Assay developments for detection of biological analytes

Tan, Jiajun

2019

Tan, J. (2019). Assay developments for detection of biological analytes. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/143757>

<https://doi.org/10.32657/10356/143757>

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Assay Developments for Detection of Biological Analytes

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A thesis submitted to the Nanyang Technology University and Universität für Bodenkultur Wien in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Jiajun Tan

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Date

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Sierin Lim

Authorship Attribution Statement

This thesis contains material from two papers published and one accepted and still in press paper in the following peer-reviewed journals in which I am listed as an author.

Chapter 2 contains materials published as:

1. Y Fang, J Tan, H Choi, S Lim, DH Kim. Highly sensitive naked eye detection of Iron (III) and H₂O₂ using poly-(tannic acid) (PTA) coated Au nanocomposite. *Sensors and Actuators B: Chemical* 2018 259, 155-161;

as well as

2. Y Fang, J Tan, T Lan, SGF Foo, DG Pyun, S Lim, DH Kim. Universal one-pot, one-step synthesis of core-shell nanocomposites with self-assembled tannic acid shell and their antibacterial and catalytic activities. *Journal of Applied Polymer Science* 2018 135 (6), 45829

Chapter 3 contains materials published as:

3. J Tan, V Zaremska, S Lim, W Knoll, P Pelosi. Probe-dependence of competitive fluorescent ligand binding assays to odorant-binding proteins. *Analytical and Bioanalytical Chemistry* 2020 412, 546-554

The contributions of the co-authors are as follows:

For the first paper:

Y Fang and J Tan planned the experiments, conducted the experiments and wrote the paper. H Choi conducted experiments and wrote the paper. S Lim and DH Kim planned the experiments and wrote the paper.

For the second paper:

Y Fang and J Tan planned the experiments, conducted the experiments and wrote the paper. T Lan and SGF Foo conducted experiments. DG Pyun conducted experiments and wrote the paper. S Lim and DH Kim planned the experiments and wrote the paper.

For the third paper:

P Pelosi and W Knoll conceived and supervised the study. J Tan and V Zaremska designed and performed experiments. W Knoll provided new tools and reagents. S Lim and P Pelosi analyzed data. P Pelosi and W Knoll wrote the manuscript. All authors revised and approved the manuscript.

15/11/2019

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Date

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Jiajun Tan

DEDICATIONS

Many shall run to and fro, and knowledge shall be increased – Daniel
12:4

I dedicate this thesis to my family members – parents and sister, as well as to my girlfriend, for they have ran to and fro during my candidature period, in order that I may discover, learn, and improve.

The secret things belong unto the LORD our God: but those things which are revealed belong unto us and to our children for ever – Deuteronomy 29:29

I also dedicate this thesis to God.

ACKNOWLEDGEMENTS

First, I would like to thank my thesis supervisor, Professor Sierin Lim from NTU and Professor Wolfgang Knoll from AIT. They have been instrumental in helping me succeed in this thesis, scientifically, logistically and administratively. Second, I would like to thank my collaborators who have helped me to further my scientific world view: Professor Paolo Pelosi, AIT, Professor Fang Yan, who is now at Nanjing Tech University, and Professor Erik Reimhult, BOKU. They have provided not only scientific help, but also have been a friend. Third, special mention to Dr Darren Tan from BOKU who has provided me scientific counsel from a ‘third person’ point of view, which was very necessary at times. Fourth, I would like to thank my collaborators who have published papers with me, Prof Richie Kim, SKKU, Prof Soh Siow Ling, NUS, Dr Do Gi Pyun, Ms Lan Tian, Mr Stanley Foo, Mr Hyunjun Choi, and Ms Valeriia Zaremska, AIT. Fifth, thank you to Prof Klaus Erik Karjalainen, NTU, and Prof Nicole Borth, BOKU, for their very crucial advices on the design of the CAR. Sixth, thank you to my thesis advisory committee, Prof Duan Hong Wei and Prof Ali Miserez, both from NTU.

I would like to thank the various people from the many labs that I have been to. From AIT: Ms Zhu Jiao, Ms Chiara D’Onofrio, Ms Isabella Fischer, Mr Patrik Aspermair, Dr Jakob Andersson, Dr Johannes Binting, Mr Ulrich Ramach, Dr Jokub Dostalek, Mr Stefan Fossati, Mrs Daria Golebiowski, Ms Simone Hageneder, Ms Simone Katharina, Ms Vanessa Priscilla, Ms Anne Schuchanegg, Mr Anil Bozdogan, Mr Gonzalo Fenoy, Dr Filippo Fedi, Mr Nestor Imodaba, Dr Priya Venugopalan, Dr Simone Fortnati, Mr Estaban

Piccinini, Dr Mark Kreuzera, and administrator, Ms Krissalis Alexandra. They were like a home away from home. From BOKU, Ms Andrea Scheberl, Dr Monika Debreczeny, Dr Markus Tomek, Dr Arturo Lopez, Dr Susanne Bloch, Dr Martina Schroffenegger, Ms Fiona Hager, Ms Valentina Mayer, and Dr Christina Schaeffer. From Biotrans department in ASTAR, Dr Nic Lindley, Dr Ann Koay, Dr Seetoh Wei Guang, Ms Guo Wei Mei, and Ms Tan Shi Min. And most importantly, from the labs in NTU, Dr Ambrish Kumar, Dr Yu Kang, Dr Herlina Arinaita Dewi, Ms Thinzar Win, Mr Vishnu Vadan, Dr Sagar Regmi, Dr Mridul Sarker, Dr Rupali Reddy, Mr Sathya Bhaskar, Ms Xu Xiaohan, Dr Fu Afu, Dr Geraldine Chiew, Dr Ma Shijun, Ms Sam Ravi, Ms Juhi Singh, Dr Usha Rani, Ms Tabitha Tan, Dr Pham Thao, Dr Kate Qi, and Dr Johnathan Wolfe. Thank you also to two good friends who journeyed with me up from our undergraduate days to also partake in doing a PhD program, Ms Su Chengxun and Mr Brendan Sieow. They have been good listening ears, giving helpful scientific discussions.

Thank you very much also to the persons who are/were in this International Graduate School joint degree program with me. You guys were not only friends and fellow professionals, but hardened sojourners. Vielen Dank. This is to: Dr Stefanie Hackl, Dr Thomas Zapf, Dr Oliver Bixner, Mr Nikolaus Leitner, and Dr Bo-Kyeong Yoon. Also thank you to Prof Bo Liedberg for being the program coordinator.

Lastly, thank you to my parents and sister. And also my girlfriend, Elaine Lum for having made the journey sweeter!

Dankeschön für alles. Ohne euch alle hätte ich das nicht geschafft.

SUMMARY

Various ligands and analytes interact with biological systems in many ways. In certain cases, they may cause harm to the body. The detection of these ligands and analytes are therefore crucial. Their different physical and chemical properties require unique strategies and methods for the detection of these substances. This thesis demonstrates three different methods for different classes of substances such as ions and molecules of sizes below 100 Daltons to proteins in the range of 100 kilo-Daltons.

Iron ions are important as intermediaries in bodily chemical reactions. Furthermore, they also play a crucial role in haemoglobin for oxygen transport in blood. Distinction between the two oxidation states is crucial as the homeostasis of iron ions is crucial for bodily functions. Here, a tannic acid shell, gold core nanocomposite was developed to detect Iron (III) ions. The nanocomposites are synthesized by a one-step one-pot synthesis method wherein the reduction of gold salt and oxidation of tannic acid to poly tannic acid shell takes place simultaneously. The chelation of Iron (III) (but not Iron (II)) to tannic acid results in the aggregation of the nanocomposites driving a distinct colour change from red to blue that is visible with the naked eye at a limit of detection of 5 μM . The chelation is further exploited to detect H_2O_2 as H_2O_2 oxidizes Iron (II) ions to Iron (III), driving the chelation. The naked eye visible colour change for H_2O_2 detection is 0.4 μM . While tannic acid-gold core nanocomposites show good utility for iron ion detections, other chemicals such as odorants require different approaches.

Odorants are hydrophobic and do not chelate with tannic acid. In nature, these chemicals require the odorant binding protein for detection where they enter the binding pockets of odorant binding protein. To detect these odorants outside their natural environment, pig odorant binding protein (OBP) was produced recombinantly using an *E. coli* expression system. We hypothesized that the deletion of the alpha helix tail domain in pig OBP would improve binding of odorants to the pig OBP and increase the pig OBP's sensitivity to the odorants. This is because the tail is positioned at the entrance of the binding pocket and appears to act as a gate hindering odorants from readily entering the binding pocket. DNA cloning was done to delete the alpha helix tail. We found that the absence of the tail domain in fact lowers the binding affinity and increases the dissociation constant by between 32% and 434%. This data suggests that the alpha helix tail is crucial in providing stability for the ligand attachment to the binding pocket of the protein. In other words, removing the tail lowers the entropy of the system.

Detection of more complex molecules, such as protein biomarkers will require proteins other than odorant binding proteins as these molecules are too large to enter the binding pocket of the OBPs. Their binding to other proteins that act as receptors also depend on their geometry charges and hydrophilicity. Here we worked to develop a cell-based assay for detecting such molecules. Cell-based assays are more advantageous than conventional system such as SPR and ELISA as it eliminates signals from unspecific binding. The cell-based assay is operated by presenting a CAR on the surface of the cell. The scFv antibody domain of the CAR serves as the receptor site. When a molecular biomarker binds to the CAR, it should cause a cross-linking and

phosphorylation of the CAR followed by an intracellular signalling that can be coupled to various outputs (e.g. calcium influx, gene transcription). Here, our designed CAR is intended to bind the ligand epidermal growth factor receptor (EGFR-CAR). We show using confocal images that the CAR is presented on the surface of HEK293FT cells. Additionally, EGFR also binds to the expressed CAR. However, the expected calcium influx and phosphorylation upon ligand binding were not apparent suggesting that the absence of intracellular cell signalling taking place. The data suggest challenges for connecting the extracellular binding event of the EGFR-CAR to the intracellular signalling in the intended cell that need to be overcome for future development of cell-based assay.

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1. Y Fang*, **J Tan***, H Choi, S Lim, DH Kim. Highly sensitive naked eye detection of Iron (III) and H₂O₂ using poly-(tannic acid) (PTA) coated Au nanocomposite. *Sensors and Actuators B: Chemical* 2018 259, 155-161
2. Y Fang*, **J Tan***, T Lan, SGF Foo, DG Pyun, S Lim, DH Kim. Universal one-pot, one-step synthesis of core-shell nanocomposites with self-assembled tannic acid shell and their antibacterial and catalytic activities. *Journal of Applied Polymer Science* 2018 135 (6), 45829
3. Y Fang*, **J Tan***, S Lim, S Soh. Rupturing cancer cells by the expansion of functionalized stimuli-responsive hydrogels. *NPG Asia Materials* 2018 10 (2), e465
4. **J Tan***, V Zaremska*, S Lim, W Knoll, P Pelosi. Probe-dependence of competitive fluorescent ligand binding assays to odorant-binding proteins. *Analytical and Bioanalytical Chemistry* 2020 412, 546-554
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1. 3rd Place Poster Award at 6th DocDay 2018, University of Natural Resources and Life Sciences, Vienna
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Figure 1.2: The activities of PAH towards DNA. Figure 1.2 was cited from: Drwal, E., Rak, A. and Gregoraszczyk, E. L. (2019). Review: Polycyclic aromatic hydrocarbons (PAHs) – Action on placental function and health risks in future life of newborns. *Toxicology*, 411, p.132 – published by Elsevier B.V.

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Figure 3.1: Model of pOBPM2. The structure is ubiquitous to all lipocalins.

Figure 3.2: Sequence alignment of various OBPs, hOBP, human OBP; rOBP, rabbit OBP; pOBP, pig OBP; bOBP, bovine OBP. The figure is cited from: Schiefner A., Freier R., Eichinger A. and Skerra, A. (2015). Crystal structure of the human odorant binding protein, OBPIIa. *Proteins: Structure, Function, and Bioinformatics*, 83(6), pp.1180-1184 – published by the Wiley Periodicals, Inc.

Figure 3.3: a) The OBP's interaction with fluorescent probe and odorants as they are added to the protein. Upon addition of fluorescent probe, some fluorescent probe enters into the OBP leading to fluorescence being present. The fluorescence is represented by the yellow rays. When odorants are added, it competes with the fluorescent to bind with the OBP. Less fluorescent probe now binds to the OBP and the total fluorescence intensity drops. b, c) Docking simulation result of positioning of fluorescent probe 1-NPN (b) and 1-AMA (c) in the pOBPM2 protein.

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Figure 3.5: SDS Page gels for pOBPM2 (a) and pOBPM2Δ121 (b). Proteins pOBPM2 and pOBPM2Δ121 have an apparent molecular weight of 23kDa and 14kDa respectively. Actual theoretical weight however is 17.8kDa and 13.5kDa for pOBPM2 and pOBPM2Δ121 respectively. The lanes in the SDS Page gels are ladder (1), non-induced with IPTG (2), induced with IPTG (3), soluble fraction after sonication (4), insoluble fraction after sonication (5).

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34 to 39, circled in red were pooled together and applied to the column a second time for further purification.

Figure 3.7: a) Chromatogram for pOBPM2 Δ 121 purification. d) SDS Page gels of collected fractions from pOBPM2 Δ 121 purification. The lanes are as follow: Lane 1, Ladder. Lanes 2 to 10, Fractions 16, 20, 21, 22, 23, 26, 31, and 36. Fraction 22, circled in red was used for binding property analysis.

Figure 3.8: a) Fitted curves with Hill equation of 1-NPN (Red) and 1-AMA (Blue) binding to pOBPM2. b) Dissociation constants based on fitted curves.

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Figure 3.12: The pET5b-pOBP plasmid. The gene is cloned in between the EcoRI and NdeI restriction sites and driven by the T7 promoter for expression.

Figure 4.1: Signals received by the receptor in a cell based bioassay are transduced either through the cell's native signalling pathway or artificial signalling pathway. The former allows for signal amplification through kinase cascades (Purple). The latter allows for design of logic gate circuits. Output signal could be through detection of calcium influx or expression of reporter genes.

Figure 4.2: The B cell receptor and its downstream signalling pathway whereby the one of which results in an influx of calcium ions. Figure 4.2 was cited from: Young, R. M. and Staudt. L. M. (2013) Targeting pathological B cell receptor signalling in lymphoid malignancies Nature Reviews Drug Discovery 12, p.229– published by Springer Nature.

Figure 4.3: The envisioned therapeutic B cell based bioassay. Step 1: CAR is at rest. Step 2: Ligand crosslinks CAR. Step 3: Intracellular signalling pathway activated. Step 4: Calcium ion influx into cells. Step 5: NFAT response element results in secretion of therapeutic antibodies.

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Figure 4.6: HEK293FT cells transfected with plasmid that contains the gene for both design one CAR and mCherry. Successfully transfected cells show mCherry expression (red coloured) but no expression of CAR. The ruler represents 20 μm .

Figure 4.7: a) Design two CAR transfected HEK293FT cells stained with EGFR chimerically fused with 6x His-tag followed by anti-His alexa fluor 488 antibody. Cells fluoresced under confocal microscopy observation proving that EGFR does indeed bind to design two CAR. b) Design two CAR transfected HEK293FT cells stained with only EGFR chimerically fused with 6x His-tag. c) Design two CAR transfected HEK293FT cells stained with only anti-His alexa fluor 488 antibody. The ruler represents 20 μm .

Figure 4.8: Graphs of calcium influx assays for a, b, c) Untransfected HEK293FT with 0.1 $\mu\text{g mL}^{-1}$, 1 $\mu\text{g mL}^{-1}$, and 10 $\mu\text{g mL}^{-1}$ of EGFR introduced. d) Untransfected HEK293FT with only HBSS introduced. e, f, g) Untransfected HEK293FT with 0.1 $\mu\text{g mL}^{-1}$, 1 $\mu\text{g mL}^{-1}$, and 10 $\mu\text{g mL}^{-1}$ of anti-Myc antibody introduced. h) Untransfected HEK293FT with 5 μM ATP introduced. i, j, k) Transfected HEK293FT with 0.1 $\mu\text{g mL}^{-1}$, 1 $\mu\text{g mL}^{-1}$, and 10 $\mu\text{g mL}^{-1}$ of EGFR introduced. l) Transfected HEK293FT with only HBSS introduced. m, n, o) Transfected HEK293FT with 0.1 $\mu\text{g mL}^{-1}$, 1 $\mu\text{g mL}^{-1}$, and 10 $\mu\text{g mL}^{-1}$ of anti-Myc antibody introduced. p) Transfected HEK293FT with 5 μM ATP introduced.

Figure 4.9: a, b) Samples were untransfected or transfected HEK293FT cells respectively being treated with 30 minutes EGFR (lane 1), 30 minutes EGFR followed by 1 minute of anti-His antibody (lane 2), 30 minutes EGFR followed by 2 minutes of anti-His antibody (lane 3), 30 minutes EGFR followed by 3 minutes of anti-His antibody (lane 4), 30 minutes EGFR followed by 5 minutes of anti-His antibody (lane 5), 30 minutes EGFR followed by 10 minutes of anti-His antibody (lane 6). c, d) Samples were untransfected or transfected HEK293FT cells respectively being treated with 1 minute anti-Myc antibody (lane 1), 2 minutes anti-Myc antibody (lane 2), 3 minutes anti-Myc antibody (lane 3), 5 minutes anti-Myc antibody (lane 4), 10 minutes anti-Myc antibody (lane 5), 30 minutes anti-Myc antibody (lane 6). e) Transfected HEK293FT (lane 1) and untransfected HEK293FT (lane 2) cell lysates who's blots were stained with anti-Myc antibody conjugated with horseradish peroxidase. This sample shows us the apparent position of CAR in the western blot. g, h) Normalized relative intensity of CAR bands to the sample lane for blots in blot

(b) and blot (d) respectively. The Green boxes represent presence of CAR band and red boxes the absence.

Figure 4.10: a) Untransfected HEK293FT, stained with anti-Myc FITC antibody. b) Transfected HEK293FT with L21C CAR, stained with anti-Myc FITC antibody. The ruler represents 20 μm .

Figure 4.11: Construct of pLenti6/V5 directional TOPO Plasmid with crucial restriction site enzymes circled in red, BamHI, XhoI, XmaI and KpnI.

LIST OF ABBREVIATIONS

1-AMA	1-Aminoanthracene
1-NPN	N-phenylnapthalen-1-amine
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
BCR	B Cell Receptors
CAR	Chimeric Antigen Receptor
DLS	Dynamic light scattering
DNA	Deoxyribonucleic Acid
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked immunosorbent assay
FPLC	Fast Protein Liquid Chromatography
FTIR	Fourier transform infrared
gFET	graphene Field Effect Transistor
GPCR	G-protein coupled receptor
H ₂ O ₂	Hydrogen Peroxide
Ig	Immunoglobulin
IPTG	Isopropyl-Beta-D-thiogalactopyranosid
ITAM	Immunoreceptor tyrosine-based activation motif
LSPR	Localized Surface Plasmon Resonance
NFAT	Nuclear factor of activated T-cells
NPs	Nanoparticles
OBP	Odorant Binding Protein
PAH	Polycyclic Aromatic Hydrocarbons
PB	Phosphate buffer
PBS	Phosphate buffered saline
pOBP	pig Odorant Binding Protein
PPs	Plant polyphenols
PTA	Poly-tannic acid
ROS	Reactive Oxygen Species
ScFv	Single chained Fragment variable
SPR	Surface Plasmon Resonance

TA	Tannic Acid
TBS	Tris buffered saline
TBST	TBS with 0.2% Tween
TRuC	T cell Receptor Fusion Construct
Uniprot	Universal Protein Resource
UV	Ultraviolet

“There is Plenty of
Room at the
Bottom”

– Richard Feynman

CHAPTER 1

Introduction

1.1 Introduction

Many analytes and ligands interact with the human body in many ways. These substances range from the sizes of ions and molecules below 100 Daltons to proteins in the range of 100 kilo-Daltons. Their interactions could be either beneficial or harmful to the human body. Bioassays for the adequate detection of these substances are essential for further understanding of their functions in the human body.

1.2 Iron

Iron plays an essential role in the human body. For example, in red blood cells, iron is essential for enabling the haemoglobin protein to transport oxygen to various parts of the body [1]. The enzyme ribonuclease reductase requires iron as cofactor in the catalysis of ribonucleotides to deoxyribonucleotides for deoxyribonucleic acid (DNA) synthesis [2, 3]. The synthesis of iron-sulphur clusters in mitochondria for iron-sulphur proteins also requires iron [4-7]. For these functions, iron in the ferrous form (Fe^{2+}) is required. However, Fe^{2+} also results in the formation of harmful reactive oxygen species (ROS) through Fenton reaction [8-10]. The conversion of iron from ferrous to ferric form enables the homeostatic regulation of iron concentration in its various forms [11-14].

The intracellular ferritin protein serves as the main iron storage molecules in the body by catalysing the conversion of iron from its ferrous to

ferric form and vice versa depending on the body's needs [15]. Other proteins involved in iron homeostasis are the iron cell efflux protein, ferroportin, and for influx, the divalent metal transporter [16-18]. Extracellularly, ferrous iron exists in soluble form partaking in various reactions [19-22]. Ferric iron, however, is complexed with transferrin to ensure that it remains inert when being transported through the human body [23]. Transferrin protein with ferric iron (holo-transferrin) can be transported into the cells via transferrin receptors [24, 25].

The inability to regulate iron or the deficiency of iron has been linked to various diseases. Iron deficiency causes anaemia [26], whereas iron overload has been linked to type-2 diabetes [27, 28]. In the brain, beta-amyloid formations are encouraged when too much iron is present, giving rise to Alzheimer's disease [29-31]. Excess iron is also linked to cancer. Within the cell, too much iron results in free-radical accumulation, causing DNA damage [32, 33]. Outside the cells, increased iron increases activity of metalloprotease, causing the degradation of extracellular matrices, encouraging tumour metastasis [34]. During inflammation, iron is kept within the cell to prevent pathogen utilization [35-37]. Excess iron also causes haemochromatosis.

Figure 1.1: Movement and transport of iron within and through the cell. Figure 1.1 was cited from: Drakesmith et al. [16] – published by Elsevier Inc.

To detect and measure the concentration of iron, we exploit iron ion's chemical properties, specifically Fe^{3+} ability to coordinate with tannic acid. During the coordination of Fe^{3+} with tannic acid, tannic acid is brought together in close proximity. Therefore, we hypothesize that replacing the tannic acid with a gold nanocomposite with a poly-tannic acid shell, Fe^{3+} would be able through forming coordinate bonds bring the gold nanocomposite together. This will render us an optical sensor for the presence of Fe^{3+} .

1.3 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is an important redox signalling molecule in the human body. Intracellularly, H_2O_2 is involved in both cell-survival and cell-death pathways. Through the NOX signalling pathway, H_2O_2 generated within the cell induces expression of proliferation and cell migration genes [38, 39]. Yet H_2O_2 also triggers the mitochondria apoptosis pathway through significant calcium influx into the cell that alters the activities of mitochondrial permeability transition pore, ultimately leading to cell death [40, 41].

Extracellularly, H_2O_2 is involved in the innate immune response [42]. Damaged cells in wounds secrete H_2O_2 creating a decreasing gradient of H_2O_2 concentration as the distance from the wound site increases. The H_2O_2 gradient serves as a signal to recruit leukocytes to the wound site [43]. Apart from signalling functions, H_2O_2 also performs antiseptic role in the human body [44]. However, excessive H_2O_2 being an ROS is a mutagen known to damage DNA with potential oncogenic consequences [45, 46].

Diffusion of H_2O_2 and intracellular scavenging of H_2O_2 helps maintain the homeostasis of H_2O_2 in cells. Depending on the make-up of membrane lipid concentration, the diffusion of H_2O_2 through the membrane can either occur freely or be hindered [47]. Aquaporin are also involved in the efflux of H_2O_2 out of the cells [48]. Scavenging proteins such as peroxidases and catalases regulate H_2O_2 levels by removing it [49-52].

Excessive H_2O_2 levels can result in diseases. High amounts of H_2O_2 in the thyroid due to it being a by-product in the synthesis of hormones have been associated with thyroid cancer [53, 54]. H_2O_2 providing oxidative stress could also be a contributing mechanism to the formation of beta-amyloids in Alzheimer's disease [55, 56].

H_2O_2 is an oxidizing agent capable of oxidizing Fe^{2+} to Fe^{3+} . We thus hypothesize that this oxidizing property of H_2O_2 , could oxidize Fe^{2+} to Fe^{3+} . In turn, by extension, the Fe^{3+} would coordinate with the tannic acid shell on the gold nanocomposite, driving the gold nanocomposites to coordinate. Hence, this yields an optical sensor for H_2O_2 .

1.4 Polycyclic Aromatic Hydrocarbons

Polycyclic Aromatic Hydrocarbons (PAH) are organic particles made up of two or more aromatic rings. PAH with four rings or less are called light PAHs while the bigger ones with more than four rings are heavy PAHs [57].

When PAH enters the body, various reactions drive the PAH into carcinogenic molecules. For example, the epoxigenation by epoxide hydrolase generates DNA adducts [58]. Additionally, peroxidase catalyses PAH into radicals that depurinate DNA, while aldo-keto reductases produces catechols from PAH, that reacts to generate ROS [59]. High levels of PAH is also associated with decreased respiratory function [60-62]. Prenatal exposure to PAH have been linked to decreased cognitive function in children [63]. This could be due to the impaired placenta development caused by PAH interference [64].

Figure 1.2: The activities of PAH towards DNA. Figure 1.2 was cited from: Drwal et al. [64] – published by Elsevier B.V.

The burning of fossil fuels in the power generators is a source of PAH in the environment [65, 66]. PAH are also generated from vehicular engines, leading to high PAH levels in urban settings [67-69]. Industrial waste is another

source of PAH. Leaching of PAH from solid sludge into liquid phase is a source of environmental pollution especially in the aquatic environment [70, 71]. Lastly, cigarette smoking is another source of PAH into the human body [72, 73].

Treatment to remove PAH from the environment is done by the addition of various oxidizing agents [74, 75]. However, some oxidizing agents such as chlorides react also with other organic matters, deriving harmful substances [76, 77]. Photolysis of PAH using ultraviolet (UV) light is another removal method. However, this method risks generating free radicals [78, 79]. Carbon nanomaterials are also employed to remove PAH through absorption onto its surface [80, 81]. Recently, bacteria degrading PAH has also been discovered and isolated, providing potential uses for microorganism based PAH removal [82, 83].

1.5 Odours

The olfactory system regulates the sense of smell and is responsible for the detection of odours. Briefly, the olfactory system is made up of the outer nose and the inner nose which is also known as the nasal cavity. Inhaled air first past through the outer nose, then the nasal cavity, before entering the windpipe leading to the lungs [84]. The ceiling of the nasal cavity contains the olfactory mucosa layer. The layer separates the rest of the nasal cavity from the olfactory receptor neurons. Odours diffuse through the mucosa layer to stimulate these neurons [85]. The neuron axons end at the olfactory bulb which consolidates the signal for further processing in the amygdala of the brain [86, 87]. This gives rise to olfactory perception in humans.

Odours serve important role in human activities ranging from warning signs to moods alterations. The gradual decomposition of fish causes the breakdown of odourless trimethylamine-N-oxide into pungent trimethylamine [88]. The pungent smell provides an indication to the declining freshness and hence the edibility of the fishes. Leakage of hazardous products such as gases, in industrial and home settings, is first detected through smell [89]. Taste perception is altered by the presence of differing odours that concurrently affect appetite. Chemicals from bodily secretions from one person may also be picked up by the olfactory receptors of another [90-92]. This allows for interpersonal chemo-signalling. Men sniffing of women's tear have their testosterone level reduced [93].

The odours of volatile organic compounds emitted by the human body are known to serve as biomarkers for various diseases [94]. Infection of the respiratory tract system caused by various pathogens is known to cause foul smells in one's breath [95]. Patients suffering from cholera discharge sweet smelling faeces due to the presence of dimethyl disulphide [96]. Various cancers such as breast and neck cancers emit dimethyl trisulphide, resulting in a rotten smell [97]. Breathes of lung cancer patient contain various benzenoid compounds [98-100]. Artificial noses ranging from gas chromatography techniques to electronic smell sensors are being developed or improved to detect such odour markers. However, reproducibility, especially carried out in different surrounding environments remains a challenge [101-103]. Sniffer dogs able to detect particles at part per trillion lower detection limits are currently also being trained to act as odour detectors [104, 105].

As both PAH and odours are hydrophobic molecules, they are able to bind into the hydrophobic core of odorant binding proteins (OBP). We hypothesize that by mutating the OBP, we can increase the association of PAH and odours to the OBP, rendering a more sensitive bioassay. Specifically, we look to the removal of the tail domain of the OBP as it appears to act as an obstructive gate to the entrance of the hydrophobic core.

1.6 Biomarkers

Another class of ligands are biomarkers. Biomarkers are helpful in the discovery and diagnosis of diseases. Infections result in the generation of antibodies that target the foreign pathogens. Briefly, upon the entrance of antigen into the blood, antigens are bound to specific B cell receptors (BCR). B cells with bound BCR endocytose the antigens, digesting them before presenting the antigen fragments in a Major Histological Class II Complex. Thereafter, T helper cells further activate these B cells to secrete antibodies [106, 107]. Detection of these antibodies that target the foreign pathogens helps confirm the presence of diseases. In auto-immune diseases, autoantibodies that target the person itself are detectable in the blood [108, 109]. For example, the presence of anti-citrullinated antibodies is detectable before symptoms of rheumatoid arthritis are present [110, 111].

Figure 1.3: The interactions of T cell towards B cells and its effect in B cells.

Figure 1.3 was cited from: Crotty, S. [112] – published by Springer Nature.

In cancer, various proteins are overly secreted by the cancerous cells. For example, in prostate cancer, prostate-specific antigens levels are increased in the blood [113]. High levels of CA-125 in blood serum also serve as indicators of ovarian cancers [114, 115]. However, not all upregulated proteins are secreted and detectable in the blood stream. Many of these upregulated proteins are in fact membrane receptor proteins. Epidermal growth factor receptors (EGFR) are found on triple negative breast cancers [116, 117]. CD19 receptors are overly expressed in lymphoma [118, 119]. However, to test for the receptors on the cells, blood samples are insufficient. A tissue biopsy is required to examine the cells for receptor over expression.

Antibodies and its fragment portions such as the single chained Fragment variable (scFv) are able to bind to these biomarkers. We hypothesize that by incorporating these biomarkers as chimeric receptors and expressed on

the surface membrane of a cell, we could develop a cell based sensor that could detect the presence of these biomarkers.

In summary, not only do these analytes and ligands differ in their molecular weights, their chemical properties such as coordinating bonds, oxidation energy, hydrophobicity and tertiary structure, are different too. These chemical properties will be exploited to develop different strategies for their detections.

1.7 Thesis Outline

In the first chapter, we discussed about five different analytes and ligands, and their impacts to human health. Adequate detection of these substances is necessary to better establish and safeguard against them. Literature reviews for each type of detection strategy is incorporated in each of the next three chapters.

Chapter two describes the development of naked-eye detection of iron ions. Gold nanocomposites with poly-tannic acid shell are used for the detection of Fe^{3+} as well as H_2O_2 analytes. By harnessing the unique ability of Fe^{3+} to coordinate with three galloyl groups as opposed to two in other metal ions, we hypothesized that gold nanocomposites were able to aggregate. This then gave rise to naked eye visible change in colour. Furthermore, the oxidizing property of H_2O_2 was used to oxidize Fe^{2+} into Fe^{3+} . This reaction drove the nanocomposites to aggregate. As such, a H_2O_2 detection system was also developed. This chapter is an adaptation of two papers published in Journal of Applied Polymer Science, and Sensors and Actuator B: Chemical.

An attempt to improve the binding of odorants in pig odorant binding protein (OBP) is described in Chapter three. The kinetic binding effects upon domain deletion of the pig OBP were investigated. The pig OBP (pOBP) has been shown to bind to various hydrophobic PAH and odours. Here, we delete the tail domain of the protein as we hypothesized that the tail domain functions as a hindrance to the entering of these molecules. However, our result shows otherwise. The removal of the tail in fact leads to a drop in binding activity of the molecules to the protein. The tail deletion lowers the entropy of the molecule binding pig OBP complex. This chapter is an adapted from a paper published in Analytical and Bioanalytical Chemistry.

In chapter four, we designed a chimeric antigen receptor (CAR) for detection of EGFR. The CAR was successfully expressed on the surface of the HEK293FT cells in the correct orientation with the intended extracellular domains on the external of the cells. EGFR was also shown to bind onto the CAR. We hypothesized that the binding of EGFR to the CAR would thus trigger a downstream signalling resulting in a calcium influx into the cell. However, calcium influx assay performed showed that the EGFR was unable to trigger a downstream signalling cascade within the HEK293FT cells that culminates into a calcium influx into the cell. Subsequent western blot study showed that the phosphorylation level of the tyrosines on the CAR did not alter even when the presentation time of EGFR ligand increased.

In the last chapter, we present the thesis conclusion and the future outlook for bioassays.

CHAPTER 2

Poly-(tannic acid) coated gold nanocomposite for naked eye detection of

Iron (III) ions and H₂O₂

2.1 Introduction

Detection of iron is crucial as iron plays multiple roles in the human body. Clinically relevant range of iron is between 7 to 30 μM [120]. Homeostasis of iron at the right level is necessary for proper bodily functions. The detection of the Fe^{3+} ion is one method for iron detection. Current method for clinical detection of iron concentration in blood uses Ferrozine to complex with Fe^{2+} ions yielding a photometric complex with measurable absorbance at 577 nm. Briefly, addition of Ascorbic acid to blood serum lowers the pH, liberates Fe^{3+} ions from transferrin and reducing the ions to Fe^{2+} ions. Thereafter, the Fe^{2+} ions are complexed with Ferrozine. Additionally, to remove false signals arising from the presence of copper (II), thiourea is added to complex with copper (II) [121, 122].

The current clinical method requires an additional step of reducing Fe^{3+} to Fe^{2+} and is not a direct measurement of Fe^{3+} concentration. However, various methods have now been developed to directly detect Fe^{3+} ions by exploiting its chemical properties. For example, its reduction property to Fe^{2+} , allows for stable coordination with bilirubin [123]. As an octahedral high spin $3d^5$ transition metal ion that allows for photo induced electron transfer between energy orbitals, it is able to quench fluorescence [124-127]. Fe^{3+} also coordinates well with the galloyl groups of catechol [128]. These methods have been exploited for Fe^{3+} ion detection.

To exploit these chemical properties of Fe^{3+} to detect Fe^{3+} presence, various molecules have been developed with varying degrees of efficiency based on its limit of detection. Fluorophores probe molecules were synthesized in two methods whereby the fluorescence is quenched in the presence of Fe^{3+} . Here, the limits of detection are 0.5 ppm and 0.1 nM respectively [124, 127]. Copper nanoclusters were also synthesized in one method. Here, fluorescence is also quenched when Fe^{3+} is present [126]. The limit of detection is 2 μM . To exploit the coordination with gallyol groups, catechol filled molecules were synthesized. The presence of Fe^{3+} drives a colour change with limit of detection at 3.52 μM . This assay also has the highest sensitivity of $7.33 \times 10^{-7} \text{ M}$ as compared to the other assays [128].

Despite their low limits of detection, the synthesizing of these molecules and the copper nanoclusters, require multiple steps for synthesis. The synthesis methods also make use of lab equipment rendering them more difficult for synthesis. These challenges are overcome in our method where the gold nanocomposite is produced in a one-pot one-step method. Lastly, the use of fluorophore in some assays means that the fluorophore molecules are susceptible to photo bleaching.

Here, we exploit the coordination chemistry between Fe^{3+} ions and tannic acid to induce poly-(tannic acid) coated gold nanocomposite (Au@PTA) to aggregate. This thus causes a red-shift in plasmon bands, and extinction wavelengths, resulting in naked eye visible colour change from red to blue.

Additionally, H_2O_2 , another crucial biological analyte was also detected by utilizing its oxidation of Fe^{2+} to Fe^{3+} ions in the presence of the Au@PTA . H_2O_2 is noted to be clinically dangerous when it reaches level at above 50 μM

[129]. When in Fe^{2+} state, the Au@PTA did not aggregate. However, when oxidized to Fe^{3+} , the Au@PTA aggregated. The current method for detecting H_2O_2 and measuring its concentration is through the use of titration with potassium iodide [130]. When potassium iodide is added to the colourless H_2O_2 solution, the iodide is oxidized to iodine, turning the solution dark purple.

2.1.1 Nanoparticles

The IUPAC has defined nanoparticles as a particle of any shape with at least two dimensions between 1 nm and 100 nm [131]. As metal particles are reduced to such sizes, they begin exhibiting optical properties different from those of bulk metals. When light propagates towards the surface of metal, the electric field in the electromagnetic waves drives surface free electrons to vibrate, generating an electric field that propagates along, and oscillates in and out of the air metal interface. These vibrating electrons are known as plasmons. In metal nanoparticles however, when the wavelength of electromagnetic wave propagated is larger than the nanoparticles, the plasmons generated do not propagate but are instead confined within the nanoparticle. This creates the phenomenon of Localized Surface Plasmon Resonance (LSPR) [132]. The wavelengths in which the electromagnetic waves are able to resonate with the plasmons, and thus be absorbed by the metal nanoparticles are dependent on the nanoparticles' sizes, shapes and dielectric constants. This can be calculated using the Mie model, a solution to the Maxwell equations. The characteristic extinction UV-vis spectra of metal nanoparticles give it its characteristic blue shift in colour as the metal nanoparticles increase in sizes or aggregates [133].

Figure 2.1: a) Colour of Gold nanoparticles of varying diameters and b) the corresponding extinction curves. Figures 2.1a and 2.1b are cited from: Subara et. al. [134] – published by the Indonesian Society for Knowledge and Human Development.

2.1.2 Nanoparticles Synthesis

Nanoparticle synthesis typically relies on the Turkevich method pioneered in 1951 [135, 136]. Briefly, the gold salt, gold hydrochlorate diluted in solution is reduced by citrate or ascorbic acid at 100 °C or room temperature respectively under stirring condition, yielding gold nanoparticles. Since then, other reducing agents such as sodium borohydride, glutamic acid, or hydroquinone have also been utilized [137-139]. To stabilize and ensure monomeric dispersion of the nanoparticles, capping agents such as citrate, gallic acid, or surfactants including DAXAD 19 were added to the formed nanoparticles [133, 135, 140-142]. To yield polymer shells, further addition of cross-linkers or UV irradiation is done to polymerize the monomers [143].

Recently, there has been an increasing trend of replacing synthetic polymers with plant polyphenols (PPs) for bionanomaterial synthesis [144-148]. The use of PPs, specifically tea polyphenol, and tannic acid (TA) for synthesizing metal@polymer nanocomposites has been reported by Fei et al. and Zeng et al. in 2014. Fei et al.'s method, although a one-step process,

requires microwave irradiation, while Zeng et al.'s method, a conventional two-step method, needs the addition of Fe^{3+} ions as cross-linkers [149-154].

TA has been shown to undergo oxidative self-polymerization to PTA before coating onto graphene oxide surface [155]. Furthermore, TA has also been used as a sole reducing and capping agent in heating-free green synthesis of metal nanoparticles [156, 157]. These two properties make TA an advantageous candidate for the synthesis of metal@polymer nanocomposite. Nevertheless, current methods of synthesizing metal@polyphenol nanocomposite with TA shell as described by Zeng et al. still relied on Fe^{3+} ions to induce coordination between TA to form the shell [151].

In our method, the reduction of gold salt as well as TA polymerization occurred concurrently, thus allowing for a one-pot, and one-step synthesis method for Au@PTA NP.

Figure 2.2: Schematic depicting the metal@PTA nanocomposites formation.

The TA shell is the result of polymerized TA.

2.2 Result and Discussion

2.2.1 Nanocomposite Synthesis

Firstly, to determine the optimal pH for Au@PTA synthesis, TA polymerization was carried out at varying pHs. The estimated pKa value of TA is 8.5 [158]. TA monomer was found to be stable in solutions of $\text{pH} \leq 7.0$ as oxidation of TA is inhibited in acidic condition. However, as the pH increases > 7.0 , TA undergoes oxidation with atmospheric oxygen, during which, self-polymerization of TA takes place spontaneously. The TA solution turns faint yellow (Figure 2.3). When pH increases beyond $\text{pH} > 8.5$, the speed of TA oxidation and self-polymerization would be sped up and the polymerized TA would be further oxidized and disassembled into smaller soluble molecules [159]. As such the shell would be unable to assemble on the AuNP core. These observations infer to us that in the final Au@PTA nanocomposite product, the TA was cross-linked through oxidation of HAuCl_4 and oxygen dissolved in the solution before self-assembling into the shell surrounding the AuNP cores. pH 7.8 was thus determined to be the optimal pH for Au@PTA nanocomposite synthesis.

Figure 2.3: A photograph of the TA solutions under different pH conditions after a 4 hours incubation at room temperature.

Au@PTA nanocomposite was synthesized by one-pot synthesis mixing of Au^{3+} and TA under mildly alkaline condition and characterized through TEM imaging and dynamic light scattering (DLS) (Figure 2.4a). The Au@PTA nanocomposites were comprised of two components with distinct electronic densities as compared to bare gold nanoparticles (AuNPs) (Figure 2.4b) [151]. The average diameter of the Au@PTA core was ~ 20 nm, while the shell thickness was ~ 5 nm. The solution of Au@PTA nanocomposites exhibited a red colour similar to that of typical Au colloids [160-162]. The UV-vis spectra of an Au@PTA solution showed an extinction peak at around 550 nm (Figure 2.4c). The slight red-shift in the extinction peak of Au@PTA in comparison to that of 20 nm AuNPs (extinction peak of ~ 520 nm) was due to the increase of refractive index around AuNPs arising from the PTA coating on AuNPs [145,

151, 163]. The corresponding element linear mapping of a single nanocomposite (Figure 2.4d, e) showed the distribution of the various elements in the nanocomposite, comprising Au (green), C (red), and O (black), indicating that the nanocomposites were composed of Au at its core and PTA as its shell [154]. Fourier transform infrared (FTIR) spectra confirmed that TA was oxidized by HAuCl_4 (Figure 2.4f), which is in good agreement with previous reports [164]. Integration of the PTA shell onto the surface of the Au cores decreased the zeta potential from -11.5 ± 0.5 mV to -17.2 ± 0.5 mV due to the presence of acidic galloyl groups in TA.

Figure 2.4: a) Table recording the relevant physical measurements of the different NPs in varying synthesis conditions. b) UV-vis spectra of dispersed Au@PTA and AuNPs. c) STEM images of Au@PTA. d) Element linear mapping of Au@PTA. e) FTIR spectra of TA and Au@PTA. f) STEM image of a single Au@PTA with inset, bare AuNPs.

Time-course experiment was conducted to determine the growth mechanism of Au@PTA nanocomposites by monitoring the morphology of the nanocomposites using TEM at synthesis time points of 5, 10, and 20 minutes. Similar to other nanoparticle synthesis techniques, the electronic density at the beginning of the synthesis process (5 minutes) was not uniform and the nanoparticles aggregated in a “like-attracts-like” manner (Figure 2.5a). The white halo around the nanoparticles may be attributed to the presence of partially polymerized PTA. We may attribute the “like-attracts-like” aggregation of nanoparticles to the fusion between metal nanoclusters, which would grow into larger mesocrystals, as observed in many biomineralization processes [165-167]. At 10 minutes, although we observed capping of PTA onto the mesocrystals, uniform Au@PTA nanocomposites were still not fully assembled (Figure 2.5b). After 20 minutes, capping of PTA is completed and a shell of thickness of ~ 5 nm was observed around the Au core (Figure 2.5c).

Figure 2.5: TEM images of Au@PTA nanocomposites at varying time points of its synthesis, a) 5 minutes, b) 10 minutes, c) 20 minutes. Corresponding photographs of the nanocomposite at the varying time points of its synthesis is attached at the bottom of each TEM images.

2.2.2 Fe³⁺ Detection

The synthesized Au@PTA was applied as a sensor to detect iron (III) ions. When Fe³⁺ was added to the Au@PTA solutions at room temperature, naked eye visible colour change from red to blue was observed (Figure 2.6a). UV-vis spectroscopy showed an extinction band shift from 550 nm to 600 nm (Figure 2.6b). The shift of which was caused by the aggregation of Au@PTA was confirmed via SEM imaging (Figure 2.6c, d).

Figure 2.6: a) Photograph of the solution containing only Au@PTA nanocomposites (left) and the same solution after the addition of Fe^{3+} (20 μM) (right). b) UV-vis spectra obtained from solutions of Au@PTA nanocomposites (solid black line) and after 30 min incubation with Fe^{3+} (dashed red line). Solid black line: Au@PTA nanocomposites without Fe^{3+} , dashed red line: Au@PTA nanocomposites with Fe^{3+} . c) SEM images of Au@PTA nanocomposites. d) SEM image of the Au@PTA nanocomposites after incubation with Fe^{3+} .

To evaluate the minimum aqueous concentration of Fe^{3+} ions required for visible colour change, we added Fe^{3+} into the Au@PTA solution at final Fe^{3+} concentrations ranging from 1 μM to 10 μM . After 5 minutes of reaction time, obvious colour changes with $[\text{Fe}^{3+}] \geq 5 \mu\text{M}$ were observed (Figure 2.7a). Upon analysis of the UV-vis spectra of Au@PTA incubated at varying

Fe^{3+} concentrations, we observed the presence of two sets of linear dynamic ranges (Figure 2.7b). Between concentrations of 5 μM and 40 μM , the increase in Fe^{3+} concentrations corresponded linearly to an increase in red-shift of the peak up to a maximum of 50 nm (Figure 2.7c). The sensitivity is 1.29 nm μM^{-1} . From Fe^{3+} concentrations of 40 μM to 200 μM , we observed that the intensity of absorbance at 600 nm (A_{600}) decreased linearly as the Fe^{3+} concentration increased (Figure 2.6d). The sensitivity is 0.002 μM^{-1} . This difference was due to the nature of aggregation. Consequently, the changes in the extinction curve that had taken place were different for the two different dynamic ranges. Below concentrations of 40 μM , coordinate bonds between Fe^{3+} and Au@PTA resulted in the formation of small perforated aggregates. At these ranges, the extinction is mainly dominated by absorbance. Thus changes in size results in shift of extinction peak. However, above 40 μM concentrations, not only did these small perforated aggregates form, they also clustered together to form larger aggregates. The extinction of Au@PTA at this size ranges is now dominated by scattering. As such, the increase in aggregation results in the broadening of extinction peak and decrease in extinction.

Figure 2.7: a) Photograph of the solutions containing Au@PTA incubated for 5 minutes with different concentrations Fe^{3+} (1 – 10 μM). b) UV–vis spectra of solutions of Au@PTA incubated with varying concentrations of Fe^{3+} . c) Calibration curve corresponding to part a (5 – 60 μM). d) A calibration curve corresponding to part a (40 – 200 μM). Values were normalized by subtracting from the A_{600} value at 35 μM Fe^{3+} concentrations. Error bars represent the standard deviations of three replicates. The lines represent best linear fits for the respective curves. Furthest points are omitted in the fitting because they do not fit the linear trend.

We also tested the selectivity of this assay for Fe^{3+} ions by testing the assay with various, monovalent, divalent, or trivalent ions at final concentrations of 50 μM . Results show that only the sample containing Fe^{3+} induced a noticeable colour change in Au@PTA (Figure 2.8). None of the other metal ions interfered with the assay. Even when the concentrations of these ions were increased to ten times the concentration of Fe^{3+} added (i.e., up to 200 μM), no distinct visible colour changes or precipitates were observed in the Au@PTA solutions (data not shown). Furthermore, no colour changes (except for Ag^+ and Hg^{2+}) or aggregations were observed after incubating the samples

for four weeks, indicating the long term stability of the Au@PTA based detection system. Conclusively, Au@PTA can be conveniently used to visually detect Fe^{3+} in a highly sensitive and selective manner.

Figure 2.8: a) Photograph of solutions containing Au@PTA incubated with different metal cations. Ion concentration of Na^+ , K^+ , Rb^+ , Ag^+ , Mg^{2+} , Ca^{2+} , Zn^{2+} , Fe^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} , Mn^{2+} , Cd^{2+} , Hg^{2+} , Al^{3+} , Cr^{3+} , Nd^{3+} , Gd^{3+} , and Dy^{3+} is 50 μM . $[\text{Fe}^{3+}] = 20 \mu\text{M}$. b) UV-Vis spectra obtained from solutions of Au@PTA after adding different metal ions.

Tannic acid is a polydentate molecule. However, unlike Ethylenediaminetetraacetic acid, the molecule does not chelate onto only one metal ion. Instead, tannic acid is able to form multiple coordination bonds with multiple metal ions, at each of its galloyl groups [168, 169]. The metal ions are able to further form coordination bonds with other tannic acid through their galloyl groups (Figure 2.9a). This results in the aggregation of tannic acid giving rise to the formation of PTA. However, only Fe^{3+} is able to form tri-catecholate complexes [170, 171]. Other metal ions tested are only able to form mono-catecholate or bi-catecholate complexes [172]. This would explain the more efficient aggregation of Au@PTA for Fe^{3+} as compared to the other metal

ions and thus the selectivity (Figure 2.9b, c). Modelling using density functional theory by Ponce et al. and Zhang et al. showed that Fe^{3+} complex with galloyl groups forms a six-coordinate distorted octahedral tris complex. Whereas metal ions such as Co^{2+} form a four-coordinate planar bis complex (Figure 2.9d, e). Two water molecules coordinate along the Z-axis to stabilize the complex essentially giving rise to an octahedral coordinate geometry shape [173, 174]. The distorted geometry for the tris complex may be attributed to steric hindrance caused by the large size of galloyl groups as compared to that of two water molecules that gives rise to a non-distorted octahedral shape.

Figure 2.9: a) Tannic acid contains multiple galloyl groups which five of them are able to form coordinate bonds with metal ions. This makes tannic acid polydentate. b) Each galloyl group of the polydentate tannic acid is able to form a coordination bond with a metal ion. For some metal ion, they can form coordination bonds with two additional galloyl groups. This forms tri-

catecholate complexes c) In other metal ions, coordination bond only forms with one additional galloyl group. This results in bi-catecholate complexes being form. d) Simulation with density functional theory showing Fe^{3+} coordinating with three tannic acids forming a distorted octahedral tris complex. e) Simulation with density functional theory showing Co^{2+} coordinating with two tannic acids forming a planar bis complex. Two water molecules also coordinate along the Z-axis plane with the Co^{2+} ion. Figure 2.9a and 2.9b were cited from: Wei et al. [175] – published by Wiley Periodicals, Inc. Figure 2.9c and 2.9d were cited from: Zhang et al. [173] – published by The Royal Society of Chemistry.

2.2.3 H_2O_2 Detection

As H_2O_2 oxidizes Fe^{2+} to Fe^{3+} (Equation (1)), we made use of the Fe^{3+} produced from the oxidation of Fe^{2+} when H_2O_2 was added to form coordination bonds with the galloyl groups of the PTA shell causing the Au@PTA to aggregate immediately. When H_2O_2 or Fe^{2+} was added separately to the Au@PTA mixture, no colour changes in the assay were observed even after 30 min. However, when H_2O_2 and Fe^{2+} ions were added together in the assay system, the colour of Au@PTA solution turned blue (Figure 2.10a). The presence of Fe^{2+} was crucial for the assay to detect H_2O_2 . Other monovalent, divalent, or trivalent ions do not induce any colour shifts (Figure 2.10b). UV–vis spectroscopy analysis showed that the absorbance at 550 nm decreased and the peak broadened with marked increase at ~ 650 nm (Figure 2.10c). To show that the change in colour is due to the oxidation of Fe^{2+} to Fe^{3+} by the addition of H_2O_2 , we compared the initial reaction rates for the production of Fe^{3+} after

the addition of H_2O_2 to Fe^{2+} to that of the production of Fe^{3+} Au@PTA aggregates. The extinction peak of Fe^{3+} (350 nm) and Au@PTA aggregates (550 nm) was measured over a course of 300 seconds after the addition of H_2O_2 to Fe^{2+} ions, and Au@PTA to Fe^{3+} respectively. We observed that the absorbance value for Fe^{3+} starts to stabilize only after 200 seconds whereas the value for the Fe^{3+} Au@PTA aggregate stabilizes within the first 50 seconds (Figure 2.10d). This shows that the rate limiting step for the reaction is the formation of Fe^{3+} after the addition of H_2O_2 to Fe^{2+} .

Figure 2.10: a) Au@PTA nanocomposites after adding Fe^{2+} (20 μM) with no oxidizing agent, Fe^{2+} (20 μM) with H_2O_2 as the oxidizing agent, and H_2O_2 , with no Fe^{2+} added. b) Photograph of solutions containing Au@PTA nanocomposites incubated with different metal cations in the presence of H_2O_2 for 1 minute. Ion concentration of Na^+ , K^+ , Mg^{2+} , Zn^{2+} , Ca^{2+} , Cu^{2+} , Mn^{2+} , Co^{2+} , Ag^+ , Hg^{2+} , Ni^{2+} and Fe^{2+} is 20 μM . $[\text{H}_2\text{O}_2] = 1 \mu\text{M}$. c) UV-vis spectra of

Au@PTA nanocomposites (solid black line), and after 30 min in the presence of Fe^{2+} and H_2O_2 (dotted red line). d) Graphs showing time dependent growth in absorbance at 350 nm of Fe^{3+} after the addition of H_2O_2 to Fe^{2+} as well as the fast aggregation of Au@PTA after the addition of Fe^{3+} at the extinction peak of 550 nm for Au@PTA.

To investigate the sensitivity of our Au@PTA based sensor for the detection of H_2O_2 , aliquots containing varying concentrations of the analyte H_2O_2 were added into Au@PTA nanocomposite solutions mixed with Fe^{2+} , and the result were observed both visually and with the use of spectrometry (Figure 2.11a). Minimum concentration of H_2O_2 required for the Au@PTA solution to turn visibly blue was 0.4 μM . Between H_2O_2 concentrations from 0.4 μM to 2.0 μM , an increase in the intensity of blue colour was seen as the added H_2O_2 concentration increased. Our naked eye H_2O_2 minimum detection concentration of 0.4 μM was lower than the reported minimum visual detection limit for H_2O_2 concentration of 0.5 μM reported in the horseradish peroxidase-dependent nanoflower system developed by Lin et al. [154]. To our knowledge, this makes our system the lowest detection level via naked eye visualization [176-179]. By observing the UV-vis spectrum with varying concentrations of H_2O_2 added, we observed that our assay allowed for a large linear dynamic range between H_2O_2 concentrations of 0.01 μM and 0.14 μM . We observed that the absorbance at ~ 650 nm increased linearly as the concentration of H_2O_2 increased (Figure 2.11b, c). Our assay's linear dynamic range was much wider as compared to other previously reported sensors (2.5–11.2 mM, 0.979–17.6 mM, and 0.02–0.5 mM) when similar experimental conditions were used for H_2O_2 detection [154,

176, 179]. As the H_2O_2 concentration reached the saturation limit of 1.4 μM in our assay, the normalized absorbance at 650 nm increases at a slower rate as compared to between concentrations of 0.01 μM and 0.14 μM .

Figure 2.11: a) Different concentrations of H_2O_2 were added to solution of Au@PTA incubated with Fe^{2+} to observe changes in colours and intensities. Concentration of H_2O_2 increased from 0.1 μM to 2.0 μM from left to right. b) UV-vis spectra of solution after 5 minutes after different concentrations of H_2O_2 were added. The extinction peak red shifts when the concentration of H_2O_2 is 0.1 μM or higher. c) Graph showing absorbance at ~ 650 nm of the various samples reacted with different concentrations of H_2O_2 . Values were normalized by subtracting from the A_{650} value at 0 μM H_2O_2 concentration. Error bars represent the standard deviations of three replicates.

Interestingly, while comparing the UV-vis spectrometry of our two assays, we realized that as increasing H_2O_2 was added to the H_2O_2 detection assay, a second peak appeared at ~ 650 nm with increasing intensity (Figure

2.7b, 2.11c). This peak was absent in the Fe^{3+} assay regardless of the concentration of Fe^{3+} added. This is because the Fe^{3+} induced aggregation is a bulk clustering of Au@PTA. However, in the H_2O_2 detection assay, the clustering as observed under SEM is “wire-like” (Figure 2.12). This results in two different planes of LSPR, producing two extinction peaks similar to that of nanorods and nanowires [180, 181]. To account for the “wire-like” oligomer structure present in the H_2O_2 detection assay but absent in the Fe^{3+} detection assay, we examined the chemistry taking place for each assay. In the Fe^{3+} detection assay, the addition of Fe^{3+} to Au@PTA resulted in an aggregation induced by the coordination between Fe^{3+} and the galloyl groups of PTA. However, when H_2O_2 was added to the mixture of Au@PTA and Fe^{2+} ions, a multi-step reaction took place. When added, H_2O_2 induced oxidation of Fe^{2+} to Fe^{3+} which was accompanied by the generation of $\cdot\text{OH}$ free radicals (Equation (1)). The $\cdot\text{OH}$ free radicals then reacted with the galloyl groups of PTA by capturing proton from them (Equation (2–3)) converting the polyphenol into phenoxyl radicals. Unlike galloyl groups which coordinated efficiently with Fe^{3+} , no coordination takes place between phenoxyl radicals and Fe^{3+} ions. The absence of galloyl groups which had been replaced with phenoxyl radicals resulted in the slowing down of Au@PTAs aggregation. Thus, the formation and aggregation of oligomers was more favourable than the aggregation of Au@PTAs.

Figure 2.12: a) SEM images of Au@PTA nanocomposites incubated with Fe^{2+} .
b) SEM image of Au@PTA incubated with Fe^{2+} and H_2O_2 with low and high magnification (inset).

Equations:

2.3 Conclusion

In conclusion, we have developed a highly sensitive Fe^{3+} and H_2O_2 sensor based on Au@PTA core@shell nanocomposites. Au@PTA was prepared by one-pot synthesis mixing of TA and gold salt together in mildly alkaline pH condition under constant vortex for 20 min at room temperature. TA reduced the gold salt to form AuNP and itself oxidized into PTA. The PTA then assembled onto the AuNP to form Au@PTA nanocomposites.

Relying on Fe^{3+} coordination to galloyl groups on TA, we developed a highly selective and sensitive sensor for detecting Fe^{3+} . The minimum detection

limit for naked eye colour change from red to blue in the presence of Fe^{3+} only is at a hitherto unreported low of 20 μM . Leveraging on the oxidation of Fe^{2+} to Fe^{3+} by H_2O_2 , we were able to develop our sensor further to detect H_2O_2 when Au@PTA was mixed together with Fe^{2+} . The naked eye detectable colour change for this assay was 0.4 μM of H_2O_2 . The high sensitivity of the H_2O_2 assay maybe explained by the formation of “wire-like” oligomer formed due to the presence of phenoxyl groups and the absence of galloyl groups.

As iron and its ions are important biological minerals while H_2O_2 is an important chemical intermediary in various industries, it is imperative that a highly sensitive, selective and easily visible read-out is available to detect the presence of Fe^{3+} and H_2O_2 [176-179, 182-187]. Herein, we have developed a PTA coated Au core@shell nanocomposite that fulfils these criteria. Coupled with its green, one-pot and one-step synthesis method, our assay is highly advantageous.

Future work will involve testing our Au@PTA nanocomposites for use in clinical settings for quantifying Fe^{3+} or H_2O_2 levels. Possible bodily fluids for testing may include blood, urine, saliva and cerebral spinal fluids [188-190]. One challenge to overcome is that the additional colour from the bodily fluid sample may create noise signals in our visual detection system. Colourless samples such as saliva will not pose a problem. Neither will urine samples that are yellow in colour as the intensity of the Au@PTA assay will overcome the yellow colour. Blood however, is red in colour and because Au@PTA is also red in colour, blood samples will certainly interfere with the Au@PTA assay. One method to overcome this is to only test the platelet poor plasma fraction of the blood sample [191]. Platelet poor plasma fraction of blood can be obtained

by centrifuging the blood sample. Another challenge to overcome is that of the formation of protein corona by proteins in the clinical samples on the Au@PTA nanocomposites when these samples are presented to the assay [192, 193]. Protein coronas are known to prevent the aggregation of nanoparticles [194]. As the assay relies on aggregation of Au@PTA to drive the assay's colour change, protein corona formation could pose a challenge to the assay from working ideally. Further investigation is required to examine if Fe^{3+} is able to overcome the protein corona's effect and still drive Au@PTA to aggregate before the assay can reach clinical standard.

Nonetheless, tannic acid is unable to form coordinate bonds with hydrocarbons and odours. To detect these particles, proteins, specifically odorant binding proteins are used. Attempts to improve the detection of these particles are discussed in the next chapter.

2.4 Materials and Methods

Materials

Hydrogen Tetrachloroaurate Hydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), Hydrogen Peroxide (H_2O_2), Trisodium Citrate, Tannic Acid (TA), Sodium Borohydride (NaBH_4), Silver Nitrate (AgNO_3), Rubidium Chloride (RbCl), Calcium Chloride (CaCl_2), Manganese Chloride Tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), Cobalt(II) Nitrate Hexahydrate ($\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), Nickel Sulphate Hexahydrate ($\text{Ni}_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$), Copper(II) Sulphate Pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), Zinc Sulphate Heptahydrate ($\text{Zn}_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$), Cadmium Chloride (CdCl_2), Mercury(II) Chloride (HgCl_2), Aluminium Potassium Sulphate Dodecahydrate ($\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$), Potassium Chromium(III) Sulphate Dodecahydrate

($\text{CrK}_2\text{O}_8\text{S}_2 \cdot 12\text{H}_2\text{O}$), Neodymium(III) Chloride Hexahydrate ($\text{NdCl}_3 \cdot 6\text{H}_2\text{O}$), Gadolinium(III) Chloride Hexahydrate ($\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$), Dysprosium(III) Chloride Hexahydrate ($\text{DyCl}_3 \cdot 6\text{H}_2\text{O}$), Iron (III) Chloride Hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), and Iron (II) Chloride (FeCl_2), were purchased from Sigma-Aldrich, Sodium Chloride (NaCl), Potassium Chloride (KCl) were purchased from Merck. Magnesium Sulphate Anhydrous (MgSO_4) was purchased from MP Biomedicals. All chemicals were used as received. Phosphate buffer (PB), sodium salt ($\text{pH} = 7.8$, 10 mM) was used for synthesizing PTA-based core@shell nanocomposites. Water used in all experiments was deionized and ultrafiltered to $18 \text{ M}\Omega \cdot \text{cm}$ using a Milipore Milli-Q gradient system.

Synthesis of core-shell metal@PTA nanocomposites

Au@PTA nanocomposites were prepared by pipetting 50 μL of TA (40 mg mL^{-1}) into 10 mL $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (1 mM) in 10 mM PB ($\text{pH} = 7.8$) at room temperature in Corning conical tube. It is to be noted that synthesis in other tubes (e.g. BD Falcon) did not yield any nanoparticle. The suspension was then vigorously mixed with a vortex mixer for 20 minutes. The colour of the solution turned red and gradually deepened until no further colour change was observed. The obtained Au@PTA were collected by centrifugation (9000 $\times g$ for 15 minutes) and rinsed with water twice. Bare AuNPs were synthesized using the same method, except that PB solution ($\text{pH} = 7.8$, 10 mM) was replaced with DI water. When conducting pH-dependent experiments, various buffer systems were used to adjust the pH value: $\text{pH} = 3.3$, glycine – HCl (10 mM); $\text{pH} = 6.0$, 7.0, 7.8, PB (10 mM); $\text{pH} = 8.5$, Tris-HCl (10 mM).

Characterization

The size and morphology of the synthesized materials were characterized using field-emission scanning electron microscopy (FE-SEM, JEOL JSM-6700F) at an acceleration voltage of 5 kV, transmission electron microscope (TEM, Hitachi H-7500), and HRTEM with an energy dispersive X-ray spectrometry (HRTEM-EDX, FEI, Tecnai G2 F20). Zeta-potential measurements were conducted in water by a potential analyser (Zetasizer Nano ZS, Malvern Instruments). UV-vis absorption tests were carried out on Shimadzu UV-2450 spectrophotometer in transmission mode.

Highly selective visual detection of Iron (III) cations

The standard procedure is as follows. Briefly, aliquots of 1 mM Fe^{3+} were added to the aqueous Au@PTA solutions (1 mL) to yield the final concentration series (1.0 μM – 400 μM). We also investigated the selectivity of our new approach for Fe^{3+} over other metal ions (Na^+ , K^+ , Rb^+ , Ag^+ , Mg^{2+} , Ca^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} , Hg^{2+} , Al^{3+} , Cr^{3+} , Dy^{3+} , Gd^{3+} , Nd^{3+}) under the same conditions. The colour change from red to blue indicates successful detection of specific ions. It is to be noted that aggregates may form after a few days. UV-vis spectra were acquired to investigate the interaction between the ions and Au@PTA.

Visual detection of H_2O_2

Au@PTA solution was added to a 96-well plate to a volume of 100 μL . Subsequently, Fe^{2+} was added to final concentration of 20 μM . The reaction was started by adding various volume of 100 μM H_2O_2 .

Chapter 3

On the odorant binding properties of the truncated pig Odorant Binding Protein

3.1 Introduction

For the detection of PAH and odorants, aggregation of Au@PTA is an unsuitable strategy as these compounds are hydrophobic and do not coordinate with the galloyl groups of tannic acid. Instead, the pig Odorant Binding Protein (pOBP) is utilized. Previous studies have shown the binding of pOBP to PAH and odorants [195]. Diphenyl binds to mutated pOBP with a dissociation constant of 0.14 μM , while anthracene has a dissociation constant to the mutated pOBP at 1.0 μM . In fact, the pOBP has also been used as a receptor protein and immobilized on various systems such as the graphene field effect transistor (gFET) to render a bioassay [196, 197]. In this chapter, we examined the impact which the deletion of a domain in the pOBP has on the protein's binding capacity towards different odorants. As the pOBP binds to several ligands, it is of potential use as a receptor protein in bioassays and can be immobilized on various systems such as the gFET [196, 197].

The current method of measuring PAH and odorant concentrations is through the use of analytical equipment such as the gas chromatography, high performance liquid chromatography or mass spectrometry [198-202]. These methods yield low limit of detection of down to 0.001 $\mu\text{g mL}^{-1}$. However, in order to eliminate the need for cumbersome equipment, research is now turning towards developing biosensors that can be miniaturized onto chips. These biosensors utilize olfactory receptors for the receptor components [203-205].

These are the closest biosensors that mimic the function of olfactory receptors. However, as these olfactory receptors are membrane proteins, refolding in lipid membrane is required. This is unlike OBPs which are soluble proteins and thus advantageously do not require refolding in a lipid membrane.

In this project, we study the effects in the binding properties of pOBP upon the deletion of the alpha helix tail, beginning from amino acid: 121, glycine (pOBP Δ 121). Instead of using pOBP wild type, we used a previously examined mutant pOBPM2 that bears a tryptophan in position eighty-eight instead of phenylalanine. pOBPM2 had been mutated from pOBP wild type to have a higher affinity to PAH [195]. Our truncated protein will thus be known as pOBPM2 Δ 121. Till date, however, no study in the properties of the deletion in the alpha helix tail has been conducted for pOBP or any lipocalin. Additionally, we also hypothesize that the absence of the tail, which acts as a gate, will allow for stronger ligand interaction with the inner core of the beta barrel. As such, the dissociation constant of ligands to pOBPM2 Δ 121 ought to decrease. In the context of bioassays, we expect the sensitivity to therefore increase. Minimum detection limit ought to also decrease.

3.1.1 Odorant Binding Proteins

The vertebrate OBPs, which include pOBP, are a member of the lipocalin family of protein. They are structurally conserved with eight stranded anti-parallel beta barrel and an alpha helix tail. A disulphide bridge is formed at the end of the alpha helix tail with the fourth strand of the beta barrel [206, 207]. This structure of lipocalin (and OBPs) is the same in most members of the family except for the bovine OBP, which lacks the disulphide bridge [208-214].

Bovine OBP protein engages in domain swapping to form a dimer and thus stabilizes the structure. Mutations of pOBP's cysteines in the alpha helix tail and beta barrel to remove the disulphide bridge as well as the deletion of glycine at position 121, yields a pOBP physiologically similar to the bovine OBP [210, 211, 214, 215].

Hydrophobic molecules bind to the core of the beta barrel of lipocalins despite their diverse roles and tissue expression levels [207, 216]. Lipocalins found in tears are postulated to either scavenge for or deliver lipids to the eyes [217]. Major urinary proteins, another sub-group of lipocalins, are thought to deliver pheromones into the environment [218, 219]. Boar salivary lipocalins, binding to steroid male pheromones, are thought to be involved in sexual arousal [220, 221].

Figure 3.1: Model of pOBPM2. The structure is ubiquitous to all lipocalins.

The first vertebrate OBP was discovered in 1982 by Pelosi et al. in cow olfactory mucosa while searching for olfactory receptors [222]. Since then, orthologues were found and isolated in sheep, mouse, rabbit, pig, human, and other vertebrates [223-226]. The crystal structure for some of the OBPs has also been solved [206, 227]. Advent of whole organism gene sequencing has led to the identification of six panda OBPs [227]. With the genomic sequencing and subsequent discovery and functional characterizations of olfactory receptors, these proteins were recognized as those responsible for recognising odorants and starting the signal processing cascade [228-231]. Thereafter, the role of OBP was proposed to be an aider in the transportation of hydrophobic odorants across the hydrophilic nasal mucosa to the olfactory receptors on the cilia of olfactory neurons [232, 233]. High concentrations of odorants in the nasal cavity drive the binding of odorants into the OBP. Upon travelling through the nasal mucosa to the olfactory bulb, low concentrations of odorants in the surrounding olfactory bulb drives the OBP to release the odorants. The odorants in turn bind onto the olfactory receptors [234]. Therefore, the dissociation constant of odorant to OBP ought to be higher than that of the olfactory receptor. Some OBPs perform similar roles by delivering pheromones to the vomeronasal organ [235].

Figure 3.2: Sequence alignment of various OBPs, hOBP, human OBP; rOBP, rabbit OBP; pOBP, pig OBP; bOBP, bovine OBP. The figure is cited from: Schiefner et al. [236] – published by the Wiley Periodicals, Inc.

Nevertheless, reverse chemo ecology experiments have shown that OBPs bind a wide variety of odours [237]. This renders OBPs suitable for use as the receptor modulus of bioassays. In fact, two OBPs, pOBP and insect *A. mellifera* (structurally different from lipocalins) have already been developed for use in gFET sensor [196, 197]. Initial studies of binding experiments relied on the use of radioligands to conduct competitive binding assays with various odours for determination of dissociation constants [237, 238]. Thereafter, the radioligands were replaced with fluorescent probes such as 1-Aminoanthracene (1-AMA), or N-phenyl naphthalen-1-amine (1-NPN) [239, 240].

Figure 3.3: a) The OBP's interaction with fluorescent probe and odorants as they are added to the protein. Upon addition of fluorescent probe (yellow), some fluorescent probe enters into the OBP leading to fluorescence being present. The fluorescence is represented by the yellow rays. When odorants (dark blue) are added, it competes with the fluorescent to bind with the OBP. Less fluorescent probe now binds to the OBP and the total fluorescence intensity drops. b, c) Docking simulation result of positioning of fluorescent probe 1-NPN (b) and 1-AMA (c) in the pOBPM2 protein.

The exact factors and their contribution to causing the odorants and PAH to have different binding properties to the OBP are not yet known. However, we do know for certain that size is one of the factors. Molecules are to be small enough to enter the binding pocket of the OBP. Another factor is hydrophobicity. Only hydrophobic molecules can enter the binding pocket of

the OBP. An interesting factor is chirality. Different chirality of a molecule can have different binding properties to the OBP [241]. Recently, it has been shown that the mutation of the amino acids in the binding pocket of the OBP can reverse the binding properties of the chiral molecules [242].

3.1.2 Expression of Recombinant Proteins in *E. coli*

Proteins are polypeptide chains with at least the molecular weight of 10 kDa [243]. The pOBP has a theoretical molecular weight of 17.7 kDa. Protein sequences are encoded by DNA sequences, where each amino acid is encoded by three base pairs. According to the central dogma of molecular biology, in the cells, DNA is first transcribed into mRNA with RNA polymerase. With the aid of ribosomes, the mRNA is translated into the protein sequence [244-246].

To synthesize the proteins recombinantly through the use of bacteria (especially *E. coli*) cells, nucleotide sequences has to be obtained. One method is through the use of Edman degradation [247-249]. Certain protein genes, already sequenced may be obtained from gene banks such as Universal Protein Resource (Uniprot) [250]. These genes may then be synthesized artificially (genetic engineering) and cloned into an expression plasmid vector.

Plasmids, which are circularized DNA, normally function to enable horizontal gene transfers between bacteria through the pili [251]. However, in genetic engineering of plasmids for protein synthesis, they are outfitted with three crucial functions: an origin of replication, an antibiotic resistance gene and a promoter sequence for expression of the protein of interest, the pOBP gene in our case [252-254]. Origin of replications enables *E. coli* to replicate the plasmid DNA enabling mass production of plasmids. Antibiotic resistance

gene when expressed in respective organism confers survivability of organisms in the presence of antibiotics. This allows for selection of colony clones containing the plasmids.

To clone the gene of interest into the expression plasmids, restriction enzymes cleave the ends of the gene of interest and the expression plasmid separately [255, 256]. This is followed by ligation of the gene of interest into the expression plasmid before transformation of the ligated plasmid into chemically competent *E. coli* [257, 258]. For storage or plasmid amplification purposes, DH5 α strain of *E. coli* is used. DH5 α *E. coli* is mutated to deactivate its endonuclease protein rendering it suitable as glycerol stock for plasmid storage purposes [259, 260]. For production of protein, the BL21 (DE3) *E. coli* and its derivative strains are commonly used as these strains contain the lac operon T7 promoter in its genome [261-264].

Activation of T7 promoter is done by addition of isopropyl-Beta-D-thiogalactopyranosid (IPTG). The IPTG is analogous to lactose, and its introduction inhibits the repressor's activity, allowing for the activation of the Lac Operon situated upstream of the T7 promoter in the native *E. coli* genomic DNA. Thus, the T7 promoter is synthesized and drives the expression of the gene of interest in the plasmid [261-264].

After expression, proteins that are extracted from the *E. coli* cytoplasm through sonication or by using the French press. Alternatively, proteins directed to the periplasm by the incorporation of PelB leader sequence are extracted via osmotic lysis [265-267]. Thereafter, the proteins are purified by running through separation columns usually with the aid of a Fast Protein Liquid Chromatography (FPLC) system. Proteins are separated based on intrinsic

characteristics, such as charges, hydrophobicity, mass, and tag presence. Ion exchange chromatography separates proteins based on charges [268-272].

For the pOBPM2 and pOBPM2 Δ 121, the GE Healthcare Life Sciences HiTrap QFF anion exchange column is used for purification. As the theoretical isoelectric points of the proteins are 4.37 and 4.51 respectively, the two proteins would be of negative charge when buffered in pH 7.4 buffer and thus binds to the positively charged anion column. The gradual increase of salt results in the dislodging of the protein from the column as the salt competes with the protein in binding to the column. As such, the protein of interest is separated from other proteins and is thus purified.

Figure 3.4: Overview steps for protein production and purification. a, b) Gene of interest and double digested vector backbone are ligated into a full plasmid. c, d) Ligated plasmid with gene of interest are transformed into competent *E. coli* cells. e) The transformed *E. coli* cells are grown and IPTG is added to

induce protein production. f, g) Proteins are collected and cleaned up through application in purification columns.

3.1.3 Binding Properties

In a mixture of receptors and ligands, which in this chapter would be specifically pOBP, and PAH or odorants respectively, there will exist three species of substances: the receptor, the ligand, and the receptor-ligand complex. The model is as such:

The ratio of the concentrations receptor-ligand complex against the product of receptor and ligand concentrations at equilibrium is termed the association constant and describes the affinity of the ligand to the receptor. Conversely, the reciprocal of the association constant is termed the dissociation constant.

$$K_D = \frac{[R][L]}{[R.L]} \quad (1)$$

As observed from equation (1), as per Le Chatelier's principle, the disturbance in concentration of either receptor or ligand will result in the establishment of a new equilibrium with a new concentration of receptor-ligand complex. However, the dissociation constant value remains the same. The value is unique to each binding pair.

In the end point binding studies between ligands and receptors, it is possible to determine ligand concentration, receptor-ligand complex concentration, and total receptor concentrations. It is not possible to determine ligand-free receptor concentration. As such, equation (1) requires

rearrangement to eliminate the unmeasurable ligand-free receptor concentration.

Rearranging equation (1) gives rise to the Hill Equation [273-276]:

$$[R.L] = [R]_{Total} \frac{[L]}{K_L + [L]} \quad (2)$$

In a competitive end point binding assay, another species of competitive ligand will be present and the model will be as such:

Resolving this model gives the formula:

$$[R.L] = [R]_{Total} \frac{[L]}{K_L(1 + \frac{[C]}{K_C}) + [L]} \quad (3)$$

This formula is also the precursor to the commonly used formula reported in various papers for the calculation of K_C [195, 227, 277].

$$K_C = [IC_{50}] \frac{K_L}{K_L + [L]} \quad (4)$$

Nonetheless, the dissociation constant between a ligand and a receptor is also dependent on the temperature as expounded by the Arrhenius equation.

The dissociation constant of the ligand is related to the reaction standard state Gibbs free energy by the equation:

$$\Delta G^0 = -RT \ln K_D \quad (5)$$

Here, R is the ideal gas constant and T, the temperature in Kelvins.

At equilibrium, the ratio of the concentrations receptor-ligand complex, receptor, and ligand concentrations will result in the minimization of Gibbs free

energy. The first-order differentiation of total Gibbs free energy with respect to ‘reaction progress’, meaning, the ratio concentration of the receptor-ligand complex, will give the equation [278]:

$$\Delta G^0 + RT \ln K_D \quad (6)$$

Thereafter, since it is a minima value, the first-order differentiation will be equal to zero. As such, we equate equation (6) to zero:

$$0 = \Delta G^0 + RT \ln K_D \quad (7)$$

The rearrangement of equation (7) will have us arrive at equation (5).

The Gibbs free energy equation is stated below:

$$\Delta G^0 = \Delta H^0 - T \Delta S^0 \quad (8)$$

3.2 Results and Discussions

3.2.1 Expression and Purification of Proteins

We first ran SDS Page gels to confirm that the proteins pOBPM2 and pOBPM2Δ121 were expressed (Figure 3.5). The protein pOBPM2 was found in the soluble and insoluble fractions. However, pOBPM2Δ121 was only found in the insoluble fraction.

Interestingly, we observed that the bands for lanes 1 and 2 to be very faint for pOBPM2 as compared to pOBPM2Δ121. The volumes of bacterial cell culture used to prepare the SDS Page samples were equal. This meant that the density for pOBPM2 cell culture was much lower than that of the pOBPM2Δ121. A possible reason for this is that the bacterial cells containing the pOBPM2 plasmids were growing at a slower rate than the bacterial cells containing the pOBPM2Δ121 plasmids. The pOBPM2 plasmid being longer

would have more nucleotides, requiring more nutrients for its replication. Henceforth, lesser nutrients were available for bacterial cell replication. For lanes 3 and 4, this problem was absent as the samples were equalised to have the same amount of overall protein. This was possible as the cells were already sonicated and the proteins were extracted and thus measurable.

Figure 3.5: SDS Page gels for pOBPM2 (a) and pOBPM2Δ121 (b). Proteins pOBPM2 and pOBPM2Δ121 have an apparent molecular weight of 23kDa and 14kDa respectively. Actual theoretical weight however is 17.8kDa and 13.5kDa for pOBPM2 and pOBPM2Δ121 respectively. The lanes in the SDS Page gels are ladder (1), non-induced with IPTG (2), induced with IPTG (3), soluble fraction after sonication (4), insoluble fraction after sonication (5).

Next, urea was used to solubilize the pOBPM2Δ121 which underwent three rounds of dialysis to refold in urea free soluble fraction. The soluble fraction of protein pOBPM2 was directly taken for purification.

The solubilized fractions of the two proteins were purified through a HiTrap QFF anion exchange column with increasing salt concentration gradient wise. For pOBPM2, we were able to obtain high purity. However, for pOBPM2Δ121, many contamination proteins are present in the eluted fractions (Figure 3.6).

Figure 3.6: a) Chromatogram for pOBPM2 purification. b) SDS Page gels of collected fractions from pOBPM2 purification. The lanes are as follow: Lane 1, Ladder. Lanes 2 to 10, Fractions 20 to 27. Fraction 23, circled in red was used for binding property analysis. c) Chromatogram for pOBPM2Δ121 purification. d) SDS Page gels of collected fractions from pOBPM2Δ121 purification. The lanes are as follow: Lane 1, Ladder. Lanes 2 to 10, Fractions 32 to 39. Fractions 34 to 39, circled in red were pooled together and applied to the column a second time for further purification.

To further purify the pOBPM2Δ121 protein, fractions from the first run containing the protein were pooled together. This was then applied into the column with increasing salt concentration step wise, with five columns volume for each step (Figure 3.7). Purified pOBPM2Δ121 fractions were obtained.

Figure 3.7: a) Chromatogram for pOBPM2Δ121 purification. d) SDS Page gels of collected fractions from pOBPM2Δ121 purification. The lanes are as follow: Lane 1, Ladder. Lanes 2 to 10, Fractions 16, 20, 21, 22, 23, 26, 31, and 36. Fraction 22, circled in red was used for binding property analysis.

3.2.2 End Point Binding Studies of Proteins

End point binding studies were done on Purified pOBPM2 and pOBPM2Δ121 to determine the dissociation constant of various ligands. We first incubated the proteins with varying concentrations of fluorescent probes to establish the kinetics of the proteins with the fluorescent probes. Thereafter, endpoint competitive binding assays of respective ligands with the fluorescent probes were done. 1-AMA was initially chosen as past experiments relied on the use of 1-AMA as the fluorescent probe [195, 240]. However, it was soon realized that 1-AMA does not bind at all to pOBPM2Δ121. Thereafter, 1-NPN, which has never been used to study pOBP or pOBPM2, was used as an additional probe (Figure 3.8). Fluorescent probe assays were modelled upon:

The model gives rise to the Hill equation as observed in equation (2). The formula is thus:

$$[P.F] = [P]_{Total} \frac{[F]}{K_F + [F]} \quad (9)$$

Figure 3.8: a) Fitted curves with Hill equation of 1-NPN (Red) and 1-AMA (Blue) binding to pOBPM2. b) Dissociation constants based on fitted curves.

To elucidate the dissociation constants of the various odorants, appropriate curve fittings were utilized (Figure 3.9). Assuming that there only was a single binding site and only one molecule (probe or odour) can fit in at each instances, the model would be as such:

Resolving this model gives rise to equation (3). The formula is thus:

$$[P.F] = [P]_{Total} \frac{[F]}{K_F(1 + \frac{[O]}{K_O}) + [F]} \quad (10)$$

Figure 3.9: Fitted curves for competitive binding assay with various ligands with the fluorescent probes 1-NPN (a) and 1-AMA (b), binding to pOBPM2. c) Apparent ligand dissociation constants based on fitted curves.

The discrepancy in dissociation constants was unexpected. Albeit having used two different fluorescent probes, the dissociation constants ought not to be at a difference of two orders of magnitude. (In fact it is now appropriate to use the term apparent dissociation constant instead of just dissociation constant.)

One possible explanation for the difference in apparent dissociation constants is that the competing odorants did not displace the fluorescence probe from the binding pocket. Instead, the odorant and 1-NPN probe coexisted simultaneously in the binding pocket. The binding pocket could have been huge allowing for the ligands to interact with different regions of the huge binding pocket. This train of thought would lead us to wonder if there could in fact be

an allosteric regulation where the binding mechanism of the ligands are sequential rather than independent [279].

Despite also using 1-NPN as fluorescent probe, competitive binding studies with pOBPM2Δ121 shows immediate decrease in fluorescence when various odorants were presented. This suggested to us that neither the cohabitation of the probe and ligand, nor the multiple probes in binding pocket situation, is true for pOBPM2Δ121 (Figure 3.10). Apparent dissociation constant were thus tabulated using the following model:

Figure 3.10: a) Fitted curve with Hill equation of 1-NPN binding to pOBPM2Δ121. b) Fitted curves for competitive binding assay with various ligands with the fluorescent probes 1-NPN binding to pOBPM2Δ121. c) Apparent ligand dissociation constants based on fitted curves. d) Calculation for change in Gibbs free energy of binding for ligand to respective proteins at

300K. Dissociation constant used for calculation are obtained from Figure 3.9c and Figure 3.10c.

We observed here an increase in dissociation constants (and thus increase in change in Gibbs free energy for binding) of the various odorant ligands and fluorescence probe to the truncated protein as compared to the non-truncated protein.

To further confirm that the tail deletion was responsible for the decrease in association of the odorants to the protein, we tested the binding of various odorants to a highly mutated pOBPM2 and its truncated version (pOBPMM2 and pOBPMM2 Δ 121). The mutations included three amino acids in the binding pocket (V80A, V90A, and N102K). Firstly, we observed that the binding of the fluorescent probe 1-NPN to pOBPMM2 was lower than that of pOBPM2 by an order of magnitude. Among the odorant ligands that exhibited binding properties to the pOBPMM2 and pOBPMM2 Δ 121, we saw immediate decrease in fluorescence, this suggested to us that the pOBPMM2 and pOBPMM2 Δ 121 have neither the properties of two fluorescence probe nor fluorescent probe and ligand coexisting in the binding pocket. We also observed that certain odorant ligands did not bind to the two proteins and their introduction brought about a stark increase in fluorescence. Amongst the odorants that can bind to both proteins, we notice an increase in dissociation constant for binding to pOBPMM2 Δ 121 as compared to pOBPMM from between 32% to 434% depending on the odour presented (Figure 3.11). Here, the apparent dissociation constant for competitive binding was fitted with the following curve:

$$[P.F] = [P]_{Total} \frac{[F]}{K_F(1 + \frac{[O]}{K_O}) + [F]} \quad (10)$$

Figure 3.11: a) Fitted curve with Hill equation of 1-NPN to pOBPMM2 (Red) and pOBPMM2 Δ 121 (Blue). b) Fitted curves for competitive binding assay with various ligands with the fluorescent probes 1-NPN binding to pOBPMM2. c) Fitted curves for competitive binding assay with various ligands with the fluorescent probes 1-NPN binding to pOBPMM2 Δ 121. d) Table of apparent dissociation constants and change in Gibbs free energy of binding for the each fluorescence probe or odorant ligand to respective proteins at 300K.

3.3 Conclusion

Here, we have shown clearly that the hypothesis that the tail acts as a gating mechanism to ligand entry and thus its removal would allow greater

ligand interaction to the pOBP binding pocket is false. Instead, the tail is crucial for providing stability to the ligands in the binding pocket. The entropy for the pOBP without the tail is lower than that of the pOBP with the tail.

Nevertheless, we have uncovered here a pitfall in the liberal use of fluorescent probes in competitive binding assays. The calculation of IC_{50} and apparent dissociation constants verily depends on the interactive model of the ligands and proteins [280].

One limitation to using pOBPs or OBPs in general as receptors for detecting odorants or PAH is that the pOBP is unspecific. As observed in our experiment, albeit at different binding constants, the pOBP binds to a host of odorants. Recent research has shown promise in altering the binding specificity of the pOBP by mutating the amino acids with the side chains facing the binding pocket of the pOBP [242]. Further work in mutating different OBPs can be done to confer more specificity to OBP binding.

Future work through the use of X-ray crystallography with protein in complex with various ligands ought to be performed to determine the binding site and capacity of the various odorants and fluorescence probe to the pOBP and the truncated pOBP. Partial truncation of the tail domain as well as the replacement of the alpha helix in the tail domain to perhaps GALA peptide will also be helpful to investigate the tail's importance to the pOBP's structure. Nonetheless, care must be taken with the use of X-ray crystallography in observing protein ligand interactions. For example, the binding of various alcohols to the LUSH OBP (structurally not a lipocalin) was proven to be false despite X-ray crystallography data showing otherwise [281, 282]. Meanwhile, it is therefore shown that the only proven method to enhance the binding of

ligands to pOBP for use as receptor on 2-dimensional transducer surfaces is through the directed evolution and mutation of the amino acids in the pOBP binding pocket [195].

Odorants and PAH interact in many ways with the human body. Their detection by the pOBP is due to its ability to enter the binding pocket of the pOBP. Other analytes such as biomarkers are much bigger or are not hydrophobic in nature. They are unfortunately unable to enter the binding pocket. Other strategies such as chimeric antigen receptor are required and will be discussed in the next chapter.

3.4 Materials and Methods

Synthesis of pOBPM2 Δ 121 and pOBPMM2 Δ 121 genome

The pOBPM2 and pOBPMM2 genes cloned into pET-5b plasmid were amplified using Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific) with the forward primer: 5'-atatacatATGcaagagcctcaacctgagca-3' and reverse primer: 5'agctcgaatTCATCAttgcccacagtcctcg-3'. Thereafter, the PCR product was purified and digested with the restriction enzymes: NdeI and EcoRI (Thermo Fisher Scientific). The digested PCR product was purified and ligated back into the pET-5b plasmid vector with T4 ligase (Thermo Fisher Scientific), yielding pOBPM2 Δ 121 and pOBPMM2 Δ 121 respectively.

Figure 3.12: The pET5b-pOBP plasmid. The gene is cloned in between the EcoRI and NdeI restriction sites and driven by the T7 promoter for expression.

Protein Expression and Purification

The pET-5b plasmid sub-cloned with recombinant pOBPM2 or pOBPM2Δ121 were transformed into *E. coli* strain BL21 (DE3) Star. Transformed bacteria were grown at 37 °C, shaken at 200 RPM in Lysogeny Broth, Miller supplemented with 50 mg L⁻¹ ampicillin to OD₆₀₀ 0.6-0.8. Thereafter protein expression was induced by the addition of 0.4 mM IPTG, and incubated for another 2 hours.

Sonication in Tris buffer, pH 7.4 was applied to the bacteria to extract the proteins. pOBPM2 is soluble whereas pOBPM2Δ121 was found in the inclusion body. Therefore pOBPM2Δ121 was incubated in Tris buffer, pH7.4

supplemented with 6M Urea and 1mM Dithiotheritol for solubilisation before undergoing dialysis three times for refolding.

Proteins were cleaned up by FPLC through a HiTrap Q FF anion exchange chromatography column with either gradient or step function protocol. Protein purity was confirmed with SDS Page analysis. Protein concentration was determined by UV-vis by multiplying absorbance at 280 nm with protein extinction coefficient.

Fluorescence binding assay

Fluorescent probes and odorant ligands were first prepared as stock solution by dissolving in methanol at 1mM concentration. Thereafter, affinity of fluorescence probe to protein was measured by incubation in a Perkin-Elmer LS45 Spectrofluorometer. Proteins were diluted to 1 μ M or 2 μ M concentration while fluorescence probes were added incrementally between 0.2 μ M and 16 μ M. For competitive binding assays, 2 μ M fluorescence probes were added to 2 μ M proteins before incremental addition of odorant ligands from 2 μ M to 16 μ M. When using 1-AMA fluorescence probe, excitation wavelength used was 375 nm with emission spectra recorded between 460 nm and 550 nm. Maximum emission peak at 500 nm was used for determining apparent dissociation constant. For 1-NPN fluorescence probe, excitation wavelength was 337 nm while emission spectra recorded was between 380 nm and 450 nm. Maximum emission peak at 410 nm was used for determining apparent dissociation constant. Curve fitting was done on software OriginPro 8.5 with appropriate curves. Error bars represent standard deviation of sample.

Chapter 4

Expression of Chimeric antigen receptors on the surface membrane of HEK293FT cells in the accurate orientation

4.1: Introduction

The detection of soluble biomarkers has to rely on a new strategy and cannot be done using Au@PTA or pOBP proteins. Biomarkers are unable to form coordinate bonds with tannic acid, neither are they able to enter the binding pocket of pOBP proteins. Here, we have designed a Chimeric antigen receptor (CAR) for use as a cell based bioassay.

4.1.1: Cell based bioassays

Cell based bioassays rely on the whole cell *in vitro* or *in vivo* to act as the receptor and readout. Typically, intracellular or membrane receptors detect the presence of extracellular ligands. This in turn couples unto an intracellular signalling pathway, transducing and amplifying a signal through the cell, resulting in the activation of a gene that acts as the output signal [283, 284]. The intracellular pathway transducing the signal may be either native to the cell or an artificially constructed circuit [285-290]. Since signal transduction can only occur through the activation of the receptors, cellular bioassays are advantageous in preventing unspecific binding as interactions between the ligands and the membrane will result in no signalling [277, 291-293]. This is unlike in the case of enzyme-linked immunosorbent assay (ELISA) where unspecific binding can occur, resulting in false signals.

Apart from ELISA, other methods used to detect ligand analytes include graphene Field Effect Transistor (gFET), Biolayer interferometry, Surface Plasmon Resonance (SPR), and Quartz Crystals Microbalance [294-298]. In general, these methods also rely on surface modification with receptors that binds onto the analytes [299-306]. As such, these techniques like ELISA also can result in false signals generated by unspecific binding. Various methods have been proposed to reduce unspecific binding, however, cell based sensor is able to eliminate the possibility of even unspecific binding generating signals [307-312].

Figure 4.1: Signals received by the receptor in a cell based bioassay are transduced either through the cell's native signalling pathway or artificial signalling pathway. The former allows for signal amplification through kinase cascades (Purple). The latter allows for design of logic gate circuits. Output signal could be through detection of calcium influx or expression of reporter genes.

Examples of protein receptor for cell based bioassay include the G-protein coupled receptor (GPCR) and Chimeric Antigen Receptor (CAR).

GPCRs are a class of eukaryotic seven trans-membrane receptor proteins that bind to a wide range of ligands [313-317]. The binding of ligand to GPCR, causes a conformational change of the GPCR. This results in the activation of G-protein, leading to a variety of signalling cascades within the cell resulting in a gene activation response [318-321]. The wide range of available ligand target makes it a good candidate for receptor purposes. However, the folding and assembly of GPCR cell membrane requires an endoplasmic reticulum and chaperone proteins [322-326]. Thus GPCR can only be expressed natively in eukaryotic cells.

CARs are composed of an extracellular single chained Fragment variable (scFv) recognition element, and an intracellular tyrosine containing immunoreceptor tyrosine-based activation motif (ITAM) domain with a transmembrane domain in between [293, 327]. Upon ligand binding, the CAR crosslinks with the tyrosine in the ITAM phosphorylating and activates a native signalling pathway.

As the signalling pathway in which these receptors converge unto brings forth calcium influx into cells, presence of calcium influx is an indication of the receptor's activation, and conversely shows ligand presence. Electrophysiology techniques coupling cells onto electronic chips may be used to detect the calcium influx [328]. Conversely, calcium indicator dyes fluorescing due to calcium chelation are also employed [329-331]. The fluorescence may be detected through flow cytometry, plate reader or even observed under confocal microscopy [332-334]. Calcium influx into cells has also been known to

activate NF κ B and Nuclear factor of activated T-cells (NFAT) promoters [335-337]. As such, by coupling luciferase genes or fluorescent protein genes after the promoters, calcium influx can be observed through the expression of these genes [338-341].

Recent advances in molecular biology, metabolic engineering and synthetic biology have aided the advancement of cell based bioassay. Directed evolution of receptor molecules has led to increased sensitivity and selectivity [342-344]. Possible false response signal generated by cross talks in signalling pathways can be knocked down by small interfering RNA or CRISPRi [345-347]. With the aid of synthetic biology, artificial signalling pathways have also been designed for signal transduction [286, 288, 289, 348-350]. These pathways are designed to reflect electrical circuits, enabling the incorporation of logic gates. For example, using AND gate, the expression of two transcription factors due to activation of two receptors, result in the expression of the actuator proteins [285, 288, 351, 352]. This is especially so for CAR immunotherapy where the use of AND gate ensures that only cancer cells, not healthy cells are triggered to undergo apoptosis [351]. Nevertheless, mammalian cell based sensors has also been engineered to circumvent the signalling pathway. Schwarz et al. replaces the cytoplasmic domain of the CAR with a protease and transcription factor. Crosslinking of CAR cleaves the transcription factor from the CAR freeing it to drive the transcription of the response gene [292]. This eliminates the possibility of cross talking and false signalling.

Other cell based sensors have been developed in other organisms. In prokaryotes, transmembrane protein localized in the periplasm of Gram negative *E. coli* and *V. Cholerae* or outer membrane of Gram positive *L. Lactis*

have been used to detect sugar molecules such as galactose and maltose, or quorum sensing analytes [353-361]. Reporter genes such as fluorescent proteins help generate an output signal. Yeast and *Xenopus* oocyte are examples of eukaryotic non-mammalian cell based sensors that have been developed [277, 343, 362-365]. Expression of reporter genes are used as output signals in yeast. However, for *Xenopus* oocyte, electrophysiology techniques such as patch clamp are used to measure chloride ions influx which acts as the output signal. Calcium dependent chloride ions channels are opened upon receptor activation, generating a measurable influx of chloride ions [366, 367].

Despite the advantageous use of cell based sensor in removing unspecific binding in bioassays, there are still various limitations unique to biosensors. For example, as live cell cultures are used, experiments need to be conducted in biosafety facilities. Additionally, precaution has to be taken to prevent contamination of genetically modified cells into the environment [368, 369].

4.1.2: Chimeric Antigen Receptors and Immunotherapy

CAR expressed in Cytotoxic T cells has also been used for therapeutic purposes. Specifically in immunotherapy where the CAR is designed to target cancer cells *in vivo*. The scFv domain of the CAR is engineered to bind to membrane proteins overexpressed on cancer cells. Thereafter, the crosslinking of the CAR activates the Cytotoxic T cell to induce apoptosis in the targeted cell [370, 371].

The design of such chimeric receptors in T cells has undergone various changes and enhancements over the years. Nonetheless, a simple description of

the CAR may be portrayed as such: outer membrane antigen detection unit, usually a scFv antibody, a transmembrane domain, and finally an intramembrane signalling module, consisting the ITAM [372-374].

The T cell receptor comprises 8 sub units of polypeptides: 1 alpha, 1 beta, 2 epsilon, 1 gamma, 1 delta, and 2 zeta polypeptides. The epsilon, gamma and delta subunits are transmembrane and each contains an ITAM domain. The zeta polypeptide is cytoplasmic, and extends into the membrane region of the cell, where they are joined together via a disulphide bridge. Each zeta sub unit contains 3 ITAM domains. The alpha and beta subunits are located on the extracellular region of the cell with a transmembrane portion. They are joined together via a disulphide bridge in the extracellular portions and they act as the targeting modularity for the T cell receptor. Together, these 8 sub units interact with each other through hydrophobic interaction in the cell membrane to yield a functional T cell receptor [375].

The first work to direct the targeting modularity of the T cell to a specific target was through chimerically replacing the binding site of the alpha and beta sub units with the variable region (heavy chains) of antibodies. The result is a T cell that now targets an antigen similar to that of the antibody. Prove of the chimeric T cell functionality is shown when interleukin-2 secretion levels are increased upon antigen presentation [376, 377]. Furthermore, these chimeric receptors were also injected into mice embryos resulting in transgenic mice producing T cells expressing the chimeric receptors and hence, are programmed for specific target.

However, these chimeric receptors still rely on the assembly of the sub units into T cell in order for its functionality. In 1993, two separate groups

published results in which an scFv was genetically fused to either the gamma or zeta subunit of the T cell receptor. Not only did the chimeric receptor localize at the cell membrane after its expression, its functionality was also confirmed when interleukin-2 or interleukin-3 secretion levels increased upon antigen presentation [378]. To increase the exposure of the scFv to antigens presented, Karjalainen et al. included a CD8 hinge region in between the scFv and zeta subunit of the T cell receptor [379].

Moreover, it is known that T cell activation relies not only on T cell receptors stimulation, but also a host of other T cell receptors such as CD28 and CD137. Activation of CD28 results in increased interleukin secretion, whereas for CD137, its activation causes increased T cell proliferation and survival. As such, to enhance the intracellular chimeric T cell signalling, CD28 and, or CD137 cytoplasmic tails containing ITAM domains were genetically fused to the chimeric receptors already with the zeta domain. Generation two chimeric T cell receptors have the additional CD28 domain while generation three chimeric receptor has additional CD28 and CD137 cytoplasmic tail domain [380-382].

To date, the CAR in T cells has undergone four generations of development [383]. The addition of different cytoplasmic domains for co-stimulatory purposes is the difference between generations. For example, the addition of CD28 domain results in proliferation of cytotoxic T cells upon stimulation [370]. Recently, Baeuerle et al. has developed a full T cell receptor fused with receptor scFv. This ensures that all signalling pathways of a native T cell receptor are retained upon activation of this CAR also known as T cell Receptor Fusion Construct (TRuC) [384]. Numerous clinical trials for CAR immunotherapy have already been conducted to treat various cancers [385,

386]. Two third generation CAR products targeting CD-19 for B cell lymphoma treatment, KymriahTM and YescartaTM are already approved by the Federal Drug Administration [387]. Despite these developments, strategies for delivering CAR expressing Cytotoxic T cells to solid tumours are still being researched [383, 388]. Various adverse effects and T cell exhaustion needs also to be overcome [389-391].

However, such CAR has only been used once in B cells and only for the alpha tail domain. Furthermore the transmembrane domain used in the B cell's CAR is native to that of T cell receptors; chimeric B cell receptor with B cell receptor transmembrane domain has not yet been developed [392]. This is not surprising as B cell receptor's transmembrane domain is known to be overly hydrophilic as compared to T cell receptor's transmembrane domain and hence requires interaction with other transmembrane domain in order to be expressed on the cell membrane surface.

The B cell receptors are composed of four protein subunits, Immunoglobulin (Ig) light chain, Ig heavy chain, Ig alpha, and Ig beta. Two Ig heavy chains and two Ig light chains are covalently linked by disulphide bonds forming the extracellular domain of the receptors. Differing specific amino acid sequences of the extracellular Ig domains confers binding specificity of the BCR to various different antigen, ensuring accurate selectivity of the BCR and for each B cell. The heavy chain extends into the cell membrane via the transmembrane region to interact with the Ig alpha and Ig beta domains.

The Ig alpha and beta are also covalently joined together by a disulphide bond forming the intracellular cytoplasmic domain of the receptors. The Ig alpha is a 220 amino acid protein, with position 113 amino acid cysteine (C113)

responsible for forming the disulphide bond with the Ig beta. Ig beta is a 228 amino acid protein, with position 135 amino acid cysteine (C135) responsible forming the disulphide bond with the Ig alpha. The cytoplasmic amino acids containing the crucial ITAM domains are 160-220 and 181-228, for Ig alpha and Ig beta respectively.

Upon binding of the BCR by antigens, two BCR will undergo dimerization resulting in the phosphorylation of the ITAMs. The phosphorylation of ITAMs activates the BCR resulting in a signalling cascade within the B cells which ultimately leads to an influx of calcium ions into the cell [393-401].

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Figure 4.2: The B cell receptor and its downstream signalling pathway whereby the one of which results in an influx of calcium ions. Figure 4.2 was cited from: Young, R. M. and Staudt, L. M. [402]– published by Springer Nature.

Experimenting of BCR with the removal of the intracellular Ig alpha and Ig beta domain, results in the inability of the BCR to be expressed on the

surface of the B cell. However, when the tyrosine and serine amino acids within the transmembrane region is mutated to two hydrophobic amino acids valine, the BCR is able to be expressed on the surface of the B cell in the absence of the intracellular Ig alpha and Ig beta domains. Subsequent experiments involving genetic fusion of either Ig alpha or Ig beta cytoplasmic domains to the intracellular end of the Ig heavy chain shows not only localization of the B cell receptor on the B cell membrane surface, but also functionality of the B cell receptor through influx of calcium ions [403-405].

Unlike Cytotoxic T cells, B cells do not cause the apoptosis of targeted cells. Instead, B cells in nature, secrete high volume of antibodies when activated. As such, it is possible to utilize B cells for antibody therapy via B cell activation using chimeric BCR. In nature, in a single B cell, the antibodies secreted by the B cells for antigen targeting are similar to the antigens B cell receptors target. However, with the use of chimeric BCR, the B cells can be trained to secrete antibodies that targets different antigen from the receptors. A possible application for this would be to direct the B cells to secrete antibodies targeting cancer cells when it detects markers secreted by the tumour [406].

Another characteristic of B cells are that their activation require the helper T cells to co-stimulate it for antibody secretion. One way to circumvent this is to use a non-constitutive promoter that can be induced by the downstream signalling process as a result of BCR activation. As calcium influx takes place when B cells are activated, an NFAT promoter which activates in the high concentration of calcium can be used for secondary signal output after the activation of the chimeric BCR [112].

4.1.3: Chimeric Antigen Receptors for Detecting Soluble Antigens

Recent advances in CAR development has allowed the CAR to respond to soluble antigens. CAR has also been ported into HEK293 cells, allowing it to serve as a cell-based bioassay [291, 293].

Here we replaced the extracellular Ig heavy and Ig light domains with a scFv as design for our CAR. Our scFv, B10, is specific to target Epidermal Growth Factor Receptor (EGFR) which is overly expressed in triple negative breast cancer cells including MDA-MB-231. The intracellular domain would be that of the BCR alpha tail in order that the ITAM may couple onto the B cell signaling pathway. To stimulate localization of the CAR, we added a cleavable murine Ig kappa chain signal peptide as the leader sequence [407].

Epitope tags such as the 6x His-tag or Myc-tag were also introduced at the outer membrane location of the CAR to serve as an epitope marker in order for confocal microscopy to be done to confirm the expression, localization and orientation of the CAR.

The design of the CAR was built upon various past works. Firstly, Sanchez et al., Mitchell et al., and Stevens et al. showed that it was possible to produce a functional truncated BCR when conjoining the tail portion of Ig Alpha to that of the membrane region of the BCR [395, 403, 405]. Although in their design, they still retained the full antibody as the antigen detection outer domain rather than change it to a scFv. However, it was necessary to mutate the YS amino acids to VV in the membrane domain of their BCR, else the truncated BCR would not be expressed successfully. This mutation was carried forward into our CAR design. The outer domain was built upon past work of Brocker et al. and Eshhar et al. which were the first developments having an

scFv with a T cell receptor tail, essentially the first CAR. The design which also incorporated with a hinge region to increase the scFv's binding capacity was used in our CAR (with BCR Ig alpha tail) [379, 408].

We envisioned that CAR displaying B cells would serve as a cell-based bioassay coupled with therapeutic purposes. The activation of CAR of the B cells would result in a downstream signaling cascade similar to that which occurs in a B cell. Thereafter, this signaling cascade will drive calcium influx into the cells, wherein, an NFAT response element would result in the cell secreting therapeutic antibody genes (Figure 4.3).

Figure 4.3: The envisioned therapeutic B cell based bioassay. Step 1: CAR is at rest. Step 2: Ligand crosslinks CAR. Step 3: Intracellular signalling pathway activated. Step 4: Calcium ion influx into cells. Step 5: NFAT response element results in secretion of therapeutic antibodies.

4.2 Results and Discussions

As a first step to the development of the envisioned B cell based bioassay, we tested the system on HEK293FT cells. Two constructs for the

gene were first cloned into the pLenti6/V5 directional TOPO vector backbone to be expressed by the CMV promoter (Figure 4.4). Thereafter, the plasmids were transfected into HEK293FT cells for expression.

Figure 4.4: a) Designs of the different CAR. Design one has 6x His-tag located at the N-terminus of the CAR while design two has a Myc-tag placed between the scFv and hinge region. b) The CAR gene is placed after the signal peptide and driven by a CMV promoter.

4.2.1 Expression of CAR

Confocal microscopy imaging confirms that the expression level for CAR design one was very low, about two or three in the million seeded cells. However, for design two, the number of cells expressing CAR was comparable to that of the number of cells expressing the enhanced green fluorescent protein

(EGFP) control (Figure 4.5). For negative control, untransfected cells were also stained with the antibody and showed no fluorescence signal.

Figure 4.5: a) Untransfected HEK293FT cells stained with anti-His alexa fluor 488 antibody. b) Design one transfected HEK293FT cells stained with anti-His alexa fluor 488 antibody. c) Untransfected HEK293FT cells stained with anti-Myc FITC antibody. d) HEK293FT cells transfected with EGFP gene. e) Design two transfected HEK293FT cells stained with anti-Myc FITC antibody. The ruler represents 20 µm.

The reason for the low amounts of cells expressing the CAR could be either due to the tag positioning or due to transfection inefficiency. To test if transfection inefficiency is the cause, we introduced a mCherry gene in between the XmaI and KpnI position in the pLenti6/V5 directional TOPO where the mCherry gene expression is driven by the SV40 promoter.

After transfection and staining with the anti-His alexa fluor 488 conjugated antibody, we observed that there are cells expressing the mCherry

gene, but not stained with the antibody (Figure 4.6). This tells us that the transfection is efficient but the expression of the design one CAR is not present. The positioning of the epitope tag being right at the N-terminus hindered the proper chaperoning of the protein through the endoplasmic reticulum onto the cell surface membrane leading to the degradation of the protein. The position of the epitope tag in design two was in fact similar to the design from Ho et al. in which a scFv was displayed on the cell surface [407].

Figure 4.6: HEK293FT cells transfected with plasmid that contains the gene for both design one CAR and mCherry. Successfully transfected cells show

mCherry expression (red coloured) but no expression of CAR. The ruler represents 20 μm .

4.2.2 Antigen binding to CAR expressed on HEK293FT cells

Next, we examined if the intended targeting ligand, EGFR is able to bind onto the successfully expressed design two CAR. HEK293FT cells transfected with design two were stained first with EGFR chimerically fused with 6x His-tag. Thereafter, anti-His alexa fluor 488 conjugated antibody was stained as if a secondary antibody to the cells. Results showed that the design two expressing HEK293FT cells did fluoresce when viewed under confocal microscopy, proving that the EGFR does indeed bind onto the design two CAR (Figure 4.7). For negative control, cells were stained with either only EGFR chimerically fused with 6x His-tag or only the anti-His alexa fluor 488 conjugated antibody.

Figure 4.7: a) Design two CAR transfected HEK293FT cells stained with EGFR chimerically fused with 6x His-tag followed by anti-His alexa fluor 488 antibody. Cells fluoresced under confocal microscopy observation proving that EGFR does indeed bind to design two CAR. b) Design two CAR transfected HEK293FT cells stained with only EGFR chimerically fused with 6x His-tag.

c) Design two CAR transfected HEK293FT cells stained with only anti-His alexa fluor 488 antibody. The ruler represents 20 μm .

The successful binding of the antibodies to the outer membrane epitope tag as well as the analyte to the outer membrane scFv domain of the CAR proves to us that the scFv and epitope tags are located on the outer membrane domains of the CAR. Therefore, the CAR is in the accurate orientation with the intended outer membrane domains located externally of the cell. The transmembrane domain of the CAR would have led to the arrestment of the translocation of the CAR polypeptide across the ER membrane [395, 409]. The inner domains of the CAR are therefore certainly in the HEK293FT cells.

4.2.3 Functionality of CAR

Next we tested the functionality of design two CAR by testing for the presence of calcium influx when presenting the transfected HEK293FT cells with EGFR. If the CAR is functional, EGFR ought to crosslink the CAR. The ITAM domain would thereafter be phosphorylated resulting in a signalling cascade. This would thus culminate in a calcium influx into the cell.

Fluo-4 was taken up into transfected and untransfected cells through an incubation period. If calcium ions were present, the calcium ions would chelate with Fluo-4, resulting in a measurable emission at 525 nm wavelength. Conversely, if calcium was not present, no emission at 525 nm would be detected.

When B cell receptors are activated, the first kinases being activated are the Lyn kinase followed by the Syk kinase. These two kinases are present in

HEK293FT cells and thus we could expect the CAR with a B cell receptor tail and ITAM to trigger a signalling cascade resulting in calcium influx when in the HEK293FT cell [410, 411]. If a T cell receptor tail is used instead, we would not expect any signalling as the first kinase T cell receptor phosphorylates is ZAP 70 kinase. The ZAP 70 kinase is not present in HEK293FT cells [412, 413].

Post Fluo-4 incubated transfected and untransfected HEK293FT cells were presented with varying concentrations of EGFR. We also presented the cells with anti-Myc antibodies. We were concerned that EGFR being a monomer would only bind to a single CAR instead of two CARs which would be necessary for CAR crosslinking to take place. Anti-Myc antibodies in contrast are dimeric and therefore would certainly crosslink the CARs. For positive control, adenosine triphosphate (ATP) that stimulates the P2Y receptors whose signalling results in calcium influx was used. For negative control, buffer free of EGFR, antibodies or ATP were used.

We observed when ATP is applied to the cells in the positive control sample, a spike in emission intensity occurs. This shows that the ATP does indeed stimulate a calcium influx signalling response (Figure 4.8h, p). However, we do not observe an emission signal spike in the samples where EGFR and antibodies were added to the cells. The signal is the same as that of the negative control. This suggests that no calcium influx has taken place.

Figure 4.8: Graphs of calcium influx assays. The Y-axis is the emission intensity and the X-axis is time. For a, b, c) Untransfected HEK293FT with $0.1 \mu\text{g mL}^{-1}$, $1 \mu\text{g mL}^{-1}$, and $10 \mu\text{g mL}^{-1}$ of EGFR introduced. d) Untransfected HEK293FT with only HBSS introduced. e, f, g) Untransfected HEK293FT with $0.1 \mu\text{g mL}^{-1}$, $1 \mu\text{g mL}^{-1}$, and $10 \mu\text{g mL}^{-1}$ of anti-Myc antibody introduced. h) Untransfected HEK293FT with $5 \mu\text{M}$ ATP introduced. i, j, k) Transfected HEK293FT with $0.1 \mu\text{g mL}^{-1}$, $1 \mu\text{g mL}^{-1}$, and $10 \mu\text{g mL}^{-1}$ of EGFR introduced. l) Transfected HEK293FT with only HBSS introduced. m, n, o) Transfected HEK293FT with $0.1 \mu\text{g mL}^{-1}$, $1 \mu\text{g mL}^{-1}$, and $10 \mu\text{g mL}^{-1}$ of anti-Myc antibody introduced. p) Transfected HEK293FT with $5 \mu\text{M}$ ATP introduced.

The reason for the absence of calcium influx ought to be investigated. It could either be due to the absence of CAR crosslinking or that the CAR crosslinking failed to transmit the intracellular signalling adequately to drive the calcium influx.

Here, we performed time dependent western blots to examine if crosslinking occurred. Transfected and untransfected cells were incubated with EGFR, EGFR with anti-His antibody, or anti-Myc antibody. As we were concerned that EGFR as a monomer was itself unable to cross-link the CAR, we decided to add anti-His antibody that could bind to the 6x His-tag chimerically fused to the EGFR. As the anti-His antibody is dimeric it would bring about the cross-linking in the event EGFR itself is unable to do so.

After incubation with respective ligands for the respective period of time, the cells were lysed and the lysate was used for western blot analysis. For western blot, anti-phosphotyrosine antibody is used to probe for tyrosine phosphorylation.

As observed in the analysis of the western blot data, the relative intensity of the protein band for the CAR does not increase as ligand incubation time increases or compared to the ligand free sample (Figure 4.9). This suggests to us that no crosslinking of the CAR is occurring or the initial kinase Lyn was not functioning optimally.

Figure 4.9: a, b) Samples were untransfected or transfected HEK293FT cells respectively being treated with 30 minutes EGFR (lane 1), 30 minutes EGFR followed by 1 minute of anti-His antibody (lane 2), 30 minutes EGFR followed by 2 minutes of anti-His antibody (lane 3), 30 minutes EGFR followed by 3 minutes of anti-His antibody (lane 4), 30 minutes EGFR followed by 5 minutes of anti-His antibody (lane 5), 30 minutes EGFR followed by 10 minutes of anti-His antibody (lane 6). c, d) Samples were untransfected or transfected HEK293FT cells respectively being treated with 1 minute anti-Myc antibody (lane 1), 2 minutes anti-Myc antibody (lane 2), 3 minutes anti-Myc antibody (lane 3), 5 minutes anti-Myc antibody (lane 4), 10 minutes anti-Myc antibody (lane 5), 30 minutes anti-Myc antibody (lane 6). e) Transfected HEK293FT (lane 1) and untransfected HEK293FT (lane 2) cell lysates who's blots were stained with anti-Myc antibody conjugated with horseradish peroxidase. This sample shows us the apparent position of CAR in the western blot. g, h) Normalized relative intensity of CAR bands to the sample lane for blots in blot (b) and blot (d) respectively. The Green boxes represent presence of CAR band and red boxes the absence.

4.2.4 Modularity of CAR

Lastly, we examined the modularity of design two CAR by switching the existing B10 scFv to another L21C scFv, albeit also binding to EGFR. However, under confocal microscopy viewing, the protein is not expressed at all (Figure 4.10). As both genes contain the cleavable murine Ig kappa chain signal peptide, we do not suppose that the signal peptide is the reason for the failure of the CAR to be expressed and localized on the membrane surface of the HEK293FT cells. Instead, the difference in initial amino acid sequences of the two scFv may explain the reason why one gets expressed and localized while the other does not. The first ten amino acids of the B10 scFv are QLQLQESPGG whereas for L21C, they are EVKLQQSGAE.

Figure 4.10: a) Untransfected HEK293FT, stained with anti-Myc FITC antibody. b) Transfected HEK293FT with L21C CAR, stained with anti-Myc FITC antibody. The ruler represents 20 µm.

In this project we intended for the cross-linking of our receptor to phosphorylate the CAR ITAM domains. This in turn would phosphorylate the

Lyn and Syk kinase. These events phosphorylate other kinases downstream to drive calcium influx which then activates the NFAT promoter. We did not however observe any calcium influx. We also did not observe any phosphorylation of the CAR ITAMs either. The presence of Syk kinase is crucial for the phosphorylation of the CAR ITAMs [414]. The HEK293FT cell expresses a low level of Syk protein as opposed to other blood cells [410]. The low presence of Syk kinase could be the reason for the absence of CAR ITAM phosphorylation.

Another possibility for the CAR cross-linking not giving rise to calcium influx is that the cross-linking of the CAR at the extracellular domain does not drive the intracellular domains to cross-link sufficiently. Previous work by Sanchez et al. has developed a chimeric BCR similar to our design except that the scFv domain is replaced with an Ig domain that when cross-linked can induce a calcium influx [395]. The Ig domain is larger than the scFv domain and thus has greater steric hindrance and would be harder for the intracellular domains to cross-link when the extracellular domains are cross-linked. Since, the Ig domain chimeric BCR could show intracellular cross-linking proven by calcium influx, the CAR with scFv domain will not have a problem inducing intracellular cross-linking when the extracellular domains are cross-linked. One method to test for this is to fuse a fluorescent protein at the C terminus of the CAR. Upon extracellular domain cross-linking we should observe that the emission intensity for each fluorescent spot is increased if the intracellular domains are cross-linked bringing the fused fluorescent proteins together. If intracellular domains are not cross-linked, the fluorescent proteins would

remain separate and would register at lower intensity as compared to when cross-linked and brought close together.

4.3 Conclusion

In conclusion, we have here expressed a CAR successfully on the membrane of the HEK293FT cell in the correct orientation and shown that the intended ligand EGFR does bind onto the surface CAR. The Au@PTA and pOBP protein systems are unable to bind to EGFR and therefore will not be able to even function as a cell based biosensor.

Nonetheless, despite the binding of EGFR to the CAR, we observe no downstream signaling process that could result in a calcium influx taking place. As a result, the sensor system is present, however, the response signal is absent.

As our final product aims to have the CAR functional in B cells, we are confident that the system would work because our CAR's tail and ITAM domain are obtained from B cell receptor domains. The CAR would certainly be able to transmit the extracellular binding event into intracellular crosslinking signal. Future work would have the CAR expressed in B cells to render the B cell a therapeutic bioassay. Its functionality would be tested through calcium influx assay and antibody secretion.

4.4 Materials and Methods

Molecular cloning of EGFP, CAR B10 design one, CAR B10 design two, CAR L21C, and mCherry into expression plasmid

Genes for EGFP, CAR B10 design one, CAR B10 design two, CAR L21C, and mCherry were purchased as Gblock from IDT DNA. They were

subsequently amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) before double digestion with BamHI and XhoI restriction enzymes (Thermo Scientific). The digested gene fragment inserts are then ligated into doubly digested pLenti6/V5 directional TOPO vector backbone (Thermo Scientific) at the BamHI and XhoI restriction sites. For mCherry, the Glock was not amplified, but instead digestion directly with XmaI and KpnI. The plasmid pLenti6/V5 directional TOPO were also digested with XmaI and KpnI. The restriction enzymes XmaI and KpnI were also purchased from Thermo Scientific. Ligation is done with T4 ligase (Thermo Scientific). Post-ligation, recombinant plasmids were transformed into Stbl3 *E. coli* cells (Thermo Scientific) for plasmid amplification.

Primers used for the Gblock amplification are:

EGFP, Forward Primer:

TCTAGAGGGATCCACCATGGTTAGTAAAGGCGAAGAGTTGT

EGFP, Reverse Primer:

CTAGACCTCGAGTTATTACTTGTACAATTCGTCCATGCCAAGG

B10 Design one, Forward Primer:

TCTAGAGGGATCCACCATGGAAACAGATACTCTCCTGC

B10 Design one, Reverse Primer:

CTAGACCTCGAGTTATTATGGCTTTTCCAGCTGAGCGTC

B10 Design two, Forward Primer:

TCTAGAGGGATCCACCATGGGAGACTGACACGCTCCTTCTTT

B10 Design two, Reverse Primer:

CTAGACCTCGAGTTATTAGGGCTTCTCCAATTGTGCGT

L21C, Forward Primer:

TCTAGAGGATCCACCATGGAAAC

L21C, Reverse Primer:

CTAGACTCGAGTTATTACGGCTTCT

Figure 4.11: Construct of pLenti6/V5 directional TOPO Plasmid with crucial restriction site enzymes circled in red, BamHI, XhoI, XmaI and KpnI.

Transfection of genes in HEK293FT cells and its imaging

HEK293FT cells were trypsinized with 0.05% trypsin (Thermo Scientific) and plated on a D35-10-1-N cell culture dish (Cellvis) at 1×10^6 cells in 2 mL of Dulbecco's Modified Eagles Medium (DMEM) medium (Thermo Scientific) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Scientific). The cells were incubated under 37 °C, 5% CO₂ condition.

Complexing of Lipofectamine 3000 (Thermo Scientific) was done by incubating 7.5 μ L of Lipofectamine 3000 in 125 μ L Opti-MEM at room temperature for 15 minutes before a mixture of 2.5 μ g recombinant expression vector, and 5 μ L of P3000 reagent in 125 μ L of Opti-MEM was pipetted in. Thereafter the mixture was further left to stand a further fifteen minutes before pipetting into the HEK293FT cells.

Forty-eight hours after transfection, cells were fixed by incubating at room temperature for one hour with 2% paraformaldehyde. Fixed cells were then washed three times with phosphate buffered saline (PBS) before incubating with 3% bovine serum albumin (BSA), 0.3 M glycine dissolved in PBS for 1 hour in room temperature. Thereafter, cells were stained with antibody overnight at 4 °C with antibody diluted in 1% BSA. The antibody used was either the D3I10 anti-His antibody conjugated with alexafluor 488, (Cell Signaling Technology #14930) or the 9E10 anti-Myc antibody conjugated with FITC (Thermo Scientific #13-2511).

After incubating overnight, stained cells were washed three times with PBS and stained with DAPI diluted in PBS at 300 nM concentration for 15 minutes. Cells were washed three times again and kept in PBS solution. The Leica TCS SP5 Confocal microscope was then used to examine the cells at 63x magnification.

For testing EGFR binding to transfected HEK293FT cells, the protocol was modified by having the EGFR chimerically fused with 6x His-tag (Acrobiosystems) stain the cells overnight at 4 °C with antibody diluted in 1% BSA. Thereafter, cells were washed three times with PBS before staining at

room temperature with the D3I10 anti-His antibody conjugated with Alexa Fluor 488.

Calcium influx assay

HEK293FT cells were plated in 384 well plates at a density of 2.4×10^4 cells per well supplemented with 30 μL of DMEM supplemented with 10% FBS. After incubation overnight, cells were transfected with respective recombinant plasmids. Thereafter the cells were left to further incubate for another 48 hours.

Cell medium were removed and the cells were incubated for four hours with 30 μL of Hank's buffered saline solution (HBSS), pH 7.4, supplemented with 100 mM of Fluo-4. Thereafter, the cells are ready for calcium influx assay.

Calcium influx assay was carried out using the fluorescence imaging plate reader (FLIPRTM, Molecular Devices). The volume of ligands such as EGFR chimerically fused with 6x His-tag (Acrobiosystems) and 9E10 anti-Myc antibody (Thermo Scientific #13-2500) be added to the cells was fixed at 25 μL diluted in HBSS. Since the cell medium was 30 μL , the total volume would be 55 μL . In essence, it would be a 2.2 times dilution of ligands. To achieve the final ligand concentration of $0.1 \mu\text{g mL}^{-1}$, $1 \mu\text{g mL}^{-1}$, and $10 \mu\text{g mL}^{-1}$ of ligands in 55 μL , stock solutions of the ligands needed to be of the concentrations of $0.22 \mu\text{g mL}^{-1}$, $2.2 \mu\text{g mL}^{-1}$, and $22 \mu\text{g mL}^{-1}$. For ATP, as the final concentration is 5 μM , the stock solution concentration is 11 μM .

Detection of phosphorylation using western blot assay

HEK293FT cells were plated in 24 well plates at a density of 1×10^5 cells per well supplemented with 500 μL of DMEM supplemented with 10% FBS. After incubation overnight, cells were transfected with respective

recombinant plasmids. Thereafter the cells were left to further incubate for another 48 hours.

Cell medium was removed through pipetting and ligands were added to the cells for respective periods of time. Ligands were diluted with PBS to a concentration of $10\ \mu\text{g mL}^{-1}$, and 100 μL were added to each cell well. When the respective time is up, 100 μL of 2 x Laemmli buffer supplemented with 5% beta mercaptoethanol were added to the cells to lyse the cells and prepare the lysate for SDS Page.

The lysates were collected and incubated at 95 °C for 10 minutes before running in an SDS Page gel. Thereafter, the proteins on the gel were transferred onto a nitrocellulose blot through the use of semi-dry transfer method at a current of 120 mA for one hour.

Transferred blots were washed twice with pH 7.4 Tris buffered saline (TBS) before blocking with 3% BSA dissolved in TBS with 0.2% Tween (TBST). Blocking was done for one hour at room temperature under shaking conditions. Thereafter, blocked blots were stained with anti-phosphotyrosine antibody conjugated with horseradish peroxidase (Cell Signaling Technology #5465) or anti-Myc antibody conjugated with horseradish peroxidase (Thermo Scientific #MA1-980-HRP). Antibodies were dissolved in 3% BSA supplemented TBST before applying on the blot overnight at 4 °C.

Thereafter, the stained blots were washed three times with TBST to remove unbounded antibody and developed with chemiluminescence reagent (Cell Signaling Technology #6883) and viewed under Invitrogen gel doc system. Photographed images were analyzed using ImageJ to determine the pixel grayscale density. Relative intensity was obtained by dividing absolute intensity

of the protein band to that of all the proteins in the lane. Normalized relative intensity was obtained by dividing each lane's respective relative intensity value with that of negative control sample where cells were not exposed to any ligand.

CHAPTER 5

Conclusion

Bioassays play an important role in the detection of various biologically relevant analyte. Depending on the chemical and physical properties, different methods and strategies for detection are required. A one-size-fits-all method lacks the ability to distinguish the complexity of the analytes. This thesis describes three distinct attempts to detect ionic, small molecule odorants by exploiting their coordinating chemistry, hydrophobicity and size respectively.

The coordination of Iron (III) ions by tannic acid allows for tannic acid-gold core nanocomposites to be aggregated together. Hydrogen peroxide's oxidation of Iron (II) to Iron (III) is exploited for the detection of hydrogen peroxide since Iron (II) does not result in aggregation of tannic acid-gold core nanocomposites but Iron (III) does. As odorants and polycyclic aromatic hydrocarbons enter the binding pocket of the pig odorant binding protein, we tried to modify the protein to increase binding affinity to these substances. However, our results show otherwise. Diseases biomarkers are too large and hydrophilic to enter the binding pocket of pOBP or coordinate with tannic acid. We developed a chimeric antigen receptor based cell-based sensor to detect the protein biomarkers.

Improvements to the performance of the bioassays are required for subsequent translations. Performance of the bioassays may be summed up with three 'S' – selectivity, sensitivity, and stability [415-417].

Selectivity pertains to the sensor's ability to detect only the target ligand of interest (absence of promiscuity) while the presence of other contaminants

does not generate any noise signal. This thus ensures a desirable high signal to noise ratio [298, 418-420].

The sensitivity of a biosensor refers to the ratio of the signal output to ligand concentration [416]. As the formula is a ratio, sensitivity is usually reported as a linear fit [416]. Although not related to sensitivity, the minimum detection limit (as low as possible), and saturation limit (as high as possible) of the ligand by the sensor is also crucial in determining the sensor's performance.

The stability of the biosensor deals with the reusability of the sensor. For example, the long-term stability of the reusable biosensor, calculated as the number of cycles to failures, is determined by factors such as the degradation of the proteins in the sensor, and leaching of transducer surface [415, 416]. Continuous operation stability is another consideration [421]. Frequent calibrations of equipment between each reading will promote reproducibility while a drifting baseline may be an indication of malfunctioning equipment [422]. Batch to batch data reproducibility in the biosensor's performance is equally crucial.

Specifically to bringing Au@PTA from the bench to commercialization requires additional challenges besides the three 'S'. For example, a suitable packing method should be designed for transportation and storage of the Au@PTA, such that its environment such as temperature can remain constant. This needs to be overcome to render the Au@PTA as a bioassay commercially viable.

For each bioassay in the thesis, we plan further work to either enhance the performances of the bioassays or to better understand the functions of the proteins.

From chapter two, we envision the synthesis of gold-silver hybrid core metal@PTA nanoparticle. This is so as to achieve a high sensitivity colour shift prescribed by Chen et al. [423]. Other forms of protein receptors, such as antibodies will also be conjugated onto the nanoparticle to confer other targets to our bioassay [424].

From chapter three, we plan to further the deletion of the alpha helix tail to not just pOBP but to other lipocalins, especially the bovine OBP. The bovine OBP, unlike other lipocalins, has its tail engage in domain swapping with other monomers for stability [214] By mutating other lipocalins, it will provide a better insight to the role of the alpha helix in the stability of the protein.

In the purpose of developing bioassays, we will continue to mutate the amino acids in the beta barrel of the different OBPs to confer better sensitivity and selectivity to the OBPs to different PAHs and odorants. These mutated OBPs will be examined for biosensor activity through incorporation onto platforms such as the gFET.

Nonetheless, the challenge of unspecific binding of the odorants to the transducers remains to be overcome.

From chapter four, we aim next to express the CARs on the surface of the B cell and engineer the B cell to secrete therapeutic antibodies upon the activation of the CARs. We thus will formulate a therapeutic *in vivo* bioassay.

As opposed to adherent cells, traditional transfection methods such as using lipofectamine to drive the gene of interest into the suspension cells are not possible. Instead, viral transduction strategy such as using lentivirus for transduction is required. The first step in our work will be to optimize lentivirus transduction into the suspension B cells. An EGFP gene instead of the CAR

gene will be used. Thereafter, we will deliver the CAR gene into the B cell allowing the cell to express the CAR protein.

Antibody staining of the transduced B cells will be done to examine if CAR is successfully expressed on the surface of the B cells. Thereafter, calcium influx assay will be conducted to examine if the CARs are functional by exposing the transduced B cells to the CAR binding ligands.

In summary, by improving the strategy from the work in each chapter, we gradually bring the standard of biological analytical detection closer to that of what the biological body is able to achieve. In addition to improving the three 'S' – selectivity, sensitivity, and stability, robustness of detection will become more paramount. The ability to detect a single molecule is the ultimate goal for future biological analytics and will require the development of novel approaches that straddles between molecular and device engineering.

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Appendix I

Supplementary information of Chapter Three

Amino Acid Sequence for pOBPM2

M QEPQPEQDPF ELSGKW**ITSY IGS**SDLEKIG ENAPFQ**VFMR**
SIEFDDKES**K** **VYLNFFSKEN** **GICEEFSLIG** **TKQEGN****TYDV**
NYAG**NNK****WV** **SYA**SET**ALII** **SNINV**DEEGD **K****TIMTGLL****GK**
GTDI**EDQDLE** **KFKEVTRENG** IPEENIVNII ERDDCPA

Bold and underlined Tryptophan is Phenylalanine in wild type pOBP.

Green highlights are beta barrel antiparallel strands.

Yellow highlight is the alpha helix in the pOBPM2 tail.

Amino Acid Sequence for pOBPM2Δ121

M QEPQPEQDPF ELSGKW**ITSY IGS**SDLEKIG ENAPFQ**VFMR**
SIEFDDKES**K** **VYLNFFSKEN** **GICEEFSLIG** **TKQEGN****TYDV**
NYAG**NNK****WV** **SYA**SET**ALII** **SNINV**DEEGD **K****TIMTGLL****G**

Green highlights are beta barrel antiparallel strands.

Amino Acid Sequence for pOBPMM2

M QRASPEQDPF ELSGKF**ITSY IGS**SDLEKIG ENAPFQ**VFMR**
SIEFDDKES**K** **VYLNFFSKEN** **GICEEFSLIG** **TKQEGN****TYDA**
NYAG**NNK****WVA** **SYA**SET**ALII** **SNIKV**DEEGD **K****TIMTGLL****GK**
GTDI**EDQDLE** **KFKEVTRENG** IPEENIVNII ERDDCPA

Green highlights are beta barrel antiparallel strands.

Yellow highlight is the alpha helix in the pOBPM2 tail.

Amino Acid Sequence for pOBPMM2Δ121

M QEPQPEQDPF ELSGKF **ITSY IGS** SDLEKIG ENAPFQ **VFMR**
SIEFD DKES **K** **VYLNFFSKEN** **G** **ICEEFSLIG** **T** KQEGN **TYDA**
NYAG **NNKWVA** **SYA** **SETALII** **SNIKV** DEEGD **K** **TIMTGLLG**

Green highlights are beta barrel antiparallel strands.

Formula derivation of model:

$$[P.F] \rightleftharpoons P + F$$

$$K_F = \frac{[P][F]}{[P.F]} \quad [P]_{Total} = [P.F] + [P]$$

$$K_F = \frac{\{[P]_{Total} - [P.F]\}[F]}{[P.F]}$$

$$K_F[P.F] = [F][P]_{Total} - [F][P.F]$$

$$K_F[P.F] + [F][P.F] = [F][P]_{Total}$$

$$[P.F]\{K_F + [F]\} = [F][P]_{Total}$$

$$[P.F] = [P]_{Total} \frac{[F]}{K_F + [F]}$$

Formula derivation of model:

$$[P.F] + O \rightleftharpoons P + F + O \rightleftharpoons [P.O] + F$$

$$K_F = \frac{[P][F]}{[P.F]} \quad K_O = \frac{[P][O]}{[P.O]} \quad [P]_{Total} = [P.F] + [P.O] + [P]$$

$$\frac{K_F}{K_O} = \frac{\frac{[P][F]}{[P.F]}}{\frac{[P][O]}{[P.O]}}$$

$$K_F[P.F][O] = K_O[P.O][F]$$

$$K_F[P.F][O] = K_O\{[P]_{Total} - [P] - [P.F]\}[F]$$

$$K_F[P.F][O] = K_O[F][P]_{Total} - K_O[F][P] - K_O[F][P.F]$$

$$\frac{[O]}{[K_O]}[P.F] = \frac{[F]}{[K_F]}[P]_{Total} - \frac{[F]}{[K_F]}[P] - \frac{[F]}{[K_F]}[P.F]$$

$$\begin{aligned}
\frac{[O]}{[KO]} &= \frac{[F]}{[KF]} [P]_{Total} - [P.F] - \frac{[F]}{[KF]} [P.F] \\
\frac{[O]}{[KO]} [P.F] &= \frac{[F]}{[KF]} [P]_{Total} - \frac{[F]}{[KF]} [P] - \frac{[F]}{[KF]} [P.F] \\
\frac{[O]}{[KO]} [P.F] + [P.F] + \frac{[F]}{[KF]} [P.F] &= \frac{[F]}{[KF]} [P]_{Total} \\
\left\{ \frac{[O]}{[KO]} + 1 + \frac{[F]}{[KF]} \right\} [P.F] &= \frac{[F]}{[KF]} [P]_{Total} \\
\left\{ \frac{[KF][O]}{[KO]} + [KF] + [F] \right\} [P.F] &= [F][P]_{Total} \\
[P.F] &= \frac{[F][P]_{Total}}{\left\{ \frac{[KF][O]}{[KO]} + [KF] + [F] \right\}}
\end{aligned}$$

$$\begin{aligned}
[P.F] &= \frac{[F][P]_{Total}}{[KF] \left\{ \frac{[O]}{[KO]} + 1 \right\} + [F]} \\
[P.F] &= \frac{[F][P]_{Total}}{[KF] \left\{ \frac{[O]}{[KO]} + 1 \right\} + [F]} \\
[P.F] &= [P]_{Total} \frac{[F]}{[KF] + \frac{[KF][O]}{[KO]} + [F]} \\
[P.F] &= [P]_{Total} \frac{[F]}{\{[KF] + [F]\} \left\{ 1 + \frac{[KF]}{[KO]} \frac{[O]}{[KF] + [F]} \right\}} \\
[P.F] &= [P]_{Total} \frac{\frac{[F]}{[KF] + [F]}}{\left\{ 1 + \frac{[KF]}{[KO]([KF] + [F])} [O] \right\}}
\end{aligned}$$

Fitting into Reciprocal curve:

$$y = \frac{A}{1 + Bx}$$

$$B = \frac{[KF]}{[KO]([KF] + [F])}$$

$$[KO] = \frac{[KF]}{B([KF] + [F])}$$

Formula derivation for Gibbs free energy formula:

For spontaneous process, the Gibbs free energy ought to be negative.

This is arrived from the equation:

$$\Delta S^{\circ}_{Surrounding} + \Delta S^{\circ}_{System} > 0 \quad (1)$$

In a spontaneous process, the entropy of the surrounding and its system increases. We can manipulate the equation to become:

$$dS_{System} + \frac{-dU - p_{External}dV}{T_{Surrounding}} > 0 \quad (2)$$

Here, U is the internal energy of the system, $p_{External}$, pressure of the surrounding, V volume of the system, $T_{Surrounding}$, temperature of the surrounding, and S_{system} , entropy of the system.

This ultimately gets rearranged to become:

$$dU + pdV - TdS < 0 \quad (3)$$

Now, in an environment of constant pressure and constant temperature, as seen in chemical reactions, equation (3) gets rearranged to be:

$$d(U + pV - TS) < 0 \quad (4)$$

In fact, Gibbs free energy is then the terms in the brackets:

$$U + pV - TS = G \quad (5)$$

Since $U + pV = H$, Gibbs free energy will be defined as:

$$H - TS = G \quad (6)$$

Appendix II

Supplementary information of Chapter Four

Amino Acid Sequence for EGFP

MVSKGEELFT	GVVPILVELD	GDVNGHKFSV	SGEGEGDATY
GKLTCLKFICT	TGKLVPWPWT	LVTTLTYGVQ	CFSRYPDHMK
QHDFFKSAMP	EGYVQERTIF	FKDDGNYKTR	AEVKFEGDTL
VNRIELKGID	FKEDGNILGH	KLEYNYNSHN	VYIMADKQKN
GIKVNFKIRH	NIEDGSVQLA	DHYQQNTPIG	DGPVLLPDNH
YLSTQSALSK	DPNEKRDHNV	LLEFVTAAGI	TLGMDELYK

Amino Acid Sequence for mCherry

MVSKGEEDNM	AIKEFMRFK	VHMEGSVNGH	EFEIEGEGEG
RPYEGTQTAK	LKVTKGGLP	FAWDILSPQF	MYGSKAYVKH
PADIPDYLKL	SFPEGFKWER	VMNFEDGGVV	TVTQDSSLQD
GEFIYKVKLR	GTNFPDGPV	MQKKTMGWEA	SSERMYPEDG
ALKGEIKQRL	KLKDGGHYDA	EVKTTYKAKK	PVQLPGAYNV
NIKLDITSHN	EDYTIVEQYE	RAEGRHSTGG	MDELYK

Amino Acid Sequence for B10 Design One

METDTLLLWV	LLLWVPGSTG	DHHHHHMAQ	LQLQESGPGL
VKPSQTLSTL	CTVSGGSISS	GDYYWSWIRQ	PPGKGLEWIG
YIYYSGSTNY	NPPLKSRVTM	SVDTSKNQFS	LKLSSVTAAD
TAVYYCARVK	IVVGAFDIWG	QGTMTVTVSSS	ASGAELGGGG
SGGGGSGGGG	STSEIVMTQS	PATLSLSPGE	RATLSCRASQ
SVSSYLAWYQ	QKPGQAPRL	IYDASNRATG	IPARFSGSGS
GTDFTLTISR	LEPEDFAVYY	CQYGSSPIT	FGQGTRLEIK
RSENEGYYFC	SVISNSVMYF	SSVVPVLQKV	NSTTTKPVLR

TPSPVNLWAT ASTFIVLFL SLFVVTVTL FRKRWQNEKF
 GVDMPDDYED ENLYEGLNLD DCSMYEDISR GLQGTQYQDVG
 NLHIGDAQLE KP

Amino Acid Sequence for B10 Design Two

METDTLLLWV LLLWVPGSTG DQLQLQESGP GLVKPSQTLS
 LTCTVSGGSI SSGDYYWSWI RQPPGKGLEW IGYIYYSGST
 NYNPPLKSRV TMSVDTSKNQ FSLKLSSVTA ADTAVYYCAR
 VKIVVGAFDI WGQGTMTVTS SSASGAELGG GSGGGGSGG
 GGS TSEIVMT QSPATLSLSP GERATLSCRA SQSVSSYLAW
 YQQKPGQAPR LLIYDASNRA TGIPARFSGS GSGTDFTLTI
 SRLEPEDFAV YYCQQYGSSP ITFGQGTRLE IKRSS EQKLI
 SEEDLENEGY YFCSVISNSV MYFSSVVPVL QKVNSTTTKP
 VLRTSPSPVNL WATASTFIVL FLLSLFVVTT VTLEFRKRWQN
 EKFGVDMPDD YEDENLYEGL NLDDCSMYED ISRGLQGTQYQ
 DVGNLHIGDA QLEKP

Amino Acid Sequence for L21C

METDTLLLWV LLLWVPGSTG DEVKLQQSGA ELVRPEASVK
 LSCKTSGYIF TNYWIHWVKQ RSGQGLEWIA RIYPGNGSTY
 YNEKFKGKAT LTADKSSSTA YMQLSSLKSE DSAVYFCARS
 TSDSSLPYWY FDVWGQGTTV TVSSGGGGSG GGGSGGGGSD
 IELTQSPTIL STSPGEKVTV TCRATLGVSY MHWYQQKPGS
 SPKPWIYATS NLASGVPARF SGSGSGTSYS LTISRVEAED
 AATYYCQQWI SNPPTFGGGT KLEIKRAEQK LISEEDLENE
 GYYFCSVISN SVMYFSSVVP VLQKVNSTTT KPVLRTSPSPV
 NLWATASTFI VLFLLSLFVV TTVTLFRKRW QNEKFGVDMP
 DDYEDENLYE GLNLDDCSM YEDISRGLQG TYQDVGNLHI GDAQLEKP

The colour key is as follows:

Signalling peptide

scFv

Linker domain

Hinge region

Transmembrane domain

Intracellular ITAM domain

Epitope tag

Details of our CAR design are as follows:

- 1) Signal peptide: Murine Igk chain signal peptide [407].
- 2) Hinge region: CD8 hinge region, Uniprot accession number: P01731 (AA 122-164) [379, 408].
- 3) Transmembrane domain is from B cell receptor with a YS/VV mutation [395, 405].
- 4) Cytoplasmic domain is a truncated CD79a, Uniprot accession number: P11911 (AA 160-220) [403].
- 5) The scFv sequence is a B10 antibody sequence [425, 426].

“What I cannot
create, I do not
understand”

– Richard Feynman