrolysis efficiency were studied in this research.

The objectives of this research are listed below.

1) Synthesize highly robust biocatalysts *via* engineering the cell surfaces with polymers coating;

2) Understanding how such coatings enhance their stability and catalytic performance;

3) Fundamental studies of cell-polymer interfaces at the cellular level;

4) The ultimate goal is to facilitate the design and fabrication of robust and effective reagents at cellular level.

2.2. Experiment

2.2.1. Materials and Instruments

Chitosan from crab shells with 75-85% deacetylated, Medium molecular weight and Brookfield viscosity 200–800cps in 1% wt. solution with 1% wt. acetic acid, 25°C, methoxy poly (ethylene glycol) with molecular weight 5000, poly (ethylene glycol) with molecular weight 10000, succinic anhydride, N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC) and

fluorescein isothiocyanate (FITC) were all purchased from SIGMA-ALDRICH and all used as received. Fluorescence images of cells were obtained with a fluorescence microscope (Zeiss, Observer.Z1). UV-Visible adsorption was acquired with a Bechman Coulter DU®730 UV/Vis Spectrophotometer.

2.2.2. Methods

Synthesis of mPEG(5000)-Chitosan (CS-mPEG): The mPEG (5000)-chitosan was prepared by the method described by Prague et al. [30] First, mPEG (5000)-butyric acid was prepared by oxidation of mPEG (5000) with toluene/succinic anhydride. After mPEG (5000) completely dissolved in anhydrous toluene, succinic anhydride was added into the mixture under a nitrogen atmosphere. The molar ratio of succinic anhydride to mPEG (5000) was 1.5:1. The mixture was stirred and heated at reflux under a nitrogen atmosphere for 18h. After cooling to room temperature, the solution was precipitated with excess diethyl ether. Separated the precipitate from the solution and reprecipitated twice from toluene solution with diethyl ether. After drying under vacuum, white mPEG (5000)-butyric acid powder was obtained.

Second, after white mPEG (5000)-butyric acid powder dissolved in anhydrous dichloromethane, N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were added into the solution. The molar ratio of mPEG (5000)-butyric/NHS/DCC was 1:1.25:1.25. The mixture was stirred for 24 hours at room temperature and precipitated with excess diethyl ether. The precipitate was separated from the solution and reprecipitated twice from dichloromethane solution with diethyl ether. After drying under vacuum, white mPEG (5000)-NHS ester powder was obtained. The synthesis of NHS-PEG (10000)-NHS was very similar to mPEG (5000)-NHS, except the molar ratio of butyric-PEG (5000)-butyric/NHS/DCC was 1:2.5:2.5.

And at last, CS-mPEG was prepared by alkylation of chitosan followed by Schiff base formation. mPEG (5000)-NHS ester powder and chitosan with a molar ratio of 0.2:1 were added into a mixture of aqueous hydrochloric acid solution (pH=5.6). The mixture was stirred for 24 h at room temperature. The resultant mixture was dialyzed with a dialysis membrane (MW 12000–14000 cut) against distilled water. CS-mPEG was obtained by removal of unreacted mPEG (5000)-NHS ester from resultant mixture. After lyophilized, white CS-mPEG powder was obtained.

FITC-Labelled mPEG (5000)-Chitosan: The conjugation of chitosan with the labelling probe was carried out by mixing 50μg of FITC per mg of CS-mPEG solution (1%, wt). This solution was stirred overnight in the dark, at room temperature. To eliminate the unbound FITC, the labelled CS-mPEG mixture was dialyzed with a dialysis membrane (MW 12000–14000 cut) against distilled water for 24 hours in the dark.

Performed fluorescence microscopy observations with a Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany) which was equipped with a Zeiss AxioCam HRc attached camera (Zeiss, Oberkochen, Germany) and used the AxioVision 3.1 software (Zeiss, Oberkochen, Germany). All images were acquired at 1300×1030 pixels and 24 bits color depth (8 bits per channel).

Bacterial Strains Growth and Expression: The recombinant strains of E. coli with the constructed plasmid PJK33 were grown at 37°C, in LB medium containing ampicillin (100μg/ml) with vigorous agitation. When cells reached an optical density of 0.8 at 650nm, IPTG (1mg/ml) was added and incubated with vigorous agitation for 15 hours at 16°C. After 15 hours incubation, the medium was collected by centrifugation. Then, the thick precipitation of recombinant cells was collected for lyophilization.

Lyophilization of Recombinant E.coli Cells: A thick precipitation of recombinant E. coli cells, was placed in Petriplates, frozen in liquid nitrogen for 10 minutes and lyophilized without any lyo/cryoprotectant in a Lyophilizer (Lyospeed, Genevac, United Kingdom) at 0.07 mbar for 48 h and stored in vials, at room temperature without applying vacuum, until further use. Viability of lyophilized cells was determined before and after lyophilization by resuspending (1 mg/mL) in distilled water. For paraoxon hydrolysis studies, lyophilized cells powder were prepared and used immediately.

Preparation of Encapsulation of Lyophilized E.coli Cells: For Preparation of encapsulation of the lyophilized E.coli cells, 4 mg/mL lyophilized cells powder were prepared in 600 ml distilled water and then 200ml CS-mPEG solution (1%, wt) was added to resuspended lyophilized E.coli cells mixture. After 30 minutes, 200 ml crosslinker (5%, wt), NHS-PEG (10000)-NHS, was added to the mixture and stored at room temperature for 1 hours. Positive charged CS-mPEG attached to the negatively charged lyophilized cells surface. The “priming” layer was facilitated by electrostatic-driven self-assembly and created a desired microgel coating on the single cells and led to the formation of a microgel in which a single E.coli was encapsulated within the CS-mPEG.

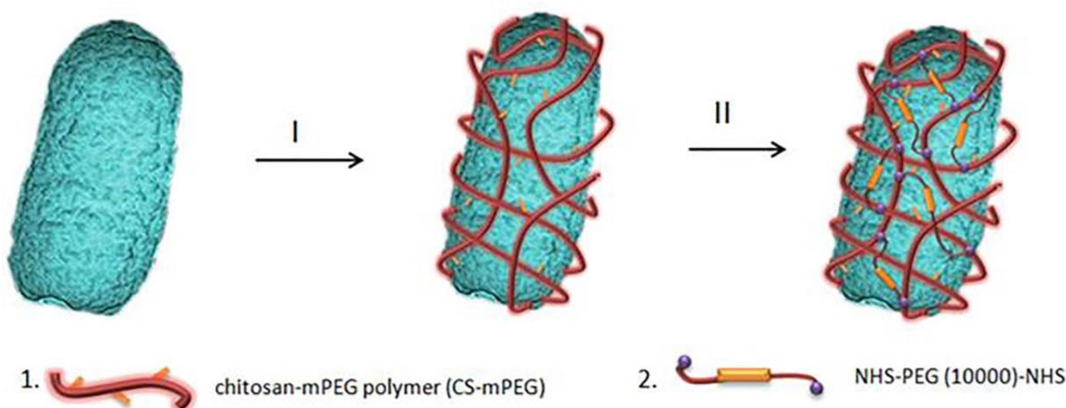


Figure 3 Schematic of a simple, effective approach to encapsulating a single bacterial cell with a protective polymer layer

Enzyme Activity Assay: The activity of the produced enzyme was determined by a colorimetric assay using a UV-VIS spectrophotometer, paraoxon for OPH as the substrate. OPH hydrolyzed the phosphorus-ester bonds in paraoxon, producing a yellow colored p-nitrophenol that can be detected by measuring the absorbance change at 405nm. The reaction mixture for enzyme activity assay included 10μl sample, 485μl 100 mM borate buffers (pH 9.24) and 5μl 75 mM paraxon-ethyls as substrate were added to the reaction mixture at last. The activity assay was preceded immediately after the addition of substrate. The UV-VIS spectrophotometer was used to detect the absorbance change of the reaction mixture at 405nm at 10s intervals for 2 minutes. The hydrolysis rate for free or encapsulated OPH-cells based enzyme was obtained.

Buffers and pH Time Study: 0.1 M solutions of Na_3PO_4, NaH_2PO_4 and Na_2HPO_4 were used to make the buffers from pH 3 to pH 12 and adjusted to the desired pH with 1M NaOH solution. The determinations were made at pH 3, 4, 7, 9, 11 and pH 12. For the pH study, measurements were made, every 10 minutes, in the first 1 hour, and then every 1 hour, in the following 3 hours. Because of the frequent measurements during the first 4 hours, the mixture was stored in cuvettes

at room temperature and room light. After the first 4 hours, they were stored at room temperature sealed with Parafilm (American National Can, Chicago, IL) and tested every 4 hours. Incubation time was 30 minutes at pH 3, 4 h at pH 12 and at the other pH, 12 hours.

Michaelis Constant: For Michaelis Constant assays, free or encapsulated lyophilized cells solution (0.4 mg/ml) was added to paraoxon solution with paraoxon concentrations varying from 0.75μM to 0.75mM. Rate of change of absorbance was measured at 405nm using a UV-VIS spectrophotometer. Data collection at 10s intervals for 2 minutes was allowed for each sample. GraphPad Prism Software was used to get Michaelis Constant K_m.

All reported values were obtained from experiments repeated at least three times, wherein variation between the experiments was less than 15%. Each treatment was carried out with three replicates and the values reported were means with standard error.

2.3. Results and Discussions

The fabrication of the biocatalyst is through a three-step process. OPH is overexpressed by recombinant E.coli. The synthetic approach for E.coli microgels is illustrated in Figure 4. After recombinant E.coli with OPH lyophilized (Step 1), chitosan-mPEG polymer (CS-mPEG) is absorbed on the bacterial surface (Step 2). A following crosslinking step with NHS-PEG (10000)-NHS stablizes the polymer protection layer (Step 3), yielding E.coli microgels.

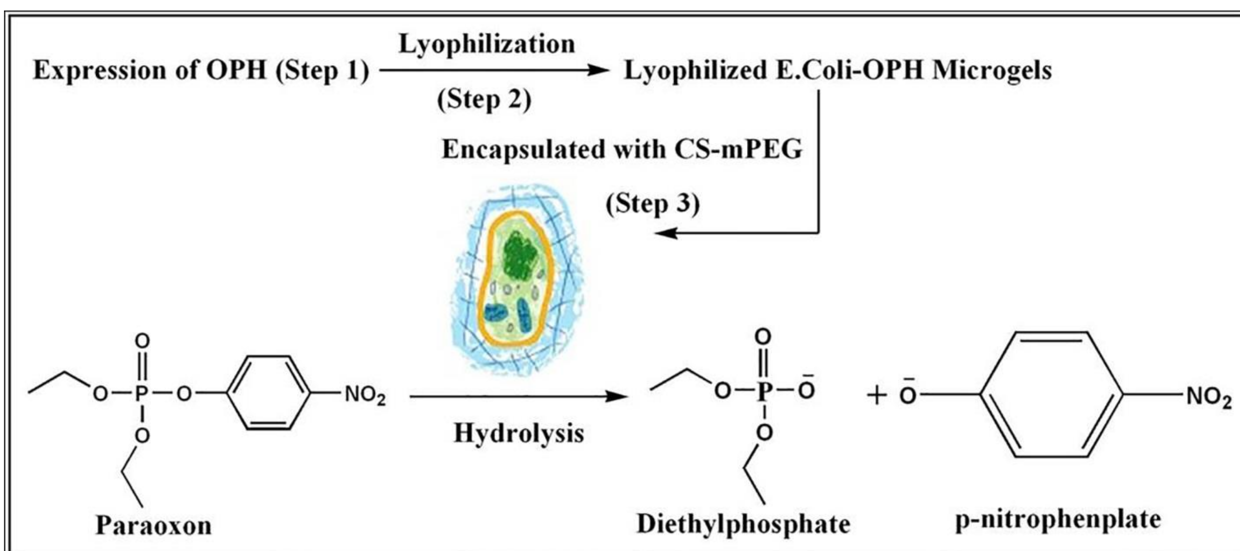


Figure 4 Schematic of encapsulating E. coli single cells using a three-step approach

Deposition of the "priming" layer was facilitated by electrostatic-driven self-assembly. Polymerization process created the desired protective coating on the lyophilized cells. Figure 5 shows a fluorescence image of E.coil microgels. The treated cells were visualized under optical microscope. Because the length scale of the polymer-cell composite was ~2μm, we referred to these as microgels in contrast to the nanogels.

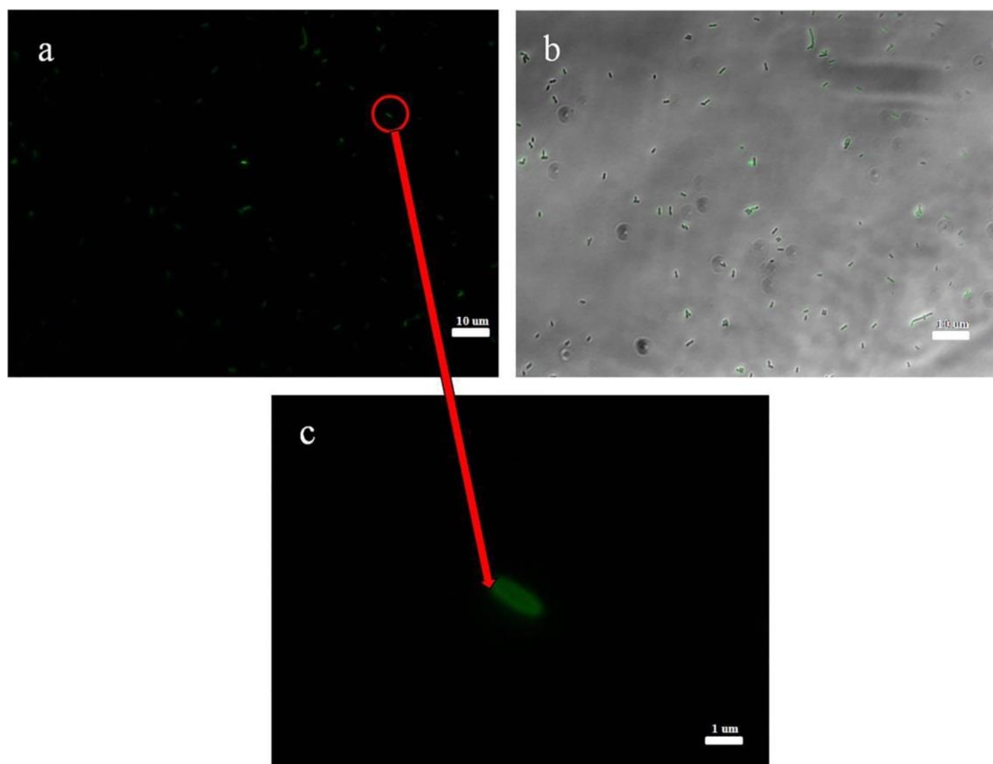


Figure 5 Fluorescence microscopy images (FITC-Labeled) of encapsulated lyophilized E,coli with CS-mPEG

Paraoxon, a model organophosphate nerve agent, is around 70% as potent as the sarin. In addition to paraoxon, other commonly used organophosphates, such as diazinon, coumaphos, and methylparathion are also hydrolyzed efficiently by OPH. Herein, paraoxon is as a sample substrate in this research.

The activity test was based on monitoring UV-Vis spectrum change of the hydrolyzed product p-nitrophenol from enzyme substrate paraoxon-etheyl at 405 nm. Typically, 10 μl 1 mg/ml E.coli microgels containing solution were added to 100 μl pH 8.5 HEPES buffer with 0.75 mM paraxon-ethyl. The light absorption change was monitored for 5 min and the rate of the reaction was determined by measuring the slope of ABS vs time plot. The molar coefficient absorbance of p-nitrophenyl was considered 1.32 x 10^4 $M^{-1}cm^{-1}$ to calculate the enzyme activity.

Hydrolysis of paraoxon by lyophilized E.coli cells followed typical Michaelis-Menten kinetics as also observed for encapsulated lyophilized E.coli cells. The estimated Michaelis–Menten parameters for expressed OPH in unencapsulated (R^2=0.9831) and encapsulated lyophilized E.coli cells (R^2=0.9690) were as follows: unencapsulated lyophilized E.coli cells: K_m, 0.3856 mM; encapsulated lyophilized E.coli cells: K_m, 0.2669 mM. Therefore, the encapsulated process allows rapid transport of substrates into the biocatalyst without significant activity compromise.

Figure 6, Figure 7 and Figure 8 show the OPH stability at different temperature. As shown in Figure 6, the OPH activity in unencapsulated and encapsulated lyophilized E.Coli microgels did not change during the first days, but then increased slightly in the following 4 days. The slight activity increase may be due to an increase in membrane permeability.

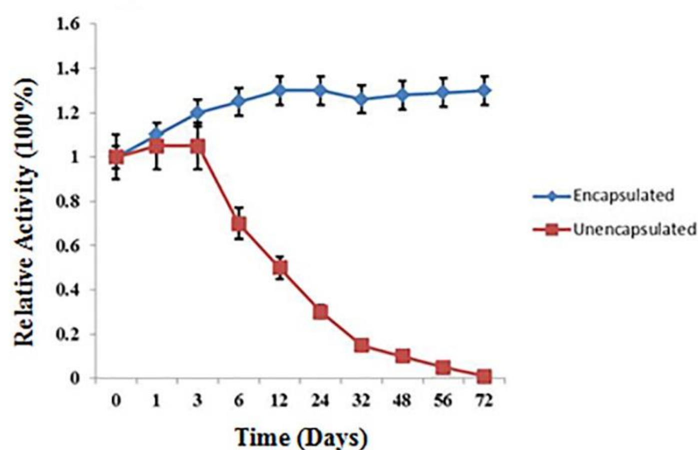


Figure 6 Relative activity of encapsulated and unencapsulated lyophilized E.Coli microgels containing engineered OPH at 25℃ for 72 days in distilled water

As shown in Figure 6, a slight increase in activity of lyophilized E.coli microgels through 6 days was followed by a noticeable decrease through the remaining time period, and became almost zero after 60 days. However, unencapsulated lyophilized E.Coli microgels were effective in its

detection of paraoxon for up to 72 days. This experiment shows that encapsulation leads to no loss of OPH activity within the microgels and the activity is maintained over an extended period of time at aquatic environment at ambient temperature.

Encapsulation of lyophilized E.Coli microgels containing engineered OPH not only affords ease of handling and usage but also prolongs shelf life of the enzyme. Thus, encapsulation of lyophilized E.Coli microgels impart enhanced long time stability which is an important consideration in an environmentally safe biotechnology for contaminated sites.

The stabilities of lyophilized E.coli microgels containing engineered OPH encapsulated with CS-mPEG and free lyophilized E.coli microgels bearing OPH were also investigated at an elevated temperature. Figure 7 shows relative activity of encapsulated or unencapsulated lyophilized E.coli microgels at 42℃ for 48 hours in distilled water. Whereas free lyophilized E.coli microgels with OPH was inactivated after 4 hours, and the activity was decreased to zero after 48 hours (Figure 7). Lyophilized E.coli microgels containing engineered OPH encapsulated with CS-mPEG showed a vivid stabilization effect after 48 hours (Figure 7). Such polymer coatings enhance their stabilities and catalytic performance in high temperature (42℃).

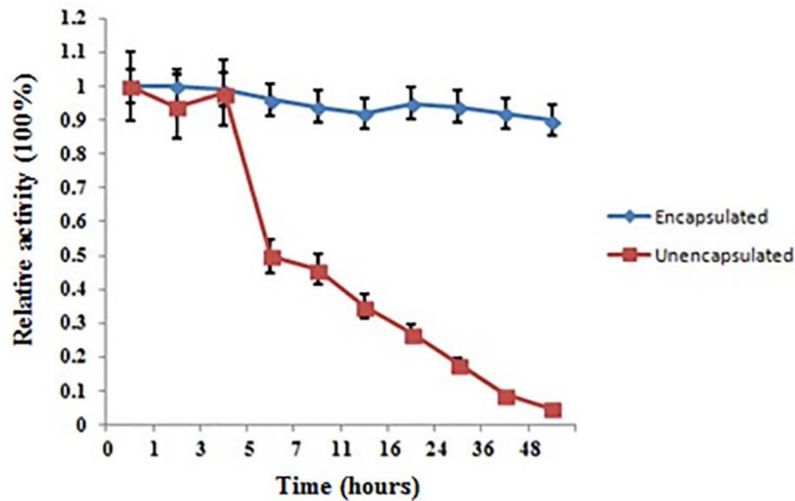


Figure 7 Relative activity of encapsulated and unencapsulated lyophilized E.coli microgels containing engineered OPH at 42°C for 48 hours in distilled water

The protective polymer coating can significantly increase biocatalyst viability in harsh thermal or aquatic environment, while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. Compared with the conventional catalytic materials prepared by encapsulating multiple enzyme aggregates within a polymer, such robust single encapsulated cells exhibit significantly improved catalytic stability and activity.

In addition to the stability of unencapsulated or encapsulated lyophilized E.coli microgels containing engineered OPH in aqueous solutions, the stabilities of lyophilized E.coli powder were investigated by measuring the activity in the following 10 days at 25°C and 42°C. Lyophilized E.coli powder containing engineered OPH showed a vivid activity after 10 days (Figure 8a) at 25°C and 42°C (Figure 8b). Lyophilized recombinant cells could be stored at 4°C, without significant loss of activity for up to 6 months (data not shown).

Lyophilization, without the use of any lyo/cryoprotectant and storage of cells under ambient conditions without vacuum achieved the desired goal. The aforesaid treatments preserved cell integrity, which is essential for maintaining the activity of OPH. This method has three advantages: (a) easy handling and storage of cells at room temperature or high temperature; (b) encapsulation extending shelf life without activity loss and (c) impressively high activity of engineered OPH.

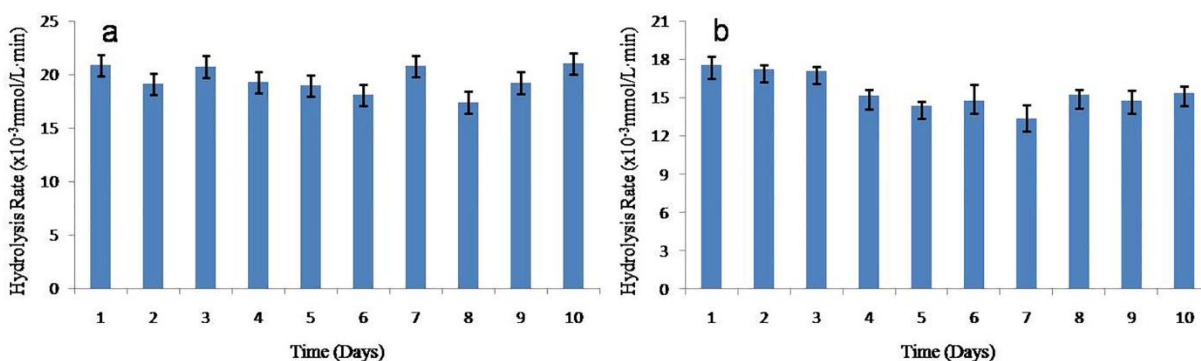


Figure 8 Paraoxon hydrolysis rate by lyophilized E.coli cells powder containing engineered OPH at 25℃ (a) and 42℃ (b) for 10 days

Exciting results were also observed in Figure 9. Excellent enzyme activity was observed in organic solvent DMSO. 87% activity of enzyme was maintained for the encapsulated lyophilized E.coli microgels in 40% DMSO solution compared to the encapsulated lyophilized E.coli microgels in distilled water (Figure 9). Whereas, the free lyophilized E.coli microgels with OPH retained only 48% activity in 40% DMSO solution, as shown in Figure 9a. It may be due to some lysis of the bacteria cell membrane and OPH enzyme leaking out. The leaking naked OPH enzyme loses their activity quickly in DMSO solution.

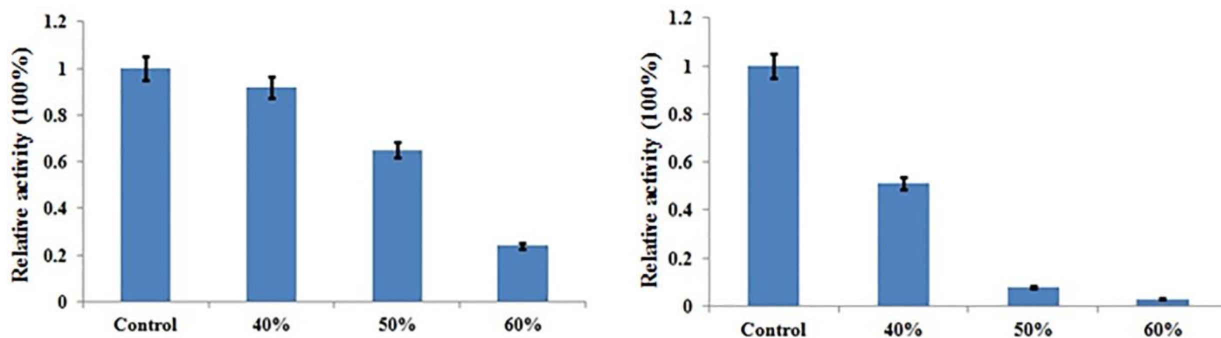


Figure 9 Relative activity of encapsulated or unencapsulated lyophilized E.coli-OPH in various solvent conditions: water (Control), 40% DMSO, 50% DMSO and 60% DMSO

The enhanced stability and catalytic performance in organic solvents could be attributed to the fact that OPH molecules are entrapped in the microscale confined space by the protective layer. Because of the space confinement, protein unfolding (i.e., denaturing) as traditionally caused by organic solvents is significantly restricted. Thereby, significant retention of enzyme activity is achieved. Such polymer coatings enhance their stability and catalytic performance in organic solvent.

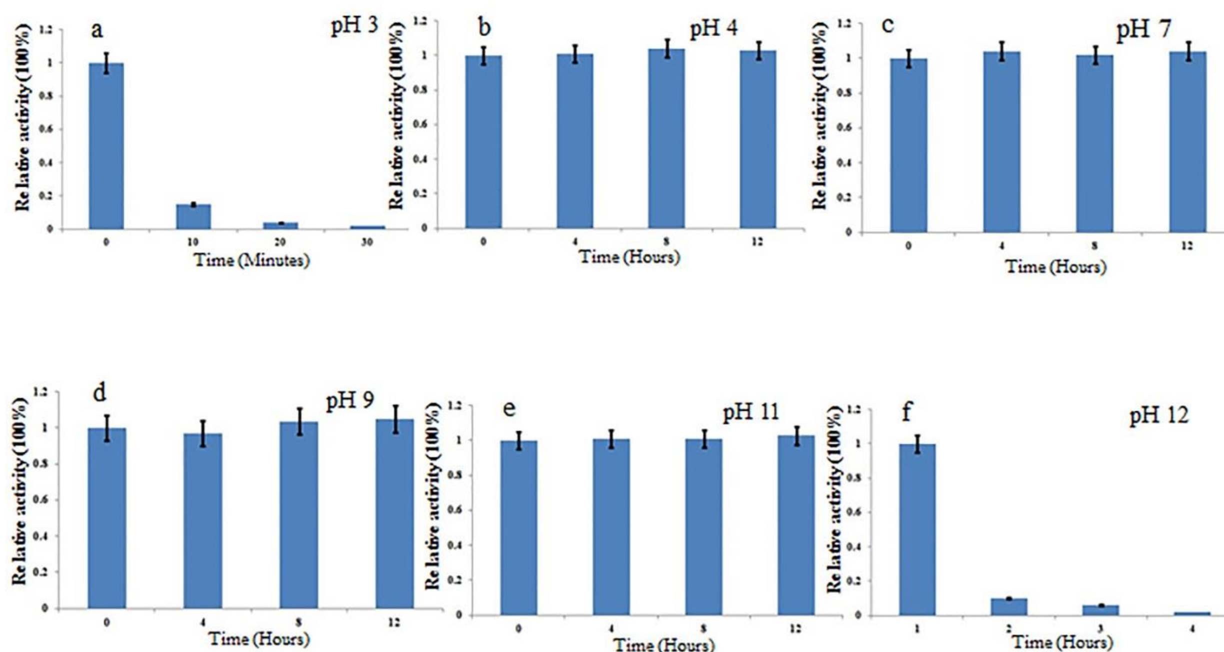


Figure 10 The stability of engineered OPH within the lyophilized E.coli microgels in different pH solution: a) pH 3, b) pH 4, c) pH 7, d) pH 9, e) pH 11 and f) pH 12

The activity in different pH solution is also an important consideration in an environmentally safe biotechnology for contaminated sites. At room temperature, lyophilized E.coli microgels were relatively stable in the pH range of 4 to 11. No effect of pH on the chemical stability was observed (Figure 10b-Figure 10e) within this pH range. Whereas, a sharp decreases in stability was observed at pH 3 and 12 (Figure 10a and Figure 10f). At pH values lower than 3.0 and higher than 12, the stability of lyophilized E.coli microgels was reduced quickly (Figure 10a, Figure 10f). Encapsulated lyophilized E.coli microgels showed enzyme stability very similar to that of unencapsulated lyophilized E.coli microgels. (Data not shown) The results indicate that lyophilized E.coli microgels have a good stability in the pH range from 4 to 11, sufficient to survive in most contaminated sites and suitable for decontamination of Chemical and Biological Warfare (CBW).

2.4. Summary

It becomes clear that the long-term stability of encapsulated lyophilized E.coli microgels, particularly at elevated temperatures (42°C), is stable. This observation could be applied in decontamination of CBW with long-term stability in wet or dry contaminated sites. Therefore, the encapsulated lyophilized E.coli cells bearing OPH are much more robust and resolve the problems associated with the environmental safety. This process retains the activity of enzyme without disturbing the structure of enzyme and the protective polymer coating on the lyophilized E.coli cells can significantly increase biocatalyst viability and stability in harsh thermal, different pH, aqueous environment and organic solvents, while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. Moreover, encapsulated lyophilized E.coli microgels harboring engineered OPH not only afford ease of handling and usage but also prolong shelf life of the enzyme. The use of genetically engineered E.coli microgels offers an environmentally safe biotechnology for detoxification of organophosphorus neurotoxic pesticides and Chemical Warfare Agents from contaminated sites.

The purification contributes to the majority of cost for recombinant protein. Furthermore, the activity of enzyme is compromised in the process of purification and fabrication. Lyophilization, without the use of any lyo/cryoprotectant, preserves cell integrity, which is essential for maintaining almost 100% activity of enzyme. Furthermore, the encapsulation process maintains the activity without disturbing the structure of enzyme.

The microgels maintained native conformation and original enzymatic activity of expressed enzymes. This method is used as a model for the use of genetically engineered, non-viable

encapsulated cells. Recombinant E.coli or yeast is a high-level expression vector to obtain the desired enzyme economically. This method can extend to other recombinant E.coli or yeast. For example, we can use recombinant E.coli to overexpress OPAA and encapsulated lyophilized E.Coli cells to serve as a defensive agents, which is very similar to OPH mentioned in this charter. We also can use yeast to overexpress urate oxidase or β-galactosidase and then encapsulated it with polymer to treat metabolic arthritis or lactose intolerance throught oral administration.

Chapter 3. Enzymatic Level: Encapsulation of Enzymes with Zwitterionic Monomers

3.1. Introduction

Combining of oppositely charged moieties, zwitterionic polymer has behaved high resistance to non-specific protein adsorption [31]. The polymer has been tested as an anti-fouling material widely, which has both positive and negative charged groups but no net charge. It shows that graft the phosphor-lipid zwitterionic polymer on surface produce effective proteins or platelet adhesion resistance [32-34]. According to this characteristic, quick recognition of immune system is not valid to zwitterionic polymer, and therefore, blood clearance exhibits delay. This can be used as hydrophilic components of nano-particles in drug delivery applications [35].

3.1.1. Existing Methods

In order to achieve this objective, surface modification of enzymes is generated. This objective can be concluded as minimizing undesired interactions to reduce cell or protein adsorption [36]. The traditional method to modify the enzyme with zwitterionic polymers is grafting the hydrophilic zwitterionic polymers to the enzyme surface thought the covalent bonding. The covalent bonding of polymer chains to enzymes surface can be achieved through [37]: (i) “grafting-to” reaction, and (ii) “grafting-from” reaction.

“Grafting-to” reaction: Coupling polymer molecules are used to take reaction of “Grafting-to” the enzyme surface. The process of combining surface of enzymes with zwitterionic polymers functional layers can be completed through: 1) Coupling to the enzyme reactive sides directly. 2)

Vinyl, carboxyl or other reactive groups are introduced to the enzyme surface. The reaction of enzymes with acyl chloride or carboxylic groups is described to attach zwitterionic monomers to enzyme surface. [38-39].

"Grafting-from" reaction: Initiation is used to polymerize monomers at the enzyme surface. Note that the reaction of "Grafting-from" through: 1) Physical excitation with radiation or plasma; 2) Initiators in solution and radical transfer; radicals in solution may initiate a homopolymerization and leading to grafting, and 3) Adsorption of a photoiniator on the surface and selective UV excitation [40].

It has been proved by Jiang and his research group that zwitterionic polymer is potential alternatives to PEG for nanoparticle coating and protein conjugation. Note that Mukherjee and his research group proved that even though reticuloendothelial system solely clear positively or negatively charged nano-particles, gold nano-particles show enhanced tumor accumulation and prolonged circulation time, together with zwitterionic material coating [41]. Since the Enhanced Permeability and Retention effect (EPR effect), nano-paritcles can be accumulated in solid tumors passively [42].

3.1.2. Limitations of Existing Methods

The chemical immobilization can encapsulate the enzyme effectively but the immobilization is often achieved at the expense of the enzyme activity during the immobilization process. The distribution of enzyme structure may cause rapidly decrease of the enzyme activity. The chemical immobilization method is not universal method for all enzymes. Naturally uncharged monomers are biocompatible and nontoxic, but they are incapable of encapsulating the enzyme.

The charged monomers can attach to the enzyme surface thought the electrostatical interaction and encapsulate the enzyme effectively. But the charged polymers have the cytotoxicity. If we want to use the uncharged monomers to encapsulate the enzyme, we need to modify the enzyme surface in order to give the enzyme reactive site and then conjugate polymers onto enzymes. This method can encapsulate the enzyme effectively but it easily denatures the enzyme during the encapsulation process. The contradiction between the encapsulation efficiency and the enzyme activity (or cytotoxicity) impedes the development of a universal method to encapsulate the enzyme with high enzyme activity. In this charter, we developed a universal immobilization method for most of enzymes, especially for unstable enzymes.

Ultra-hydrophilicity of zwitterionic polymer is given by the combination of oppositely charged moieties, which maintains the neutrality of overall charge contemporarily. Therefore, due to the non-specific protein adsorption and minimizing mammalian cell or bacterial adhesion, zwitterionic polymer is treated as an alternative to the expansively used PEG for non-fouling applications [43-44]. The neutral zwitterionic polymers are biocompatible and nontoxic, but they are incapable of interaction with enzymes. However, upon the addition of divalent metal cations even zwitterionic polymers can interact with negative DNA or biomacromolecule and then form a new complex with a new charge balance [45- 46].

Between DNA and lipid bilayers consist of dipalmitoylphosphatidylcholine (DPPC), there are evidences to prove that the calcium-induced formation of complexes [47-49]. More complicated formations have been observed with other kinds of lipids, such as 1, 2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) with other divalent cations, including Mg^{2+}, Mn^{2+} and others [50-55].

Considerable diversity is investigated in structure, stability and phase behaviors, depending on the option of the lipids and divalent cations [40].

3.1.3. Innovation

Zwitterionic monomers or polymers were used in this research to encapsulate positive or negative charged enzymes and maintain enzyme activities during the encapsulation process. Upon the addition of divalent metal cations, the zwitterionic monomers or polymers acquire the ability to form complexes with enzymes. And then, crosslinkers were introduced to stabilize the complex. New complexes have an efficient antibiofouling property, which may apply to biomedical field. In this charter, we used zwitterionic monomers (with divalent metal cations-induced) to encapsulate different enzymes and studied the retaining of their activity, stability and antifouling property.

3.2. Experiment

3.2.1. Materials

MPC (2-methacryroyloxyethylphosphorylcholine) is the most common zwitterionic monomer for free-radical polymerization. It is our most basic monomer for nanogel synthesis. However, MPC has a critical drawback. It is incapable of encapsulating the enzyme without vinyl-covered modification because of neutral charged property. Upon the addition of divalent metal cations, MPC acquire the ability to form complexes with enzymes. And then, crosslinkers was added to stabilize the complex. New complexes have an efficient antibiofouling property, which is suitable for biomedical applications.

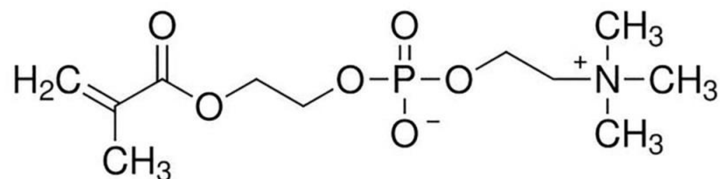


Figure 11 The molecular structure of 2-methacryroyloxyethylphosphorylcholine (MPC)

3.2.2. Methods

As mentioned above, the method we proposed could be applied to most enzymes, especially for unstable enzymes, to form antibiofouling surfaces. Dynamic Light Scattering (DLS) was used to characterize the zeta potentials and sizes distribution of the nanogels.

Encapsulation Procedure: In order to minimizing disturbance of enzyme structure, we herein envisioned a novel delivery platform based on zwitterionic polymer layer nanocapsules that consist of a single-protein core and thin antibiofouling polymer shells. Generally, the preparation of nanogels is a two-step procedure (Figure 12). Zwitterionic monomers are added into the reaction solution to allow attaching to the protein surface with electrostatic attraction effect induced by divalent metal cations. Then, crosslinkers (BIS), initiators (APS) and redox pair (TEMED) are added to the reaction solution and in-situ polymerization was carried out at room temperature with redox pair initiated aqueous free radical polymerization. Both two steps are conducted in aqueous environment. Applying an additional crosslinking process after complex formation stabilizes the complexes. With this protocol, we synthesized a series of nanogels with various enzymes.

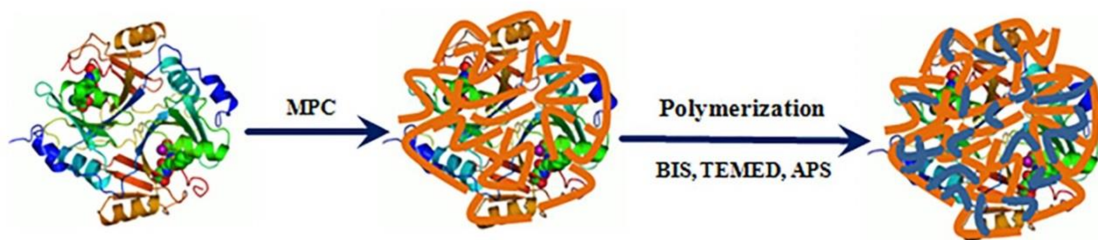


Figure 12 Schematic of encapsulating enzymes through polymerization reaction

Agarose Gel Electrophoresis Test: Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most molecular biology research laboratory routinely uses agarose gel electrophoresis for the analysis of proteins and nanogels. It has been pointed out that the movement of nanogel can be affected by different factors, including the electrical field strength, the agarose concentration, and more importantly, the sizes of protein or nanogel. Meanwhile, the speed of nanogel movement is anti-proportional to the size. In this charter, agarose gel electrophoresis was used to determine the presence and electric property of nanogels.

In Vitro Study: Reducing nonspecific absorption of biomacromolecules since they attract immune system cells and increase non-specific uptake of circulating nanoparticles by macrophages and organs in the mononuclear phagocyte system (MPS), such as the liver and the spleen, which lead nanoparticles to be removed from circulation before reaching their tumor targets, resulting in a substantial reduction in targeting efficiency in nanoparticle-based diagnostics and therapeutics [56-59]. To reduce non-specific binding, zwitterionic polymers with an optimal molecular weight were introduced onto the enzyme surface. Meanwhile, encapsulation was an effective method to stabilize enzymes in aqueous medium through steric effect.

Macrophages were selected for testing non-specific cell uptake of the polymer-coated enzymes. Cells were seeded into a 96-well chamber slide and left overnight. After exposure to nanogels (label with rhodamine B) at concentrations of 0.1 mg/ml for 3 h at 37 ℃, cells were washed with PBS. After being washed three times with PBS, the image was visualized via fluorescence microscopy.

3.3.Results and Discussions

Agarose Gel Electrophoresis Test: In this experiment, we investigated the electric property of nanogels with zwitterionic polymers surface. Proteins we used in this experiment are negative charged proteins (ovalbumin (OVA), Enhanced Green Fluorescent Protein (EGFP) and Bovine Serum Albumin (BSA)) and positive charged protein (lysozyme). From the results of agarose gel electrophoresis, we can see that negative charged proteins (ovalbumin, EGFP and BSA) move to the positive electrode and positive charged protein (lysozyme) move to the negative electrode. However, all nanogels stay in the gel hole without moving compared to the native proteins. The results indicate that the zwitterionic polymers can shield the protein charge and give the enzyme neutral charged property. These neutral nanogels may increase the circulation time of proteins in blood circulation system and reduce the immune response.

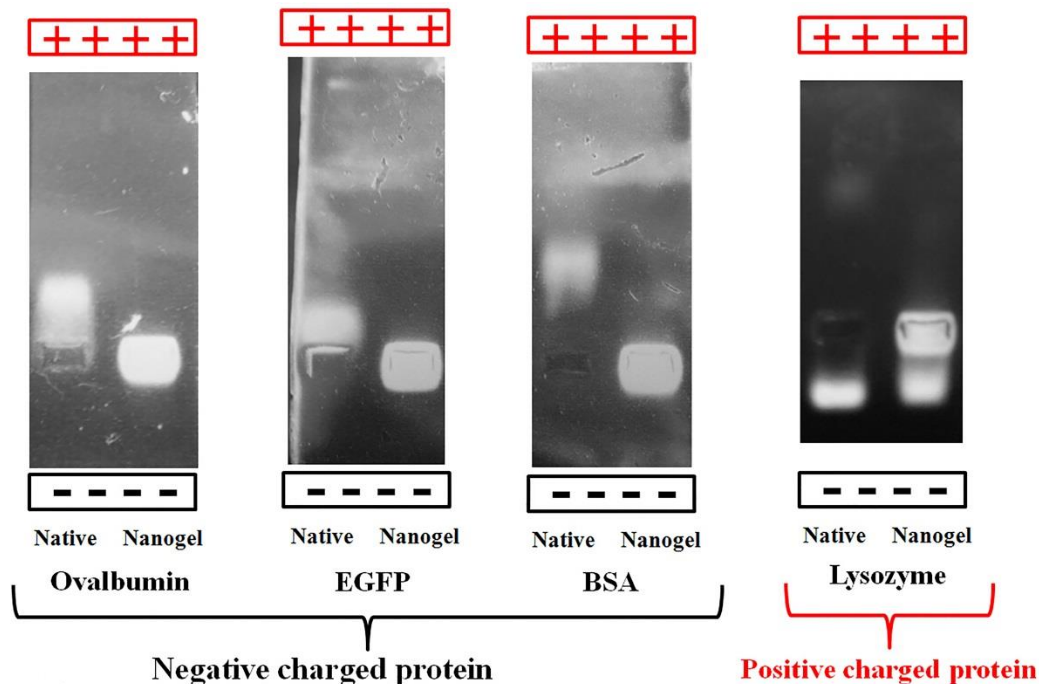
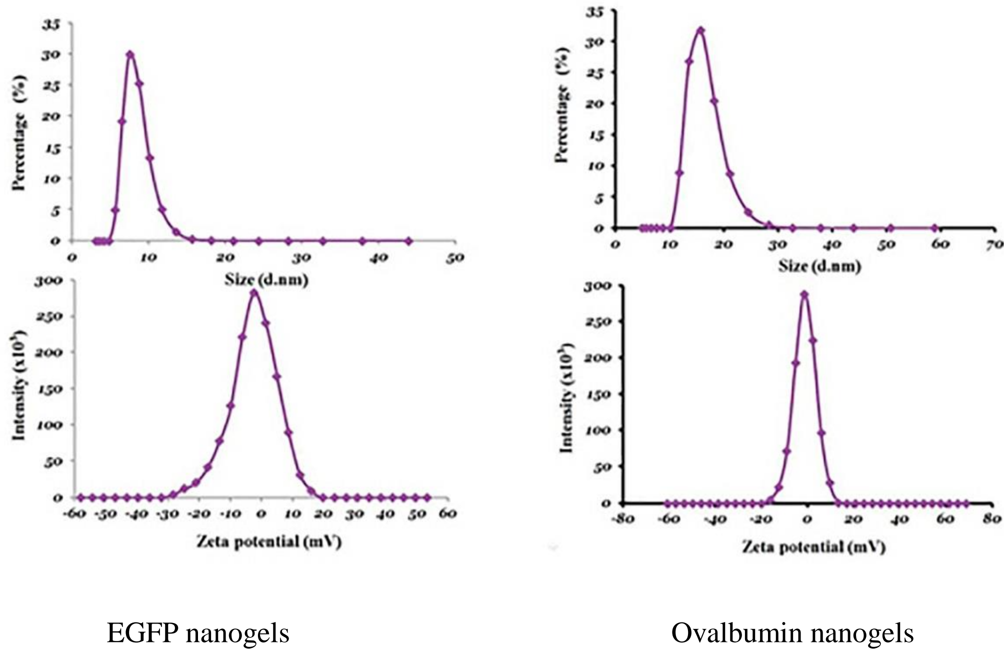


Figure 13 Agarose gel electrophoresis for the analysis of proteins and nanogels electric property

Zeta Potentials and Sizes Distribution: Zeta potentials and sizes distribution of nanogels were tested by Dynamic Light Scattering. Figure 14 shows the size and zeta potential distribution of EGFP and ovalbumin nanogels. From the Figure 14, we can see that the zeta potentials of nanogels are all around 0 mv and the sizes distribution of nanogels are between 10 nm and 30 nm. The neutral property and sizes distribution is suitable for long circulation in blood circulation systems. The surface charge also could be engineered. We can adjust the zeta potentials of the nanogels from negative to positive. Both anionic and cationic monomers can be incorporated onto polymer shells around enzymes. Furthermore, by varying the ratio of ionic monomer versus neutral monomer, we could tune the surface charge. The adjustable surface charge offers us more chance of engineering our enzymes to fit certain application purposes. However, in this charter, we don't discuss positive or negative nanogels and only discuss neutral hydrophilic nanogels for long circulation purpose.



EGFP nanogels Ovalbumin nanogels

Figure 14 Size distribution and zeta potential of EGFP and ovalbumin nanogels

Activity Test: We herein envisioned a novel delivery platform based on non-covalent bonding. We encapsulated the luciferase, catalase and uricase with MPC through free radical polymerization. Figure 15 provides the data that this strategy can preserves 75% of the original activity for the luciferase and up to 95% of the original activity for the catalase and uricase after encapsulation. For all the enzymes we've tested, this strategy gives a satisfactory residual activity. This procedure is generally applicable to most of enzymes, especially for unstable enzymes.

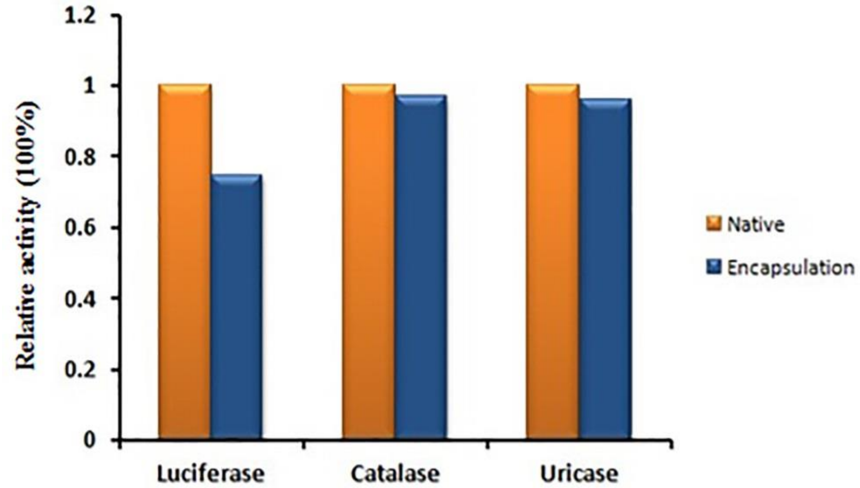
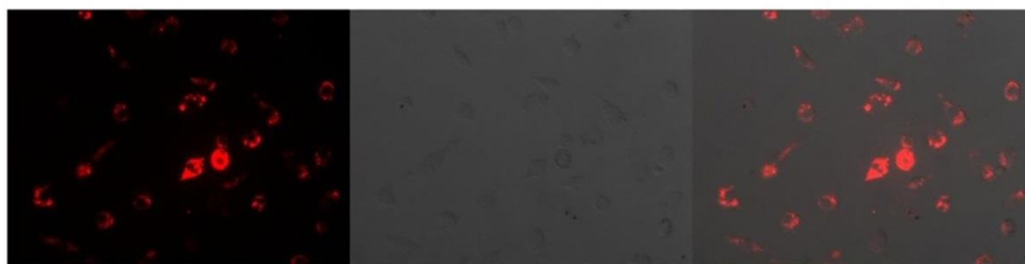
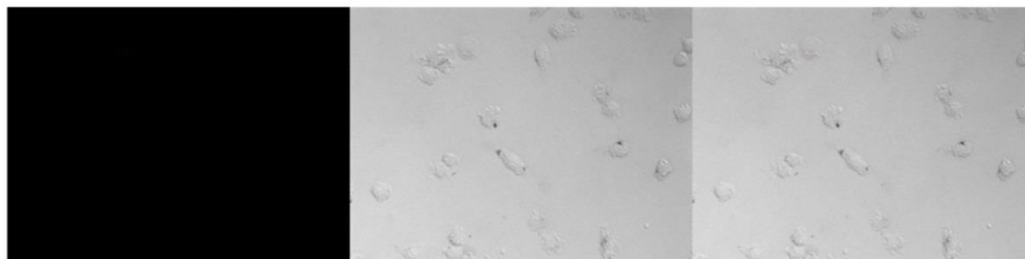


Figure 15 The relative activity of the luciferase, catalase and uricase after encapsulation

In Vitro Study: From the macrophages fluorescence microscopy, we can see that, the macrophage can phagocytose native OVA-rhodamine B; however, it cannot phagocytose OVA-rhodamine B nanogels. The evidence shows that the OVA-rhodamine B nanogels have the macrophage-resistant property which is due to the nonspecific absorption surface.



(a) Fluorescent image of Macrophages treated with OVA-Rhodamine B



(b) Fluorescent image of Macrophages treated with OVA-Rhodamine B nanogels

Figure 16 The macrophages fluorescence microscopy for OVA-rhodamine B and OVA-rhodamine B nanogels

3.4. Application

3.4.1. Introduction

It has been shown in recent experiments of bioluminescent proteins that high spatial resolution of 1 mm can be used as imaging agent. In addition, biological tissue is allowed to be imaged with a depth of centimeters, which is able to capture different organs [60]. As one of its medical imaging application, luminescent proteins are investigated. Meanwhile, neither luciferase nor its substrate has been observed to be toxic to mammalian cells. Because electromagnetic radiation signals are attenuated the biological tissue through the "absorption" wavelength range, just some of luciferase proteins are captured in imaging application. Together with the emission of light over

the wavelengths vary from 540 to 615 nm, this process lies outside the absorption wavelength of tissue.

In this section, we present an example about the application of the antifouling nanogels. Luciferase nanogel for tumor imaging is as an example. Spatial information could be generated by bioluminescence imaging, which is able to capture the kinetics of tumor growth, regression and relapse. Angiogenesis is important to investigate tumor progression and the administration of anti-angiogenesis based therapy in the vivo assessment of tumor. It is possible to measure tumor cell early in the disease course at the stages of minimal residual disease in living animals by bioluminescence imaging [61]. The determination of dynamics of biological processes in vivo can be applied in many fields.

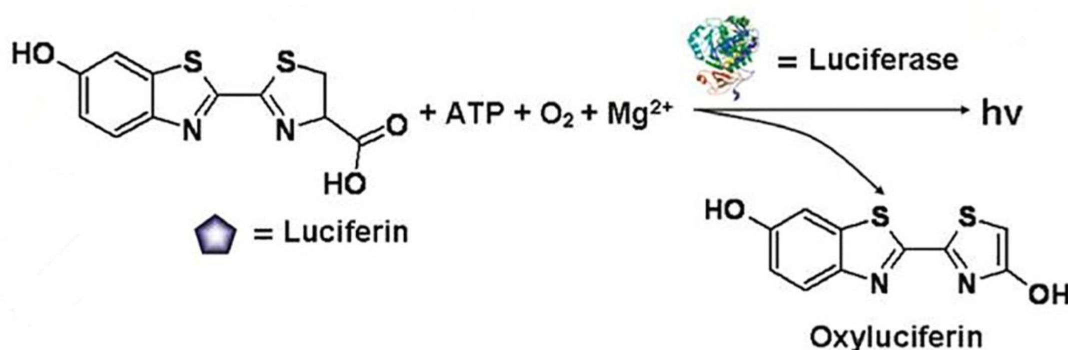


Figure 17 The generation of bioluminescence stimulated by the chemical reaction catalyzed by firefly luciferase

However, the minimization disturbance of luciferase structure may lead to protein denaturation. Naturally forms of luciferase generally have poor thermal stability which compromise practical in vivo imaging applications [62]. Therefore, based on gene transferation, luciferase-luciferin is limit to live animal imaging modality and reporter-gene strategy filed. In animal models, this

technology has been expansively applied to follow tumorigenesis and response of tumors. As a high-throughtput and sensitive imaging modality, bioluminescence imaging is a great tool to monitor tumor growth, as well as the progress of anti-tumor therapies in animal models.

A central limitation of all reporter-gene strategies and live animal imaging modality is the need to introduce the reporter gene construct into the cell populations of interest. Gene transfer using viral vectors or non-viral strategies have been useful; however, they suffer from variable efficiency, especially when attempting to introduce genes into certain primary cells. And this imaging modality is impractical to use in patients.

A new approach to improve biocatalyst stability, while maintaining their activities was envisioned. We focused on surface coating at the single enzyme level without disturb the structure. The monomers used in this experiment were MPC, containing either zwitterionic terminal group, which have shown high resistance to non-specific protein adsorption. Due to this, nanogels with zwitterionic polymers can avoid quick recognition by the immune system and exhibit delayed blood clearance from the body, as such being promising candidates for the hydrophilic components of nanogels in drug delivery applications.

3.4.2. Experiment

Characterizations: Figure 18 is some characterizations of luciferase nanogels, which includes agarose gel electrophoresis, activity test, size distribution and zeta potential distribution.

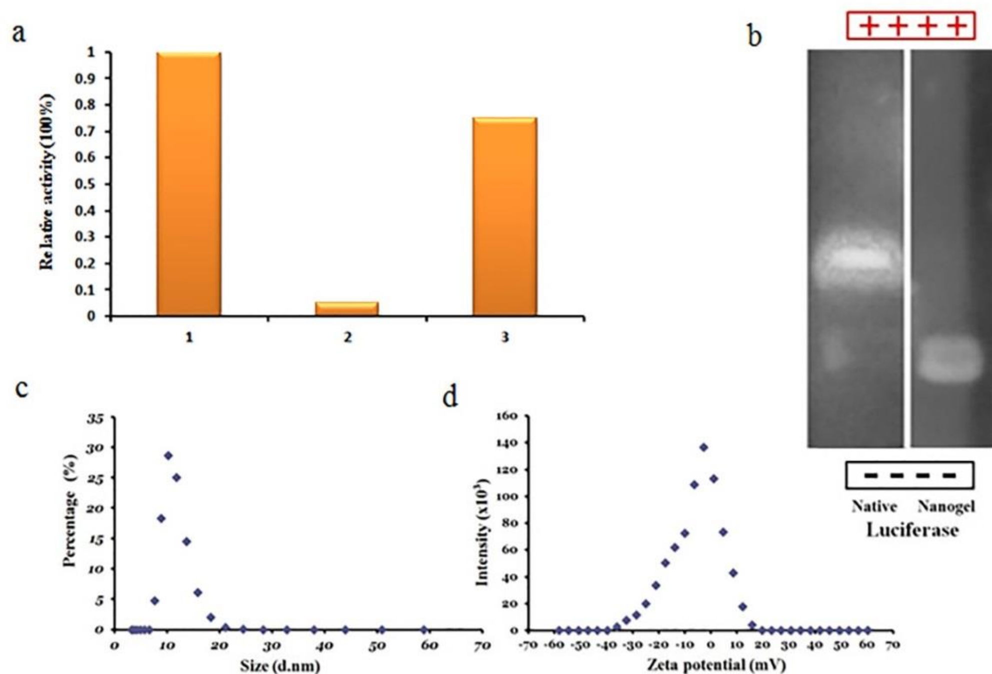


Figure 18 Relative activity, agarose gel electrophoresis, size distribution and zeta potential distribution of luciferase nanogels

Figure 18b is the agarose gel electrophoresis for native luciferase proteins and luciferase nanogels, which provides data on zeta potential for native luciferase proteins and luciferase nanogels. From the results, we can see that the native luciferase proteins are negative charge. After encapsulation, the electric charge of native luciferase proteins is shield by the zwitterionic polymers and luciferase nanogels are neutral charge, which is consistent with the result of zeta potential distribution in Figure 18d. The size of luciferase nanogels is about 12 nm (Figure 18c), which is suitable for long circulation in human body. This method also can retain up to 80% of its original activity. This method is superior to the traditional encapsulation method. For example, if we conjugate vinyl group to the luciferase surface and then encapsulate it with polymers through polymerization reaction, the remaining activity of luciferase is below 5% of its original activity. Luciferase nanogels with neutral surface are ready for tumor imaging.

In Vivo Study: Breast cancer (4T1 cell) model was used for animal studies. The 4T1 mammary carcinoma is a transplantable tumor cell line originally isolated by Fred Miller and colleagues. 4T1 cells were selected without mutagenesis. The 4T1 tumor is highly tumorigenic and invasive and, unlike most tumor models, can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone. We use Luc-FA nanogels to target breast cancer. To modify the luciferase nanogel with folic acid, N-(3-Aminopropyl) methacrylamide hydrochloride (APM) linked folic acid is conjugated to the protein shell via an aqueous free radical polymerization. After the reaction is complete, the conjugated protein is purified via dialysis. We studied the biodistribution of Luciferase-Folic Acid nanogel (Luc-FA nanogel) in mice using IVIS spectrum with intravenous (IV) and intraperitoneal (IP) administration of Luc-FA nanogels and luciferrin. Imaging was performed at time t = 30mins, that is subsequent to the IV administration of Luc-FA nanogels. The image in Figure 19(b) was obtained after 30 mins IV administration of Luc-FA nanogels and shows that the highest accumulation can be observed in the tumor and the liver. Figure 19(c) was obtained 18 hours after the IV administration of Luc-FA nanogels. The luciferase was still in the liver and tumor. In addition, the strong bioluminescence from the liver and tumor of the mouse prove that the encapsulation enhance the thermal stability of the luciferase in mice.

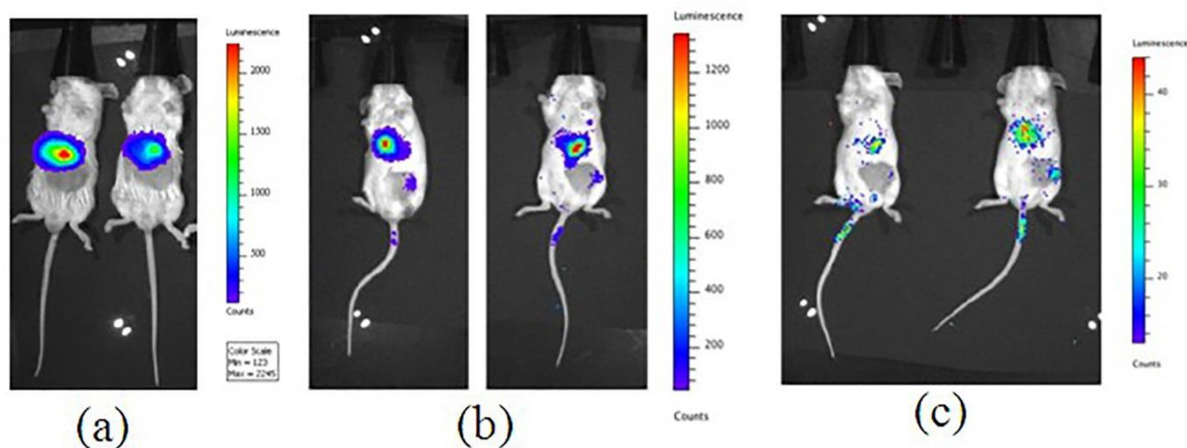


Figure 19 (a) 30 mins post IV administration of Luc; (b) 30 mins post IV administration of Luc-FA nanogels; (c) 18 hours post IV administration of Luc-FA nanogels

Luciferase with zwitterionic polymers coating shows prolonged circulation time. With a prolonged circulation time, the nanoparticles may passively accumulate in solid tumors due to the EPR effect. Therefore, the protective polymer coating can significantly increases biocatalyst viability in harsh thermal while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. Such robust single enzymes exhibit significantly improved catalytic stability, activity and antibiofouling property.

In this section, we investigated the potential application of MPC nanogels to protect and local delivery luciferase for tumor imaging. As a high-throughtput and sensitive imaging method, luciferase imaging is a great tool to detect or monitor tumor growth, as well as the progress of anti-tumor therapies in animal models. However, luciferase by itself is not stable which largely hinder the application of this method. MPC were used in this experiment to encapsulate luciferase in order to maintain the activity during the encapsulation process. From the results of agarose gel electrophoresis, we can see that these luciferase nanogels are around 12 nm in size and the electric charge of native luciferase proteins is shield by the zwitterionic polymers to yield

a neutral charge surface. In vivo studies, folic acid conjugated luciferase nanogels showed that these nanogels have been circulated in the blood for increased time and accumulated in the tumor sites.

3.4.3. Suggestions for Future Experiments

According to previous in vivo study of folic acid conjugated luciferase nanogels, we found out the first limitation of using this method was the fact that the accumulation of these luciferase nanogels in liver was too high. Future study may focus on decreasing the accumulation of these nanogels in liver by enhancing the antibiofouling property of these nanogels. To achieve this goal, different polymers with dramatic antibiofouling property could be tested and screened to encapsulate enzymes.

Another improvement of these luciferase encapsulated nanogels is to tune the emission wavelength by conjugating potential fluorophores onto the surface of these nanogels by taking advantage of Bioluminescence Resonance Energy Transfer (BRET). Conjugating fluorophores may push the responsive wavelength of these luciferase nanogels to much longer wavelength. Long wavelength light has much greater penetration of the tissue so even tumors in deeper tissue could be detected outside the body, which is especially important for detecting tumors in deep tissue. For example, Quantum Dots (QDs) of CdTe could be used to conjugate to the luciferase nanogel surface. These water soluble QDs having broad emission wavelengths of 696 nm, 724 nm, and 754 nm could be attached to the luciferase nanogels surface to create a series of QDs conjugated nanogels to broaden the emission wavelength range. This method could make it possible to use Luc-FA nanogels to detect and monitor tumors even in much deeper tissues.

Another future experiment is to track the metastasis of 4T1 breast cancer cells by using Luc-FA nanogels in a long-term circulation time frame. In our previous in vivo Luc-FA nanogel studies, we have used the 4T1 breast cancer cells as our animal models. 4T1 breast cancer cells are a transplantable tumor cell line originally studied by Fred Miller and his colleagues. One advantage of using 4T1 breast cancer cells is that these cells are highly tumorigenic and invasive. These properties make these cancer cells spontaneously metastasize from the primary tumor sites into multiply distant sites such as bones, brain, liver, lung and especially lymph nodes and blood. This in turn provides a way to target metastasize breast cancers by using Luc-FA nanogels. It would be worthy to study whether luciferase nanogels could accumulate in these distant sites besides primary tumor sites to provide a way to observe the metastasis of these 4T1 breast cancer cells. Our last imaging was at a relatively early stage of the breast cancers. However, by using Luc-FA nanogels, we may be able to detect the metastasis signals at the late stage of the tumor progression. Then, a lot of useful information could be collected and analyzed to understand the cancer progression.

3.5. Summary

In this charter, we focused on surface coating with hydrophilic zwitterionic polymers at the single enzyme level without disturb the enzyme structure. The protective polymer coating can significantly increases biocatalyst viability while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. The zwitterionic polymers layers have an efficient antibiofouling property which reduce the protein or cell adsorption, reduce immune response and prolong the circulation time of enzyme-based nanogels in blood circulation system. The antibiofouling property is important in biomedical field. These studies indicated that this

method can largely maintain the activity of enzymes during the encapsulation process and give the enzyme antibiofouling property which increases nanogels' circulation time in blood circulation system and accumulation in tumor sites, which provide us a powerful tool for enzyme-based cancer therapy.

Chapter 4. Conclusions

Enzymes are always considered as the great gifts from nature. The increasing development of modern industry and medical science calls for new functional enzymes to meet their demand. Existing methods to modify enzymes' surface are easily to demolish enzymes' activity. The minimization disturbance of enzymes' structure may lead to denaturation. The contradiction between the encapsulation efficiency and enzymes' activity always exists.

As a summary, I successfully developed two novel strategies that simultaneously maintain the activity of enzymes during the encapsulation processes, improve enzymes' stability and introduce enzymes with new functionality at cellular or enzymatic level.

At cellular level, a bacteria encapsulation approach is envisioned to maintain their activities during the encapsulation process, while improving biocatalyst stabilities in harsh environment. I introduced polymers shell to the bacteria cell surface under non-denaturing, aqueous conditions. The protective polymer coating significantly increases biocatalyst viability in harsh thermal, different pH, or organic solvent environment, while allowing rapid transport of substrates into the biocatalyst without significant activity compromise. Enzymes are kept in their biological conditions without disturbing their structure, resulting in highly retaining enzymes' activity. Purification steps, which contribute to the majority of cost for recombinant protein, are eliminated. This strategy greatly reduces the production cost, making the large scale enzymes-based production possible. Bacteria microgels, I fabricated in this research, are very suitable for the application of detection or decontamination field.

At enzymatic level, I used zwitterionic monomers (with divalent metal cations-induced) to encapsulate different enzymes successfully. In this strategy, there is no covalent bond between the enzymes' surface and polymers shell and the enzymes' structure is maintained at the maximum. Therefore, the retaining of enzymes' activity is achieved during the fabrication process. Meanwhile, zwitterionic polymers shell increases enzymes' stability and gives enzymes antifouling property. The electrical property of enzymes' surface can be tuned from positive to neutral or negative, which extend the application of enzymes. In this thesis, the luciferase nanogel with zwitterionic polymers shell is as an example to explain this strategy. This strategy can apply to almost all enzymes. Nanogels with zwitterionic polymers shell have long circulation property which is the key point of cancer therapy. Therefore, this method has wide application in bio-medical field.

Overall, my research establishes two novel strategies to stabilize enzymes and create new surface. There is no covalent bond between the enzyme and the polymer shell. Therefore, without disturbing the enzyme structure, the resulted macrogels or nanogels maintain enzymes' activity at the maximum. The resulted macrogels or nanogels also gain enhanced stability and various new functionalities. This work opens an avenue for various enzymes-based applications, such as therapeutics, analysis and bio-catalysis.

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