The Development of Carbon Nanodots as Fluorescent Receptor for the Detection of Organophosphate Pesticides

by

Melissa Chang May Fung

A thesis submitted to the
Faculty of Engineering, Computing and Science
Swinburne University of Technology Sarawak Campus,
Malaysia
In fulfilment of the requirements for the degree of
Master of Science by Research

2016
Abstract

Excessive applications of pesticides have been a concern to the community because very few monitoring measures are available to conduct on-site pesticide residues inspection in the environment. The use of organophosphate pesticide in Malaysia continues to prevail, thus, there is a need for a substantial detection system to be installed. This thesis describes the production of carbon dots (C-dots) with unique fluorescent property that can be applied as optical detection probes for pesticide. In this work, C-dots were synthesised via bottom-up approach and further developed into a sensing probe for the detection of organophosphate (OP) pesticides. Sucrose was selected as starting precursor and converted into yellow emitting C-dots (C-dots\textsubscript{yellow}) via acid carbonisation process using strong phosphoric acid as the dehydrating agent. C-dots\textsubscript{yellow} was used to investigate the effects of OP pesticides towards its photoluminescence property. The sensing of three organophosphate pesticide standards, malathion, methyl parathion, and paraoxon-ethyl, and one commercial product, MAPA MALATHION EC 57 (MM57), via C-dots\textsubscript{yellow} was examined via various approaches. First approach uses C-dots\textsubscript{yellow} solely for the detection which has successfully detected paraoxon-ethyl and methyl parathion. The second sensing approach involves utilising C-dots\textsubscript{yellow} with direct addition of Paraoxonase 1 (PON 1) enzyme which can be used for malathion and paraoxon-ethyl sensing. The final approach was performed by tagging PON 1 onto C-dots\textsubscript{yellow}. The new composite denoted as C-dots – PON 1 can be utilised to sense for methyl parathion and paraoxon-ethyl. The sensing of MM57 was possible via utilising C-dots\textsubscript{yellow} with direct addition of PON 1 and C-dots – PON 1. The signals obtained from each respective sensing were analysed and recorded. From the signals obtained, this allows the calibration of future electronic sensing device for pesticide detection. The sensing potential of C-dots\textsubscript{yellow} for various analytes was further investigated and evaluated in this study.
Acknowledgments

First and foremost, I wish express my sincere gratitude to my coordinating supervisor, Dr. Ng Sing Muk, for the opportunity and support to complete my research. Your knowledgeable guidance and continuous succor had made this possible. Thank you for being patient with me when time was running out despite your hectic schedule. It was a pleasure working with you.

I also wish to extend my appreciation to my co-supervisors, Professor Maria Ngu-Schwemlein and Dr. Irine Runnie Ginjom. Prof. Maria, thank you for your caring and constructive opinions on my work. It was pleasure meeting you. Dr. Irine, thank you once again for helping me complete another chapter of my life. You have been a great mentor to me.

I would also like to thank Dr. Paul Nielson for chairing my thesis defense and for your help during my candidature. I would also like to extend my special thanks to the dedicated lecturers of Swinburne University of Technology, Associate Professor Peter Morin Nissom, Dr. Moritz Müller, Dr. Farouq Twaiq, Dr. Daniel Tan and Ms. Ting Lik Fong for your helpful advice and suggestions on my project.

Special thanks to the Research and Consultancy Office of Swinburne University of Technology Sarawak for the golden opportunity and SUTS Postgraduate Research Studentship (SPRS) award bestowed upon me. I would also like to extend my gratitude for the financial support through the Swinburne Sarawak Research Grant, Phase 1/2013 (2-5509).

I would like to thank Dr. Alvin Chai from Agricultural Research Centre (ARC), Semongok, Sarawak, Malaysia for the supply malathion and methyl parathion pesticide stocks.

I am also grateful to the science laboratory staffs; Dayang Rafika, Nurul Arina and Chua Jia Ni. Thank you for your kind and considerate treatment. Many thanks for lending materials and equipment required for the project. I would also like to extend my gratitude to all my colleagues for their continuous support and guidance.
To Jessica Fong and Diana Choo, thank you for guiding me with the essentials from the start of my candidature. It was a great pleasure meeting you girls and working together under the same supervisor.

To Tan Xian Wen and Shirley Bong, thank you for sharing beneficial information and resources. It was a great experience exploring new knowledge with you. Your advice and thoughts were greatly appreciated.

And last but definitely not least, to my parents. You have always been there for me, through ups and downs, but you never gave up. You have inspired me, gave me the courage to move on and support me all throughout my life. You are my pillar of strength. Thank you and I love you.
Declaration

I hereby declare that this thesis contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due references is made in the text of the examinable outcome. To the best of the candidate’s knowledge contains no material previously published or written by another person except where due reference is made in the text of this thesis. Where the work is based on joint research or publications, I have disclosed the relative contributors of the respective workers or authors.

Melissa Chang May Fung
# Glossary of Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>BChE</td>
<td>Butyryl cholinesterase</td>
</tr>
<tr>
<td>C-dots</td>
<td>Carbon dots</td>
</tr>
<tr>
<td>C-dots_yellow</td>
<td>Yellow emitting carbon dots</td>
</tr>
<tr>
<td>CNP</td>
<td>Carbon nanoparticles</td>
</tr>
<tr>
<td>CVD</td>
<td>Coronary vascular disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3 - (3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme – linked immunoabsorbant assays</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agricultural Organization</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>FRET</td>
<td>Forster resonance energy transfer</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HDL</td>
<td>High – density lipoprotein</td>
</tr>
<tr>
<td>HMDA</td>
<td>1,6-Diaminohexane</td>
</tr>
<tr>
<td>HPLC</td>
<td>High – performance liquid chromatography</td>
</tr>
<tr>
<td>IMS</td>
<td>Ion mobility spectrometry</td>
</tr>
<tr>
<td>IPC</td>
<td>Ion pair chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LDL</td>
<td>Low – density lipoproteins</td>
</tr>
<tr>
<td>HDL</td>
<td>High– density lipoproteins</td>
</tr>
</tbody>
</table>
LOD    Limit of detection
MM57   MAPA MALATHION 57 EC
MRL    Maximum residue levels
MS     Mass spectroscopy
MW     Molecular weight
NHS    N-Hydroxysuccinimide
nm     Nanometres
OP     Organophosphate
OPH    Organophosphorus hydrolase
PBS    Phosphate – buffered saline
PEG    Polyethylene glycol
PL     Photoluminescence
PLQY   Photoluminescent quantum yield
PNP    p – nitrophenol
PON 1   Paraoxonase 1
PON 2   Paraoxonase 2
PON 3   Paraoxonase 3
ppb    Parts per billion
ppm    Parts per million
PVA    Polyvinyl alcohol
QDs    Quantum dots
QY     Quantum yield
rpm    Rounds per minutes
SAW    Surface acoustic wave
SWCNT  Single – walled carbon nanotubes
TRIS   Tris (hydroxymethyl) aminomethane
UV     Ultraviolet
Publications Arising from this Thesis


Early work has been presented in the following conferences and contributed to the content presented in this thesis:


Table of contents

Abstract .......................................................................................................................... ii
Acknowledgments ........................................................................................................ iii
Declaration .................................................................................................................. v
Glossary of Acronyms ................................................................................................. vi
Publications Arising from this Thesis ........................................................................ viii
Contents ...................................................................................................................... ix
List of Figures ............................................................................................................. xii
List of Tables ............................................................................................................... xviii

1. INTRODUCTION ............................................................................................... 1
   1.1 Introduction ..................................................................................................... 1
   1.2 Problem statement ....................................................................................... 2
   1.3 Rationale ....................................................................................................... 2
   1.4 Objectives ..................................................................................................... 3
   1.5 Outline of the thesis .................................................................................... 4
   1.6 Scope and Delimitation .............................................................................. 5

2. LITERATURE REVIEW ....................................................................................... 6
   2.1 Introduction ................................................................................................... 6
   2.2 Carbon dots ................................................................................................. 6
      2.2.1 Synthetic method ................................................................................ 10
      2.2.2 Principles of fluorescence ................................................................... 12
      2.2.3 Characterisation of C-dots .................................................................. 19
      2.2.4 Quenching of fluorescence .................................................................. 20
      2.2.5 Surface modification strategy ............................................................. 22
      2.2.6 Fluorescence sensing .......................................................................... 24
      2.2.7 Sensor .................................................................................................. 25
   2.3 Pesticides ...................................................................................................... 27
      2.3.1 Toxicology effects of pesticides ......................................................... 29
      2.3.2 Conventional detection of pesticides ............................................... 30
      2.3.3 Biosensors for pesticide detection ..................................................... 31
      2.3.4 Organophosphorus hydrolase (OPH) ............................................... 33
      2.3.5 Paraoxonase 1 (PON 1) .................................................................... 35
   2.4 Heavy metal .................................................................................................. 37
2.5 Sustainable technology.................................................................39
3. RESEARCH METHODOLOGY .........................................................43
  3.1 Introduction..................................................................................43
  3.2 Materials.....................................................................................45
  3.3 Instrumentation...........................................................................45
  3.4 Personal protective equipment ...................................................46
  3.5 Synthesis of C-dots......................................................................46
    3.5.1 Acid dehydration of carbohydrate source.............................46
    3.5.2 C-dots\textsubscript{yellow} stock preparation............................48
    3.5.3 Saccharide pyrolysis...............................................................48
  3.6 Surface modification of C-dots....................................................49
  3.7 Salting out...................................................................................49
  3.8 Photoluminescent quantum yield (PLQY)......................................49
  3.9 Limit of detection (LOD)...............................................................50
  3.10 Stern-Volmer modelling..............................................................51
  3.11 Pesticide detection......................................................................52
    3.11.1 HPLC analysis of pesticides................................................52
    3.11.2 PON 1 enzyme stock preparation.........................................53
    3.11.3 PON 1 enzyme activity.........................................................54
    3.11.4 Sensing of pesticide standards............................................55
    3.11.5 Sensing of commercial sample............................................57
  3.12 Heavy metal................................................................................57
  3.13 Immobilisation of C-dots with polyvinyl alcohol............................58
4. RESULTS AND DISCUSSION..........................................................59
  4.1 Introduction..................................................................................59
  4.2 Optical properties.......................................................................59
    4.2.1 Optical properties of C-dots..................................................60
    4.2.2 Effects of acid for C-dots\textsubscript{yellow} production..................63
    4.2.3 Carbonisation mechanism....................................................64
    4.2.4 Optimisation of synthesis condition.....................................65
    4.2.5 Optimisation of sensing condition.......................................69
  4.3 Saccharides pyrolysis.................................................................71
  4.4 Surface modification of C-dots....................................................75
List of Figures

Figure 2-1: Unique properties of C-dots with some potential applications. .......................................................... 10

Figure 2-2: Colour spectrum of visible light wavelengths between 200 nm to 800 nm. .......................................................... 10

Figure 2-3: A brief illustration of the Jablonski diagram. .......................................................... 13

Figure 2-4: Absorbance, fluorescence and phosphorescence spectra of tryptophan respectively in a glass at low temperature. Revised from Cioni and Strambini (2002). .......................................................... 15

Figure 2-5: Graphical representation of C-dots and the active sites available on the surface. .......................................................... 17

Figure 2-6: Illustration of electronic absorption and emission bands of excited fluorophore (Herman et al., 2009). .......................................................... 18

Figure 2-7: Optical properties of blue, green, yellow and red carbon nanoparticles with digital images of solution under excitation (Bhunia et al., 2013). .......................................................... 18

Figure 2-8: Comparison of collisional and static quenching for a system with increased temperature. .......................................................... 22

Figure 2-9: Brief physio-chemical treatments of C-dots production that can be used for chemical or biochemical sensor. .......................................................... 23

Figure 2-10: Major routes of pesticide to could reach and contaminate the surrounding environment. .......................................................... 28

Figure 2-11: The inhibition activity of OP pesticide compounds on AChE in the nerve synapse. .......................................................... 30

Figure 2-12: Molecular structures of (a) methyl-parathion, (b) paraoxon-ethyl and (c) malathion. .......................................................... 33

Figure 2-13: Paraoxon pathway hydrolysed by PON 1 producing diethyl phosphoric acid and p-nitrophenol. .......................................................... 34
Figure 2-14: Biological assembly image of serum paraoxonase by directed evolution at a resolution of 2.2 Å. Protein chains are depicted by rainbow (spectral) colour gradient from N-terminal to C-terminal adapted from (Harel et al., 2004). ......................................................... 36

Figure 2-15: A crystal structure of PON 1 (pdb. 1v04) adapted from Bigley and Raushel (2013) and Harel et al. (2004). A) Side view of β-propeller fold with extended HDL anchoring helices. B) View of metal centres and ligating residues of PON 1. C) View of substrate binding pockets of PON 1. ................................................................. 36

Figure 2-16: Carcinogenic mechanism pathway of chromium (III) compound (Henkler et al., 2010). ........................................................................................................... 39

Figure 2-17: The basic reduction elements of green chemistry adapted from Clark (2005). .................................................................................................................. 41

Figure 3-1: Summary of pathway adopted for C-dots_{yellow} production via H_3PO_4 hydrolysis. .................................................................................................................. 47

Figure 3-2: Synthesis and analysis pathway of C-dots_{yellow} via acid dehydration by H_2SO_4 or H_3PO_4. ............................................................................................................ 47

Figure 3-3: Front and back snapshot of MM57 supplied by local vendor in Malaysia. Instructions of preparation were stated on the label. .................................................................................. 53

Figure 4-1: Photos of (a) ultrapure water, (b) sucrose and (c) C-dots_{yellow} observed under UV light exposure. ................................................................. 60

Figure 4-2: Spectrum of C-dots_{yellow} (a) excitation, (b) emission and (c) absorbance, derived from carbonisation of sucrose by concentrated H_3PO_4. .................................................................................. 61

Figure 4-3: Fluorescence spectra of neutral C-dots_{yellow} condition (a) excitation and (b) emission and acidic C-dots_{yellow} condition (c) excitation and (d) emission. .................................................................................. 62
Figure 4-4: Different emission spectra of C-dots produced by H$_2$SO$_4$ and H$_3$PO$_4$ hydrolysis of sucrose. (a) H$_2$SO$_4$ oxidation of 250 mg/mL sucrose, (b) H$_2$SO$_4$ oxidation of 500 mg/mL sucrose, (c) H$_3$PO$_4$ oxidation of 250 mg/mL sucrose, (d) H$_3$PO$_4$ oxidation of 500 mg/mL sucrose.

Figure 4-5: Normalised intensity of C-dots$_{yellow}$ with increasing sucrose concentration used as starting precursor.

Figure 4-6: Cumulative concentration of H$_3$PO$_4$ increases fluorescence intensity of C-dots$_{yellow}$.

Figure 4-7: PL intensity trend of C-dots$_{yellow}$ emission at various temperature intervals.

Figure 4-8: Correlation of C-dots$_{yellow}$ intensity obtained from heat treatment of 85°C for a period of 60 minutes.

Figure 4-9: Effects of different pH conditions on PL intensity of C-dots$_{yellow}$.

Figure 4-10: Influence of high ionic strength by KCl on C-dots$_{yellow}$ of PL intensity.

Figure 4-11: Visual pictures captured after sucrose pyrolysis treatment under respective temperature for 10 minutes.

Figure 4-12: Fluorescence spectrum of 5 mg/ml sucrose pyrolysis at different temperature for 10 minutes. (a) 300°C, (b) 250°C, (c) 350°C and (d) 200°C.

Figure 4-13: Intensity pattern of C-dots emission of increasing carbonisation temperature from 200 to 350°C.

Figure 4-14: A combination of C-dots spectrums obtained from a selection of saccharides, (a) starch, (b) glucose, (c) sucrose and (d) melezitose, resulting from thermal carbonisation.
Figure 4-15: Excitation and emission spectrums of C-dots and functionalised C-dots after 5 days of functionalisation. (a) functionalised C-dots excitation, (b) functionalised C-dots emission, (c) C-dots excitation and (d) C-dots emission. 76

Figure 4-16: Maximum intensity of functionalised C-dots yellow by HMDA, registered for each respective day. 76

Figure 4-17: Snapshot of liquid-liquid extraction separation; mixture of C-dots yellow and pure acetone in 1:1 ratio settled after vigorous shake via vortex. 77

Figure 4-18: Excitation and emission spectrum of C-dots prior extraction and after extraction via acetone. (a) C-dots excitation, (b) C-dots emission, (c) isolated C-dots excitation and (d) isolated C-dots emission. 78

Figure 4-19: HPLC chromatogram of 605.40 µM malathion standard stock. 79

Figure 4-20: Standard curve analysed via HPLC of malathion expressed in peak height of respective concentration. 80

Figure 4-21: HPLC chromatogram of 809.26 µM paraoxon-ethyl standard stock. 81

Figure 4-22: Standard curve analysed via HPLC of paraoxon-ethyl analysed in terms of peak height of respective concentration. 81

Figure 4-23: Effects of respective components present in PON 1 storage and activation buffer on the fluorescence intensity of C-dots yellow. 83

Figure 4-24: PON 1 quenching effects on C-dots yellow with increasing enzyme concentration expressed in mg/mL. 84

Figure 4-25: Standard curve plotted in increasing PNP concentration for determination of PON 1 activity. 85

Figure 4-26: Combination of sensing signal plots for the detection of malathion, paraoxon-ethyl and methyl parathion by C-dots yellow. 86
Figure 4-27: Illustration depicting C-dots – PON 1 interaction for sensing of OP substrate. Fluorescence analysis was performed on bare C-dots_{yellow}, during C-dots_{yellow} binding to PON 1 and OP substrate binding on PON 1, replacing C-dots_{yellow}. ................................................................. 87

Figure 4-28: Combination of sensing signal plots for the detection of malathion, paraoxon-ethyl and methyl parathion by C-dots_{yellow} with PON 1. ........................................................................................................... 89

Figure 4-29: C-dots – PON 1 tested for malathion, paraoxon-ethyl and methyl parathion sensing respectively. ................................................................. 90

Figure 4-30: A scatter plot displaying the results obtained for malathion standard compared to MM57 sensing via C-dots_{yellow} after direct addition of PON 1. ........................................................................................................... 92

Figure 4-31: Linear trend of malathion and MM57 before the peak of vertical curve. ........................................................................................................... 92

Figure 4-32: Signal trend obtained for MM57 sensing by C-dots – PON 1 composite. ........................................................................................................... 93

Figure 4-33: Downward quenching standard curve observed for MM57 sensing via C-dots – PON 1. ................................................................. 94

Figure 4-34: The effects of various metal ions with concentration of 5 µM respectively on the fluorescence intensity of C-dot_{yellow} under neutral condition. ........................................................................................................... 95

Figure 4-35: The effects of various metal ions with concentration of 5.0 µM respectively on the fluorescence intensity of C-dots_{yellow} under acidic condition. ........................................................................................................... 96

Figure 4-36: Stern-Volmer plot of the quenching effect caused by Cr(III) ions towards the fluorescence intensity of C-dots_{yellow} under acidic condition. ........................................................................................................... 97

Figure 4-37: Schematic drawing of PVA-C-dots_{yellow} film formation. ................. 99
Figure 4-38: Snapshot of PVA – C-dots\textsubscript{yellow} film before inducing quencher spot on the film. ................................................................. 99

Figure 4-39: Snapshot of PVA – C-dots\textsubscript{yellow} film after inducing quencher spot on the film. .................................................................................. 99

Figure 5-1: Snapshot of the portable sensing device assembly utilising C-dots as optical receptors. ................................................................. 105
List of Tables

Table 2-1: Nanomaterial based biosensors established for several OP pesticides detection (adapted from Zhang et al. (2014))...32

Table 3-1: Buffer recipe for the preparation of different pH buffers...48

Table 3-2: Final concentrations of malathion expressed in molarity for HPLC analysis...52

Table 3-3: Final concentrations of paraoxon-ethyl expressed in molarity for HPLC analysis...52

Table 3-4: PNP standards set up for calibration curve plot...55

Table 3-5: Respective metal ions weight prepared from metal salts for 0.1 M stock...58

Table 4-1: Pyrolysis reaction temperature with the respective C-dots emission obtained for each temperature...72

Table 4-2: Malathion, expressed in molarity, with respective peak height based on the HPLC chromatograms obtained...80

Table 4-3: Paraoxon-ethyl, expressed in molarity, with respective peak height based on the HPLC chromatograms obtained...81

Table 4-4: MM57 contents in respective concentrations (%) correlated with malathion standard curve to determine actual molarity of malathion present in commercial sample...82
1. INTRODUCTION

1.1 Introduction

Pesticide industry is one of the most important provision industries in Malaysia’s crop plantation due to the significant contribution towards the economy (Abdul Rani, 2002). Due to this, it is indisputable that the usage of pesticides is of interest to increase the yield of crops. Land for agricultural activities is getting limited in Malaysia due to the conversion of land to other non-agricultural usage. Henceforth, the use of pesticides continues to be part of an important contribution to increase crop intensity to meet demands of the growing population. Apart from agricultural use, organophosphate (OP) insecticides such as malathion are also used to control dengue fever under large scale spraying programs. Most common method of applying pesticides in Malaysia’s crop plantation is by using knapsack sprayer. This is inefficient because out of the 20% that reaches the plant; only less than a percent of the pesticide chemical contributes to pest control. As a result, the remaining pesticides will contaminate the surrounding environment (MADI, 1996, Abdul Rani, 2002). An increasing amount of pesticide is applied due to the emergence of resistant pest strains. With the increased usage, the chances of accidental or intentional discharge of pesticide rinses and waste from farm area and manufacturing plants increases, thus will lead to environmental contamination. Henceforth, non-targeted organisms will be exposed to the toxic effects of excessive pesticide that present in the environment (Abdul Rani, 2002).

Currently, there are very few nationwide or district monitoring measures available to conduct pesticide residues inspection on-site in the environment. The uses of pesticide in Malaysia continues to prevail, thus, there is a need for a substantial residue detection system to be installed. The European Union (EU) is monitoring various kinds of pesticide residues in order to improve the administration of legislation for pesticide residue content in food products (European Food Safety Authority, 2014). The analysis is performed in comparison to the maximum residue levels (MRL) legal limits. Among the third world country, Malaysia was found to have the highest MRL exceedance rates (approximately 38%) as compared to other countries such as Cambodia, India,
Kenya, and China (European Food Safety Authority, 2014). World Health Organization (WHO) and EU has set the MRL of pesticides to be 10 ppm for most food products and 0.1 ppb for drinking water (Ragnarsdottir, 2000). In addition, Malaysian agricultural products by local farmers do not undergo a level of assurance by the authorities to certify the level of pesticide is within the regulation before the products are marketed.

Thus, this has motivated the interest of this study to develop a substantial device for pesticide detection. This study will report on the signals obtainable when novel optical sensing probes are used for the detection of OP pesticide. The approach will be utilising carbon dots (C-dots) as optical nanoparticles that will be synthesised via sustainable method and corresponding signals obtained when OP pesticides are added will be analysed.

1.2 Problem statement

Based on reports by the EU, there are concerns regarding the methods available to assist the authorities to determine the amount of pesticide in Malaysian agricultural products. Are the agricultural products safe for consumption in Malaysia? Can the identification and quantification of pesticides be done instantly? This has become the questions probed by the community because they do not have the full confidence in the current enforcement system. These fundamental problems have caused doubts due to the quality assurance standards in Malaysia. A challenge faced by the enforcement bodies is the lack of simple and portable devices available to determine the amount of pesticides on-site particularly OP pesticides found in food products. The presence of pesticide in agricultural products and the environment continue to be the concern of the public and government. To achieve this, regulation of pesticide content used on agricultural products must be activated via novel, robust and rapid sensing device. Such attempt will greatly help the vast nation to have safer agricultural products.

1.3 Rationale

With environment safety playing such a key role for a healthy and sustainable community, it is therefore logical to develop sensors to monitor and regulate the amount of pesticide used for agricultural activities. Ultimately, the
idea is that a novel sensor platform will be designed and evaluated for a rapid monitoring of OP compounds. The work in this dissertation focuses on a sustainable approach for developing an optical sensor utilising C-dots for the detection of OP pesticides. During the course of this research, the activities involve synthesising C-dots, characterising C-dots and analysing the effects of pesticide on C-dots response for analytical application.

1.4 Objectives

This project undertakes on the idea to develop C-dots from economical natural sources as optical sensing receptor for detection of OP pesticide. Bare and surface modified C-dots will be synthesised and the main focus of this study is to characterise the interaction between C-dots and pesticide compounds for sensing application. The pesticide group of interest is OP due to the common used in Malaysian agricultural sector. The change of optical property of C-dots will be recorded and analysed in order to develop the system into sensing probe. This study embarks on the following objectives:

1. The synthesis of bare and surface modified carbon dots from sustainable natural resources.
2. Characterisation of both bare and surface modified carbon dots optically using standard optical spectroscopy methods.
3. Study the changes in optical emission property of the carbon dots in the presence of pesticide compounds and correlate the changes into readable signals for sensing application.
4. Generation of novel scholarly ideas on nano-biosensors for the detection of pesticides that can contribute towards the current and future improvements of nanotechnology, biotechnology and environmental sustainability.
5. Explore potential application of C-dots as alternative sensing platform for other analytes.

This research is based on the hypothesis that the presence of OP pesticide will cause a measurable change of optical properties of C-dots synthesised by a sustainable approach. The modelling of the change of signal can provide information for pesticide quantification.
1.5 Outline of the thesis

This dissertation comprised of the following chapters.

Chapter 1 introduces the motivation of this study and the underlying hypothetical orientation.

Chapter 2 provides general background and more details on carbon nanoparticles, C-dots fluorescence principles and pesticides. In this chapter, the synthetic method of production, optical properties and surface modification approaches of C-dots will be reviewed. Furthermore, the quenching of fluorescence mechanism and sensing application will be deliberated. Lastly, the pesticide toxicology effects, detection method, organophosphorus hydrolase (OPH) and Paraoxonase 1 (PON 1) will be introduced in this chapter.

Chapter 3 contains description of experimental methods including synthesis, surface modification, passivation, immobilisation, and determination of quantum yield of C-dots. This followed by the methodologies for high-performance liquid chromatography (HPLC) analysis of OP pesticides, PON 1 enzyme and OP sensing via C-dots. Furthermore, the detection of cationic metal ions by C-dots will also be described which will showcase the ability of C-dots as sensing particle for other target analytes.

Chapter 4 presents the results and discussion of this dissertation. The parameters set for production of yellow emitting C-dots and their characterisation via different approaches will be compared and presented followed by particle response towards different OP pesticides standards and a commercial sample. A detailed analysis of OP pesticide sensing will be evaluated and presented. A linear signal of C-dots and pesticide response will be demonstrated. Finally, metal ion sensing will also be discussed as an alternative target analyte besides OP pesticide, employing C-dots as sensing receptor.

Chapter 5 concludes the thesis with a summary of the results collected, contribution of this work to the sensing application of C-dots and followed by potential recommendations for further work of this research project.

A reference list followed after Chapter 5.
1.6 Scope and Delimitation

The study will focus on the synthesis of C-dots and its application as a sensing receptor. There are several colours of C-dots emission reported and this work has chosen to work on yellow emitting C-dots (C-dots\textsubscript{yellow}) because there are insignificant reports of utilising C-dots\textsubscript{yellow} in OP pesticide sensing. Sustainable source will be used for C-dots production via acid dehydration method. Surface modification on C-dots will be performed via covalent coupling aided by crosslinking reagents mainly 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). A quick acetone passivation method will be administered on bare C-dots and the change of properties will be analysed, followed by immobilisation of C-dots via polyvinyl alcohol (PVA).

Sensing of OP pesticide will involve three major pesticide species; malathion, methyl parathion and paraoxon-ethyl. The commercial product MAPA MALATHION 57 EC, denoted as MM57, available in the market was used for commercial product analysis. Alternative to OP pesticide, the sensing potential for metal ions will be performed to examine the sensitivity and selectivity of C-dots towards several species of heavy metal. The list of heavy metal ion species includes aluminium (III), cobalt (II), copper (II), chromium (III), lead (II), mercury (II), nickel (II), tin (II) and zinc (II) which are commonly found in heavy metal contaminated water bodies.

Due to some limitations of facilities, not all general characterisation data of the C-dots are obtainable. Thus, some characterisations will be sourced from other institutions as presented in this thesis. The scope of this study will also cover the potential application of C-dots for the detection of various analyte. Fabrication of pesticide sensor is technically more challenging, thus, an initial attempt of immobilising C-dots will be performed which will be part of the scope for this study. The recommendations for major component set up will also be provided for further research.
2. LITERATURE REVIEW

2.1 Introduction

This chapter will focus on providing the background information on the components and techniques related to the development of OP pesticide sensor. The contextual information on C-dots will be discussed which will ultimately lead to the discussion of the synthetic method available for production and principles of the fluorescence properties. Besides, the characterisation, quenching mechanism and surface modification strategy of C-dots will also be discussed. The topic of discussions such as the fluorescence sensing system and sensor devices will also be deliberated.

The motivation of this study focuses on the detection of pesticide because OP pesticides are considered as health threatening compounds. Background reviews on OP pesticide, toxicology effects, detection method and pesticide hydrolysing enzymes will be discussed. In this study, a series of OP pesticide was selected for the sensing application.

Finally, a brief introduction on the heavy metals will be provided in this chapter. Then, this is followed with the fundamentals of sustainable technology.

2.2 Carbon dots

Nanoparticles have gained extensive attention since it was first pioneered by Micheal Faraday in 1857 through the investigation of the thin films of gold (Faraday, 1857). Particles in nano-size have extremely large surface-area-to-volume ratio. This property enables nanoparticles to have unique characteristics compared to bulk materials (Christian et al., 2008, Alivisatos, 1996). With the large surface area available, target molecules can interact and bind easily on the surface active sites (Lahiff et al., 2010). Due to this, the surface plays an important role in portraying the unique features of a nanoscale material (Christian et al., 2008). Nanoscale dimension of nanoparticles, generally from 1 to 100 nanometres (nm), enables them to exhibit unique characteristics and functions which drew significant attention by researchers (Lahiff et al., 2010, Lalena and Cleary, 2005, Medina et al., 2007). An assortment of materials were used to fabricate nanoscale particles including
gold, carbon and platinum (de Dios and Díaz-García, 2010). Carbon based materials, for example, graphene nanosheets, porous carbon and fullerenes have promising applications in nanoelectronic, sensors, drug delivery and bioimaging (Chandra et al., 2011, Berger et al., 2006, Li et al., 2010b, Stoller et al., 2008, Kim et al., 2008, Bourlinos et al., 2008a, Neugart et al., 2007). The applications of nanoparticle are dependent on the surface properties which can be functionalised to exhibit selective properties. This provides potential for a wide variety of substrate detection via nanoparticle based methodologies. Nanotechnology have grown evidently in the biosensors field because being small sized, one billionth of a metre, provides great interface sensitivity due to their substantial surface-to-volume ratios. Various nanomaterials are available for admirable optical or electrical properties making them highly suitable as responsive signal transducers for biosensors (Liu et al., 2008). All in all, nanotechnology is an effective approach for economical multi analyte detection systems.

Carbon nanoparticles (CNP) were discovered and the earliest known for its use is as prehistoric wall art pigment in France (Ospitali et al., 2006). CNP provides many benefits comprising high surface area, a great number of reactive adsorption sites and good conductivity (Macdonald et al., 2008, Rassaei et al., 2008).

Carbon element is among the most abundant elements in the Earth’s crust. It is naturally nontoxic and became rather important in nanochemistry due to the fluorescent property with relatively high quantum yield (QY). Fluorescent carbon quantum dots, are also known as C-dots. C-dots are photoluminescent dots which produces measurable fluorescence signal when exposed to ultraviolet (UV) light source. C-dots have fundamental luminescent properties (Yang et al., 2012a) and they can be an excellent alternative to other carbon nanomaterials because the production is simple, rapid and inexpensive. C-dots have risen to become one of the most important nano-carbon members having excellent properties of being benign, abundantly available and economical (Wang and Hu, 2014). In 2004, they are categorised as a new class of nanomaterial and since then, many methods of production have been reported.
Carbon is commonly known to be black coloured in physical appearance and only until recently being discovered to dissolve in water and show fluorescence (Esteves da Silva and Gonçalves, 2011). Bulk carbon materials have unequivocally different properties when synthesised as nano sized C-dots as compared to their macroscopic structure. These nanoparticles are chemically inert with high photobleaching resistance (Wang and Hu, 2014). Besides, C-dots have comparable photochemical stability and photo-physical performance to that of semiconductor quantum dots (QDs), plus, having non blinking fluorescence emission and readily soluble in water.

In the past, QDs are fluorescent nanoparticles perceived as inorganic semi-conductive nanocrystals made from group III-V and II-VI elements of the periodic table (Volkov, 2015). They have been developed and used as fluorescent labels. QDs have unique properties such as strong fluorescence, broad excitation spectra, high photostability against photobleaching, good biocompatibility and tunable excitation and emission wavelength (Mattoussi et al., 2000, Li et al., 2007, Xie et al., 2004, Chen et al., 2008, Lewinski et al., 2008, Tan et al., 2007, Mao et al., 2010). A major drawback of QDs for biological application is their potential toxicity. Classically, QDs are made of cadmium core encapsulated with bioinert zinc sulphide (ZnS) shell structure. Further coating is required for biological application such as with chitosan, silica and amphillic polymers for better biocompatibility and water solubility. The core size of typical QDs ranged from 2 to 5 nm and with the added coating, the hydrodynamic diameter may reach 25 nm. With the increased size, the kidneys could not clear the particles from the body and this result in long term circulation of QDs in the body. Consequently, the shell structure breaks down, causing metal ions to leak in the cytoplasm, causing cytotoxic effects (Kumar, 2010). Till date, slight leaching are still being reported despite the coating (Fang et al., 2012).

The concerns surrounding the toxicity of QDs have become a major roadblock for the biological application of QDs and this has placed C-dots in the major advantage for important biological applications such as in vivo bioimaging (Esteves da Silva and Gonçalves, 2011). Pan and Feng (2009) developed a strategy utilising QDs conjugated with folate to improve imaging of breast cancer cells. In vitro cellular uptakes were observable for both cancer cells and
normal healthy cells. The cells viability after QDs induction, with and without passivation, was evaluated to be below 90% (Pan and Feng, 2009). In another similar study, Yang et al. (2015) utilises simple, low-cost one step microwave treatment of folic acid (FA) for production of C-dots to distinguish HeLa cancer cells from normal healthy cells. Their approach produces blue emitting C-dots with high quantum yield of ca. 25%. The C-dots showed excellent biocompatibility and good specificity to target folate receptor (FR) expressed predominantly in cancer cells. In addition, the viability of normal and cancer cells remained above 90% after the incubation with the C-dots. These results proved that C-dots is a better alternative of QDs which can be used as a probe for cancer diagnosis and treatment (Yang et al., 2015).

C-dots have been attracting a significant amount of research interest as compared to nascent QDs when cost, size and biocompatibility are of concern. Up to date, there are many advances made in developing these nanolight particles (Baker and Baker, 2010). Most C-dots have the ability to be functionalised by a selection of organic, inorganic, biological or polymeric species. This is possible due to the presence of many carboxylic acid and oxygen moieties seemingly available on the surface of C-dots. It is a noteworthy fact that C-dots are excellent eco-friendly and low toxic replacements of highly toxic metal based QDs.

C-dots are excellent candidates for a diverse array of practical applications such as a chemical sensors, biosensors, drug deliveries, bioimaging and also as dyes (Xu et al., 2014). A simplified illustration of the unique properties of C-dots with its common uses is shown in Figure 2-1. C-dots exhibit strong optical absorption in UV region and their most captivating feature is their photoluminescence (PL) properties. However, it is still not clearly determined whether the PL origin is caused by different quantum effects due to size or emissive traps present on the surface of C-dots. There are plenty of prodigious advantages of C-dots, nevertheless, there are also distinct limitations of C-dots. According to Bhunia et al. (2013), one of the limitations is synthetic methods produces weak fluorescent of C-dots with QY of less than 1%. Besides, there are plenty of blue and green emitting C-dots but only a few methods report on yellow and red emitting C-dots.
Different colour emitting C-dots have distinctive optical properties according to their respective emission colour. Blue, green, yellow and red C-dots with their respective most intense emission is 440 nm (excitation at 370 nm), 500 nm (excitation at 400 nm), 560 nm (excitation at 425 nm) and 600 nm (excitation at 385 nm) respectively (Bhunia et al., 2013). Different C-dots emission colours are referable based on visible light colour spectrum (Figure 2-2).

**Figure 2-1:** Unique properties of C-dots with some potential applications.

**Figure 2-2:** Colour spectrum of visible light wavelengths between 200 nm to 800 nm.

**2.2.1 Synthesis method**

One of the first synthesis methods of carbon nanoparticle reported was published by Birrenbach and Speiser (1976). Their study investigated on nanoparticle ability as drug carriers via water and hexane-based micellar system (Birrenbach and Speiser, 1976). There are several types of CNP mainly carbon nanotubes (Ajayan, 1999, Liu et al., 2010, Yang et al., 2010), graphene sheets (Novoselov et al., 2004, Liu et al., 2010, Rao et al., 2009, Yang et al., 2010).
nanodiamonds (Greiner et al., 1988, Liu et al., 2010) and carbon nanodots (Xu et al., 2004, Baker and Baker, 2010, Fan and Chu, 2010, Bourlinos et al., 2008b, Hu et al., 2009). The discovery of CNP was first reported by Xu et al. (2004) by analysing a fast moving high luminescent band during gel electrophoresis of single-walled carbon nanotubes (SWCNT) suspension. Fractions of SWCNTs and nanoparticle impurities were collected and analysed. Two isolated products were identified as fluorescent carbon nanoparticles and short tubule carbon species. Under 365 nm excitation, green-blue, yellow and orange fluorescence were observed. In addition, Fourier transform infrared spectroscopy (FTIR) scan performed on the products revealed the presence of carboxylic groups on the nanoparticles (Xu et al., 2004).

An important part of research on C-dots is to determine an optimal method to prepare homogenous C-dots; obtaining a best way to produce C-dots. Benign C-dots have been successfully prepared although intrinsic mechanism of the fluorescence properties is not clearly understood. Presently, there are many blue and green emitting C-dots reported but they have lower emission compared to QDs (Chandra et al., 2011). However, it is still an ideal research field due to the environmental friendly nature of C-dots compared to QDs with higher toxicity that limits its application in cell or biological imaging. Numerous synthetic pathways and C-dots applications have been discovered and developed (Baker and Baker, 2010, Fan and Chu, 2010). Synthetic route for C-dots production could be divided into three major steps. First step will be the synthesis of raw C-dots, followed by passivation process of the raw C-dots and finally, functionalisation of C-dots.

Synthesising raw C-dots can be obtained via two types of approaches, mainly top-down or bottom-up (Esteves da Silva and Gonçalves, 2011). Different method could result in varying the physicochemical properties of the nanoparticles. Top-down synthetic method could be defined as the process of forming C-dots from bulk carbon sources. In brief, it is the process of producing smaller products that exhibit fluorescent physiognomies from larger structures of graphene or graphite. Top down methods include laser ablation of carbon targets (Bourlinos et al., 2008b, Sun et al., 2006, Cao et al., 2007, Sun et al.,
2008, Wang et al., 2009, Yang et al., 2009b, Yang et al., 2009a), laser irradiation of carbon particles (Li et al., 2011b), arc discharge soot (Xu et al., 2004), thermal decomposition (Yu et al., 2013) and electrochemical synthesis. On the other side, bottom up method involves the formation of C-dots from molecular precursors. It involves the ‘burning’ of carbogenic materials to produce C-dots under controlled condition. Bottom up methods include acid dehydration of carbohydrates (Peng and Travas-Sejdic, 2009), thermal combustion (Bourlinos et al., 2008b, Bourlinos et al., 2008a, Liu et al., 2009, Wang et al., 2010), candle or natural gas burners soot (Liu et al., 2007, Ray et al., 2009, Tian et al., 2009), ultrasonic treatment and microwave methods.

Alternatively, synthesis methods can also be classified as chemical and physical method. The chemical method involves microwave, acid oxidation, hydrothermal, thermal treatment and electrochemical methods. Physical methods involve laser ablation, plasma treatment and arc discharge. Among all these methods, acidic oxidation is proved to be an effective and convenient way for synthesis of C-dots with high QY (Xu et al., 2014).

Synthesis of phosphate functionalised C-dots with green and yellow PL of narrow size distribution has rarely been reported as mentioned by Xu et al. (2014). Carbonaceous materials are products resulting from carbonisation of small organic molecules by strong oxidising agents such as acids (Ray et al., 2009, Tian et al., 2009, Qiao et al., 2010, Dong et al., 2010, Peng and Travas-Sejdic, 2009, Lu et al., 2015). More often, carbonisation of carbohydrates via sulphuric acid at 50 to 100°C produces C-dots with blue and green emission. However, red and yellow C-dots could be obtained by using phosphoric acid based carbonisation of carbohydrates at 80 to 90°C (Bhunia et al., 2013).

2.2.2 Principles of fluorescence

The use of fluorescence in biological science has been expanding during the past 20 years, via fluorescence spectroscopy and time resolved fluorescence as the primary tools to understand the biochemistry and biophysics. Some common methodologies using fluorescence approach are deoxyribonucleic acid (DNA) sequencing, flow cytometry, genetic analysis and forensics. As opposed to most biochemical measurements that requires
radioactive traces; highly sensitive fluorescence detection will not require those difficult protocols. As stated by Lakowicz (2006), luminescence is the emission of light by electronically excited states from any substance and has two categories, which are fluorescence and phosphorescence. Typically, an electron in excited orbital of excited singlet states is paired to a second electron in the ground state orbital, subsequently, causing emission of photon when returning to ground state orbital. Generally, emission rate of fluorescence is $10^8$ s$^{-1}$, therefore, the fluorescence lifetime is usually near to $10$ ns ($10 \times 10^{-9}$ s). Lifetime is the average rate of time spent of the molecules in the excited state. The processes that took place during light absorption and emission could be illustrated by the renowned Jablonski diagram. The diagram is often applied to deliberate starting point of light absorption and emission of molecular processes occurring in excited states. A schematic diagram is as shown in Figure 2-3.

![Jablonski diagram](image)

**Figure 2-3:** A brief illustration of the Jablonski diagram.

Singlet ground, first and second electronic states are represented by $S_0$, $S_1$ and $S_2$ respectively with vibrational energy levels depicted by 0, 1 and 2 existed in fluorophores. Transitions between states are illustrated by vertical lines. Following absorption of light energy, several processes occur. Fluorophore generally excited to higher vibrational level such like $S_1$ or $S_2$. Some molecules are exceptional because in their condensed phases, they rapidly relax to lowest vibrational level, $S_1$. The process is known as internal conversion which takes place within $10^{-12}$ s or less. However, internal
conversion is generally completed prior to emission because fluorescence lifetimes are generally $10^{-8}$ s. Hence, fluorescence is emitted when electrons return to singlet ground state, $S_0$ from the lowest energy vibrational state of $S_1$.

Moreover, molecules in $S_1$ state can also undergo spin conversion to first triplet state. $T_1$ is termed as phosphorescence and is typically shifted to longer wavelengths which require lower energy. This occurrence is known as intersystem crossing whereby $S_1$ was converted to $T_1$. It is forbidden for $T_1$ to undergo transition into singlet ground state thus, resulting in smaller magnitude of rate constants compared to fluorescence. Examples of phosphorescent are molecules containing bromine and iodine because they are considered heavy atoms. Not only could heavy atoms facilitate intersystem crossing, they could also enhance quantum yields of phosphorescence.

Based on Jablonski diagram, it was observable that energy of emission is less than that of absorption. This is reflected by fluorescence at longer wavelengths or lower energies, a clear indication of energy losses between excitation and emission. This occurrence was perceived by Sir. George G. Stokes in 1852 at University of Cambridge (Stokes, 1852) and one usual reason of Stokes shift is the rapid energy deterioration of fluorophore to the lowest vibrational level of $S_1$. Moreover, solvent effects, complex formation and energy transfer can cause further Stokes shifts.

The emission of light from triplet excited state is known as phosphorescence. For this process, the same spin orientation of the electron as the ground-state electron occurs in the excited orbital. Phosphorescence lifetimes are usually in the range from milliseconds to seconds because transitions to ground state are forbidden thus, resulting in slow emission rate. In some “glow-in-the-dark” toys, longer lifetime phosphorescence substances were used and observed. In these toys, after light exposure, the substances glow after several minutes before the excited substances return to ground state. In fluid solutions at room temperature, many deactivation processes such as quenching processes and non-radiative decay exists which competes with the emission, therefore, phosphorescence is usually not seen in fluid solutions. With that, the quantum yields of phosphorescence are relatively low in fluid solutions.
at room temperature. The long phosphorescent lifetime, presence of other quenchers, impurities and dissolved oxygen are reasons to the absence of phosphorescence in fluid at room temperature. Low temperatures are preferable to decrease rate of non-radiative decay, comparable to emission rates of phosphorescence.

Fluorescence spectra typically exist at shorter wavelengths and phosphorescence spectra are usually at the longer wavelengths. Phosphorescence spectra are generally more structured as compared to fluorescence spectra. A phosphorescence spectrum is shown separately from fluorescence spectrum in Figure 2-4, adapted from (Cioni and Strambini, 2002), depicting fluorescence and phosphorescence of tryptophan at low temperatures in a glass. The phosphorescence spectrum displays well resolved vibrational structure and red shifted relative to the fluorescence spectrum (Cioni and Strambini, 2002).

![Figure 2-4: Absorbance, fluorescence and phosphorescence spectra of tryptophan respectively in a glass at low temperature. Revised from Cioni and Strambini (2002).](image)
Intermolecular interactions affect energies of both excited and ground states. Stronger interaction will result in lower level energy scale, and vice versa. Often, during excitation, it is not just the energy that changes; spatial distribution of electrons also drastically changes. These states will cause different interaction with surrounding molecules. When interaction with the surrounding is stronger during the ground state, molecules that absorb and emit light of higher energy between these states will have their spectrum shifted to shorter wavelengths. However, if the interaction is stronger during the excited state, the energy between the ground and excited state is lower, thus, the spectrum will be shifted to longer wavelengths (Demchenko, 2008).

Surrounding environment and solvent polarity causes recondite effects on fluorophore spectral properties. The origin of C-dots PL could have been from the defects of surface states (Hu et al., 2009), surface groups (Fang et al., 2012), varied sized nanoparticles (Baker and Baker, 2010, Li et al., 2010a), surface passivation (Baker and Baker, 2010), fluorophores with varying degrees of π–conjugation (Mao et al., 2010, Bourlinos et al., 2012) and recombination of electron hole pairs localised within sp² carbon clusters embedded within sp³ matrix (Srivastava and Gajbhiye, 2011). C-dots adsorption shoulders on UV scan attributed by π – π* transition of C═C bonds, n – π* transition of C═O bonds or others. Most C-dots are composed of sp² carbon atoms hybridised with ample oxygenous residues and also other small conjugated aromatic structures of carbon clusters. Visible fluorescence of the C-dots would be generated by suitable band gaps and radiative recombination by isolated aromatic structures (Fang et al., 2012). C-dots are carbon based nanomaterials constitute different families of nanomaterials and mainly carbon with sp² hybridisation characteristic of monocrystalline graphite with relatively high oxygen content (Baker and Baker, 2010, Esteves da Silva and Gonçalves, 2011, Fan and Chu, 2010, Xu et al., 2004). In Figure 2-5, an illustration is provided to display an example of active sites suggested to exist on C-dots surface.
Measurements and analysis of fluorescence spectroscopy can be applied to wide range of chemical and biological sciences. They provide information of molecular processes such as solvent interaction with fluorophores, conformational changes, distance between molecules and binding interactions. Fluorescence technologies are increasing in numbers due to numerous simple methodology and economical raw materials available for developing this technology. Emission spectrum is measurable using most spectrofluorometers at a particular constant excitation wavelength. The fluorescence spectrum is generally presented as emission intensity versus the wavelength in nm or in some cases, wavenumber (cm$^{-1}$).

Previously, the photon absorption of a fluorophore and relaxation to lowest vibrational energy level of excited state pathway was discussed. Due to the emission of photon which leaves the fluorophore in higher vibrational ground state, the emission spectrum is usually a symmetrical mirror image of the absorption spectrum. The probability of the election returning to the particular vibrational energy of the ground state after excitation is similar to the probability of the electron position in the ground state before excitation. In cases of photon having varying wavelength and quanta, they will have sufficient absorbed energy and thus often produce transitions from other inter-nuclear separation distances and vibrational energy levels. This will result in absorption spectrum with multiple peaks (Figure 2-6) (Herman et al., 2009).
Figure 2-6: Illustration of electronic absorption and emission bands of excited fluorophore (Herman et al., 2009).

As examples of C-dots spectra, the respective optical properties of blue C-dots, green C-dots, yellow C-dots and red C-dots are shown in Figure 2-7 (Bhunia et al., 2013). Typically, the emission wavelength of C-dots is strongly dependent on the size of the particle while the full width at half maximum (FWHM) determined from the PL spectra is dependent on the size distribution of the C-dots. According to Bhunia et al. (2013), the size of blue C-dots is less than 4 nm whereas the particle size of yellow C-dots can be within 1 to 10 nm. Therefore, as shown in Figure 2-7, when blue C-dots are compared with yellow C-dots, the emission spectrum was blue-shifted and narrower due to the smaller particle size.

Figure 2-7: Optical properties of blue, green, yellow and red carbon nanoparticles with digital images of solution under excitation (Bhunia et al., 2013).
The measurement of fluorescence intensity at a single wavelength is a sensitive and simple method to obtain information from fluorescence reporters (Demchenko, 2008). The fluorescence QY can be described as the ratio of quanta number emitted to the amount of quanta absorbed (Demchenko, 2008). There are several aspects that influence emission spectra and QY which include internal charge transfer, solvent polarity and viscosity, conformational changes, proton transfer and excited state reactions. Normally, more than one factor will simultaneously affect the optical property of a fluorophore.

### 2.2.3 Characterisation of C-dots

One method that could characterise the surface properties of C-dot is via infrared spectroscopy. Infrared spectroscopy encompasses the study of infrared light interaction with matter. It works by expressing vibrational modes of molecular covalent bonds. Generally, infrared spectra could determine the type of functional groups present in a sample. Infrared radiation is an alternative name for heat. Through infrared radiation, chemical bonds in materials vibrate because heat has been absorbed by the matter. Different functional groups will have different tendency to absorb infrared radiation of the same wavelengths, regardless of the molecular structure the functional group is on. As an example, different compounds such as carboxylic acids, ketones and aldehydes have C═O stretch of the carbonyl group at 1700 cm$^{-1}$. The wavenumbers are used to correlate the properties of the molecular structure with the absorbed infrared radiation. Unknown molecules are then identifiable based on the infrared spectrums obtained. An infrared spectrometer is an instrument that can be used to measure infrared spectrum. The instrument uses an interferometer that can analyse a sample either by a reflectance or transmittance mode.

FTIR spectroscopy enables easy, non-destructive and sensitive analysis of molecules (Etzion et al., 2004). A majority of spectrometers works within the mid-infrared radiation region, defined as light within 4000 to 400 cm$^{-1}$. A great number of molecules have strong absorbance within this region. Infrared spectrum generally displays the plot of measured infrared radiation intensity usually in absorbance versus the wavenumber in cm$^{-1}$. Absorbance in the FTIR spectrum is typically measured in peak area, peak height, peak area ratio or peak height ratio. The wavenumber at which the infrared absorption measured
can be correlated with molecular structure thus, identifying the functional groups in an unknown sample. Peak intensities are generally affected by the concentration of sample while the width is sensitive to sample chemical matrix including hydrogen bonding and pH. Besides, infrared spectra can also quantify the amount of molecules in the sample via Beer’s Law. A variety of sample types that can be identified by FTIR includes biological materials, liquid, solids, gases, powder, semi-solids, polymers and inorganics (Smith, 2011, Smith, 1995, Thomas et al., 2008). As mentioned earlier, FTIR scan have been performed by Xu et al. (2004) on the carbon nanoparticle products that they found. The scan has proven the presence of carboxylic groups on the surface of the nanoparticles.

### 2.2.4 Quenching of fluorescence

Fluorescence phenomenon is the emission of light quanta by fluorophore. The outcome of this phenomenon usually leads to the production of bright measurable emission. When this emission is switched from emissive to less emissive state, the change can be easily observed and recorded by a detector, such as a spectrofluorometer. It is relatively easy to understand a sensing event based on the enhancement or quenching response of the fluorophore (Demchenko, 2008).

Fluorescence quenching, as stated by Lakowicz (2006), refers to the process that reduces fluorescence intensity of the sample (Lakowicz, 2006). Fluorescence quenching is a phenomenon when the fluorescence intensity showed a decrease due to some disturbance of the emission mechanism. Molecular interactions such as excited state reactions, energy transfer, collisional quenching and molecular conformational change, in addition, chemical or electronic mechanism such as electron exchange, photoinduced electron transfer and intersystem crossing can result in quenching. There are two different types of quenching whereby both requires molecular contact between quencher and fluorophore; firstly, collisional quenching which is also known as dynamic quenching and second, static quenching. Quenching measurements enables determination of fluorophore accessibility to quenchers. A variety of quenchers are available. Therefore, fluorophore-quencher combinations can be identified for a specific purpose. Although quenching
investigation is forthright, one should always examine fluorescent impurities and consider absorption spectra of quenchers.

One common kind is the collisional quenching. Collisional quenching occurs when the quencher encounters the fluorophore during lifetime of excited-state. The fluorophore then returns to ground state without photon emission. In this situation, quenching does not cause permanent change in the molecule. Stern-Volmer equation could be used to describe collisional quenching.

\[
\frac{F_0}{F} = K_{SV}[C] + 1
\]

Stern-Volmer bimolecular quenching constant was represented by \( K_{SV} \) while \([C]\) is concentration of quencher. The quenching constant represents sensitivity of fluorophore towards a quencher. When \( K_{SV} \) value is low, it could mean fluorophore is inaccessible by the water soluble quenchers. Similarly, larger \( K_{SV} \) values can be obtained if fluorophore is well exposed to its surrounding. It is crucial to identify that a linear Stern-Volmer plot does not always verify collisional quenching of fluorescence. Examples of molecules that can acts as collisional quenchers include oxygen, amines and halogens. Different fluorophore-quencher pairs can cause different mechanisms of quenching. For example, heavy atoms and halogens can cause quenching through intersystem crossing to the triplet state and spin-orbit coupling. In another case, acrylamide causes quenching on indole could have been caused by electron transfer, from indole to acrylamide. Besides collisional quenching, complex formation with quenchers, also known as static quenching, can also occur (Lakowicz, 2006).

In the category of static quenching, the quencher and fluorophore forms a complex and the complex becomes non fluorescent. It was denoted as static quenching because it does not rely on molecular collision and it occurs on the ground state. This complex returns to the ground state immediately when it absorbs light without emission of a photon. Observable fluorescence comes from the un-complexed fluorophores. The dependency of fluorescence intensity based on quencher concentration is derivable by deliberating association constant for the complex formation. Static quenching eliminates a portion of the fluorophore from observation.
In some cases, static quenching can be represented in Stern-Volmer plots. Based on dependence on temperature, viscosity and lifetime, static and collisional quenching can be differentiated. In the case of temperature dependence, an example is depicted in Figure 2-8. Higher temperature causes rapid diffusion of quenchers on to fluorophore, hence, higher expanse of collisional quenching can be observed. Whereas, if smaller amount of quenching is observed from higher temperature study, it can be considered as static quenching because this involves the detachment of weakly bound complexes (Lakowicz, 2006).

![Figure 2-8: Comparison of collisional and static quenching for a system with increased temperature.](image)

### 2.2.5 Surface modification strategy

Treatments can be performed on raw C-dots to enhance the fluorescence emission intensity. These treatments such as surface oxidation to carboxylic acid groups (Xu et al., 2004, Bourlinos et al., 2008b, Sun et al., 2006, Sun et al., 2008, Wang et al., 2009) and C-dots doping and capping of particle with organic polymer. Surface passivation agents could significantly modify PL properties of C-dots and also improve solubility of C-dots in non-aqueous solvents (Baker and Baker, 2010). Amino linkages could be formed by amino terminated reagents such as ethanolamine is useful in C-dots surface passivation (Baker and Baker, 2010). The surface of C-dots is tunable via surface modification such as covalent bonding (Yang et al., 2012a, Yin et al., 2013, Zheng et al., 2014, Dong et al., 2012), sol-gel technology (Wang et al., 2011a, Mao et al., 2012), and coordination (Zhao et al., 2011) which helps selectivity and sensitivity of these particles for application.
Figure 2-9: Brief physio-chemical treatments of C-dots production that can be used for chemical or biochemical sensor.

As shown in Figure 2-9, raw materials undergo either the top-down approach or the bottom-up approach to produce C-dots. Some raw C-dots may not be fluorescent thus, chemical treatments are usually performed to passivate the surface and make the C-dots fluorescent. These treatments generally maximise and stabilises fluorescence output.

Passivation of bare carbon nanoparticles can significantly enhanced the optical properties. As an example, Wu et al. (2013) performed a study to passivate the surface of optically active carbon nanoparticles (OCN), derived from commercial honey, with hyperbranched bis-MPA polyester hydroxyl polymer (HBP). Their study has proven the significant enhancement of OCN fluorescence properties by the hyperbranched polymer as compared to linear polymer polyethylene glycol (PEG) coated OCN. Furthermore, they have also proved that extensively branched passivating agents could amplify the emission efficiency of OCN (Wu et al., 2013).

PEG can be used to functionalised C-dots for *in vivo* imaging and biosensing. Yang et al. (2009b) have explored the feasibility of utilising PEGylated carbon quantum dots (CQDs) as fluorescence contrast agent in mice. The PEG-passivated CQDs were injected into mice and fluorescence images showed sufficient contrast for imaging in both green and red emissions.
(Yang et al., 2009b). PEGylated CQDs are found to be non-cytotoxic up to concentrations which were much higher than the required amount for cell imaging and related applications (Wang and Hu, 2014).

Besides affecting the PL properties, passivation also aids in defining the functionalities of C-dots. A new organosilane-functionalised C-dots (OS-CDs) were formed by solvothermal synthesis of citric acid with N-(β-aminoethyl)-γ-aminopropylmethyl-dimethoxysilane (AEAPMS) developed by Wang et al. (2015). The OS-CDs synthesised have dual long chain functional groups, –NH₂ and –Si(OCH₃)₃ as terminal moieties which enables the particles to have multi-solvent dispersible properties and exhibit excellent selectivity and sensitivity to mercury (Hg²⁺) ion. The results obtained showed that OS-CDs passivated by AEAPMS may be used as effective Hg²⁺ sensor in practical application (Wang et al., 2015).

2.2.6 Fluorescence sensing

Chemical and biochemical analysis via fluorescence sensing has become an active area for research (Wolfbeis, 2004, Cammann, 2003, Rich and Myszka, 2005, de Silva et al., 2001, Badugu, 2005, Geddes and Lakowicz, 2005b, Geddes and Lakowicz, 2005a, Lakowicz, 1994). This upsurge for research in fluorescence sensing application fulfills the need for low cost testing method and rapid analysis for a wide range of environmental, bioprocess and clinical applications. Fluorescence is relatively preferable than absorption for high sensitivity detection because of the different way of measuring the intensity of the system.

According to Lakowicz (2006), light absorbance measures the intensity difference between light passing through reference and sample while in fluorescence, intensity is measured directly without comparing to reference beam. The advantage of utilising fluorescence studies is due to the ability to measure fluorescence relative to a dark background rather than applying absorbance measurement to compare with bright reference beam. Moreover, it has the ability to separate compounds based on their excitation or emission properties as opposed to a single spectrum. Most materials absorb light hence, in spectrophotometric techniques, it is difficult to segregate targeted analyte
from an intricate matrix. Most photomultiplier tubes in spectrofluorometer are able to detection of low levels of light. Sensing of fluorescence intensity change, lifetime or anisotropy phenomenon can be used to understand collisional or static quenching.

### 2.2.7 Sensor

In a sensor, information of a binding between target compounds with a sensing platform can be determined (Demchenko, 2008). A chemical sensor translates chemical compounds or reaction present in a system into measureable signals via a device that reads chemical stimulus (Demchenko, 2008). The output signal is very important to identify and quantify chemical analytes. A typical sensor usually consists of a recognition element. This element will then interact with analyte and its properties, optical, physical, or mechanical properties will change and recorded into a readout signal. Optical sensor works by using light as a stimulus and ability to detect changes of light intensity from the sampling system in relation to compound binding to the sensing platform (Holford et al., 2012). Fluorescent labelling technology is easy to implement and inexpensive for sensing application. This is mainly due to the fluorescence variation signals upon complex formation and direct sensor operation (Gauglitz, 2005, Demchenko, 2008).

In fluorescence-based fibre optic spectroscopy, the enhancement or quenching of fluorescence intensity at the receptor could be used to correlate to the amount of compound present (Carrere, 2006). It is of benefit to use nanoparticles as sensing receptors due to their improved performance of having low detection limits, signal amplification due to large surface areas and capability for simultaneous multiple analyte (Holford et al., 2012).

Biosensor is a recognising device integrated with a biological element (Zhang et al., 2014). Biosensor is termed as a self-contained device that recognises an analyte via biological element and converts the signal resulting from the interaction into an electronic signal (Marty et al., 1995). By contrast to conventional analytical instruments, biosensors are alternatives to be widely used for detection of OP. In recent years, a number of researches have deduced the use of biosensors that can achieve high sensitivity, low detection
limits, rapid detection, and large linear range of OP detection for on-site application.


It is also possible to immobilise the fluorescent nanoparticles on solid surfaces for developing main parts of sensing devices as the optical element (Demchenko, 2008). These particles are able to be used due to their PL properties. The change of intensity will be measured via spectrometry instrument. One of the materials that can be integrated into fluorescence based sensor is C-dots. The working mechanism will be able to contribute to the detection of pesticide compounds. Fluorescence-based sensors are devices which will produce analytical signals from photoluminescent emission process. They may utilise emission intensity, emission wavelength or fluorescence anisotropy as analytical information. C-dots can be used to identify and quantify targeted analyte by monitoring the analytical fluorescence output. Signals will arise from selective disorientations that change the fluorescence species concentration or altering polarity, pH or charge, thereby, changing fluorophore emission (Schäferling, 2006). The inexpensive raw materials in small amounts can be used for development of a sustainable biosensor (Liu et al., 2008). Such sensors are cheaper and easier to handle although shows less sensitivity in some cases.
2.3 Pesticides

During the 15th century, chemicals such as lead and arsenic were used as pesticides, added into the soil to eradicate insect species. Between the 17th to 19th century, the demand for using pesticide became stronger to increase yield of plant by reducing the damage caused by parasites (Miller, 2002, McCartor and Becker, 2010). Pesticides are known to eradicate or deter pests, in the form of plant or animal life from crops (Marty et al., 1995). According to the Food and Agriculture Organization of the United Nations (2003), the term pesticide is described as any substance intended to destroy pest or unwanted plant species that interferes with the production, storage or marketing of agricultural commodities, feedstuffs or wood products. Based on the definition, the term ‘pesticide’ applies to insecticides, herbicides, fungicides and various pest control substances.

Pesticides are used by agriculturists to protect crops from the invasion of pests before and also after harvesting the products (Marty et al., 1995). Pest attacks on agricultural crops have been a nuisance for agriculturalists and this has cause heavy usage of effective pest eradication chemicals such as the OP pesticides (Molina et al., 1994, Marty et al., 1995). Pests, such as insects, weeds, bacteria, and rodents can cause crop depletion and thus pesticides are required to control food production (Marty et al., 1995). Yield of vegetables, corn, maize, and cotton crops are improving and crop losses are significantly reduced due to the usage of pesticides (Venugopal et al., 2012). Many low and middle income countries are having more agrarian labourers migrating to urban area to acquire higher income opportunities. Usually, these countries are also food producer and exporter. Therefore, less farmers are available but the demand for food maintain which leads to the frequent use of pesticides (Ponting, 2007, McCartor and Becker, 2010). Many farmers especially at rural areas are not aware of health hazards posed by pesticides and this has contributed to the use of excessive amount of highly toxic and cheap pesticides. Millions of tons of OP are used every year to control the pests on products that feed the growing world population and this can pose serious threat on the food safety. Globally, the scale of pesticide impact on human is estimated to be around 5 to 8 million people affected by pesticide contamination.
Since 1960’s, the application of pesticides have increased enormously (Venugopal et al., 2012). Nonetheless, the downfall of these chemicals is the tremendous acute effects on human health and environment quality which raised consternations at local, national and also in global scales (Venugopal et al., 2012). Neurotoxic OP pesticide chemicals are not only used in agricultural industries but some are also used as chemical war agents in military affairs (Sahin et al., 2011, Tang et al., 2014). It is very important to ensure the pesticides in vegetables and fruits are completely degraded before marketing the agricultural products (Marty et al., 1995).

OPs have stable chemical properties turning them into persistent organic pollutants and extensively dispersed in the environment for a long period of time (Tang et al., 2014). Henceforth, living organisms are exposed to suffer the negative consequences and adverse toxic effects of pesticides (Abdul Rani, 2002, Venugopal et al., 2012). Bioaccumulation of these dangerous compounds in the food chain is possible. The dissipation pathways of pesticide residues are depicted in Figure 2-10.

![Image of dissipation pathways of pesticide residues](image_url)

Figure 2-10: Major routes of pesticide to could reach and contaminate the surrounding environment.
The major routes of water contamination by pesticide include spray drift of pesticides from agricultural areas to unintended area. Airborne particles enable pesticide residue sorption and then precipitation will wash these particles to the aquatic environment. According to Miller (2002), around 98% of insecticides sprayed on crops contaminate other sources such as food, water, soil and air instead of the targeted organisms. The harmful residues could also permeate through the soil into the ground water. Accidental spill or neglecting proper handling procedures can also cause excessive residue wash off from land and carried to the water as runoff.

2.3.1 Toxicology effects of pesticides

The presence of OP in insecticides targets the nervous system whereby it could cause irreversible inhibition acetylcholinesterase (AChE) in pests, resulting in critical neurotoxic effects (Chambers and Oppenheimer, 2004, Sahin et al., 2011). Long term exposure could lead to death due to the failure of acetylcholine (ACh) chloride reaction (Schäferling, 2006). The important function of AChE is to catalyse the hydrolysis of neurotransmitter ACh most importantly at the somatic nervous system motor division (Chen and Yang, 2013). This indirectly leads to accumulation of neurotransmitter, ACh (Hassell, 1991, Liu et al., 2008, Schäferling, 2006, Apilux et al., 2015). In Figure 2-11, the illustration portrays the act of inhibition by OP pesticide on AChE which resulted in the over-expression of ACh. AChE is vital for nerve impulses responses in the body but the toxicity of OP pesticide can cause irreversible binding of AChE and inhibition mechanism that reacts with O–H bond of the AChE serine amino acid. Therefore, they are widely used as pesticides to eradicate pests (Borriello, 2007).
Without proper precaution of applying these pesticides for agricultural use, it could also affect nervous systems of other living organisms such as domestic livestock, fish, birds, wildlife and human being (Azab et al., 2015). These chemical are carcinogenic and cytotoxic which causes adverse health effects to animals and humans when the residues enter the food chain via water, soil or air (Sassolas et al., 2012). OP pesticide residues could cause abdominal pain, seizures, paralysis, respiratory failure, and chronic health effects such as central nervous system damage, DNA damage, immune system deficits, deformities, cancer development and even death (Tran-Minh et al., 1990, Cremisini et al., 1995, Eyer, 1995, Steenland, 1996, Jamal, 1997, Ray, 1998, Liu et al., 2008, Jaga and Dharmani, 2005, McCartor and Becker, 2010).

2.3.2 Conventional detection of pesticides

The detection technique of pesticides based on sensing mechanism involves the inhibition of cholinesterase, immunoassays and OPH. Enzyme inhibition mechanisms detect initial velocity kinetic performance of the reaction catalysed by AChE. Immunosensor devices are based on indirect assay measurement coupled with detectable labels such as enzymes or fluorescent chemicals (fluoroimmunoassays) (Heldman et al., 1985). Enzyme-linked immunosorbent assay (ELISA) is a common example of a technique used for
pesticide analysis due to its low detection limits without requiring radioactive material. As stated by Viveros et al. (2006), the current available portable equipment is inaccurate and not specific to certain OP pesticides (Viveros et al., 2006). The detection of pesticides is possible via few conventional methods such as chromatographic methods with selective detectors for efficiency, sensitivity and reliability (Sassolas et al., 2012).

Different types of commercial OP compounds can be determined and quantified using advanced methods such as HPLC (Hall et al., 1997, Molina et al., 1994), liquid (LC) or gas chromatography (GC) (Leoni et al., 1991, Sherma, 1993, Sherma, 1995, Lacorte and Barcelo, 1994), mass spectroscopy (MS) (Hall et al., 1997, Mendoza, 1972) (Guo et al., 2013, Ji et al., 2005) and surface acoustic wave (SAW) (Paddle, 1996) and ion mobility spectrometry (IMS) (Steiner et al., 2002, Chen and Yang, 2013). However, these approaches require expensive instruments and complex sample pre-treatments. These make the methods less suitable for field testing of multiple specimens (Liu et al., 2008, Ji et al., 2005, Guo et al., 2013, Marty et al., 1995).

2.3.3 Biosensors for pesticide detection

Relatively, new inexpensive, sustainable, and rapid sensing techniques can be the alternative and sensitive tool for identification and quantification of OP pesticide. Therefore, developing fast automated, versatile and inexpensive sensing device is of great interest especially dealing with a large number of samples. Enzyme biosensors work by having pesticides as specific inhibitors rather than being substrates of the enzyme. The detection is based on the inhibiting capacity of the enzyme activity (Marty et al., 1995). Another type of sensor is the immunosensors whereby test kits can be developed as ‘indirect technique’, where detection of molecular interactions could be done via labeling such like fluorophore, enzyme or radioactive isotope (Marty et al., 1995). However, these kits possess disadvantages which includes slow equilibration time and irreversible binding which averts continuous or reusability application. Optical immunosensors can be developed to operate at which the sensor response to light is reformed upon binding of a specific compound. Biosensors utilising enzyme or biomarkers for pesticide sensing are listed in Table 2-1.
Table 2-1: Nanomaterial based biosensors established for several OP pesticides detection (adapted from Zhang et al. (2014)).

<table>
<thead>
<tr>
<th>Nanomaterials</th>
<th>Enzyme/Biomarker</th>
<th>Target analytes</th>
<th>Detection limit</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT, Au/cr - GS</td>
<td>AChE</td>
<td>Paraoxon</td>
<td>0.4 pM, 0.1 pM</td>
<td>(Liu and Lin, 2006, Wang et al., 2011b)</td>
</tr>
<tr>
<td>Fe$_3$O$_4$/MWCNT</td>
<td>AChE</td>
<td>Malathion, Chlorpyrifos, Monocrotophos, Endosulfan</td>
<td>0.1 nM, 0.1 nM, 1 nM, 10 nM</td>
<td>(Chauhan and Pundir, 2011)</td>
</tr>
<tr>
<td>CNT</td>
<td>AChE and CHO</td>
<td>Methyl Parathion</td>
<td>0.05 µM</td>
<td>(Lin et al., 2004)</td>
</tr>
<tr>
<td>CNT, MC/CB</td>
<td>OPH</td>
<td>Paraoxon</td>
<td>0.15 µM, 12 µM</td>
<td>(Deo et al., 2005, Lee et al., 2010)</td>
</tr>
<tr>
<td>Au/ ZrO$_2$/SiO$_2$</td>
<td>-</td>
<td>Paraoxon - ethyl</td>
<td>0.5 ng mL$^{-1}$</td>
<td>(Yang et al., 2012b)</td>
</tr>
<tr>
<td>Fe$_3$O$_4$ @ TiO$_2$, QD</td>
<td>OP- BChE</td>
<td>Paraoxon</td>
<td>0.01 nM</td>
<td>(Zhang et al., 2013)</td>
</tr>
</tbody>
</table>

The pesticide sensing system is inspired by a work conducted by (Simonian et al., 2005). Several studies have been performed to develop biosensors utilising AChE, butyryl cholinesterase (BChE), urease and glucose oxidase for OP detection (Evtugyn et al., 1996, Mionetto et al., 1994, Palleschi et al., 1992, Kulys and D'Costa, 1991). However, in each enzyme inhibition biosensor, the OP compound will deteriorate the enzyme activity due to the interaction with the active site which will cause the sensor efficacy to decrease. Henceforth, new strategies are required to overcome the shortcomings of inhibition-based biosensors. In 1996, (Rainina et al., 1996) pioneered the catalytic approach for direct detection of neurotoxins by utilising OPH (E.C.
3.1.8.1) (Simonian et al., 1997, McDaniel et al., 1988). In this approach, OPH was used to hydrolyse a variety of OP pesticides including paraoxon, parathion and sarin (Dumas et al., 1990). A wide range of OP compounds can be hydrolysed by OPH, releasing alcohol and acid which could be detected via amperometry (Wang et al., 1999) or potentiometry. The protons products catalysed by OPH in a highly specific manner in many cases is chromophoric or electroactive (Mulchandani et al., 1998b). Due to the hydrolysis of pesticides, the products will cause a change in pH detectable by conventional pH electrodes or pH sensitive fluorescent dye (Rainina et al., 1996, Russell et al., 1999) but such approaches are highly dependent on sample buffering strength. The buffer strength should be low enough for the detection of pH change due to the hydrolysed substrates but this method is difficult to control. Therefore, alternative analytical techniques harnessing fluorescence change studies of a molecule by quenching (Chen et al., 2000) or Forster resonance energy transfer (FRET) (Lichlyter et al., 2003) have been developed.

2.3.4 Organophosphorus hydrolase (OPH)

OPH is an enzyme which hydrolyses OP pesticides (Mulchandani et al., 2001). This enzyme has comprehensive substrate specificity and hydrolyses only a number of OP pesticides including paraoxon, parathion, methyl parathion, malathion and diazinon (Du et al., 2010, Lee et al., 2010, Pedrosa et al., 2010, Munnecke, 1980, Dumas et al., 1990, Dumas et al., 1989a, Dumas et al., 1989b, Makkar et al., 2013, Schofield and Dinovo, 2010, Zheng et al., 2007, Liu et al., 2008). The molecular structures of methyl parathion, paraoxon-ethyl and malathion is as shown in Figure 2-12.

Figure 2-12: Molecular structures of (a) methyl-parathion, (b) paraoxon-ethyl and (c) malathion.
OPH was originally isolated from *Pseudomonas diminuta* MG and *Flavobacterium* sp. in the 1980’s by Mulbry and Karns (1989) and Serdar et al. (1989) (Tang et al., 2014). OPH has drawn significant attention of research studies due to their greater specificity than cholinesterases and not subjected to non-specific inhibition. OPH can also be a suitable recognition element for OP compound in biosensors (Rainina et al., 1996, Viveros et al., 2006).

There are some OPs that are more likely to have phenolic leaving groups and this can be leveraged for sensing systems to detect the leaving groups due to the enzymatic reaction between pesticides and Paraoxonase 1 (PON 1) (Sahin et al., 2011). Numerous studies have been performed to analyse the presence of pesticide via AChE integrated in biosensor system. As an example, when paraoxon molecule is hydrolysed by PON 1, diethyl phosphoric acid and \( p \)-nitrophenol (PNP) will be released as hydrolytic products (Figure 2-13). AChE is not inhibited by the products due to the hydrolytic process by PON 1 which indirectly detoxifies the OP complexes (Chen and Yang, 2013, Chambers, 2008).

![Figure 2-13: Paraoxon pathway hydrolysed by PON 1 producing diethyl phosphoric acid and \( p \)-nitrophenol.](image)

In addition, the release of \( H^+ \) is possible due to the aforementioned process because diethyl phosphoric acid acted as a weak acid. According to Sassolas et al. (2012), OPH enzyme are able to cleave esters bonds of OP pesticides, especially \( P - O \), \( P - S \), \( P - F \) and \( P - CN \) bonds, resulting in products that are, usually electroactive and/or chromophoric that are detectable by biosensors (Sassolas et al., 2012, Lai et al., 1994, Mulchandani et al., 1998a, Mulchandani et al., 1998b, Constantine et al., 2003, Lee et al., 2003).
This ability makes OPH most captivating because it has the potential of hydrolysing a variety of OP compounds (Viveros et al., 2006).

As an example, Simonian et al. (2001) targeted fluorine-containing OP compounds and used OPH to selectively cleave P – F bond of fluorine. This process resulted in solution pH change, detectable by organophosphorus acid anhydrolase (OPAA)-based biosensor. This validates the potential of integrating OPH for selective, direct, simple and rapid determination of OP pesticides via electrochemical technique (Moretto and Kalcher, 2014).

2.3.5 Paraoxonase 1 (PON 1)

The family of organophosphorus hydrolase consists of paraoxonases such as paraoxonase 1 (PON 1), paraoxonase 2 (PON 2) and paraoxonase 3 (PON 3) (Chen and Yang, 2013). Human serum paraoxonase (E.C.3.1.1.2) is an enzyme that catalyses hydrolysis of organophosphate paraoxon into nontoxic products such like PNP and diethyl-phosphoric acid. Paraoxonase is an aromatic esterase that requires calcium for its activity and is inhibited by chelating agent such like ethylenediaminetetraacetic acid (EDTA) (Eckerson et al., 1983). PON 1 possesses dual functionality, first being a hydrolyser of organophosphate pesticide and other dangerous environmental agents and secondly, PON 1 aids in protecting low density lipoproteins (LDL) from oxidative variation via manipulating high density lipoprotein (HDL) ability. Therefore at low levels of PON 1, it is a prominent risk factor for coronary vascular disease (CVD) (Borriello, 2007). The crystal structures of PON 1 in different elevation were represented (Figure 2-14 and Figure 2-15).

The crystal structure (Figure 2-15) was represented by different colour coding for the structure. Catalytic calcium is represented in blue, structural calcium denoted in green and ligating residues shown in stick structures. Then, in a close up view depicted in Figure 2-15 (b) and Figure 2-15 (c), large group of pocket residues are represented in red, small group pocket denoted in purple and the leaving group is designated by yellow colour. D269 represents the catalytic aspartate and residues important for phosphotriesterase reaction.
Figure 2-14: Biological assembly image of serum paraoxonase by directed evolution at a resolution of 2.2 Å. Protein chains are depicted by rainbow (spectral) colour gradient from N-terminal to C-terminal adapted from (Harel et al., 2004).

Figure 2-15: A crystal structure of PON 1 (pdb. 1v04) adapted from Bigley and Raushel (2013) and Harel et al. (2004). A) Side view of β-propeller fold with extended HDL anchoring helices. B) View of metal centres and ligating residues of PON 1. C) View of substrate binding pockets of PON 1.
It is known as paraoxonase due to its unique ability to hydrolyse highly toxic parasympathomimetic metabolite paraoxon. Human serum PON 1 is calcium dependent esterase which splits an ester into acid and alcohol via hydrolysis reaction (Borriello, 2007). The first report on the enzymatic hydrolysis of organophosphate compound by animal tissues, primarily human and rabbit tissues begins in 1946 by Abraham Mazur (Borriello, 2007, Draganov and La Du, 2004, Pejin-Grubiša, 2012). In the 1950s, Norman Aldridge studied the rate of paraoxon hydrolysis in serum and tissues of mammalian species (Borriello, 2007, Draganov and La Du, 2004). In addition, Aldridge also proposed paraoxonase enzymes are capable of hydrolysing organophosphates and aromatic esters.

2.4 Heavy metal

Heavy metals are referring to those metals having density exceeding 5 g per cubic centimetre. Although not fulfilling this, arsenic, chromium, lead and mercury are also considered as heavy metals due to the hazardous effects to the environment (Barakat, 2011). Chemical intensive industries discharge large amounts of hazardous cadmium, chromium, copper, nickel and lead of which they are highly soluble in aquatic environments. The pipelines transporting effluents from industrial operations often run through the ground and occasionally can leak into the soil through breakage or cracks thus causing contamination (McCurtor and Becker, 2010). These pollutants can also enter the rivers and eventually some reaches the ocean. It will contaminate and accumulate in aquatic species which are also food sources for the local communities (McCurtor and Becker, 2010).

Heavy metals are very persistent and stable therefore; they are not easily destroyed nor degrade. This can result in the absorption by small living organisms which is part of the food chain. Since human sit on the top of the chain, the accumulation in large concentration in the body is possible and will cause serious health disorders (Babel and Kurniawan, 2004). Exposure of heavy metals could lead to severe health effects such as organ damage, cancer, reduced growth and also death. It is with best interest of regulatory bodies to regulate and monitor amount of heavy metal contamination and also to perform treatment on waste water prior discharge from industries. However, there is a
lack of sensing probes that can be easily used and, applying green chemistry principle as detection. It will be of best interest to be able to analyse concentration of metal ions in water efficiently with rapid, inexpensive and sensitive detection.

Chromium can be released from minerals formed naturally in the Earth, however, industrial processes such as metal processing, tanning operations and stainless steel welding made up the majority of chromium environmental releases (McCartor and Becker, 2010). Extensive mining activity such as chromite mining causes serious environmental danger through soil and water reservoir pollution. Water contaminated by chromium at catchment areas surrounding mine quarries is being discharged to the surrounding without pre-treatment (Das et al., 2013). Chromium can exist in water in different valence states of -2 to +4, however, Cr(III) and Cr(IV) are more common in the environment. Cr(III) is poisonous in high concentration. In neutral condition, Cr(III) is insoluble and can be precipitated out (Diederik, 2008). Chromium can cause immense health impacts such as respiratory, reproductive, immunological and developmental problems (McCartor and Becker, 2010).

Negatively charged DNA phosphate groups are susceptible to electrostatic interaction with cationic Cr(III) to form complex that affects the transcription and replication of DNA strands. This can lead to cancer development (Codd et al., 2001, Cervantes et al., 2001). Cross-links between DNA and proteins, glutathione or ascorbate, respectively can occur too. Cr(III) plays a crucial role in carcinogenesis and this is proven by studies conducted by Voitkun et al. (1998) and Snow (1991). Cr(III)-glutathione adduct was found to be the most potent followed by Cr(III)-His and Cr(III)-Cys in a study utilising pSP189 shuttle vector conducted by Voitkun et al. (1998). This demonstrates the influence of Cr(III)-dependent pathway in carcinogenicity induced by Cr(III) (Voitkun et al., 1998). In another study, Cr(III)-treated template was found to greatly increase the rate of DNA replication relative to controls via purified DNA polymerases. Besides, the mutation frequency was found to be dose-dependent of the Cr(III) induced. This suggested has that Cr(III) contributes to chromium mediated carcinogenesis (Snow, 1991). As shown in Figure 2-14, the formation of Cr(III)-DNA adducts is a predominantly expressed in the carcinogenic
mechanism. In parallel, hydroxyl radicals are generated because chromium ions can participate in Fenton-like reactions.

![Figure 2-16: Carcinogenic mechanism pathway of chromium (III) compound (Henkler et al., 2010).](image)

Conventional removal methods of heavy metals from contaminated effluents such as chemical precipitation, ion exchange and electrochemical removal do not remove contaminants completely. It requires high energy and produces toxic sludge in return (Eccles, 1999). In addition, conventional detection methods utilising high end sensitive equipment such as atomic absorption spectroscopy (AAS), MS and ion pair chromatography (IPC) produces specific and accurate results but these equipment requires trained technicians, costly technological equipment, time consuming and tedious sample preparation. The negative implications of heavy metal contamination have raised concerns by environmental protection bodies to standardise and monitor levels of contaminants at factory discharge point via compact measurement sensors for environmental monitoring.

### 2.5 Sustainable technology

Green chemistry is a new philosophy of innovative design in chemical processes for chemical production that could eliminate hazards while achieving goals for a sustainable development. Green chemistry involves the use of renewable resources for the conversion into chemical substances such as fine and bulk chemicals. Green chemistry has gained a strong position in areas of research and developments because it has demonstrated that ingenious
scientific design can help achieve a healthy environment and economy simultaneously for a sustainable civilisation. There are twelve principles of green chemistry developed by Paul T. Anastas and John C. Warner in 1991. These principles are adapted from Anastas and Warner (1998) and are listed as follows:

1. Prevention of waste and toxic by-products is better than to clean up afterwards.
2. Synthetic method should be designed in advance to maximise the incorporation of all materials used into the production of the final product.
3. Synthetic designs should be designed to use and generate substances that posed little or no toxicity to human health and the environment.
4. Chemical products produced should preserve the efficacy of desired function while minimising the toxicity to human and the environment.
5. Solvents, separation agents and auxiliary chemicals should be made unnecessary whenever possible and harmless when used.
6. Energy requirements of synthetic methods should be minimised and recognised for their environmental and economic impacts. It is preferable for these methods to be conducted at ambient temperature and pressure.
7. Whenever technically and economically practical, raw material feedstock should be renewable rather than depleting.
8. Unnecessary intermediate derivatisation (blocking group, protection and deprotection techniques, temporary modification of physical and chemical processes) in synthetic methods should be reduced.
9. The use of catalytic reagents with great selectivity is superior to stoichiometric reagents.
10. Chemical products should be designed so they do not persist in the environment at the end of their function and degrade into innocuous products.
11. Analytical methodologies require to be further developed to allow real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. Raw materials and chemical substances used in a chemical process should be chosen to minimise potential of chemical accidents.
From the twelve principles, green chemistry can be considered as a series of reductions which can improve the economy, environment and community. Costs can be saved by reducing waste production because waste removal has become increasingly expensive especially when it is hazardous, minimal energy consumption and reducing material consumption. The use of renewable resources will render the manufacturing industry to be more sustainable. Furthermore, reduction in risks and hazards of handling dangerous substances can benefit not only the operators, but also the local communities (Clark, 2005). A summary of basic reduction components of green chemistry are shown in *Figure 2-17*.

![Figure 2-17: The basic reduction elements of green chemistry adapted from Clark (2005).](image)

In the twentieth century, sustainable development has been accepted as an orthodoxy for global economic and environmental protection (Clark, 2005). According to Vandamme and Soetaert (2004) the term “sustainability” can be defined as fulfilling the needs of the present without compromising future generation’s ability to achieve their own necessities. By basic definition, “green carbon” relates to the availability of materials produced from highly abundant precursors with minimal energy consumption and avoid further generation of
toxic polluting substances (Titirici, 2012). The current stress amongst researchers is to support and perceive awareness of a demand to create novel technology for a progressing sustainable society (Titirici, 2012, McCartor and Becker, 2010). Nature has offered abundance of opportunities for our need to produce functional materials using the wide variety of raw materials including carbohydrate, proteins and nucleotides (Titirici, 2012). With respect to the impact of chemicals used and the utilisation of the Earth’s resources, the application of C-dots could promote continuous protection of a sustainable global environment.

Carbohydrates and molasses are examples that can be utilised from the industrial sugar sector raw material supply. C-dots can be easily produced by breaking down large carbon sources such as the carbohydrate. Carbohydrate is abundantly available because the rate of harvesting carbohydrate is less likely to exceed the rate of regeneration. Many types of carbohydrate are available from the environment and this includes glucose, fructose, lactose, sucrose, maltose and many more. Sucrose (C\textsubscript{12}H\textsubscript{22}O\textsubscript{11}) is a disaccharide organic compound composed of glucose and fructose. Physically, it exists as a white crystalline solid which is highly soluble in water. It could be naturally found in sugarcane, honey and sugar beets. Sucrose is a great candidate for sustainable production of C-dots since it is rich in carbon content. It is also nontoxic and biocompatible, qualities that are environmentally friendly.
3. RESEARCH METHODOLOGY

3.1 Introduction

The synthesis of C-dots is an important part of this study. Sucrose was selected as the main carbon source and various methods was performed to produce C-dots\textsubscript{yellow} in this project. Mainly, the acid hydrolysis method was an effective and convenient way to synthesized C-dots\textsubscript{yellow}. Small organic molecules was carbonised by an oxidising acid to produce carbonaceous materials. This simple and more environmental friendly synthetic method of C-dots\textsubscript{yellow} production was performed by one pot approach via direct phosphoric acid (H\textsubscript{3}PO\textsubscript{4}) oxidation of sucrose. The optimal conditions such as concentration of sucrose and H\textsubscript{3}PO\textsubscript{4} used, temperature applied, period of incubation, the effect of pH, and high ionic strengths was determined.

Thermal degradation, or pyrolysis, method was investigated to compare with C-dots\textsubscript{yellow} produced via acid hydrolysis. Thermal degradation was achieved via furnace assisted pyrolysis (Tan et al., 2014, Weng et al., 2015). Pyrolysis approach involves thermal decomposition of the organic matter in furnace at high temperature. Saccharide is a common name of sugars which is generally termed for carbohydrate. Sugar polymers or oligomers could be formed by monomeric carbohydrates, producing monosaccharide, disaccharide, trisaccharide, and polysaccharide which will be applied in this study.

Covalent grafting is a suitable surface functionalisation method to tag oxygen-containing groups on surface of C-dots with another molecule. Carbodiimide conjugation of C-dots with primary amines through formation of amide bond could be accomplished by the EDC carbodiimide crosslinker reaction (Montalbetti and Falque, 2005). The coupling chemistry behind the functionalisation of C-dots\textsubscript{yellow} involved a prevailing cross linking using EDC. EDC works by inducing the formation of amide bond through activation of carboxylic acid group side chain on the surface of C-dots\textsubscript{yellow} to form an active ester intermediate. With the aid of N-Hydroxysuccinimide (NHS) as an intermediate stabiliser, 1,6-Diaminohexane (HMDA), can be added to form amide bond. EDC-NHS activation reaction works best at pH 4.7 to 6 and the reactions generally performed in phosphate-buffered saline (PBS).
The sensing of pesticides; malathion, methyl parathion, and paraoxon-ethyl, was analysed with bare and functionalised C-dots\textsubscript{yellow}. The validation of the pesticides in standard stocks was performed via HPLC. This was followed by activation of PON 1 enzyme via preparing in Tris (hydroxymethyl) aminomethane (TRIS) and calcium chloride (CaCl\textsubscript{2}) because PON 1 is an aromatic esterase that requires calcium for its activity. Sensing of pesticide standards were made via two methods. First, direct sensing of pesticides was performed by means of direct addition of PON 1 enzyme without coupling agent with bare C-dots\textsubscript{yellow}. Secondly, the sensing was performed using C-dots functionalised with PON 1, denoted as C-dots – PON 1. This approach was conducted to determine the influence of PON 1 enzyme on the sensing sensitivity. A study by Benning et al. (1994) revealed that OPH have 6 primary amines on each monomer in the form of lysine residues and two sulfhydryl residues (Simonian et al., 2005). This makes the enzyme a suitable candidate for direct conjugation via EDC through carboxylates present on surface of C-dots\textsubscript{yellow} to primary amines of PON 1. C-dots – PON 1 was then used for pesticide sensing and the signal obtained was correlated with control experiments. Commercial sample will also be tested with the new sensing strategy.

Immobilisation of C-dots\textsubscript{yellow} in PVA was also investigated. This was part of the plan for fabricating a mobile sensing device. PVA hydrogels are non-toxic, hydrophilic bioadhesive gels applied for numerous biomedical and pharmaceutical studies (Hassan and Peppas, 2000). They also have exceptional physical and chemical stabilities making them an excellent material for hydrophilic membrane production. They can form film on surfaces enabling the usage for immobilising particles on a surface (Zhang et al., 2008). However, PVA is not compatible with high salt content components therefore the salting out extraction method with acetone was necessary to remove excess salt and impurities from C-dots\textsubscript{yellow}. 

3.2 Materials

All chemicals purchased were of analytical grade and used as received from suppliers without further purification. All aqueous solution was prepared using ultrapure water (18.2 MΩ, 25°C) of Millipore Milli-Q system. Sucrose (C\textsubscript{12}H\textsubscript{22}O\textsubscript{11}) and phosphoric acid (H\textsubscript{3}PO\textsubscript{4}, 85%) were purchased from Bendosen (Malaysia). Sodium hydroxide (NaOH) was purchased from Unichem (Malaysia). Starch was purchased from Fisher Chemical (United Kingdom), 1,6-Diaminohexane (HMDA) was purchased from Merck (Germany), acetone was purchased from EMSURE, Merck (Germany), and sulphuric acid (H\textsubscript{2}SO\textsubscript{4}) was purchased from Fisher Scientific (Malaysia). N-Hydroxysuccinimide (NHS), D-melezitose monohydrate, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Fluka), quinine hemisulfate salt monohydrate standard, p-nitrophenol (SUPLECO), phosphate buffered saline (PBS), paraoxon-ethyl and malathion standards were purchased from Sigma Aldrich (Malaysia). Paraoxonase 1 (PON 1) was purchased from Thermo Scientific. Tris (hydroxymethyl) aminomethane (TRIS) was purchased from 1\textsuperscript{st} Base (Malaysia), calcium chloride (CaCl\textsubscript{2}) and sodium carbonate monohydrate (Na\textsubscript{2}CO\textsubscript{3}.H\textsubscript{2}O) were purchased from HmbG (Malaysia). Metal ions; aluminium nitrate (Al(NO\textsubscript{3})\textsubscript{3}), cobalt (II) nitrate (Co(NO\textsubscript{3})\textsubscript{2}), chromium (III) nitrate (CrNO\textsubscript{3})\textsubscript{3}, copper (II) nitrate (Cu(NO\textsubscript{3})\textsubscript{2}), lead nitrate (Pb(NO\textsubscript{3})\textsubscript{2}), mercury (II) nitrate (HgCl\textsubscript{2}), nickel nitrate (Ni(NO\textsubscript{3})\textsubscript{2}), tin (II) chloride (SnCl\textsubscript{2}), and zinc nitrate (Zn(NO\textsubscript{3})\textsubscript{2}) were purchased from R & M Marketing (Malaysia). Hydrochloric acid (HCl), D-(+)-Glucose, potassium hydrogen phthalate (KHP), polyvinyl alcohol (PVA), monopotassium phosphate (KH\textsubscript{2}PO\textsubscript{4}), sodium bicarbonate (NaHCO\textsubscript{3}) and potassium chloride (KCl) were also purchased from R & M Marketing (Malaysia).

3.3 Instrumentation

Fluorescence measurements were accomplished using a fluorescence spectrophotometer (CARY Eclipse Varian). Sample was placed in a quartz cuvette of four clear windows and a path length of 1 cm for measurements. Both emission and excitation slits were set at 10 nanometres (nm). UV-Visible absorbance in the range of 200 to 800 nm was recorded using a UV-Vis Spectrophotometer (Varian Cary 50 Conc). Mettler Toledo SevenEasy pH metre was used for accurate determination of the pH value of the solution. Forced air
drying oven (TUFF/ Malaysia/ TFAC-136) was used for incubation purposes. CARBOLITE ELF 11/14 drop down door 1100°C chamber furnace was used for pyrolysis process.

3.4 Personal protective equipment

The toxicity of pesticide analytical stocks used posed high treats towards the wellbeing of the user, other laboratory users and the environment. Therefore, high precautions were taken during analysis. Stock and reagent bottles were clearly labelled with appropriate labelling system. Disposable gloves, safety goggles, respirators, and overall laboratory coat with protective boots are worn during experimentation. Chemical wastes are collected in labelled waste bottles and placed in designated area for collection by city council waste management system of Sarawak, Malaysia.

3.5 Synthesis of C-dots

3.5.1 Acid dehydration of carbohydrate source

The optimised conditions for the production of C-dotsyellow were investigated. Firstly, the concentration of sucrose required for the synthesis was optimised. To perform this, 4 stock solutions of different concentrations were first prepared by dissolving exactly 0.5, 1.0, 1.5, and 2.0 g of sucrose powder in 4 volumetric flasks, and made up to 10 mL using distilled water. Then from each flask, 3 mL of the solution was mixed with 7 mL of concentrated H₃PO₄, and the general C-dots synthesis procedure was performed. The summary of the overall process performed for the production is illustrated in Figure 3-1. The fluorescence intensity for the isolated C-dots was recorded for all the sets. Optimisation of H₃PO₄ was performed by having a fixed sucrose concentration of 100 mg/mL, while the concentrations of acid used were 5, 10, and 15 M. The effect of temperature treatment was studied by setting the oven temperatures to 60, 70, 80, 85, 90 and 95°C respectively, while the significant on incubation period was studied at an interval of 5 minutes over 60 minutes.

The properties of C-dots produced from different oxidising agents were then analysed. Concentrated H₂SO₄ and H₃PO₄ were used to carbonise sucrose. An illustration provided in Figure 3-2 portrays a flow of procedures taken for the
Initial production of C-dots\textsubscript{yellow}. The method was performed with 1 mL of sucrose of different concentrations; 250, 350, and 500 mg/mL. These stocks were then mixed with 2.5 mL of concentrated H\textsubscript{3}PO\textsubscript{4}. The mixture undergoes thermal incubation at 85ºC for 30 minutes in a drying oven. Next, the mixture was neutralised and centrifuged at 4400 rpm for 30 minutes. Finally, the suspension was collected and analysed via spectrofluorometer. The process was repeated for evaluating effects of H\textsubscript{2}SO\textsubscript{4}, substituting the aforementioned H\textsubscript{3}PO\textsubscript{4}.

**Figure 3-1:** Summary of pathway adopted for C-dots\textsubscript{yellow} production via H\textsubscript{3}PO\textsubscript{4} hydrolysis.

**Figure 3-2:** Synthesis and analysis pathway of C-dots\textsubscript{yellow} via acid dehydration by H\textsubscript{2}SO\textsubscript{4} or H\textsubscript{3}PO\textsubscript{4}.
Effects of pH was investigated using buffer solutions prepared with slight modification from the one reported by Robinson and Stokes (1965). All buffer mixtures are listed in Table 3-1. For each buffer, the final volume was made up to 100 mL. The study was performed by adding of 100 µL of C-dots_{yellow} sample into the respective buffer solutions having the final volume of 3 mL.

Table 3-1: Buffer recipe for the preparation of different pH buffers.

<table>
<thead>
<tr>
<th>pH</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.96</td>
<td>1.012 g KHP with 0.1 mL 0.1M HCl.</td>
</tr>
<tr>
<td>5.03</td>
<td>1.012 g KHP with 22.6 mL 0.1M NaOH.</td>
</tr>
<tr>
<td>6.03</td>
<td>0.681 g KH₂PO₄ with 5.6 mL 0.1M NaOH.</td>
</tr>
<tr>
<td>7.00</td>
<td>0.681 g KH₂PO₄ with 29.1 mL 0.1M NaOH.</td>
</tr>
<tr>
<td>8.00</td>
<td>0.681 g KH₂PO₄ with 46.7 mL 0.1M NaOH.</td>
</tr>
<tr>
<td>9.61</td>
<td>0.420 g NaHCO₃ with 5.0 mL 0.1M NaOH.</td>
</tr>
<tr>
<td>11.2</td>
<td>0.420 g NaHCO₃ with 22.7 mL 0.1M NaOH.</td>
</tr>
</tbody>
</table>

The fluorescence of the samples was recorded under similar instrument setting. As for the effect of ionic strength towards the optical properties of C-dots, 4.69 M KCl was prepared by dissolving 34.96 g of KCl in 100 mL of distilled water. C-dots were introduced to the KCl solution gradually until 4.69 M and the fluorescence of C-dots was recorded.

3.5.2 C-dots_{yellow} stock preparation

For C-dots_{yellow} sample preparation in acidic condition, 100 µL of acidic C-dots_{yellow} was taken straight from the storing vial and transferred into a quartz cuvette. Distilled water was added into the cuvette to make up to the final volume of 2 mL. The same steps were taken for preparation of C-dots_{yellow} sample in neutral condition. 100 µL of the C-dots_{yellow} first neutralised was taken straight from the storing vial and placed in the quartz cuvette. Next, distilled water was added to make up to the final volume of 2 mL.

3.5.3 Saccharide pyrolysis

Initial studies were performed to determine the appropriate temperature required to carbonise 1 g of sucrose on a fixed duration of 10 minutes because it was deduced in this study that longer period of 10 minutes leads to production
of ashes and no fluorescing products was obtained. The temperature of the furnace was adjusted to 200, 250, 300 and 350ºC respectively to 4 sets of sucrose samples. When the optimum temperature of pyrolysis was determined, 1 g of glucose, sucrose, melezitose and starch respectively was weighted out and placed in a crucible. The furnace was preheated to the optimum temperature and the crucibles were placed in the furnace for 10 minutes. Then, the crucible was removed from the furnace and left to cool before the contents were transferred into a separate vial. The final product was sealed tightly in the vial and kept in the desiccator. The sample was dispersed in distilled water to make final concentration of 5 mg/mL before each analysis.

3.6 Surface modification of C-dots

In 5 mL of C-dots\textsubscript{yellow} stock solution prepared in 0.1 M PBS buffer, 2 mg of EDC was added into the solution followed by 3 mg of NHS. The pH was corrected to pH 5.0 ± 0.5 with addition of HCl solution. The reaction mixture was stirred well via magnetic stirrer in sealed condition for 15 minutes at room temperature. After that, the buffer was adjusted to pH 7 using 0.1 M PBS buffer. Next, 100 mg of HMDA was added into the mixture. The reaction was left to proceed for another 2 hours at room temperature. Products were then stored in air tight vials at room temperature.

3.7 Salting out

The salting out extraction technique was employed to isolate C-dots from the mother liquor after the neutralisation process. 5 mL of filtered (5 µm, PVC, Millipore syringe filter) acetone was added into 5 mL of C-dots\textsubscript{yellow} in a tube. The tube was shaken thoroughly on a vortex for 30 minutes and was left to settle to equilibrate. Top layer supernatant was collected and placed in a separate centrifuge tube. The solution was then concentrated via vacuum concentrator for at least 6 hours followed by lyophilisation process ran overnight. The solid product was collected and stored in tightly sealed vial.

3.8 Photoluminescent quantum yield (PLQY)

Absorbance of the C-dots\textsubscript{yellow} stock was measured using ultrapure distilled water as baseline. The optical density of the C-dots\textsubscript{yellow} was recorded via UV-Vis Spectrophotometer (Varian Cary 50 Conc). The integrated
fluorescence intensity of C-dots\textsubscript{yellow} was recorded and calculated from the spectrum. The same steps were repeated using the quinine sulphate standard that was reported to have a QY of 0.546 at excitation wavelength of 366 nm (Brouwer Albert, 2011). The quinine sulphate was prepared in 0.1 M H\textsubscript{2}SO\textsubscript{4} and the concentration of sample was controlled with absorbance below 0.1 to minimise inner filter effect. PLQY measurements were based on the equation below (Eq. (2)).

\[
Q = Q_R \frac{I}{I_R} \frac{A_R}{A} \frac{n^2}{n_{R}^2}
\]  

(2)

where Q denotes as the quantum yield while QR is PLQY of standard, I as the integrated intensity, A as the absorbance value and n as refractive index.

3.9 Limit of detection (LOD)

Limit of detection (LOD) characterises information of a sensor because it provides information of the minimum analyte concentration that a sensor can reliably detect (Kellner et al., 2004, Demchenko, 2008). For an electronic instrument, the ultimate performance is determined by signal-to-noise ratio (SNR) measurements. SNR can be determined from the peak height of a fluorescence spectrum and rationing it with the level of noise on the baseline of the spectrum. Noise is usually taken as random fluctuations at the baseline. SNR can be used to verify if a signal is tangible. The intensity signal must be above 3 times of the noise to be considered real, if not, it should be disregarded. The concentration can be measured with equitable statistical certainty of 97.7%, determined via calculating the LOD. The sensitivity of the sensor equipment can be correlated with the LOD. Higher sensitivity will have lower LOD. LOD is usually expressed as the serial measurements of blank signals standard deviation without the analyte (Demchenko, 2008). LOD is deducible by an approach of measuring replicates of a blank sample. The mean value and standard deviation will be calculated based on the measurement.

The LOD standard deviation was determined based on 6 blank samples. 6 blank samples were prepared by dispersing 100 µL of C-dots\textsubscript{yellow} in distilled water to a final volume of 2 mL. All 6 readings were recorded at fixed excitation wavelength of 420 nm. The maximum peak of each peak was recorded and
standard deviation was determined. Then, the standard deviation was multiplied 3 times. To establish the LOD, a number of spiked samples with concentration expected to be within the analytical range was prepared. For every increasing analyte concentration analysis, the determination of LOD can be performed from the slope gradient. The performance of the method was validated with adding a known amount of analyte to the blank solution. This represents detection concentration of targeted samples.

The LOD was determined using Eq. (3).

\[
\text{LOD} = \frac{3\sigma}{s}
\]  

(3)

where \(\sigma\) is the standard deviation of the blank C-dots\text{yellow} sample (\(n = 6\)) and \(s\) represents the slope value of calibration plot.

### 3.10 Stern-Volmer modelling

Analysis of quenching activity was performed by consecutive addition of analyte into C-dots\text{yellow}. The analyte tested was prepared in a fixed concentration of 0.1 M. With each accumulative addition of analyte, the maximum intensity was recorded and plotted to determine quenching trend of analyte on fluorophore.

In a non-linear quenching system, Stern-Volmer equation was applied to establishing correlation of fluorophore intensity with increasing quencher concentration. The relationship was applied to describe the photoluminescence quenching profiles of the C-dots\text{yellow} in the presence of different quencher concentrations. In collisional quenching, the system could be represented by following equation (Eq. (1)):

\[
\frac{F_0}{F} = K_{SV}[C] + 1
\]

(1)

where, \(F_0\) represents the initial intensity while \(F\) is the intensity with the presence of quencher. \(K_{SV}\) will be the Stern-Volmer quenching constant and \([C]\) is the concentration of quencher.
3.11 Pesticide detection

3.11.1 HPLC analysis of pesticides

Pesticide compounds in acetone fractions were analysed using UV-Vis High Performance Liquid Chromatography (HPLC; Waters). The standard solution of malathion and paraoxon-ethyl were prepared from stock solutions of 100 mg/mL respectively. From this solution, a variation of concentration was prepared (Table 3-2 and Table 3-3).

Table 3-2: Final concentrations of malathion expressed in molarity for HPLC analysis.

<table>
<thead>
<tr>
<th>Standard sample</th>
<th>Malathion (mg/mL)</th>
<th>Molarity (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.200</td>
<td>605.40</td>
</tr>
<tr>
<td>B</td>
<td>0.100</td>
<td>302.70</td>
</tr>
<tr>
<td>C</td>
<td>0.010</td>
<td>30.27</td>
</tr>
<tr>
<td>D</td>
<td>0.001</td>
<td>3.03</td>
</tr>
</tbody>
</table>

Table 3-3: Final concentrations of paraoxon-ethyl expressed in molarity for HPLC analysis.

<table>
<thead>
<tr>
<th>Standard sample</th>
<th>Paraoxon-ethyl (mg/mL)</th>
<th>Molarity (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>0.200</td>
<td>809.26</td>
</tr>
<tr>
<td>F</td>
<td>0.100</td>
<td>404.63</td>
</tr>
<tr>
<td>G</td>
<td>0.010</td>
<td>40.46</td>
</tr>
<tr>
<td>H</td>
<td>0.001</td>
<td>4.05</td>
</tr>
</tbody>
</table>

Separation was performed on reversed phase, 150 mm, LC-18, 5µm column at 25ºC. Isocratic elution was used for the HPLC system and measured at temperature of 30ºC. The mobile phase was acetonitrile : water; 60 : 40 for malathion analysis and 50 : 50 for paraoxon-ethyl analysis. Flow rate was 0.70 mL min\(^{-1}\) with injection volume of 10 µl and separation was performed over 15 minutes for malathion analysis and 25 minutes for paraoxon analysis. The wavelength of UV-Vis detector was set at 230 nm for malathion and 270 nm for paraoxon.

The concentration of commercial pesticide sample, MM57 (Figure 3-3), that was purchased from the local market vendor was validated using the calibration curve obtained from the standards. The information label of the
chemical bottle stated malathion as the active ingredient with 57% in content and remaining 43% are made of inert chemicals. Based on the information provided for users on the back side of the bottle, the recommended dilution for the chemical was to dilute 25 mL of pure stock in 10 L. Therefore, 0.25 mL MM57 was mixed in 100 mL pure acetone.

![Front view](image1.png) ![Back view](image2.png)

**Figure 3-3:** Front and back snapshot of MM57 supplied by local vendor in Malaysia. Instructions of preparation were stated on the label.

The concentration of malathion in MM57 was determined via HPLC UV-VIS detector. The conditions set were; wavelength at 230 nm, mobile phase constituting of acetonitrile: distilled water (60 : 40), flow rate at 0.7 mL min\(^{-1}\) and at temperature of 30ºC.

### 3.11.2 PON 1 enzyme stock preparation

The PON 1 enzyme was supplied in storage buffer containing 0.1 M TRIS (pH 7), 20% glycerol and 0.1 M glycine. Preparation of PON 1 (1 mg/mL) enzyme from storage buffer was performed according to a modified method based on a study conducted by Parsaeyan et al. (2012). The enzyme stock was prepared via the addition of 20 µl enzyme to 700 µl of buffer consisting of 1 mM TRIS-HCl (pH 7) with 2 mM CaCl\(_2\).
The steps begin with preparation of buffer TRIS-HCl buffer with \( \text{CaCl}_2 \). Firstly, 0.15 g of TRIS was added into a 100 mL standard flask. Then, 50 mL of distilled water was added into the flask. The pH was adjusted to pH 7 via 0.1 M HCl. Then, TRIS-HCl buffer was made up to the mark via distilled water. This makes up the final concentration of TRIS-HCl to be 12.50 mM. Then, to prepare 0.05 M \( \text{CaCl}_2 \), 0.56 g of \( \text{CaCl}_2 \) was added into a 100 mL standard flask. Distilled water was added until the mark and the stock was mixed thoroughly by inverting the flask and shake. Next, a total volume of 5 mL buffer was prepared by mixing 400 \( \mu \text{L} \) of 12.50 mM TRIS-HCl and 200 \( \mu \text{L} \) \( \text{CaCl}_2 \) with 4400 \( \mu \text{L} \) of distilled water. Then, this was followed by obtaining 15.89 \( \mu \text{L} \) of PON 1 enzyme from storage buffer into 124.11 \( \mu \text{L} \) of the TRIS-HCl buffer and \( \text{CaCl}_2 \), which resulted in the final PON 1 concentration of 0.11 mg/mL. Following, 116.76 \( \mu \text{L} \) of 0.11 mg/mL PON 1 was further diluted in 4083.24 \( \mu \text{L} \) of TRIS-HCl buffer with \( \text{CaCl}_2 \) to a final PON 1 concentration of 3.15 \( \times 10^{-3} \) mg/mL. This final concentration was used for the pesticide sensing analysis.

3.11.3 PON 1 enzyme activity

Paraoxonase activity was assayed based on a modified protocol described by Mackness et al. (1991). Paraoxonase activity was performed at pH 8 and determined at 25°C. Paraoxonase stock (100 \( \mu \text{L} \)) of 0.05 mg/mL was prepared by diluting it in 1 mM TRIS-HCl buffer (pH 8) with 2 mM \( \text{CaCl}_2 \).

Paraoxonase 1 activity was assayed based on PNP standard curve measured at 412 nm at 25°C. The standard curve of PNP was plotted by determining the absorbance (AU) vs. concentration of PNP (nM). Stop buffer was prepared by weighing out 6.2 g of \( \text{Na}_2\text{CO}_3\cdot\text{H}_2\text{O} \) and dissolve in a 250 mL standard flask with 200 mL of distilled water. The volume was made up final volume of 250 mL with distilled water. The content in the flask was transferred into a separate bottle for storage. PNP stock was prepared by dissolving 0.03 g of PNP into 100 mL of distilled water. This makes up final PNP concentration of 2 mM. Next, 2 mM PNP was further diluted into in 1:10 ratio by transferring 1 mL of 2 mM PNP into a conical tube. Then, distilled water was added into the conical tube to make up the final volume of 10 mL. The final concentration of standard PNP stock was 0.2 mM.
For PNP standards, 11 cuvettes were labelled with known concentrations of PNP standard (Table 3-4). Distilled water was added into the cuvette respectively followed by PNP standard stock (0.2 mM). Then, stop buffer was added into the cuvette respectively and mix thoroughly by vortex. The absorbance of each standard was measured at wavelength 410 nm using cuvette number 1 as the blank.

**Table 3-4:** PNP standards set up for calibration curve plot.

<table>
<thead>
<tr>
<th>Cuvette</th>
<th>PNP (µM)</th>
<th>0.2 mM PNP (µL)</th>
<th>Distilled water (µL)</th>
<th>Stop buffer (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 (blank)</td>
<td>0</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>50</td>
<td>950</td>
<td>1000</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>100</td>
<td>900</td>
<td>1000</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>150</td>
<td>850</td>
<td>1000</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>200</td>
<td>800</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>250</td>
<td>750</td>
<td>1000</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>300</td>
<td>700</td>
<td>1000</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>350</td>
<td>650</td>
<td>1000</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>400</td>
<td>600</td>
<td>1000</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>450</td>
<td>550</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>500</td>
<td>500</td>
<td>1000</td>
</tr>
</tbody>
</table>

The molar extinction coefficient calculated was 20147 M$^{-1}$ cm$^{-1}$. 1 unit (U) of paraoxonase activity produced 1 µmol of PNP per min.

### 3.11.4 Sensing of pesticide standards

Methyl parathion standard was contributed by the Agricultural Research Centre (ARC), Semongok, Sarawak, Malaysia. The standard was readily prepared in pure acetone to the final concentration of 10 ppm. However, malathion and paraoxon-ethyl pesticide standards, readily available in 100 mg per vial, were purchased from Sigma Aldrich, prepared in analytical grade acetone. The pesticide stock of 100 mg was diluted in 1 mL of analytical grade acetone to a final concentration of 100 mg/mL. The standard stocks were stored in respective air-tight glass tubes with caps firmly wrapped with parafilm.
The detection of pesticides was conducted via two approaches. For the first approach, the system utilised bare C-dots\textsubscript{yellow} with direct addition of PON 1 right before analysis and the other involved the use of C-dot – PON 1. For the quantification of pesticide via direct analysis, 100 µL of C-dots\textsubscript{yellow} was pre-conditioned with equal volume of acetone and mixed in a quartz cuvette. Then, 100 µL of PON 1 (3.15 X 10\textsuperscript{-3} mg/mL) was added into C-dots\textsubscript{yellow} and given a thorough mix then, followed by dilution to final volume of 2 mL with distilled water. This will be the blank of the pesticide detection via direct analysis. Fluorescence analysis was performed first for the blank, C-dots\textsubscript{yellow} with addition of PON 1. Then, pesticide standards of malathion and paraoxon-ethyl respectively were prepared by diluting 0.2 mL of 100 mg/mL of standard stock in pure acetone to a final volume of 20 mL which resulting in final concentration of 1 mg/mL. C-dots\textsubscript{yellow} stock was prepared by adding 100 µL of C-dots\textsubscript{yellow} with 100 µL of acetone in a quartz cuvette, followed by distilled water to a final volume of 2 mL. The addition of acetone to C-dots\textsubscript{yellow} served to pre-condition C-dots\textsubscript{yellow} because pesticides stocks were prepared in acetone. For each pesticide standard sensing, a total amount of 1 µL from 1 mg/mL standard stock is added consecutively into C-dots\textsubscript{yellow} stock prepared in the quartz cuvette. The content of the cuvette was thoroughly mixed by slight vortex shaking. Fluorescence analysis was performed after each pesticide stock addition.

Coupling of C-dots\textsubscript{yellow} with PON 1 involved the formation of covalent bond via EDC activation. Firstly, 5 mL of C-dots\textsubscript{yellow} was placed in a vial, and added with 2 mg of EDC, followed by 3 mg of NHS. The mixture was left to stir in a confined vial for 15 minutes. Next, 5 mL of PON 1 (3.15 X 10\textsuperscript{-3} mg/mL) enzyme was added to the mixture and the contents in the vial was stirred for another 2 hours. The products were stored in vials at room temperature before sensing of pesticide via this conjugated compound. 100 µL of C-dots – PON 1 was placed in quartz cuvette and diluted to final volume of 2 mL with distilled water. For each pesticide standard sensing, a total amount of 1 µL from 1 mg/mL standard stock is added consecutively into C-dots\textsubscript{yellow} stock prepared in the quartz cuvette. The content of the cuvette was thoroughly mixed by slight vortex shaking.
3.11.5 Sensing of commercial sample

The stock solution of MM57 was prepared by mixing 0.25 mL of MM57 with 100 mL to a dilution ratio of 1:400. The dilution recommendation was stated on the directions provided on the commercial product bottle label. For the direct analysis, 100 µL of C-dots\textsubscript{yellow} was added into a quartz cuvette and 100 µL of acetone was mixed to precondition C-dots\textsubscript{yellow}. PON 1 stock was mixed thoroughly into preconditioned C-dots\textsubscript{yellow} and diluted to final volume of 2 mL with distilled water in quartz cuvette. 1 µL of MM57 was added consecutively into C-dots\textsubscript{yellow} with PON 1 and fluorescence analysis was performed after every addition. For the sensing of MM57 by C-dots – PON 1, 100 µL of C-dots – PON 1 was preconditioned by 100 µL of acetone and diluted to final volume of 2 mL. Then, 1 µL of MM57 of the stock solution was added into the preconditioned C-dots – PON 1. Fluorescence analysis was performed after each consecutive MM57 addition.

3.12 Heavy metal

In order to evaluate the analytical potential of the C-dots\textsubscript{yellow} isolated for other practical application, it was tested for metal ions sensing. The change in the optical signal intensity due to the presence of different metal ions with C-dots\textsubscript{yellow} was used as the measurable parameter. Metal ion stock solution was prepared by dissolving the respective amount of metal ions salts in accordance with the weight listed in Table 3-5.

To perform the analysis for the potential of applying C-dots\textsubscript{yellow} for the detection of metal ions, a series of fixed C-dots\textsubscript{yellow} stock were added with different metal ions from their respective stock solutions (0.1 M). In a quartz cuvette, 100 µL of C-dots\textsubscript{yellow} was added. This was followed with diluting the stock with distilled water to a final volume of 2 mL and made as blank. The analysis was first performed on this blank sample. Subsequently, 10 µL of each respective metal ion (0.1 M) were added into C-dots\textsubscript{yellow} stock. The final recorded intensity of each analysis will be relatively correlated with the initial intensity of the C-dots\textsubscript{yellow} in the absence of metal ions.
Table 3-5: Respective metal ions weight prepared from metal salts for 0.1 M stock.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical formula</th>
<th>Oxidation state</th>
<th>Weight for 0.1 M stock preparation (g) in 100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium nitrate</td>
<td>Al(NO₃)₃</td>
<td>Al (III)</td>
<td>3.75</td>
</tr>
<tr>
<td>Cobalt (II) nitrate</td>
<td>Co(NO₃)₂</td>
<td>Co (II)</td>
<td>2.91</td>
</tr>
<tr>
<td>Chromium (III) nitrate</td>
<td>Cr(NO₃)₃</td>
<td>Cr(III)</td>
<td>2.42</td>
</tr>
<tr>
<td>Copper (II) nitrate</td>
<td>Cu(NO₃)₂</td>
<td>Cu (II)</td>
<td>4.00</td>
</tr>
<tr>
<td>Lead nitrate</td>
<td>Pb(NO₃)₂</td>
<td>Pb (II)</td>
<td>3.31</td>
</tr>
<tr>
<td>Mercury (II) nitrate</td>
<td>HgCl₂</td>
<td>Hg (II)</td>
<td>3.42</td>
</tr>
<tr>
<td>Nickel nitrate</td>
<td>Ni(NO₃)₂</td>
<td>Ni (II)</td>
<td>2.91</td>
</tr>
<tr>
<td>Tin (II) chloride</td>
<td>SnCl₂</td>
<td>Sn (II)</td>
<td>2.26</td>
</tr>
<tr>
<td>Zinc nitrate</td>
<td>Zn(NO₃)₂</td>
<td>Zn (II)</td>
<td>2.97</td>
</tr>
</tbody>
</table>

3.13 Immobilisation of C-dots with polyvinyl alcohol

A 5% PVA (average molecular weight (M_W): 130000) was prepared to cast C-dots_{yellow} film for immobilisation. Firstly, 5 g PVA powder was weighed out and placed in a flask. Then, 100 mL of distilled water was added into the flask and stirred on heating mantle. The mixture was heated for 2 hours at a constant 90°C until the powder was dissolved. The heating process continues for 2 hours. The PVA film was left to cool and stored in tightly sealed sterile vials.
4. RESULTS AND DISCUSSION

4.1 Introduction

The key motivations for developing a sensor include simple and sustainable production, sensitive, rapid response time, and reproducible. This work aimed to synthesise C-dots through simple, economical, and low energy consumption method. The C-dots was further utilised to develop a sensitive optical probe for detection of contaminants in trace amounts with good reproducibility.

A brief review of synthesis work was presented, which included the motivation for surface modification and immobilisation studies. The synthesis of C-dots was performed via two bottom-up approaches. For acid hydrolysis method, two types of acids, H$_3$PO$_4$ and H$_2$SO$_4$, were used to carbonise sucrose. As for the thermal carbonisation method, different carbohydrate sources were carbonised in a furnace. In the aforementioned chapter, the interest also lied on producing C-dots$_{yellow}$ due to its novelty for sensing application. Preliminary work towards achieving this objective began with the optimisation of synthesis conditions.

C-dots$_{yellow}$ was used to detect three different types of organophosphate pesticides namely: paraoxon-ethyl, methyl parathion and malathion. Commercialised pesticide product, MM57, was also tested for validation of the proposed probe.

Furthermore, the potential of C-dots$_{yellow}$ for other application was analysed where it was tested for the sensing of various metal ions. This will further leverage C-dots$_{yellow}$ as a sensing probe for detection of metal ions.

4.2 Optical properties

The optical properties were measured and recorded by spectrofluorometer. The measurements are then relatively correlated with respect to the maximum intensity of blank samples for each analysis. All the correlated intensities are labelled in arbitrary unit (a.u.).
4.2.1 Optical properties of C-dots

In this study, \( \text{H}_3\text{PO}_4 \) was chosen as the oxidising agent to convert sucrose into C-dots\text{yellow}. It is worth mentioning that the carbonisation process was not effective when performed under normal ambient condition, as there was no significant change in the physical outlook of the sucrose solution when added with the acid. This could be due to the lower oxidation strength of \( \text{H}_3\text{PO}_4 \) as compared to \( \text{H}_2\text{SO}_4 \) or hydrochloric acid (HCl) (Lenihan et al., 2010). Thus to enhance the hydrolysis reaction, the mixture was placed into an oven with temperature set above room temperature. After 30 minutes of incubation at 85ºC, the mixture has turned from clear to homogenous red-brown solution. This has significantly indicated that the carbonisation process has taken place under the set condition. NaOH was added to terminate the carbonisation and to neutralise the acidic mixture. Such addition has turned the solution from deep red-brown to yellowish colour.

In order to isolate the C-dots\text{yellow} from the bulk carbon residue, water was employed as a dispersing agent to for the formation of colloidal suspension. Basically, colloidal solution only forms when particles size is in nanometre range and with this, the suspended aliquot of the carbonised product will be mostly C-dots\text{yellow}. The larger and bulk carbon residues were removed using centrifugation at 4400 rpm and the aliquot rich in C-dots\text{yellow} was collected. The presence of the C-dots\text{yellow} in the aliquot was confirmed by their strong yellow luminescence under UV light (Figure 4-1).

![Figure 4-1](image-url)  

**Figure 4-1:** Photos of (a) ultrapure water, (b) sucrose and (c) C-dots\text{yellow} observed under UV light exposure.
To further confirm the fluorescence property, the scan using spectrofluorometer has obtained a strong band peak at 560 nm when excited at 430 nm (Figure 4-2). All spectrum for the C-dots produced were normalised with respect to the maximum intensity of the respective category. The neutral C-dots\textsubscript{yellow} displayed strong PL band with maximum intensity at 562 nm when excited at 438 nm. All spectra were normalised with respect to the maximum intensity of the neutral C-dots.

![Figure 4-2: Spectrum of C-dots\textsubscript{yellow} (a) excitation, (b) emission and (c) absorbance, derived from carbonisation of sucrose by concentrated H\textsubscript{3}PO\textsubscript{4}.](image)

The absorption spectra showed two apparent peaks at 285 and 229 nm, where ultrapure water was used as blank for the measurement (Figure 4-2 (c)). The scan was performed from 200 nm to 800 nm. The peak perceived at 285 nm was caused by C=O bond n \rightarrow n^\ast transition and the other peak at 229 nm was attributed to the transition of aromatic sp\textsuperscript{2} bond from n \rightarrow n^\ast. These were also previously reported by Xu et al. (2014) and Luo et al. (2009).

In acidic condition, the C-dots\textsubscript{yellow} displayed a maximum emission intensity at 560 nm (Figure 4-3 (d)), excited at 350 nm (Figure 4-3 (c)). As
compared to C-dots\textsubscript{yellow} in neutral condition, it absorbed excitation light of shorter wavelength, by ca. 80 nm. During the excitation, the electron elevated to higher vibrational excited state with the high energy. Then, the excited electrons will lose vibrational energy to the surrounding and return to the lowest excited singlet state followed by relaxation back to ground state with simultaneous fluorescent emission. The emission of C-dots\textsubscript{yellow} in acidic condition was on the same emission wavelength when in neutral condition. Therefore, acidic condition caused C-dots\textsubscript{yellow} to absorb light at a higher energy as compared to neutral condition.

![Fluorescence spectra of neutral C-dots\textsubscript{yellow} condition (a) excitation and (b) emission and acidic C-dots\textsubscript{yellow} condition (c) excitation and (d) emission.](image)

The FWHM of the emission band was ca. 100 nm, which showed consistency with the report by Xu et al. (2014). FWHM often reflects on the homogeneity of the photoluminescence origin for fluorescent nanoparticles regardless from surface defect or size factors. A value around 100 nm was considerably narrow, indicating that the C-dots\textsubscript{yellow} synthesis in this study have a homogenous distribution of fluorescence origin. The PLQY was determined using quinine sulphate in 0.1 M H\textsubscript{2}SO\textsubscript{4} as reference. The PLQY of the
synthesised C-dots\textsubscript{yellow} was evaluated to be 0.18%. Although this value is low, this is consistent with the PLQY of C-dots that were recorded elsewhere on C-dots (Bhunia et al., 2013).

4.2.2 Effects of acid for C-dots\textsubscript{yellow} production

The production of C-dots\textsubscript{yellow} in previous section was performed through acid hydrolysis of sucrose by H\textsubscript{3}PO\textsubscript{4} in specific conditions. However, to further revaluate the optical profile in terms of wavelength shifting, additional work was performed to analyse the effects of different acids for hydrolysis of sucrose. To test the distinctive optical properties producible by two different acids, a comparison was made with higher sucrose concentration of 250 and 500 mg/mL. Acid hydrolysis was performed between H\textsubscript{3}PO\textsubscript{4} and H\textsubscript{2}SO\textsubscript{4} with sucrose of higher concentrations. These two acids were chosen because it is a good representative of oxidising agents. H\textsubscript{2}SO\textsubscript{4} is a diprotic acid having two acidic hydrogen atoms. It is a strong acid with the acid dissociation constant (K\textsubscript{a}) value larger than 1. H\textsubscript{3}PO\textsubscript{4} is a triprotic acid, having three acidic hydrogen atoms. H\textsubscript{3}PO\textsubscript{4} is a weaker acid as compared to sulphuric acid. Therefore, when these acids were used for carbonisation, the stronger acid will produce particles of smaller sizes while weaker acid will produce larger particles for the same duration of carbonisation (Bhunia et al., 2013).

When comparing the spectra obtained (Figure 4-4), C-dots synthesised via H\textsubscript{2}SO\textsubscript{4} fluoresced in the shorter wavelength region. It was clear that C-dots hydrolysed by H\textsubscript{2}SO\textsubscript{4} (Figure 4-4 (a) and (b)) emitted at a wavelength around 420 nm. This shows that the emission colour of the C-dots produced is blue. This supports the argument that H\textsubscript{2}SO\textsubscript{4} acid is stronger and can cause more vigorous reaction that promotes nucleation rather than particle growth during the synthesis process. In general, the rate of carbonisation restricts the particle to be less than 4 nm and smaller particles are deemed to emit in the blue region (Bhunia et al., 2013). Contrarily, larger particles tend to fluoresce at a longer wavelength. Fluorescence spectrum of C-dots hydrolysed by H\textsubscript{3}PO\textsubscript{4} (Figure 4-4 (c) and (d)) exhibited emission near 550 nm. H\textsubscript{3}PO\textsubscript{4} did not increase the rate of carbonisation thus, nucleation growth may increase leading to larger particle size.
Regardless of the varied sucrose concentration, the type of acid played a crucial role in determining specific optical properties of C-dots emission. The optical profile of C-dots$_{\text{yellow}}$ emission did not alter with extreme sucrose concentration. Therefore, a higher sucrose concentration does not affect the emission profile of C-dots$_{\text{yellow}}$ in terms of spectrum shift. This demonstrates that minimal sucrose and acid concentration can be used to synthesise and produce C-dots$_{\text{yellow}}$. This effort is beneficial in order to control minimum consumption of carbohydrate source and acid as a mean to achieve more sustainable approach. Further optimisation steps for C-dots$_{\text{yellow}}$ production will be discussed in the following section.

![Figure 4-4: Different emission spectra of C-dots produced by H$_2$SO$_4$ and H$_3$PO$_4$ hydrolysis of sucrose. (a) H$_2$SO$_4$ oxidation of 250 mg/mL sucrose, (b) H$_2$SO$_4$ oxidation of 500 mg/mL sucrose, (c) H$_3$PO$_4$ oxidation of 250 mg/mL sucrose, (d) H$_3$PO$_4$ oxidation of 500 mg/mL sucrose.](image)

4.2.3 Carbonisation mechanism

C-dots$_{\text{yellow}}$ was successfully isolated by controlling different variables during the synthesis process. The carbonisation of sucrose, a carbon-rich
source, by acid oxidation involved several steps. The first step was the hydrolysis of sucrose into glucose and fructose (Xu et al., 2014, Li et al., 2011a), which later, these monosaccharides were dehydrated into furfural intermediates (Xu et al., 2014, Ryu et al., 2010, Kwon et al., 2012). At this stage, the intermediates were readily undergo polymerisation and aromatisation resulting in the change of colour from transparent to red-brown as observed in this study. Similar observations were also reported by other groups of researchers (Xu et al., 2014, Sakaki et al., 1996, Chen et al., 2013).

The ensuring nucleation and expansion of carbon occur by cross-linking of macromolecules (Xu et al., 2014, Sun and Li, 2004). In acidic condition, the carbon particles exposed to oxidation can promote better solubility in aqueous solution (Xu et al., 2014, Li et al., 2011a). The presence of heteroatoms such as phosphate, oxygen, hydrogen, and nitrogen in the carbon precursor or the activating agent could affect the surface chemistry of carbon materials. This caused modification on the electrochemical and catalytic properties of the carbon (Puziy et al., 2008, Leon y Leon and Radovic, 1994, Puri, 1970, Pérez-Cadenas et al., 2003, Bandosz et al., 1996, Stöhr et al., 1991, Matzner and Boehm, 1998). Carbons activated by H$_3$PO$_4$ as studied are classified as phenol-like, phosphorus-containing and carboxylic groups (Puziy et al., 2002, Puziy et al., 2003, Puziy et al., 2005, Castro Muñiz et al., 2007, Puziy et al., 2007, Puziy et al., 2006). H$_3$PO$_4$ catalytic process could initiate a chemical reaction involving reduction of pentavalent phosphates with carbon resulting in production of C-dots$_{yellow}$ which was observed in this study. When H$_2$SO$_4$ was used, blue emitting C-dots was produced (Figure 4-4). H$_2$SO$_4$ has stronger oxidising property to generate O═C—O—H and C—O—H from C—H and strong dehydrating property to convert saturated C—C to unsaturated C═C, causing a blue shift fluorescence (Hu et al., 2014).

4.2.4 Optimisation of synthesis condition

The carbonisation performed was a catalytic process of acid acting upon the conversion of sucrose into C-dots$_{yellow}$. A study has been carried out to carbonise the starting precursor of different concentrations. Overall, it was observed that the optimum concentration of sucrose required for the synthesis was approximately 100 mg/mL when concentrated acid was used. When the
sucrose concentration was raised beyond this, the fluorescence intensity showed no further increase in the intensity, indicating the saturation of product (Figure 4-5). The C-dots\textsubscript{yellow} was the only species identified to show fluorescence under this study settings, thus being correlated directly to reflect the amount of C-dots\textsubscript{yellow} formed. Higher intensity indicated the presence of more amount of C-dots\textsubscript{yellow}.

![Graph showing the relationship between sucrose concentration and fluorescence intensity](image.png)

**Figure 4-5:** Normalised intensity of C-dots\textsubscript{yellow} with increasing sucrose concentration used as starting precursor for oxidation by H\textsubscript{3}PO\textsubscript{4}.

Apart from sucrose concentration, the concentration of H\textsubscript{3}PO\textsubscript{4} can also alter the carbonisation process due to the strength of the hydrolysis. The result from this study shows that the fluorescence of the isolate extracted after 30 minutes of carbonisation at 85°C have increased drastically as the concentration of the acid was increased gradually (Figure 4-6). Below the concentration of 5 M of H\textsubscript{3}PO\textsubscript{4}, the conversion was ineffective even set with higher temperature which was observed by comparing the fluorescence intensity recorded. Optimum fluorescence intensity for the C-dots was recorded at 15 M of H\textsubscript{3}PO\textsubscript{4}. The motivation of this study was also to reduce the amount of acid used in order to achieve greener synthesis approach.
Figure 4-6: Cumulative concentration of $\text{H}_3\text{PO}_4$ increases fluorescence intensity of C-dots$_{\text{yellow}}$.

Temperature will be a major parameter affecting the conversion rate and type of products formed from the reaction. As mentioned previously, heating was necessary to produce the coloured product after the addition of acid to the sucrose solution. Different incubation temperatures applied during the synthesis have also affected the intensity of the C-dots$_{\text{yellow}}$ recorded. Temperature around 85ºC gave the highest intensity for those isolates extracted after 30 minutes of carbonisation (Figure 4-7). Sufficient energy was required to assist the acid hydrolysis process to form more yield as observed from the result. However higher temperatures above 85ºC can lead to less effective nucleation of carbon with more complete hydrothermal pyrolysis that ended up forming carbon dioxide and ashes. These low fluorescent products were avoided since it will reduce the yield of the C-dots$_{\text{yellow}}$. Besides, higher temperatures can also promote rapid carbonisation, resulting in smaller sized particles that tend to emit fluorescence towards the blue region (Bhunia et al., 2013).
Figure 4-7: PL intensity trend of C-dots\textsubscript{yellow} emission at various temperature intervals.

The kinetics for the carbonisation process performed at 85°C using 100 mg/mL of sucrose with concentrated H\textsubscript{3}PO\textsubscript{4} was monitored over 60 minutes with 5 minutes interval. The aliquot of the reaction mixture was extracted and evaluated for its photoluminescence property for each of the interval. The result shows a gradual increment in intensity recorded over time, indicating that sucrose was steadily converted into C-dots\textsubscript{yellow} by the acid (Figure 4-8). The reaction took around 40 minutes before showing a plateau trend, an indication that the carbonisation reaction has completed. A similar trend was obtained when the sucrose concentration was reduced to 50 mg/mL, but with recorded intensity that was lower by approximately 40% at the plateau region. Nonetheless, shorter time was recorded to reach the maximum intensity. From all the fixed concentrations of sucrose used in this study, the time taken to complete the reaction was observed around 40 to 60 minutes.
4.2.5 Optimisation of sensing condition

The C-dots\textsubscript{yellow} prepared in this study was intended to be used as sensing probe for the detection of pesticides. Thus, it is crucial to investigate the effect of the solution condition towards the properties of C-dots\textsubscript{yellow}, particularly on the photoluminescence that will be employed as sensing signal. pH was one of those important parameters that need to be studied. This was performed by adjusting the pH of the C-dots\textsubscript{yellow} solution accordingly across a broad pH spectrum using buffer solutions and their respective fluorescence property was recorded. This study found that fluorescence intensity did not fluctuate significantly within the studied pH range from 3.96 to 11.40 (Figure 4-9). This reflected that the protonation and deprotonation of the surface functional groups have very minimal impact towards the electronic transition of the C-dots\textsubscript{yellow}, which is an advantage for sensing application due to the low interference effect even with fluctuating pH condition.

Figure 4-8: Correlation of C-dots\textsubscript{yellow} intensity obtained from heat treatment of 85ºC for a period of 60 minutes.
Figure 4-9: Effects of different pH conditions on PL intensity of C-dots$_{yellow}$.

Similar result was obtained when the salinity condition of the C-dots$_{yellow}$ was adjusted using various concentrations of KCl salt, even up to the saturation point of 4.69 M (Figure 4-10).

Figure 4-10: Influence of high ionic strength by KCl on C-dots$_{yellow}$ of PL intensity.
The C-dots\textsubscript{yellow} mixture was still homogenous with no observable aggregates under the presence of concentrated K\textsuperscript{+} cations and Cl\textsuperscript{-} anions electrolytes. This contributes to the robustness of the C-dots\textsubscript{yellow} for sensing as any change in intensity will not be due to the surrounding environment, but rather due to the real effect from the analyte of interest.

4.3 Saccharides pyrolysis

Carbohydrate pyrolysis was performed as a comparative method for C-dots\textsubscript{yellow} production. Based on visual observation (Figure 4-11), heat treatment at 200\textdegree C for 10 minutes only causes slight browning of powder whereas at 250\textdegree C sucrose caramelisation was observed. Then, 250\textdegree C of heat treatment resulted in dark brown residue and finally, heat treatment of 350\textdegree C caused a dome structure with hydrophobic crisp once broken. The optical properties of the C-dots obtained was analysed and Figure 4-12 represents a collection of spectrum from sucrose pyrolysis at different temperatures. The trend of the peak intensity obtained from the respective carbonisation temperature on sucrose is depicted in Figure 4-13. The maximum emission wavelengths corresponding to the carbonisation temperature are listed in Table 4-1. Based on the optical profile, the spectrum of C-dots produced was blue-shifted from 465 to 439 nm when temperature increases to 300\textdegree C from 250\textdegree C. However, when 350\textdegree C was applied, the spectrum was red-shifted with a 50\% loss in PL intensity. The loss of PL could have been caused by structural collapse due to the extreme heat applied. The optimum temperature with highest C-dots yield was determined to be 300\textdegree C.

Regardless of the different pyrolysis temperatures, the emission property of C-dots produced from sucrose has remained in the blue region. Higher temperature led to the production of small C-dots that will emit blue fluorescence (Milosavljevic et al., 2014). The emission colour in various regions is influenced by temperature factor. Different size, shape and defects caused by high temperature also affect the emission colour properties. The variation of density and sp\textsuperscript{2} sites of C-dots are suggested to be the cause of different optical properties. By altering the size of C-dots, the energy gap can be tuned. Lower temperature will cause C-dots to absorb at longer wavelengths. The narrowing
of band gap from π electron delocalisation due to increased size of sp² domain can cause red-shifted emission (Peng et al., 2012).

**Figure 4-11:** Visual pictures captured after sucrose pyrolysis treatment under respective temperature for 10 minutes.

**Table 4-1:** Pyrolysis reaction temperature with the respective C-dots emission obtained for each temperature.

<table>
<thead>
<tr>
<th>Reaction temperature (ºC)</th>
<th>Emission wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>Not available</td>
</tr>
<tr>
<td>250</td>
<td>465</td>
</tr>
<tr>
<td>300</td>
<td>439</td>
</tr>
<tr>
<td>350</td>
<td>455</td>
</tr>
</tbody>
</table>
Figure 4-12: Fluorescence spectrum of 5 mg/ml sucrose pyrolysis at different temperature for 10 minutes. (a) 300°C, (b) 250°C, (c) 350°C and (d) 200°C.

Figure 4-13: Intensity pattern of C-dots emission of increasing carbonisation temperature from 200 to 350°C.
Different carbohydrate sources were also converted into C-dots tested using thermal carbonisation at a fixed temperature of 300ºC and the final concentration of C-dots used for analysis was 5 mg/ml. The results obtained are shown in Figure 4-14. Monosaccharide of glucose produces C-dots with emission at 436 nm and the disaccharide of sucrose produced C-dots with emission at 465 nm. Trisaccharide was represented by melezitose which produces C-dots emitting at 544 nm and finally, pyrolysis of polysaccharide, starch results in C-dots with emission at 433 nm. Pyrolysis of melezitose presents a red-shift spectrum. The red-shift could have been caused by different oxidation effects applied at 300ºC and the number of monosaccharides connected in the chain. Oligosaccharide may have different structural size after carbonisation and could have been larger than starch, which caused the red-shift. Whereas starch breaks down into smaller structures when high temperature at 300ºC was applied. This showed potential of utilising melezitose for producing C-dots_{yellow} via simple pyrolysis method but the raw material is very expensive and will not be a sustainable option.

![Figure 4-14: A combination of C-dots spectrums obtained from a selection of saccharides, (a) starch, (b) glucose, (c) sucrose and (d) melezitose, resulting from thermal carbonisation.](image-url)
4.4 Surface modification of C-dots

The aim for this section is to identify the properties of surface modified C-dots passivated with HMDA. In this modification, nitrogenous compounds played an important role to enhance fluorescence emission. To shield C-dots\textsubscript{yellow} from the surrounding, HMDA was covalently attached on the surface. The emission spectra of C-dots expressed desirable fluorescence properties with narrow spectrum (Figure 4-15). In addition, based on the spectrum, there was no obvious shifting of optical properties. The maximum fluorescence emission as shown in Figure 4-16 was obtainable after 5 days of passivation.

The maximum intensity obtained was roughly 15 times of the initial C-dots intensity. This demonstrates the radiant efficiency increases with the presence of HMDA. This is possibly the result of increasing surface area available for light passivation as C-dots\textsubscript{yellow} coating was extensively branched (Wu et al., 2013). However, the intensity gradually dropped after 5 days. The quenching of fluorescence could have been resulted from collisional self-quenching between the passivated colloidal. The amount of HMDA passivated on the C-dots\textsubscript{yellow} surface reaches a saturation point; it could cause aggregation of particles. Once aggregation occurs, the possibility of collision between passivated C-dots\textsubscript{yellow} would quench the fluorescence emission (Liu et al., 2015). Furthermore, when the fluorescence started to decrease, it can be considered that the ligand has reached maximum particle size and quantum confinement, therefore, the reaction was stopped (Goncalves and Esteves da Silva, 2010).
Figure 4-15: Excitation and emission spectrums of C-dots and functionalised C-dots after 5 days of functionalisation. (a) functionalised C-dots excitation, (b) functionalised C-dots emission, (c) C-dots excitation and (d) C-dots emission.

Figure 4-16: Maximum intensity of functionalised C-dots_{yellow} by HMDA, registered for each respective day.
4.5 Salting out

The production of C-dots\textsubscript{yellow} involved acid dehydration by concentrated H\textsubscript{3}PO\textsubscript{4} and followed by neutralisation by using concentrated NaOH. High abundance of sodium ions is a common impurity in many compounds (Wang and Sherwood, 1995). Therefore, salting out extraction was used to remove the excess salts and impurities. This involves reducing the solubility of molecules in high ionic strength solution. The separation of hydrophilic target, C-dots\textsubscript{yellow}, from the aqueous mixture is possible with the aid of an organic solvent as extractant (Fu et al., 2015, Dai et al., 2014).

In a mixture of water and miscible organic solvent of acetone, a two phases will form when inorganic salt such as sodium phosphate is added (Majors, 2009). Acetone is polar aprotic solution with medium-high dipole moment. It contains atoms of different electronegatives such as hydrogen and oxygen but they lack N-H or O-H bonds to participate in hydrogen bonding. Acetone can be homogenised with water to extract the C-dots\textsubscript{yellow}.

![Figure 4-17: Snapshot of liquid-liquid extraction separation; mixture of C-dots\textsubscript{yellow} and pure acetone in 1:1 ratio settled after vigorous shake via vortex.](image)

As shown in Figure 4-17, there were two obvious layers. The bottom layer contains solid white precipitate while the top layer showed a clear yellow liquid. C-dots\textsubscript{yellow} are polar particles which will diffuse into the aqueous layer.
(Wang and Sherwood, 1995). The extract was evaporated and lyophilised into a solid and re-dispersed in water into respective C-dots\textsubscript{yellow} stocks. Water molecules tend to surround charged molecules to support the molecules to remain dissolve in the aqueous environment. However at high ionic concentration, the water molecules are not able to support the charges of the ion resulting in insoluble solute. When the solubility of water is decreased by adding acetone which is less polar, inorganic salt will precipitate out of the solution. With this process, neutral polar organics can be pre-concentrated and extracted. The phase separation enables polar analytes to contain in the organic phase (Majors, 2009). This provides an alternative method of separation, partial purification and concentration of C-dots\textsubscript{yellow}.

Exception for inorganic salts that are soluble in acetone are those with either one or both cation and anion from the bottom of the periodic table, as example, sodium iodide and caesium fluoride. The used of acetone are often applied for S\textsubscript{N}2 displacement reactions. In this reaction, soluble salt becomes the source of nucleophile, nonetheless, the leaving group will form insoluble salt. Ionic charge, molecular size, and surface area are among the factors that will also affect salting out process.

Figure 4-18: Excitation and emission spectrum of C-dots prior extraction and after extraction via acetone. (a) C-dots excitation, (b) C-dots emission, (c) isolated C-dots excitation and (d) isolated C-dots emission.
From the spectra plotted in Figure 4-18, it indicated that the optical emission profile of the C-dots\textsubscript{yellow} remains unchanged. After lyophilisation, the solid was re-dispersed in water and it showed good homogeneity. The C-dots has intensity loss of ca. 20% of its initial intensity. This could have been caused by further processing conditions that C-dots\textsubscript{yellow} undergo. Lyophilisation involves dehydration and freezing the material under low pressure. Series of procedures can cause loss of C-dots.

4.6 Pesticide detection

4.6.1 Calibration curve

The scope of this analysis is to validate the content of malathion, from the standard stock by plotting a standard calibration curve from the peak height against the concentration of malathion. The chromatogram recorded from the HPLC computerised system is as shown in Figure 4-19. In Table 4-2, the respective peak height for corresponding malathion concentration was listed. Consequently, the peak height values were determined and the calibration curve was presented in Figure 4-20. The correlation coefficient was 1. All measured values were within the expected range of data provided by the suppliers.

![Auto-Scaled Chromatogram](image)

Figure 4-19: HPLC chromatogram of 605.40 µM malathion standard stock.
Table 4-2: Malathion, expressed in molarity, with respective peak height based on the HPLC chromatograms obtained.

<table>
<thead>
<tr>
<th>Malathion (µM)</th>
<th>Peak Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>605.40</td>
<td>58400</td>
</tr>
<tr>
<td>302.70</td>
<td>28919</td>
</tr>
<tr>
<td>30.27</td>
<td>2911</td>
</tr>
<tr>
<td>3.03</td>
<td>269</td>
</tr>
</tbody>
</table>

Figure 4-20: Standard curve analysed via HPLC of malathion expressed in peak height of respective concentration.

The following analysis aimed to separate and plot a standard curve of paraoxon-ethyl measured at 270 nm (Figure 4-21). The maximum peak height values of each paraoxon-ethyl concentration was determined and recorded in Table 4-3. The standard curve was presented in Figure 4-22. The correlation coefficient was 0.999. All the values recorded were within the expected range of data provided by the suppliers.
Figure 4-21: HPLC chromatogram of 809.26 µM paraoxon-ethyl standard stock.

Table 4-3: \hspace{2em} Paraoxon-ethyl, expressed in molarity, with respective peak height based on the HPLC chromatograms obtained.

<table>
<thead>
<tr>
<th>Paraoxon (µM)</th>
<th>Peak Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>809.26</td>
<td>332565</td>
</tr>
<tr>
<td>404.63</td>
<td>170124</td>
</tr>
<tr>
<td>40.46</td>
<td>17691</td>
</tr>
<tr>
<td>4.05</td>
<td>1589</td>
</tr>
</tbody>
</table>

Figure 4-22: Standard curve analysed via HPLC of paraoxon-ethyl analysed in terms of peak height of respective concentration.
4.6.2 Validation of commercial sample

The MM57 was pre-treated and introduced into the HPLC system. This aimed to separate malathion from the matrix and to determine its amount present in the commercially available product. The maximum peak height measured at 230 nm for each MM57 concentration was identified as recorded in Table 4-4. All the values recorded were correlated with the malathion standard curve (Figure 4-20) and the malathion content present in the commercial product was determined. Based on the calibration curve, the malathion concentration in MM57 is 0.28 M.

Table 4-4: MM57 contents in respective concentrations (%) correlated with malathion standard curve to determine actual molarity of malathion present in commercial sample.

<table>
<thead>
<tr>
<th>MM57 (%)</th>
<th>Peak Height</th>
<th>Malathion concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.14</td>
<td>71092</td>
<td>712</td>
</tr>
<tr>
<td>0.10</td>
<td>6629</td>
<td>67</td>
</tr>
<tr>
<td>0.01</td>
<td>538</td>
<td>6.05</td>
</tr>
</tbody>
</table>

4.6.3 Effect of buffer components

PON 1 enzyme was prepared in buffer prior usage. The storage and activation buffer contains a selection of main components such as glycerol, TRIS, glycine and CaCl₂. To avoid the interference effect from these components with the C-dots<sub>yellow</sub>, tests were performed separately with the individual component (Figure 4-23). The intensity was expressed in relative to the initial C-dots<sub>yellow</sub> intensity. There was insignificant change of intensity for glycerol, TRIS and CaCl₂ but glycine, showed considerable enhancement of fluorescence. Based on the preparation steps of PON 1, the final glycine concentration calculated in 3.15 X 10⁻³ mg/mL stock is 0.32 mM only. Therefore, the effect is negligible because the concentration is much lesser than 0.05 M which will not have a significant impact on C-dots<sub>yellow</sub>. 
Next, the effect of PON 1 enzyme on the fluorescence intensity of C-dots\textsubscript{yellow} was investigated. As usual, PON 1 was prepared in the TRIS buffer with CaCl\textsubscript{2} to a final concentration of $3.15 \times 10^{-3}$ mg/mL. PON 1 was added into C-dots\textsubscript{yellow} subsequently with each interval of 10 µL and the emission change was recorded. The intensity was expressed in relative to the initial C-dots\textsubscript{yellow}. Figure 4-24 demonstrates that accumulative addition of PON 1 causes quenching of C-dots\textsubscript{yellow} emission. Therefore, it can be deduced that there was an effect when additional enzyme concentration was added. The quenching could have been caused by collisional interaction of the enzyme on the surface of C-dots\textsubscript{yellow}.
4.6.4 PON 1 enzyme activity

The PON 1 enzyme assay was performed to verify the activity of the enzyme and also to quantify its amount (Bisswanger, 2014). Through the catalyse reactions of the enzymes, the identification could be done by measuring the amount of product produced via spectrophotometer with correlation to a standard curve. In a PON 1 catalysed activity with paraoxon, the products released consist of diethyl phosphoric acid and PNP. This assay utilises paraoxon as the substrate, which is colourless, and PNP as the coloured product for measurement of enzyme activity. PNP develops a strong yellow colour under basic condition and absorbs in 410 nm of light.

The discontinuous assay was implemented in this study by stopping a reaction after a period of time and the absorbance was measured. To stop the reaction, the enzyme was deactivated by a denaturing agent, which Na$_2$CO$_3$ was used in this study. When Na$_2$CO$_3$ was added with PNP, it discontinued the reaction and the solution turned into yellow colour. The amount of PNP was proportional to the amount of yellow colour formed. A standard curve ranging from 5 to 50 nM PNP (Figure 4-25) was constructed to calculate the activity of phosphatase. The standard curve provided a direct relationship between

**Figure 4-24:** PON 1 quenching effects on C-dots$_{yellow}$ with increasing enzyme concentration expressed in mg/mL.
absorbance and concentration. The total paraoxonase activity determined was 1811 U/mL.

![Figure 4-25: Standard curve plotted in increasing PNP concentration for determination of PON 1 activity.](image)

**Figure 4-25**: Standard curve plotted in increasing PNP concentration for determination of PON 1 activity.

### 4.6.5 Sensing of pesticide standards

#### 4.6.5.1 Sensing via C-dots\textsubscript{yellow}

In a preliminary study, methyl parathion standard (10 ppm) obtained from the Agricultural Research Centre (ARC) and malathion with paraoxon-ethyl purchased from suppliers respectively were tested solely on C-dots\textsubscript{yellow}. This test was performed to determine the sensing ability of C-dots\textsubscript{yellow} with these three different pesticide standards without the use of PON 1 enzyme. All measurements via C-dots\textsubscript{yellow} were pre-conditioned with acetone before pesticides were introduced into the system. Direct addition of pesticide stock without pre-condition will cause a sudden spike of intensity due to a change of solvent properties. The initial signal generated from pre-conditioned C-dots\textsubscript{yellow} was used as a baseline without foreign components. Acetone did not cause any shifting in the optical profile of C-dots\textsubscript{yellow}.

A control experiment was conducted to investigate the influence of pesticide standards on the changes of C-dots\textsubscript{yellow} fluorescence intensity in
terms of enhancing or quenching. This has enabled one to establish an equation based on the signal plotted. This equation can be calibrated into a sensing device computer system. As shown in Figure 4-26, three respective plots of pesticide sensing via C-dots_{yellow} were plotted.

The results obtained indicate C-dots_{yellow} exhibited a change of fluorescence intensity in response to different paraoxon-ethyl concentrations. Paraoxon-ethyl quenched the intensity of approximately 20% at 470 µM. From the results, it can be deduced that C-dots_{yellow} has the potential for paraoxon-ethyl sensing. The LOD deduced from the plot for paraoxon-ethyl sensing was 17.56 ± 1.18 µM. As compared to malathion sensing, the fluorescence intensity of C-dots_{yellow} did not alter much. Methyl parathion gave a slight enhancement of intensity under 2 µM. The LOD was determined to be 0.63 ± 0.15 µM. Undoubtedly, the surface properties and size of C-dots_{yellow} influences surface resonance effects which is accountable for the fluorescence change in this system (Simonian et al., 2005).

![Figure 4-26](attachment:image.png)

**Figure 4-26:** Combination of sensing signal plots for the detection of malathion, paraoxon-ethyl and methyl parathion by C-dots_{yellow}.
4.6.5.2 Sensing via C-dots\textsubscript{yellow} with PON 1

The practicability of enzyme based biosensor for OP pesticide detection utilising C-dots as competitive inhibitor of PON 1 enzyme was examined. When PON 1 enzyme was introduced, the C-dots\textsubscript{yellow} can interact with it and caused a change of optical properties of C-dots\textsubscript{yellow}. When pesticide was present, it interrupted the interaction between C-dots\textsubscript{yellow} and PON 1 due to higher binding affinity between the enzyme and the pesticide substrate. This indirectly displaces PON 1 from C-dots\textsubscript{yellow} surface due to weaker interaction and thus, caused an alteration towards the fluorescence signal (Figure 4-27).

Figure 4-27: Illustration depicting C-dots – PON 1 interaction for sensing of OP substrate. Fluorescence analysis was performed on bare C-dots\textsubscript{yellow}, during C-dots\textsubscript{yellow} binding to PON 1 and OP substrate binding on PON 1, replacing C-dots\textsubscript{yellow}.

In the early stage of pesticide detection, PON 1 (3.15 \times 10^{-3} \text{ mg/mL}) was added to react with pre-conditioned C-dots\textsubscript{yellow}. Then, 10 \text{ µL} of pesticide standards were added subsequently into the system. The blank consisted of the C-dots\textsubscript{yellow} with PON 1. Signals resulted from C-dots\textsubscript{yellow} after addition of pesticide was correlated with the blank. Within the measured range, the intensity of C-dots\textsubscript{yellow} was quenched by the increasing concentration of PON 1 (Figure 4-28). Based on the results, malathion and methyl parathion have enhanced the intensity of C-dots\textsubscript{yellow}. A higher degree of increment was caused by malathion. The LOD for malathion sensing was determined to be 0.74 \pm 0.11 \text{ µM}. The respective equations from each slope plotted based on the best fit line can be calibrated and set as default for future computerised sensing system.
The sensing of paraoxon-ethyl showed a quenching trend of 10% when only around 12 µM was added into the system. This showed that a lower detection limit can be obtained from this system as compared to the previous system whereby only C-dots\textsubscript{yellow} was used for the sensing application. Based on the plot, the LOD determined for paraoxon-ethyl sensing based on this system is 0.93 ± 0.21 µM. A synthetic fluorophore must yield different binding affinity and displaceable by the OP compound of interest. Binding affinity of C-dots\textsubscript{yellow} on PON 1 plays an important parameter that contributes to the sensor feasibility. Some fluorophore molecules may be structurally similar to certain OP compounds and they are likely to have higher affinity for the enzyme than the OP compounds. Thus, could display minimal signal alteration. Fluorophores with high affinity for the enzyme will require more OP substrates to displace the fluorophore from the enzyme active site. However, low affinity fluorophores will result in high background noise and possibility to be displaced by other molecules. These attributes could lead to lower sensitivity and specificity of the sensor. Therefore, the best sensor performance is feasible with the optimum binding affinity of fluorophore comparative to the OP pesticide substrate of OPH enzyme (Simonian et al., 2005).

The presence of competitive and non-competitive PON 1 inhibitors in the environment will greatly affect the performance of this system, similar with other enzyme-based sensors. Thus, active enzyme levels should be determined for calibration of the sensor prior operating the system as depicted in Chapter 4.6.4. Other foreign compounds present in the environment or even in prepared samples may bind to the PON 1 enzyme. It is also possible for these compounds to change the fluorescence properties of the fluorophore, thus for sensitive applications, multiple sensing technology should be applied.
In the following system, sensing will be performed using C-dots – PON 1. The sensitivity of this approach was assessed by adding the amount of pesticides accumulatively to C-dotsyellow – PON 1. The changes in the fluorescence intensity was observed and analysed. All the results were relatively correlated to the initial intensity of C-dots – PON 1 without any analytes.

It was found that after each addition of malathion, there were no apparent fluorescence change (Figure 4-29). However, when methyl parathion was added, the intensity was greatly quenched as compared to the previous sensing systems. The relative intensity showed a quenching trend of about 10% when only about 1.80 µM of methyl parathion was added. With the new enumerated signal, it provides a better sensitivity for methyl parathion sensing with a lower LOD. The LOD for methyl parathion sensing via C-dots – PON 1 sensing system was 0.17 ± 0.001 µM.
Next, the sensing ability of C-dots – PON 1 for paraoxon-ethyl was analysed. From the results, it was deducible that 20% of the fluorescence was quenched by adding around 470 µM of paraoxon-ethyl. This trend was similar to the results plotted for the sensing of paraoxon-ethyl solely via C-dots\textsubscript{yellow}. This means that the enzyme has not contributed in enhancing the sensitivity. Thus, the presence of PON 1 can be omitted for paraoxon-ethyl detection. Therefore, direct sensing of paraoxon-ethyl can be performed by simple C-dots\textsubscript{yellow} application. The LOD of paraoxon-ethyl sensing via C-dots – PON 1 was 17.56 ± 0.83 µM.

![Figure 4-29: C-dots – PON 1 tested for malathion, paraoxon-ethyl and methyl parathion sensing respectively.](image)

Based on these results, it was possible to develop catalytic enzyme activity of PON 1 for methyl parathion sensing. This direct measurement approach is unlike the analysis via pH measurement, which is highly dependent on sample buffering capacity. This detection method did not depend on the enzyme hydrolysing ability unlike several enzyme inhibition biosensors which can degrade due to loss of enzyme activity. C-dots\textsubscript{yellow} can detect paraoxon-ethyl specifically without the use of enzyme. Furthermore, this will also save the cost for developing a portable sensor because the use of an enzyme is no
longer required. One factor that could limit the sensitivity and workability of this system was the fluorescence output properties of C-dots\textsubscript{yellow}. This is because C-dots\textsubscript{yellow} with higher quantum efficiency will display lower fluorescence change. With superior fluorescence yield, the threshold for the system's intensity increases.

Overall, the LODs obtained for the sensing of paraoxon-ethyl, malathion and methyl parathion were within reasonable agreement with the lowest amount allowed for daily consumption of these OP compounds. According to World Health Organization (WHO) (1996) and Food and Agriculture Organization of the United Nations (FAO) (1996) the MRL legal limit of methyl parathion, which is the similar as paraoxon-ethyl, on agricultural product is 0.01 mg/ kg with an acceptable daily limit (ADI) of 0.003 mg/kg. Based on European Commission (2010) report on the active substance, the ADI registered was 0.03 mg/kg bw/day for malathion. Therefore, based on the low LOD obtained, the amount of pesticides can be quantified by using the sensing system.

4.6.6 Sensing of commercial product

The sensing of commercial product, MM57, was analysed via two systems; C-dots\textsubscript{yellow} with direct addition of PON 1 right before analysis and covalent coupling of C-dots\textsubscript{yellow} with PON 1 producing C-dots–PON 1. Firstly, malathion and MM57 was analysed via C-dots\textsubscript{yellow} after addition of PON 1. The ratio was normalised based on the fluorescence intensity recorded after addition of PON 1. The results (Figure 4-30) obtained indicate a similar vertical curve trend observed for both malathion and MM57 sensing. Malathion sensing revealed an increment trend up to 6 µM whereas MM57 enhances the intensity up till around 130 µM. Subsequently, both indicated a downward trend which could signify the self-quenching effect and also dilution due to the increasing volume. From the result, more MM57 was required to achieve the similar trend because within the commercial product itself, only 57% of the sample ingredient is active malathion. The rest of the product consists of unknown ingredients which could hinder the activity of MM57 sensing by C-dots\textsubscript{yellow} with PON 1. Due to the curved trend, one may record two different concentrations at a single relative intensity obtained. Therefore, serial dilutions can be performed to examine and
analyse signals of highly diluted samples. This enables one to check if a similar signal can still be recorded from the diluted sample. If so, then, one can conclude that the first signal have a higher pesticide concentration.

Nonetheless, the curved trend does not limit the application capability of this system for MM57 sensing because a linear trend can be obtained. The plot points before saturation was taken to plot a linear graph is as shown in Figure 4-31.

![Figure 4-31: Linear trend of malathion and MM57 before the peak of vertical curve.](image)

**Figure 4-30:** A scatter plot displaying the results obtained for malathion standard compared to MM57 sensing via C-dots yellow after direct addition of PON 1.
Based on Figure 4-31, the upward trend plot could be used as a sensing signal response for the detection of malathion and MM57. The LOD of malathion sensing was $0.81 \pm 0.081 \mu M$. Then, the LOD of MM57 was $14.64 \pm 1.04 \mu M$, signifying a lower sensitivity was achieved for the detection of MM57. The unique equation from the signals plotted can be used and calibrated into a computerised system for the sensing of MM57.

Next, the system utilising C-dot – PON 1 for MM57 sensing was applied. The results obtained (Figure 4-32) showed a quenching trend induced by increasing MM57 concentration. However, a plateau trend was observed under 200 µM therefore, any signals before this concentration was disregarded.

Consequently, a downward trend was observed (Figure 4-33) when the signals past 200 µM were taken into deliberation. Based on the equation, the LOD for MM57 sensing is $97.33 \pm 4.62 \mu M$. The C-dots<sub>yellow</sub> structural properties played a major role in the ability for the sensing of MM57. Surface of C-dots<sub>yellow</sub> can be specifically modified to increase sensitivity, but also subject to change the way of interaction between the C-dots and analyte. This will indirectly cause a change in optical profile. Besides, conformational changes caused by PON 1
with substrate may also impact the emission properties of C-dots\textsubscript{yellow} by causing quenching or enhancement of intensity.

![Figure 4-33: Downward quenching standard curve observed for MM57 sensing via C-dots – PON 1.](image)

### 4.7 Heavy metal sensing

The study so far has successfully isolated C-dots\textsubscript{yellow} under optimum synthesis condition using sucrose as starting precursor, while major water parameters such as pH and ionic strength were found to have negligible effect towards the emission profile. The C-dots\textsubscript{yellow} have been investigated for pesticide sensing and now, it will be of additional merit to utilise the C-dots\textsubscript{yellow} for another real sensing application. Since the C-dots\textsubscript{yellow} were prepared via acid hydrolysis carbonisation, functional groups rich with electrons such as hydroxyl and carboxyl groups can form on the surface due to the acid oxidation process. In fact, oxidation of carbon nanoparticles was reported to be necessary to promote fluorescence. For instance, Liu et al., (2007) has reported that carbon nanoparticles collected from candle soot only portray the fluorescence once oxidised by nitric acid. Thus, the fluorescence observed from this study has directly supported the presence of those functional groups on the surface. These functional groups will act as potential sites to interact with metal ions with good affinity due to their opposite change nature. Coordination bond can form
easily by the donation of electron pair from the functional terminal to the metal ions.

In this study, commonly found metal ions in water sources and reservoirs (i.e. Al(III), Co(II), Cu(II), Cr(III), Pb(II), Hg(II), Ni(II), Sn(II) and Zn(II) ions) were chosen to be tested with the C-dots\textsubscript{yellow} system (Tan et al., 2014). The sensing potential was evaluated by identifying metal ions that generally will cause the intensity of the fluorescence to shift from the initial value recorded solely from C-dots\textsubscript{yellow}. The testing was performed particularly under two conditions; neutral and acidic surroundings. Basic condition was omitted from testing as most of the metal ions will form precipitate under high concentration of hydroxide ions. The screening was performed after the addition of 5 µM of the respective metal ions into solution containing C-dots\textsubscript{yellow}. The analytical sensitivity of C-dots\textsubscript{yellow} towards the target ion was taken based on the degree of fluctuation in the intensity. The net change of intensity will be used as sensing signal, where larger change reflects on better sensitivity. Under neutral condition, the interaction activity of the metal towards the C-dots\textsubscript{yellow} was significant where the initial intensity for majority of the metal ions tested has showed quenching or enhancement in the intensity (Figure 4-34).

![Figure 4-34: The effects of various metal ions with concentration of 5 µM respectively on the fluorescence intensity of C-dot\textsubscript{yellow} under neutral condition.](image-url)
This indicated that the metal ions has interacted with the surface and interrupted the origin of the fluorescence. The functional groups will be loosely protonated at this condition, leaving negative moieties more assessable to metal ions for interaction. Based on this observation, tuning down of interaction affinity between the functional groups and the metal ions was seen possible by shielding up the negative moieties. This can be achieved simply by increasing the pH of the solution that will promote protonation of the surface. Shielding will cause weaker electrostatic interaction across all metal ions and eventually can promote better selectivity only towards those metal ions having higher charge with small ionic size. This expected trend was observed when the pH of the tested C-dots\textsubscript{yellow} solutions was lowered to the acidic range. The intensity changes recorded for all the metal ions were below 5%, except for Cr(III) ions that have exceeded 10% (Figure 4-35).

![Figure 4-35: The effects of various metal ions with concentration of 5.0 µM respectively on the fluorescence intensity of C-dots\textsubscript{yellow} under acidic condition.](image)

This has really narrowed down the interference effect across the metal ions tested in this study. Particularly, the Al(III) ion that has similar size to charge ratio to the Cr(III) ion showed no significant change in the fluorescence intensity even at lower pH. This can be due to the comparatively more stable oxidation state of the Al(III) ion that will not undergo redox reaction that can
disturb the original electronic transitions of the C-dots\textsubscript{yellow} even at close approximate distance under the electrostatic attraction.

4.7.1 **Analytical characteristics of Cr(III) sensing**

Since the addition of Cr(III) ions has caused significant quenching on the C-dots\textsubscript{yellow} with high specificity under acidic condition, the analytical characteristics at this controlled condition were further evaluated. The relative intensity recorded at the peak wavelength compared to the initial intensity of C-dots\textsubscript{yellow} was adopted as the sensing signal for the analytical characteristic study. Single wavelength monitoring is simple and direct as compared to other approaches such as monitoring area under the spectrum. There has been no direct linear correlation between the degree on quenching to the amount of Cr(III) ions added, but definitely showed as concentration dependent quenching trend. Thus to model the analytical trend (**Figure 4-36**), standard Stern-Volmer quenching relationship (Eq. (1)) was adopted. The relationship was applied to describe the photoluminescence quenching profiles of the C-dots\textsubscript{yellow} in the presence of different concentrations of Cr(III) ions. The LOD determined for Cr(III) was calculated to be 19.12 µM.

![Figure 4-36: Stern-Volmer plot of the quenching effect caused by Cr(III) ions towards the fluorescence intensity of C-dots\textsubscript{yellow} under acidic condition.](image)

\[
y = 0.0007x + 1.0025 \\
R^2 = 0.9948
\]
A possible sensing application could be on detection of Cr(III) ions level in vegetables, since the roots of the plants usually will convert Cr(IV) ions from the soil to Cr(III) ions (Yau, 2011). The United States Environmental Protection Agency (EPA) stated that the National Research Council (NRC) identified the Estimated Safe and Adequate Daily Dietary Intake (ESADDI) of chromium to be 50-200 µg/ d (National Research Council, 1989) which is corresponding to 0.71 to 2.90 µg/ kg/ day for an adult of 70 kg (U.S. Environmental Protection Agency, 1998). Furthermore, the Food and Drug Administration (FDA) specified a Reference Daily intake for chromium of 120 µg/ day (U.S. Department of Health and Human Services, 1995). The proposed sensing probe using C-dots\textsubscript{yellow} could potentially be used to cater this need.

4.8 Immobilisation of C-dots\textsubscript{yellow} with PVA

The immobilisation of C-dots\textsubscript{yellow} was performed as an initial effort to fabricate of C-dots\textsubscript{yellow} into a portable sensing system. This study was performed to evaluate the workability of a non-ionic polymer to bind colloidal particles onto a solid surface. The particles are expected to emit fluorescence after the binding process. As an example, it can be fabricated into a designed flow cell and utilises fibre optic to transmit the source of excitation light to the C-dots\textsubscript{yellow} sample and collect emission signal which will be directed to a detector. A schematic design has been provided to illustrate the process involved for casting the PVA – C-dots\textsubscript{yellow} film (Figure 4-37). Immobilisation of C-dots\textsubscript{yellow} was performed on clean glass slide surface. A ratio of 1:1 (PVA: C-dots\textsubscript{yellow}) was mixed and casted randomly on the surface. A quick scan under UV light showed an obvious display of fluorescence by C-dots\textsubscript{yellow} (Figure 4-38). Under visual and physical observation, the film did not show any obvious signs of clump nor cracks. It formed a smooth transparent film and it was easily peeled from the glass slide surface.
To evaluate the potential of using immobilised C-dots\textsubscript{yellow} as a sensing platform, the effects of a quencher on the film was observed. From the previous experiment on metal ion sensing, lead (II) metal ion was found to be one of the
strong quencher of C-dots\textsubscript{yellow} under neutral condition. Therefore, to analyse
the consequence of adding a quenching component on the PVA – C-dots\textsubscript{yellow}
film, 10 µl of 0.1 M lead (II) ion solution was induced on the film. The spot of the
drop test was indicated by the red circles in Figure 4-39. From the visual results,
it can be deduced that the quencher specifically turned off the fluorescence
whereas other area of the film was not affected. This show that even after
immobilisation, C-dots\textsubscript{yellow} was still reactive to specific analytes. Sensing
colloidal particles were often required to be immobilised in a sensing device,
therefore, this serves as an initial approach to immobilise C-dots\textsubscript{yellow} for real
sensing probe.

4.9 Summary

The optimum conditions of producing carbon dots emitting yellow
fluorescence were thoroughly assessed in this study. The results have
demonstrated the differences of C-dots unique optical properties when different
acids were used for carbohydrate carbonisation. It was concluded that H\textsubscript{2}SO\textsubscript{4}
carbonisation of sucrose under controlled conditions will result in blue emitting
C-dots while H\textsubscript{3}PO\textsubscript{4} carbonisation will produce yellow emitting C-dots. The
synthesis method was optimised with the best yield obtained by heating of 100
mg/mL of sucrose in concentrated phosphoric acid at 85ºC for 30 minutes.
There are two conditions of C-dots\textsubscript{yellow} produced consisting of neutral C-
dots\textsubscript{yellow} that displayed strong PL band with maximum intensity at 562 nm when
excited at 438 nm and acidic condition that displayed a maximum intensity at
560 nm, excited at 350 nm. The fluorescence of the carbon dots was found
stable with minimum fluctuation under various pH conditions (pH 3.96 – 11.40)
and ionic strengths (up to 4.69 M of KCl). Pyrolysis of carbohydrates was also
conducted and the results obtained showed blue emitting C-dots were more
common. Melezitose managed to produced yellow emitting C-dots through
pyrolysis, but it will be too costly to be developed into a sensing nanoparticle.

The bare C-dots\textsubscript{yellow} undergo surface modification via HMDA through
EDC covalent coupling approach. Based on the obtained result, the
fluorescence intensity was greatly enhanced, proving that the surface was
successfully modified through activated carboxyl groups seemingly available on
C-dots\textsubscript{yellow} surface and bound to secondary amine group on HMDA. Then,
neutralised C-dots\textsubscript{yellow} was subjected to salting out extraction to remove excess salt from the nanoparticles. The quantum yield of C-dots\textsubscript{yellow} was 0.18%. Such yield is consistent with reports from literature.

The C-dots\textsubscript{yellow} were tested for sensing of OP pesticides. First, the pesticide standards were validated via HPLC for identification and quantification. Then, the commercial MM57 sample was analysed. The first approach of pesticide sensing was solely utilising C-dots\textsubscript{yellow}. The sensing of paraoxon-ethyl and methyl parathion was possible with C-dots\textsubscript{yellow} without presence of PON 1. The respective calibration equation for the sensing of paraoxon-ethyl is 
\[ y = -0.0005x + 0.998 \]
and methyl parathion is 
\[ y = 0.0139x + 0.996 \]. The LOD of paraoxon-ethyl sensing without PON 1 was 17.56 ± 1.18 µM whereas for methyl parathion, it was 0.63 ± 0.15 µM.

The next sensing approach was utilising C-dots\textsubscript{yellow} with direct addition of PON 1 into the colloidal particles and sensing of pesticide was analysed using this method. The sensing of malathion and paraoxon-ethyl were conceivable by using this method. The equation from the standard curve of malathion sensing is 
\[ y = 0.0119x + 1.001 \]
while the equation of paraoxon-ethyl sensing standard curve is 
\[ y = -0.0094x + 1.0043 \]. The LOD of malathion was 0.74 ± 0.11 µM and the LOD of paraoxon-ethyl was 0.93 ± 0.21 µM.

The final approach was performed by tagging PON 1 onto C-dots\textsubscript{yellow}. The new composite denoted as C-dots – PON 1 was used for the pesticide sensing. By using this approach, the sensing of methyl parathion and paraoxon-ethyl was feasible. The equation from the standard curve plotted for methyl parathion sensing was 
\[ y = -0.0516x + 0.9891 \]
while the equation for paraoxon-ethyl sensing was 
\[ y = -0.0005x + 0.9909 \]. The LOD calculated from the standard curve was 0.17 ± 0.001 µM for methyl parathion and 17.56 ± 0.83 µM for paraoxon-ethyl sensing.

The approach of utilising C-dots\textsubscript{yellow} by direct addition of PON 1 and C-dots – PON 1 for MM57 sensing was conducted. The data collected for showed that the sensing of MM57 via C-dots\textsubscript{yellow} with PON 1 concludes the LOD of 14.64 ± 1.04 µM with a standard curve equation of 
\[ y = 0.0006x + 1.0038 \]. The
used of C-dots – PON 1 for MM57 sensing produced a standard curve equation, 
\[ y = 9.0 \times 10^{-5} x + 1.0315 \] with a LOD value of 97.33 ± 4.62 µM. Additional potential application of C-dots\textsubscript{yellow} was performed and evaluated for heavy metal sensing. Acidic condition of C-dots\textsubscript{yellow} could selectively sense for Cr(III) ions with a LOD of 19.12 µM. This was followed with the final stage where C-dots\textsubscript{yellow} was immobilised and the emission ability was visually analysed. The immobilisation via PVA could be performed. The C-dots\textsubscript{yellow} – PVA film was successfully casted on a clean slide displaying bright fluorescence. A drop test was conducted and it showed visible quenching of fluorescence by a quencher.
5. CONCLUSION

5.1 Summary

This study have presented: (1) the synthesis method for bare and surface modified C-dots\_yellow from sucrose, (2) characterisation of both bare and surface modified C-dots\_yellow optically using spectrofluorometer and UV-Vis spectroscopy, (3) the changes of C-dots\_yellow optical emission properties in the presence of OP compounds were studied and correlated into readable signals for sensing application, (4) generate of novel scholarly ideas on C-dots for the detection of OP pesticides which contribute towards the current and future improvements of nanotechnology, biotechnology and environmental sustainability and (5) the potential application of C-dots\_yellow have been explored as alternative sensing platform for Cr(III) metal detection.

The neutral C-dots\_yellow displayed strong PL band with maximum intensity at 562 nm and acidic C-dots\_yellow displayed a maximum intensity at 560 nm. The UV-Vis scan was performed from 200 nm to 800 nm perceived two peaks, at 285 nm and 229 nm respectively. The quantum yield of C-dots\_yellow was 0.18%. The bare surface of C-dots\_yellow was successfully modified via EDC covalent coupling approach with PON 1 enzyme and shows various response on three different OP pesticides, namely paraoxon-ethyl, malathion and methyl parathion. All the calibrated signals were obtained and used to evaluate the LODs of the respective pesticides. The detection of OP pesticides was conducted via three different approaches. First, C-dots\_yellow was solely used for the detection. This method has successfully detected paraoxon-ethyl with LOD of 17.56 ± 1.18 μM and methyl parathion with LOD of 0.63 ± 0.15 µM. Then, it was followed by direct addition of PON 1 into C-dots\_yellow and consecutive quantification of OP pesticides. Via second method, it has effectively detected malathion with LOD of 0.74 ± 0.11 μM and paraoxon-ethyl with LOD of 0.93 ± 0.21 μM. Finally, the last method involves generating a new composite denoted as C-dots – PON 1 produced by tagging PON 1 onto C-dots\_yellow. With the final method, the detection of The LOD calculated methyl parathion and paraoxon-ethyl were possible with LODs of 0.17 ± 0.001 μM and 17.56 ± 0.83 μM respectively. The approach of utilising C-dots\_yellow by direct addition of PON 1
and C-dots – PON 1 for commercial product, MM57, sensing was conducted. The LOD collected were 14.64 ± 1.04 μM via direct PON 1 addition and 97.33 ± 4.62 μM via C-dots – PON 1. The changes in optical emission property of the C-dots\textsubscript{yellow} in the presence of OP compounds were correlated into readable signals for sensing application. The equations obtained can be evaluated and used to calibrate a pesticide sensor. It was confirmed that the presence of OP pesticide causes a measurable change of C-dots\textsubscript{yellow} optical properties. The modelling of the signal change provides information of the amount of OP pesticide. The LODs determined were in reasonable range with the limit set by World Health Organization (WHO) (1996), Food and Agriculture Organization of the United Nations (FAO) (1996) and the European Commission (2010) justifying the use of these system for pesticide sensing.

Last but not least, the potential of utilising C-dots\textsubscript{yellow} for another real application was conducted for Cr(III) ion sensing. A variety of metal ions were introduced to C-dots\textsubscript{yellow} and the effects were analysed. It was determined that the selectivity for Cr(III) sensing was possible with LOD of 19.12 μM. The detection was conceivable by tuning the pH factor of C-dots\textsubscript{yellow}. Immobilisation studies were also performed to preview the possibility of immobilising this colloidal particle for the fabrication of C-dots\textsubscript{yellow} into a portable sensing device. Successively, this allows the assembly of cost saving optical component required for a sensing device because the type and amount of sources required can be attained economically.

### 5.2 Future directions

The development of a portable sensing device continues to expand and evolve. This work has shared the potential of developing fluorescence nanoparticles for the sensing of various analytes. This work has started preliminary assembly for a portable sensing device (Figure 5-1).
Figure 5-1: Snapshot of the portable sensing device assembly utilising C-dots as optical receptors.

The assembly consist of major parts such as the sample source, peristaltic pump, flow cell, and fibre optic. The sample source will include targeted compounds in liquid form which can either be injected via syringe or supplied from a larger flask. This will then flow through the system with the help of a peristaltic pump which works with minimal power source. This will then flow into a flow cell designed for this system whereby an optical fibre can be mounted perpendicular to the immobilised C-dots in the flow cell. The optical fibre will then be the source of excitation light and receiver of emission signal from the C-dots which will be transmitted to a detector.

Besides enhancing the sensing system, it would be of great value to analyse the sensing ability of C-dots yellow with more variety of pesticides and also the ability to differentiate and quantify a mixture of pesticides.
References


European Commission 2010. Malathion. *Directorate E- Safety of the food chain. EC.*


Office of the Federal Register, National Archives and Records Administration.


