Synthesis of Enzyme-based Organic-Inorganic Hybrid Nanoflower Particles

Shamini Anboo[1], Sie Yon Lau[*1], Jibrail Kansedo[1], Pow-Seng Yap[2], Tony Hadibarata[3], Azlina Harun Kamaruddin[4]

[1]Department of Chemical and Energy, Curtin University Malaysia, CDT 250, 98009, Miri, Sarawak, Malaysia.

[2]Department of Civil Engineering, Xi'an Jiaotong-Liverpool University, Suzhou, 215123, China.

[3]Department of Civil and Construction Engineering, Curtin University Malaysia, CDT 250, 98009, Miri, Sarawak, Malaysia.

[4]School of Chemical Engineering, Universiti Sains Malaysia, 14300 Nibong Tebal, Seberang Perai Selatan, Penang, Malaysia.

*Email: johnlsy@curtin.edu.my

Abstract Enzyme-incorporated hybrid nanostructures are the immobilization of enzymes and inorganic components that exhibits promising characteristics in various industries. The immobilization of enzymes onto nanomaterial is naturally based to accommodate the enzymatic activity, stability, recyclability as well as their catalytic functions. The designing of these conjugates can improve the overall enzymatic performance by imparting their novel properties onto the system in comparison to conventional free enzymes which experience drawbacks in terms of deactivation or denaturing. A facile and ultrafast method is described in this paper to synthesize a novel enzyme-incorporated lipase/$Cu_3(PO_4)_2$ hybrid nanoflower (NF). The physical properties of the hybrid NF allow easier retrieval which indicates its higher reusability and recyclability value. The enzyme loading capacity was found to be 95.1% whereas, the catalytic performance of lipase/$Cu_3(PO_4)_2$ hybrid NF at the optimal conditions resulted in a specific enzyme activity of 1752 U/g corresponding to an increment of 90.5% to that of free lipase. This indicates that the well-designed lipase/$Cu_3(PO_4)_2$ hybrid NF to be highly efficient in industrial biocatalytic applications. Meanwhile, in future work, we aim to study its operational stability and reusability to enzymatically degrade biopolymers through hydrolysis process.

Keywords: Hybrid nanoflower, Immobilized enzyme, Ultrasonication reaction

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Nanostructures have been ventured plenty as platforms to promote enzyme immobilization to ensure more efficient use of enzymes in comparison to free enzymes. This is because, free enzymes often lose their activity easily, has a higher cost often with a low reusability value [1-4]. Enzyme immobilization is the process of attaching enzymes onto a support to completely immobilize the attached enzyme [5, 6]. It is often accomplished by 4 methods, covalent bonding, adsorption, entrapment and/or cross-linking [7]. The main reasons as to why enzymes show reduced activity is that, (a) the organic solvent used is able to immobilize some parts of the enzyme, (b) ability to block the active site to reduce availability of enzyme, (c) the enzymes are less favorable when attached onto a support matrix and (d) limiting the mass transfer between enzyme and substrates [8-12]. Therefore, there is room for improvement and development of immobilization methods to acts as enzyme carriers to ensure good reusability, stability and enzymatic activity.

Ultrasonically assisted method is a fairly common method in the synthesis of NFs, whereby, the mechanism involves using sound energy to extract desired compounds from sources at ultra-sonic frequencies of greater than 20 kHz. It causes change, both physically and chemically by the forming, growing and collapse of bubbles in a solution [13, 14]. The ultrasonic method under various conditions affects the shape and size of the NFs produced [15]. Some factors which affects the final structure and performance of the synthesized NF includes the ultrasonic waves itself, whereby, high intensity waves prevent the growth of crystal nuclei despite the prolonged duration [16]. Different frequencies may also cause the difference in the structure of the nanoparticle (NP) whether it is 1D, 2D or 3D in shape [15]. Besides, the sonication temperature also affects the process. The typical sonication temperature is between room temperature and 75 ˚C [17]. Higher temperatures allow for higher aggregation of particles whereby, it improves the exfoliation, and delamination [18]. Furthermore, studies show that the ultrasonic amplitude has an effect on the morphology, size and phases of NP produced. Higher wave amplitude decreases the rate of particle growth [16, 19, 20].

Batule and co-workers (2015), developed a facile one-step method to synthesize hybrid NFs incorporated with laccase and copper sulphate as inorganic component. The study showed that the simple and quick method of synthesis produced intricate, high surface-to-volume ratio NF structures [21]. Owing to inspiration and simplicity of preparation, herein, we develop a modified method to prepare lipase/$Cu_3(PO_4)_2$ hybrid NFs.

2. Materials and Methods

Initially, copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$, Reagent grade, Merck) solution was dissolved in water to produce 0.2 M solution. The pH was measured (pH 7.4) before adding mixture to 3 mL of 0.1 M phosphate buffer solution (PBS, BioPerformance Certified, Sigma-Aldrich). Lipase from porcine pancreas was then added to the solution to achieve a concentration of 0.3 mg/mL (Sigma-Aldrich) before sonicating using ultrasonic cleaner (EW-08895-11, Cole Parmer) with frequency 40 kHz 7 mins at 30˚ C. The solution was then centrifuged (Universal 320R, Hettich) at 3600 rpm for 6 mins before vacuum freeze-drying (Labconco, Fisher). Scanning electron microscopy (SEM) (Quattro S, Thermo Scientific) imaging was conducted in order to observe the topography of the NFs. The enzyme loading or immobilization yield was analyzed by measuring protein concentration in supernatant using Bradford assay (1-1,400 μg/mL protein, Supelco) and bovine serum albumin, BSA (Reagent grade, Sigma-Aldrich) as standard solution at absorbance 595 nm using UV-Vis spectrophotometer (DR-2800, Hach). The immobilization yield was calculated using the following equation.

$$\text{Immobilization yield} = (1 - \text{remainder lipase/initial lipase amount}) * 100\% \quad (1)$$

The enzymatic activity of the lipase/$Cu_3(PO_4)_2$ hybrid NFs were conducted using olive oil substrate emulsion and cupric acetate pyridine reagent (CAPR) (Reagent grade, Merck) following the novel works of Mustafa and co-workers (2016) [22]. The chemicals used to form substrates are olive oil, 1% (v/v) Triton-X and PBS at pH 8 in the ratios of 2:1:1 respectively. The CAPR reagent was prepared by mixing

cupric acetate monohydrate (Cu (OAc)2.H2O) and pyridine (Reagent grade, Sigma-Aldrich). Absorbance was measured at 655 nm whereby oleic acid was used as standard solution.

3. Results and Discussion

3.1. Morphology and Particle Distribution of Lipase/$Cu_3(PO_4)_2$ Hybrid NFs

Flower shaped hybrid NPs were successfully obtained as shown in **Figure 1A-B**, whereby there are significant differences in structure between lipase/$Cu_3(PO_4)_2$ hybrid NFs and pure $Cu_3(PO_4)_2$ NFs. The flower architecture is much more visible in the lipase/$Cu_3(PO_4)_2$ hybrid NFs which may be due to the interaction between the inorganic component to form metal phosphate crystals and eventually "bind" onto the enzyme which acts as a precursor [23]. **Figure 1B** shows the structure of $Cu_3(PO_4)_2$ NPs without lipase prepared using the same method. It is observable that the structures have irregular flower-like shapes. In fact, the structures look more closely together and have a rather spiky architecture. This may be due to the lack of precursors for metal phosphate crystals to bind in order to form the structures [24]. The particle sizes vary between enzyme-incorporated lipase/$Cu_3(PO_4)_2$ and pure $Cu_3(PO_4)_2$ NPs, whereby, the difference is rather significant and the average particle size is shown in **Figure 1C**. It is worth noting that, the initial size of $Cu_3(PO_4)_2$ NPs was 3.62 nm whereas upon the immobilization of enzyme, the hybrid NFs grew up to 5.17 μm.

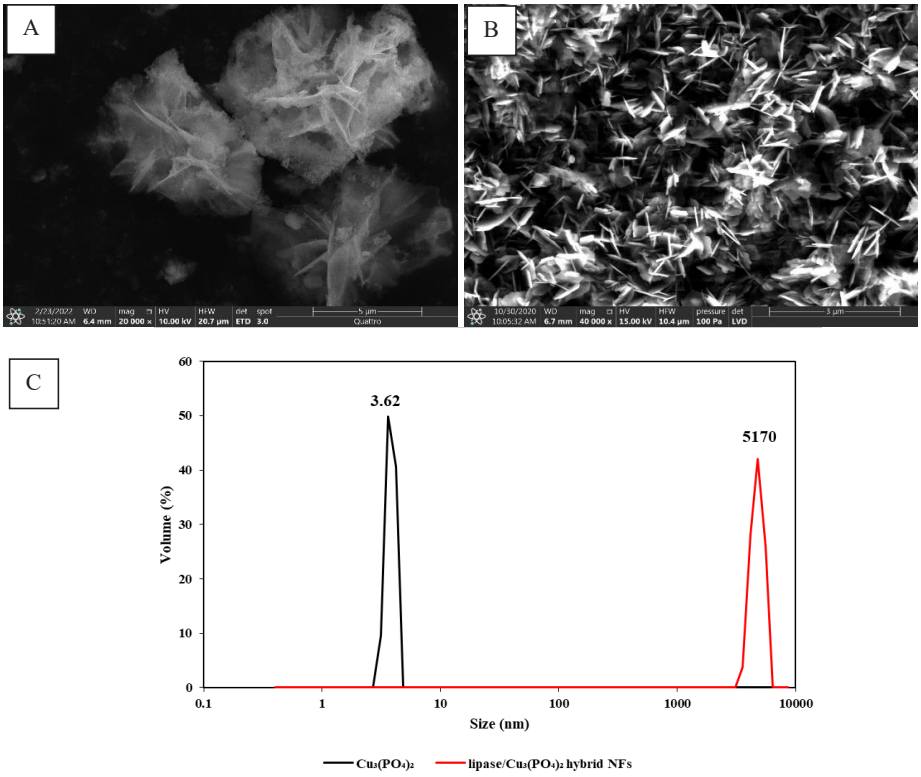

Figure 1 SEM images of NFs: lipase/$Cu_3(PO_4)_2$ hybrid (A), pure $Cu_3(PO_4)_2$ (B); Particle size analysis of lipase/$Cu_3(PO_4)_2$ hybrid and $Cu_3(PO_4)_2$ NPs (C)

There have been no known mechanisms as to how these hybrid NFs are formed to this day due to the lack of study in molecular modelling. However, the following facts can aid understanding to the formation of said materials. Firstly, the reaction between Cu^{2+} and phosphate was conducted simultaneous to the reaction between Cu^{2+} and lipase [23, 25-27]. The products of the above step were metal lipase/Cu^{2+} hybrid nanostructure, whereby lipase acts as the precursors to the formation and growth of copper phosphate crystals which formed plate-like structures. Finally, self-assembly between the plates resulted in a lipase/$Cu_3(PO_4)_2$ hybrid NF structure.

3.2. Enzymatic Activity of Lipase/$Cu_3(PO_4)_2$ Hybrid NFs

The immobilization yield of lipase/$Cu_3(PO_4)_2$ hybrid NFs based on operating conditions was calculated to be 95.1% whereby, the actual enzyme loaded was found to be 0.285 mg/mL. Therefore, the maximum amount of enzyme that can be loaded onto the NPs were found to be 0.285 mg/mL. The enzymatic activity on the other hand showed that the immobilized lipase had a much higher reactivity rate compared to that of free lipase as shown in **Figure 2**. After 40 mins of incubation, immobilized lipase had a catalytic rate of 1752 U/g which was an 90.5% increment in comparison to free lipase (165.9 U/g).

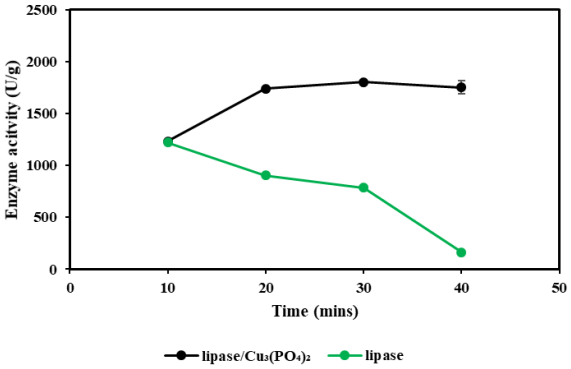

Figure 2 Enzyme activity between immobilized and free lipase

4. Conclusion

Hybrid NFs has been successfully developed using a facile one-step method via ultrasonication. It was observable that the morphology of structures varied between hybrid NFs and a pure metal-based nanomaterial. The enzyme immobilization yield was found to be 95.1% whereas the catalytic activity was found to be 1752 U/g which shows that there is much potential for hybrid NFs to act as biocatalysts. For future work, we would be studying the catalytic performance of lipase/$Cu_3(PO_4)_2$ hybrid NFs as a biocatalyst to enzymatically degrade biopolymers.

5. Acknowledgement

The authors would like to acknowledge the financial support provided by the Fundamental Research Grant Scheme (FRGS) Malaysia (Reference Code: FRGS/1/2019/TK10/CURTIN/02/2, Grant: FRGS 2019-1). The authors would like to thank Curtin University Malaysia for academic and facility support throughout this work.

References

1. Kuhad, R.C. and A. Singh, *Biotechnology for environmental management and resource recovery*. 2013: Springer.
2. Patel, R.N., *Chemo-enzymatic synthesis of pharmaceutical intermediates.* Expert Opinion on Drug Discovery, 2008. **3**(2): p. 187-245.

3. Turner, N.J., *Directed evolution drives the next generation of biocatalysts.* Nature Chemical Biology, 2009. **5**(8): p. 567-573.
4. Yu, L., et al., *Fabrication and application of enzyme-incorporated peptide nanotubes.* Bioconjugate Chemistry, 2005. **16**(6): p. 1484-1487.
5. Chagas, P.M.B., et al., *Immobilized soybean hull peroxidase for the oxidation of phenolic compounds in coffee processing wastewater.* International Journal of Biological Macromolecules, 2015. **81**: p. 568-575.
6. Anboo, S., et al., *Recent advancements in enzyme-incorporated nanomaterials: Synthesis, mechanistic formation, and applications.* Biotechnology and Bioengineering, 2022.
7. Wong, J.K.H., et al., *Potential and challenges of enzyme incorporated nanotechnology in dye wastewater treatment: A review.* Journal of Environmental Chemical Engineering, 2019. **7**(4).
8. Frenkel-Mullerad, H. and D. Avnir, *Sol−gel materials as efficient enzyme protectors: Preserving the activity of phosphatases under extreme pH conditions.* Journal of the American Chemical Society, 2005. **127**(22): p. 8077-8081.
9. Ge, J., et al., *Recent advances in nanostructured biocatalysts.* Biochemical Engineering Journal, 2009. **44**(1): p. 53-59.
10. Kim, J., J.W. Grate, and P. Wang, *Nanobiocatalysis and its potential applications.* Trends in Biotechnology, 2008. **26**(11): p. 639-646.
11. Luckarift, H.R., et al., *Enzyme immobilization in a biomimetic silica support.* Nature Biotechnology, 2004. **22**(2): p. 211-213.
12. Temoçin, Z., *Covalent immobilization of Candida rugosa lipase on aldehyde functionalized hydrophobic support and the application for synthesis of oleic acid ester.* Journal of Biomaterials Science, Polymer Edition, 2013. **24**(14): p. 1618-1635.
13. Cheaburu-Yilmaz, C.N., H.Y. Karasulu, and O. Yilmaz, *Nanoscaled dispersed systems used in drug-delivery applications*, in *Polymeric Nanomaterials in Nanotherapeutics*, C. Vasile, Editor. 2019, Elsevier. p. 437-468.
14. Santos, H.M., C. Lodeiro, and J.-L. Capelo-Martínez, *The Power of Ultrasound*, in *Ultrasound in Chemistry*. 2008. p. 1-16.
15. Bangi, U.K.H., et al., *Ultrasonically assisted synthesis of lead oxide nanoflowers using ball milling.* International Nano Letters, 2017. **7**(2): p. 149-155.
16. Nguyen, V., et al., *Effect of ultrasonication and dispersion stability on the cluster size of alumina nanoscale particles in aqueous solutions.* Ultrasonics Sonochemistry, 2011. **18**: p. 382-8.
17. Femila Komahal, F., et al., *Hierarchical zinc aluminate 3D nanostructures, synthesized by bio-inspired ultrasound assisted sonochemical route: Display and dosimetry applications.* Arabian Journal of Chemistry, 2020. **13**(1): p. 580-594.
18. Ali, F., et al., *Effect of sonication conditions: Solvent, time, temperature and reactor type on the preparation of micron sized vermiculite particles.* Ultrasonics Sonochemistry, 2014. **21**(3): p. 1002-1009.
19. Ghasemi, S., et al., *Sonochemical-assisted synthesis of nano-structured lead dioxide.* Ultrasonics Sonochemistry, 2008. **15**(4): p. 448-455.
20. Karami, H., et al., *Synthesis of lead oxide nanoparticles by sonochemical method and its application as cathode and anode of lead-acid batteries.* Materials Chemistry and Physics, 2008. **108**: p. 337-344.
21. Batule, B.S., et al., *Ultrafast sonochemical synthesis of protein-inorganic nanoflowers.* International Journal of Nanomedicine, 2015. **10**(Special Issue on diverse applications in Nano-Theranostics): p. 137-142.
22. Mustafa, A., A. Karmali, and W. Abdelmoez, *A sensitive microplate assay for lipase activity measurement using olive oil emulsion substrate: modification of the copper soap colorimetric method.* Journal of Oleo Science, 2016. **65**(9): p. 775-784.

23. Zhang, B., et al., *Preparation of lipase/$Zn_3(PO_4)_2$ hybrid nanoflower and its catalytic performance as an immobilized enzyme.* Chemical Engineering Journal, 2016. **291**: p. 287-297.
24. Zhang, B., et al., *Red-blood-cell-like BSA/$Zn_3(PO_4)_2$ hybrid particles: Preparation and application to adsorption of heavy metal ions.* Applied Surface Science, 2016. **366**: p. 328-338.
25. Ge, J., J. Lei, and R.N. Zare, *Protein–inorganic hybrid nanoflowers.* Nature Nanotechnology, 2012. **7**(7): p. 428-432.
26. Zhang, Z., et al., *A feasible synthesis of $Mn_3(PO_4)_2$@BSA nanoflowers and its application as the support nanomaterial for Pt catalyst.* Journal of Power Sources, 2015. **284**: p. 170-177.
27. Zhang, Z., et al., *Manganese(II) phosphate nanoflowers as electrochemical biosensors for the high-sensitivity detection of ractopamine.* Sensors and Actuators B: Chemical, 2015. **211**: p. 310-317.