博士論文（要約）

Development of immobilization methods for a cytochrome P450 monooxygenase system

(シトクロム P450 モノオキシゲナーゼシステムの固定化法の開発**)**

Tan Cheau Yuaan

タン　チャオ　ユエン

Note:

- **Chapter 2 has been published in its entirety (except the results on demonstration of small gel particles formation) in *Scientific Reports* 5:8648 (2015).**

- **Chapter 3 is scheduled to be published within 5 years**

Chapter 1 General Introduction & Objective

Cytochrome P450s (P450s or CYPs) represent one of the largest gene superfamily encoding heme-containing enzymes in all domains of life. The first of this enzyme was discovered in liver microsome as a pigment bound to carbon monoxide (CO) which gave an intense absorption band at 450 nm thereby founding its name. The unusual spectral features of P450s is caused by a cysteine thiolate group occupying the fifth axial ligand of the heme prosthetic group leading to P450's classification as a heme-thiolate protein. Many P450s catalyze multiple reactions, and as such, common method for naming enzymes is insufficient for this group of enzymes and a systematic nomenclature has been developed on the basis of structural homology by Nelson and co-workers.

With respect to their catalytic cycle, P450s require two electrons which are sourced externally from NAD(P)H cofactors and some P450s utilize redox partners, ferredoxin and ferredoxin reductase to transfer these reducing equivalents to the catalytic center. In general, the organization of corresponding redox partners classifies a P450 and can be divided into ten classes. Among them, the class I bacterial P450s was targeted for immobilization studies because of their extensive involvement in biotechnological applications such as drug synthesis, gene-directed enzyme prodrug therapy, horticulture industry, biosensors and bioremediation.

There were several previous attempts to immobilize class I P450s with the first report dating back to 1988 where purified P450 from *Saccharomyces cerevisiae* was co-immobilized with its corresponding reductase by entrapment in calcium alginate or in polyacrylamide. The effort continued to immobilizing CYP105D1 on ionic exchanger as solid support coupled with cofactor regeneration system to preclude the expensive NAD(P)H necessity. P450 BM3, a rare and self-sufficient natural fusion of reductase and heme domains belonging to class VIII was also immobilized on ionic exchanger and entrapped in a sol-gel matrix. Even though P450 BM3 is catalytically sufficient without the need of redox partners, its immobilization still displayed lower specific activity. These methods mentioned above, though advantageous, were proven to have major drawback of enzyme leakage due to weak physical interaction between solid supports and enzymes or uncontrollable pore size of mesoporous particles which translates to loss of activity with lower processivity. A rather innovative method to immobilize P450s while excluding the need of external cofactors and redox partners which function to transfer electrons to heme center is adsorption on electrode. The electrode was capable of supplying electrons directly to the heme center of P450cam for conversion of substrates to products. Nonetheless, inactive preparation or extreme slow turnover rate was observed with this method likely as a result of inappropriate orientation which inhibited efficient electron transfer. Therefore, these predicaments call for a novel method to immobilize class I bacterial P450s which could have great implications in biotechnological applications.

Herein, this dissertation focused on developing suitable methods for immobilization of class I P450s using P450cam monooxygenase system as model system with retention of catalytic activity and cofactor regeneration.

Chapter 2 Oligomeric proteins-assisted supramolecular assembly of cytochrome P450 and its redox partners

Further development in enzyme immobilization made significant progress such as the formation of cross-linked enzyme aggregates (CLEAs) and bifunctional enzymes with self-assembling properties giving rise to catalytic gels. Inspired by these, a similar approach has been developed to insolubilize P450cam monooxygenase system.

In a previous study, a bacterial P450, its electron transfer protein and reductase were genetically fused to a heterotrimeric proliferating cell nuclear antigen (PCNA) from *Sulfolobus sulfataricus* to yield the protein complex,

PUPPET (PCNA-utilized protein complex of P450 and its two electron transfer-related proteins). The C-termini of all the PCNA subunits are exposed on the same side of the ring giving an edge for fusion to a *Pseudomonas putida* P450 (P450cam), its electron transfer protein, putidaredoxin (PdX), and specific reductase, putidaredoxin reductase (PdR), thereby co-localizing the enzymes on the PCNA ring. The electron transfer protein carries electrons from the reductase to the P450 in the complex by shuttling them. Therefore, PUPPET works as a single enzyme with high catalytic turnover (500 min^{-1}) as a result of intramolecular electron transfer.

PCNA from *S. solfataricus* is composed of three distinct subunits, PCNA1, PCNA2 and PCNA3, which forms a ring-shaped heterotrimer in a step-wise manner. The feasibility of expression and purification of each of these subunits in *E. coli* provides an advantage for exploitation in the current system which facilitates expression and purification of separate fusion proteins. Upon mixing of the three fusion proteins, the PUPPET complex can be further stabilized by selective intersubunit disulfide bonds as the native PCNA lacks intrinsic cysteine residues.

Extension of PUPPET network to obtain a protein gel can be done by selective linkage between the PCNA rings while maintaining the enzymatic activity. Although it is difficult to selectively cross-link PCNA subunits in PUPPET with chemical reagents, introduction of more than two assembling domains into the PCNA ring would give rise to a supramolecular formation of PUPPET through a selective linkage between the PCNA subunits. Thinking of a bottom up approach, the dimerized PCNA subunits are expected to form a massive supramolecule, in which PUPPET can be found at the assembling points.

In this chapter, a facile and straightforward way is demonstrated to immobilize a bacterial P450 system simply by employing the self-assembly nature of two oligomeric proteins. The PCNA subunit proteins genetically fused to a homodimeric protein can spontaneously assemble to form a supramolecular complex. Phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri* was used to obtain homodimeric PCNA subunit proteins because it exists as a stable homodimeric protein and regenerates NADH, which is consumed by P450-catalyzed monooxygenation. The protein mixture resulted in a water-insoluble gel with monooxygenase activity and achieved a cost-effective monooxygenation process as a result of NADH regeneration by PTDH with phosphite. To date, this is the first report on a bacterial P450 which requires separate redox partners to be immobilized in order to catalyze its monooxygenation reaction with cofactor regeneration.

Chapter 3 Indirect immobilization of cytochrome P450 system using PCNA as mediator

Protein gel formation as an avenue to immobilize P450cam monooxygenase system has been shown to be successful as retrieval of gel from reaction mixture for multiple cycle catalysis coupled with cofactor regeneration was observed. Nevertheless, the catalytic gel displayed low apparent specific activity which was attributed to high diffusion limitation of substrate as evident from its kinetic constants. Lack of specific control over shape and size of the gel which contributes to its activity would limit its application on industrial scale. As such, further development along this line led to modification of the long-established method of adsorption on solid support.

The ferredoxin moiety of bacterial P450 systems functions as a shuttle molecule to transfer electrons between the specific reductase to catalytic heme in P450. This function would have been impeded if the P450 and its redox partners were to be directly immobilized on solid supports. From this front, the demand of an adaptor to immobilize P450s and their electron transfer-related partners on solid support emerged to prevent mobility inhibitory effect.

PCNA from *S. sulfataricus* was proven to be advantageous as a scaffold to enhance the monooxygenase activity of P450cam when fused to C-termini of all three PCNA subunits in our previous study. Further extension of PCNA exploitation was demonstrated in previous chapter where formation of active water-insoluble gel involved the driving force of PCNA self-assembly and PUPPET can be found at assembling points within the gel. However, the low specific surface area of the water-insoluble gel led to high mass transfer limitation thereby reducing its catalytic activity. The method of immobilizing the three-component enzymes on a solid support with a defined shape and smaller size such as surface-functionalized magnetic beads could potentially enhance the specific surface area leading to reduced mass-transfer effect. Therefore, combination of PUPPET formation and immobilization of PUPPET on pre-fabricated support such as NHS-activated magnetic beads seem as a promising strategy to the

current challenge encountered. As such, PCNA will then serve as a mediator in attaching all the three active enzymatic components on a single solid support. In addition, a mutant of PTDH from *P. stutzeri* which is thermostable is introduced into this immobilization strategy to meet the demand of stoichiometric consumption of NADH. The dodecamutant of PTDH was shown to be tolerant to high phosphite concentration in my study, a desirable trait when phosphite is needed to maintain a self-sufficient P450 monooxygenase system.

Chapter 4 Conclusion and perspectives

Immobilization of bacterial P450s and their redox partners has been a great challenge to enzymologists for decades. Several brilliant attempts including but not limited to encapsulation in mesoporous particles coupled with cofactor regeneration for sustaining reducing equivalents supplies and application of electrochemistry to drive catalysis without the need of cofactors or corresponding reductases were proven to be immensely useful for further development. A more recent study demonstrated *in situ* immobilization of P450 BM3 on poly(3-hydroxybutyrate) [P(3HB)] via phasin tag protein. These methods have, in a way or two, their respective drawbacks such as enzyme leaching or denaturation of the enzymes.

In my study, I have explored two different methods to immobilize cytochrome P450cam monooxygenase system. In Chapter 2, I have demonstrated a facile method driven by self-assembly nature of a heterotrimeric PCNA to form a supramolecular gel network. Apart from expression vector construction and protein purification which are also required in many other studies, this current method just requires simple mixing of fusion proteins to obtain immobilized preparations. Recovery of the immobilized enzymes is just as easy – by simple centrifugation. In addition, the system was made more cost-effective when PTDH regenerates NADH thereby sustaining the electron supplies. As a rough estimate, utilization of this system saved the cofactor cost by 60% based on the experiment of oxygen consumption of capped gel not to mention the ability of the gel to hydroxylate the substrate for several cycles. Additional advantage with this system is that it requires only micromolar of protein concentration to form a gel network while other studies with gel formation requires milimolar concentrations.

Meanwhile, the objective of Chapter 3 was to develop a method for overcoming the mass transfer effect of water-insoluble gel observed in Chapter 2. To achieve this, the multicomponent P450cam system was immobilized on a solid carrier via adaptor protein which has not been reported thus far. From the results obtained, I found that immobilizing the three components of P450cam monooxygenase system to magnetic beads through PCNA as an adaptor protein successfully retained the enzymatic activity with the ability to regenerate cofactor as well. The magnetic beads with smaller size offered a higher specific surface area of immobilized enzymes leading to reduced diffusion limitation. This can be interpreted as suitable modification to immobilize proteins involved in cascade reactions using the most common method of immobilization on solid carrier conquering the decades-long challenge.