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CONTRACTING ORGANIZATION: Queensland University of Technology, QLD Australia

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14. ABSTRACT

This project builds on a novel sensor platform which takes the enzyme from glucometers (PQQ-GDH), and uses synthetic biology to re-engineer it into a biosensor that requires a biomarker of choice for function. This provides sensitive and specific detection of biomarkers suitable for continuous electrochemical readout. Although pandemic related delays and a death of one of the Chief Investigators have impacted the project, we have made substantial progress. Work has centered on construction of biosensor electrodes first by entrapping developed biosensors through dialysis membrane entrapment, and subsequently employing orientated immobilization and performance evaluation of the PQQ-GDH functionalized electrodes. Performance of the developed biosensors was tested in biofluids. Most recently, modified sensor architecture using caged peptide and a switchable luminescent/fluorescent readout was developed. Our work has identified the rate of GDH biosensor activation as the critical parameter for its suitability for continuous monitoring.

15. SUBJECT TERMS
Biosensors, Continuous monitoring, Glucose dehydrogenase

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TABLE OF CONTENTS

1. INTRODUCTION:

This project aims to adapt a novel sensor platform based on glucometer technology, in order to make continuously monitoring and wearable biosensors. The work builds on a novel sensor platform which takes the enzyme from glucometers (GDH), and uses synthetic biology to re-engineer it into an artificial allosteric switch. This enables sensitive and specific detection of biomarkers suitable for continuous readout.

2. KEYWORDS:

Protein Biosensors, allosteric regulation, Continuous monitoring, Glucose dehydrogenase, electrochemistry, bioelectrodes

3. ACCOMPLISHMENTS:

What were the major goals of the project?

This report is Annual period 2 of the grant (0-12 months). Relevant approved SOW Aims are:
Major task 1:

- *Subtask 1/2*: Preparation of GDH biosensors and the wild type GDH with biorthogonal reactive groups, Biorthogonal conjugation of GDH biosensors to electrodes (0-6mo)
- *Subtask 3*: Electrochemical testing of functionalized electrodes and LOD determination (6-12mo)
- *Subtask 4*: Analysis of the analyte and glucose fluctuation on the performance of the GDH biosensors (6-12mo)

Major task 2:

- *Subtask 1:* Electrode optimization and testing in simulated biological fluids (6-18mo)
- *Subtask 2:* Construction and testing of Cystatin C biosensors and electrodes (6-18mo)

What was accomplished under these goals?

Note where relevant, content is followed by the location of the work conducted (Site 1 **QUT** or Site 2 **Clarkson**) and if relevant, **MainTask.SubTask** in the approved SOW. For e.g. (**1.1 Clarkson).**

Major activities

Note, this annual report encompasses a 1 year period of which 6 months was the original grant completion time, and 6 months was a no-cost extension period (first 6 months of 1 year). Operational tempo was significantly reduced during the non-funded extension period, and hence reduced over the entire year.

- Further electrochemical investigation using cyclic voltammetry of GDH based biosensors, including development of improved electrodes for GDH-PQQ biosensor conjugation. Additionally, work was undertaken using an alternative fluorometric detection method for the GDH-PQQ chassis (**Clarkson**).

- Development of GDH-PQQ variants with faster activation kinetics via optimization of the core molecular switch. The rate of response was identified as a key issue hampering the development of the real time monitoring systems as the rate of biosensor response needs to be significantly faster than the measured event **(QUT)**

In the first 12 months of the grant, the Covid-19 pandemic delayed construction of Cystatin specific binding modules and thereon based biosensors due to closure of the labs at **QUT** and of the contract research organizations**.** Unfortunately, Cystatin binding domains for integration into the GDH-PQQ sensor chassis have not been able to be developed subsequently, and it is considered unlikely they will be available during the remaining grant extension time. To mitigate this issue, electrochemical work (**Clarkson)** used the available surrogate GDH biosensors of calmodulin binding peptides, Cyclosporine A and methotrexate during this annual reporting period and the previous.

Specific objectives

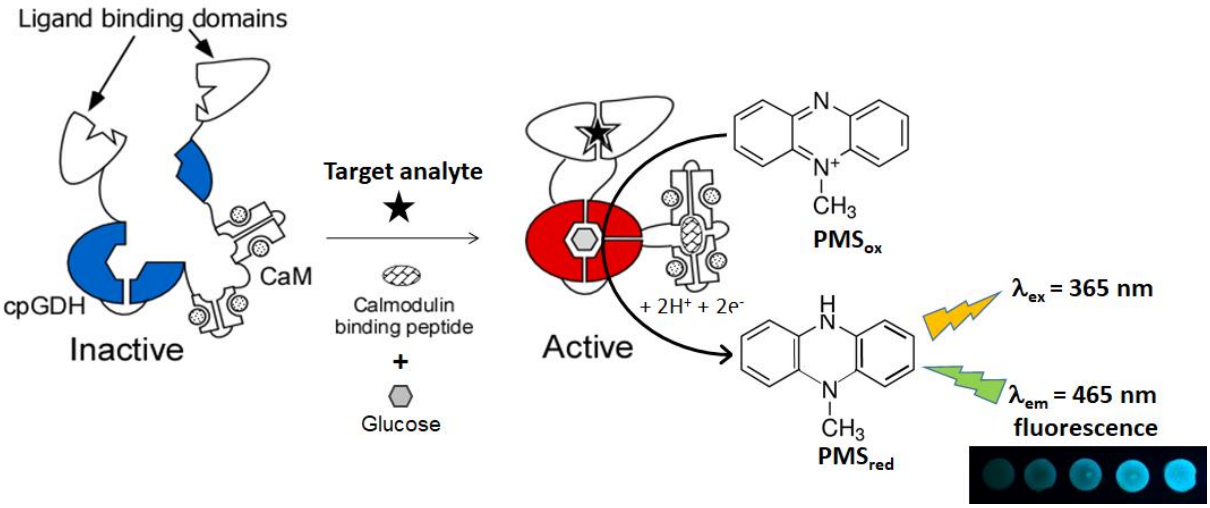
- *Alternate fluorometric readout for GDH-PQQ biosensor chassis*
- *Sensor GDH-PQQ chassis improvement to allow faster ON-OFF switching times via screening of chimera variants.*
- *Development of Cystatin binders (unsuccessful)*
- *Development of improved methods to create suitable electrode structure for sensor interfacing.*

Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative).

Alternate fluorometric readout of activity from GDH-PQQ sensor chassis

This work (**Clarkson 1.3, 2.1)** centered on interfacing the PQQ-GDH sensor to an alternate readout (Fluorometric detection via the electron mediator, phenazine methosulfate as per Figure below).

The specific sensors used were arrays of the macrocyclic immunosuppressant drugs Cyclosporine A and FK-506 This work allowed detection of the analytes in human blood, serum, urine, and saliva **(2.1)**. The work is attached as **Appendix 1** to this report ("Nanostructured Interface Loaded with Chimeric Enzymes for Fluorimetric Quantification of Cyclosporine A and FK506"). The schematic figure following is derived from this report.



The schematic of the GDH-based biosensor operation with a fluorescence output signal. The used abbreviations: cpGDH – circular permutated PQQ-glucose dehydrogenase; CaM – calmodulin; PMS_{ox} and PMS_{red} – phenazine methosulfate reduced and oxidized states, respectively.

Development of fast responding variants of the GDH-PQQ sensor architecture suitable for continuous monitoring.

This work relies on development of GDH-PQQ sensor variants with faster activation kinetics via optimization of the core molecular switch. The rate of response was identified as a key issue hampering the development of the real time monitoring systems as the rate of biosensor response needs to be significantly faster than the measured event. Because development of Cystatin binders for adaption for the GFH-PQQ sensor chassis has not been successful at this late grant stage, this sensor optimization work has been the main grant thrust at **QUT (1.4, 2.2).**

Note: Since the first annual report (0-12months) was revised during Q6 (Feb 2022), a draft version of this work was included in that report as Appendix 3). The final paper was published in Q7 of the grant and is included as **Appendix 2** to this annual report.

To improve sensor response time for the GDH biosensor chassis, we developed an *in vitro* expression-based platform for the analysis of chimeric protein libraries and screened a focused library of chimeras between PQQ-glucose dehydrogenase and calmodulin. Using this approach, we identified 50 chimeras that were activated by calmodulin-binding peptides. We analysed the performance parameters of the active chimeras and demonstrated that their dynamic range and

response times are anticorrelated, pointing to the existence of an inherent thermodynamic trade-off. We show that the structure of the ligand peptide affects both the response and activation kinetics of the biosensors suggesting that the structure of a ligand:receptor complex can influence the chimera's activation pathway. Practically, we demonstrated an improved variant with 50% activation response in 2.5 minutes, from a pool ranging from 2.5 to 30 minutes. Although further work in sensor response is warranted, this work with the related GFH biosensor for Rapamycin should port over to a rapidly responding sensors intended for the challenging continuous monitoring environment. This work was published as Ergun Ayva C, Fiorito MM, Guo Z, Edwardraja S, Kaczmarski JA, Gagoski D, Walden P, Johnston WA, Jackson CJ, Nebl T, Alexandrov K. Exploring Performance Parameters of Artificial Allosteric Protein Switches. J Mol Biol. 2022 Jun 14:167678. doi: 10.1016/j.jmb.2022.167678. Epub ahead of print. PMID: 35709893. (**Appendix 2**).

A summary of the sensor chassis variant screening data is provided in the Figure below:

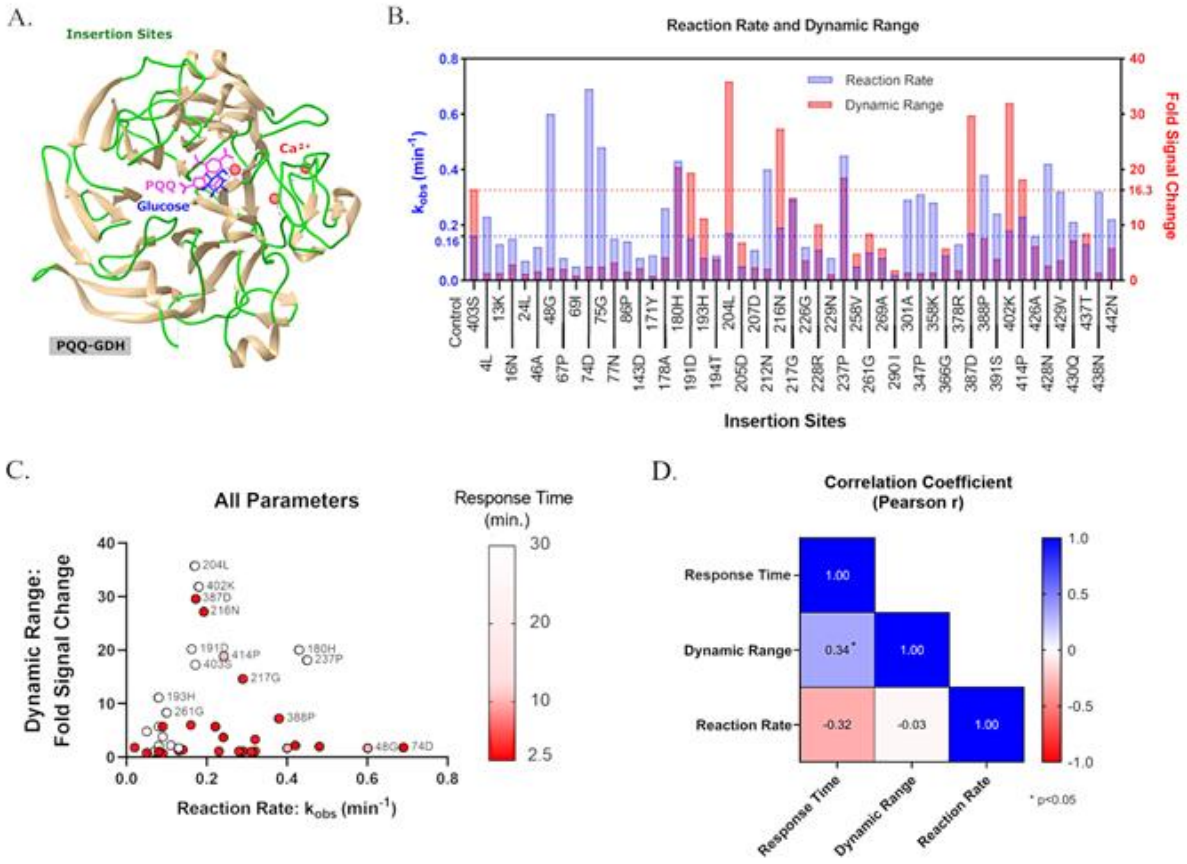
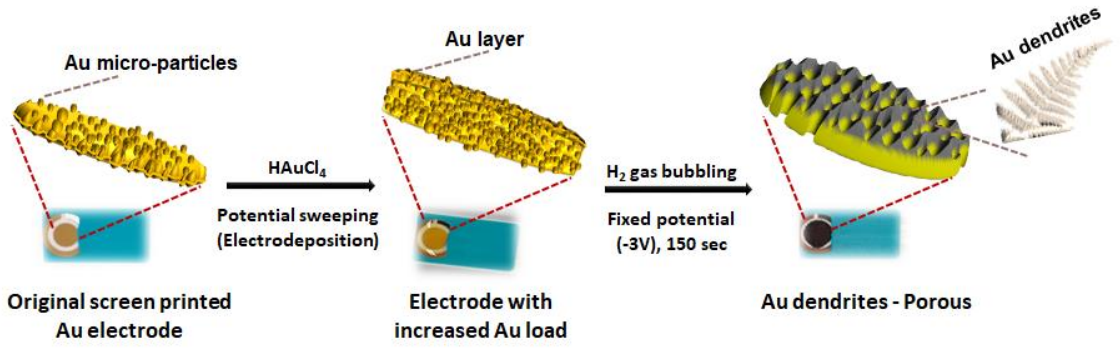


Figure 3. ***Design and analysis of the GDH-CaM chimera library*** **(A)** Ribbon representation of PQQ-GDH structure with 221 CaM insertion sites coloured in green. Glucose (dark blue), cofactor PQQ (pink), and Ca^{2+} ions (red) are displayed in ball and stick representation. **(B)** GDH activity of the CaM-BP dependent mutants are plotted according to their observed reaction rate, k_{obs} (min^{-1}) and dynamic range (fold signal change). Horizontal dashed lines represent the activity of the internal control, GDH-CaM-403S, where the blue line is for reaction rate and the red line is for dynamic range.**(C)** A plot of reaction rate, dynamic range, and the response rate of the

identified chimeras. The gradient red colour's intensity stands for response time, where red indicates the fastest response and white indicates the slowest response. **(D.)** Statistical analysis of the three parameters: response time, dynamic range, reaction rate using Pearson's correlations (sample size, n=41). Correlation matrix with a heat map shows the Pearson r values for each pair ($p<0.05$ labelled with an asterisk, *).
The data set obtained in our screening campaign was large enough (n=41) to analyse the correlation among the performance parameters. We used Pearson's correlations as a statistical method to analyse the relationship among the developed biosensors' dynamic range, response time, and maximal catalytic activity. A slight but statistically significant positive correlation (Pearson r = 0.34 in Figure 3D) between the dynamic range and response time was detected, meaning that chimeras with large dynamic range tend to require more time to activate fully. Several chimeras such as 180H and 237P displayed both large dynamic range and high reaction rate; however, these chimeras had long response times (Figure 3C). Hence, the library analysis suggests that simultaneous optimisation of all three parameters may not be possible, at least in the case of GDH-CaM chimera.

Improvement of electrode structure for interface to GDH-PQQ biosensor chassis

For the GDH-PQQ sensors to produce practical functionalised electrodes, it is necessary to have a suitable electrode matrix capable of high efficiency electron transfer **(1.3)**. Work occurred at **Clarkson** on developing an alternative electrochemically active interface suitable for the GDH-PQQ sensor chassis, based on H2 bubble based created porosity on the electrode surface. This technique was effective and is currently being trialled for the interfacing of the GDH-PQQ chassis with electrochemical readout at **Clarkson**. A report on this work is included as **Appendix 3**. The technique is also summarised in the following Figure:

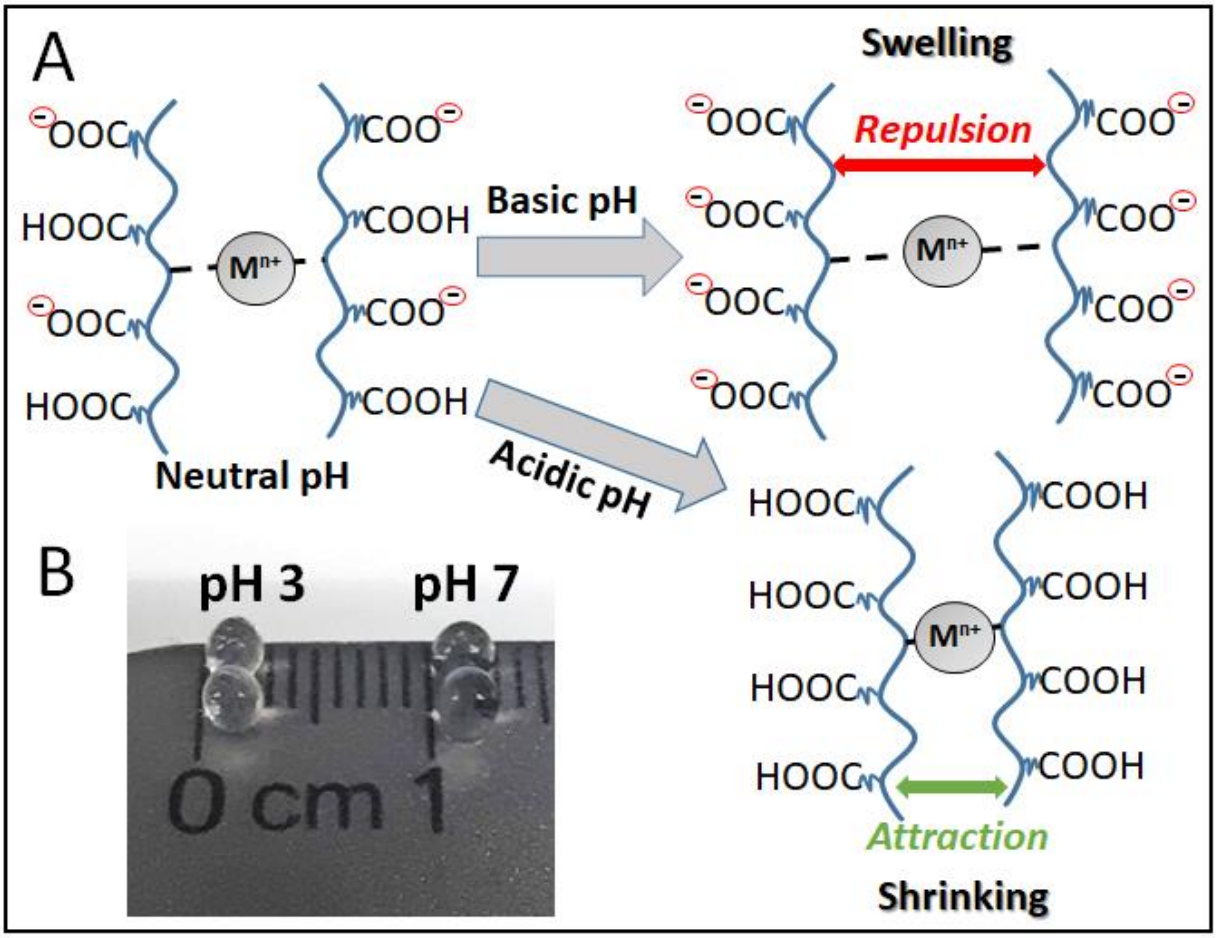


Schematics of the Au screen printed electrode (Au-SPE) modification leading first to the electrochemical deposition of a Au multilayer and then its roughening.

Electrochemical release of Biomolecules from Alginates

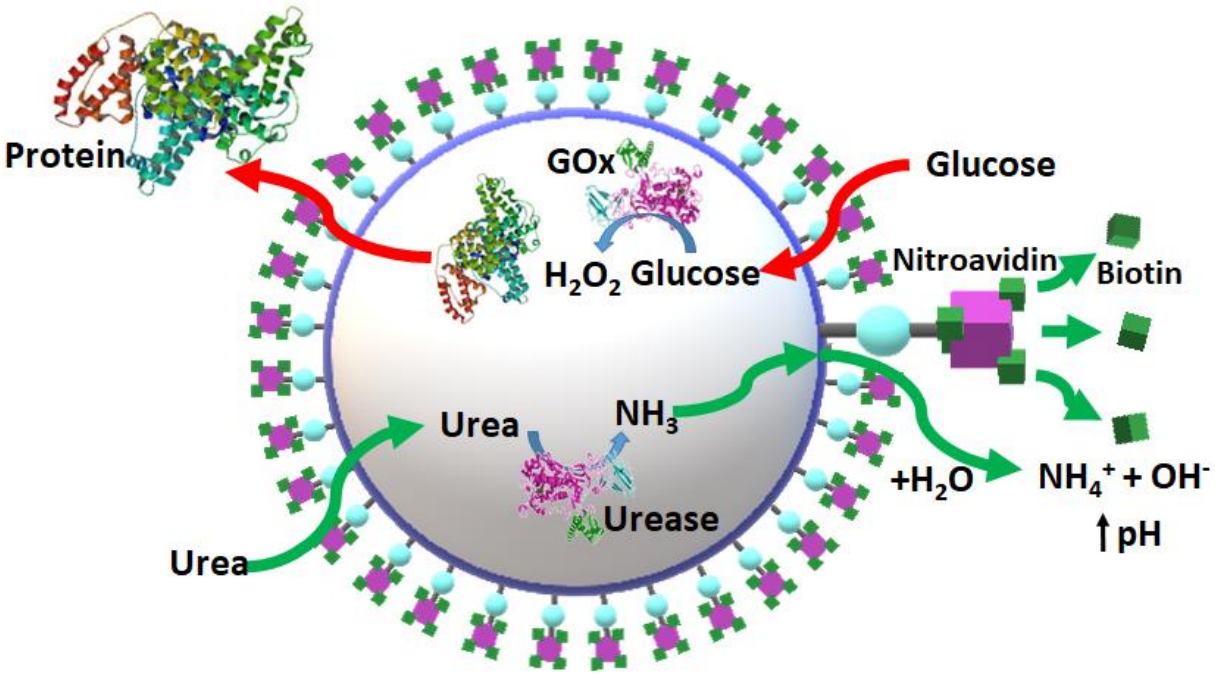
Work occurred on controllable specific biomolecule release from Alginate, both as hydrogel deposited on electrode surfaces and alginate microspheres. This occurs as a result of local pH changes causing protonation of carboxylic groups, or cleavage of affinity bonds between nitroavidin and biotin. The overall work is directed toward different biomolecule species triggered by different input signals. Although this work was adjacent to main grant SOW, it is included as it built on work at **Clarkson** during the first annual period, and will be acknowledged as such in the final publications.

This work is included as **Appendix 4** *Stimulation/Inhibition of Protein Release from Alginate Hydrogel Using Electrochemically Generated Local pH Changes* and **Appendix 5** *Multifunctional Hybrid Nanocomposite Hydrogel Releasing Different Biomolecular Species Triggered with Different Biochemical Signals Processed by Orthogonal Biocatalytic Reactions.* Sample figures illustrating the concepts are included below.



Appendix 4 Figure. (A) Schematically shown swelling-shrinking of the alginate hydrogel upon pH changes between basic and acidic solutions. While in general the cross-linking metal cations (M^{n+})

might be different cations, particularly in the present study they were Ca^{2+} cations. (B) A photo of alginate hydrogel beads after being exposed to pH 3 and pH 7, then showing smaller sizes at pH 3 because of the hydrogel shrinking. Note that the alginate hydrogel used in this study was prepared in the form of films deposited on electrodes (not as the beads shown here for visual convenience only).



Graphical Abstract Appendix 4: Release of proteins from Alginates via electrochemically induced pH change

Delays

The main delay has been in (**2.2, QUT**): *Construction and testing of Cystatin C biosensors and electrodes (6-18mo).* This is partially due to pandemic related delays that have led to receiving the 1 year no cost grant extension as described previously. We however were able to progress the main goal of the project using the surrogate biosensors of methotrexate and Cyclosporine A available to us.

As mentioned above, we have identified the rate of biosensor activation as a critical parameter. We established that the rate of response of the originally developed GDH biosensors (15-20 min.) may be too slow for monitoring rapid physiological processes. This problem is somewhat mitigated by the development of alternate readout chassis as described in the previous annual report. However, to fully solve the problem we needed to develop biosensors with response rate in the range of 2-4 minutes.

What opportunities for training and professional development has the project provided?

2 Undergraduate students have received training in enzyme electrochemistry work at Site 2 **Clarkson**

How were the results disseminated to communities of interest?

Work on faster responding sensor chassis variants at **QUT** was published: Ergun Ayva C, Fiorito MM, Guo Z, Edwardraja S, Kaczmarski JA, Gagoski D, Walden P, Johnston WA, Jackson CJ, Nebl T, Alexandrov K. Exploring Performance Parameters of Artificial Allosteric Protein Switches. J Mol Biol. 2022 Jun 14:167678. doi: 10.1016/j.jmb.2022.167678. Epub ahead of print. PMID: 35709893. **(Appendix 2 to this report)**

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The main work for the remaining grant extension time will be work on the SOW objectives as described above. In particular, work at Site 2 (**Clarkson**) will continue on subtasks: **1.3** *Electrochemical testing of functionalised electrodes*, **2.1** *Electrode optimization and testing using simulated biological fluids*. This will continue as before using existing GDH platform sensors specific for biomarkers rapamycin, MTX or CPA as appropriate, but working on additional sensors as they become available.

Discovery of suitable binders for Cystatin C has not proved successful so far as above. In the likely case that suitable binding elements cannot be found within the course of the grant extension, the main grant focus will remain on the successful rapid response chassis **(QUT)** and integration of existing biosensors into practical monitoring configurations **(Clarkson)** as previously.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The project has contributed to the field of Synthetic Biology by generating new knowledge on switchable and tuneable bioelectrochemical systems.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to report

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Initial issues with hiring and pandemic response at Site 2 (Clarkson) due to the Covid-19 pandemic initially (year 1) slowed work commencement at this site, however significant progress has been made. Delays are also occurring at Site 1 (QUT) due to periodic pandemic lockdowns, and the interrupted global supply chain of reagents as occurred in the first year.

Ongoing issues with staff hiring, travel and consumable sourcing due to the Covid-19 pandemic occurred. *Note: Due to the ongoing Covid-19 pandemic, significant delays have occurred at both grantee institutions (QUT and Clarkson), including institutional lockdowns. On the basis of these delays, we have received the 1 year no-cost extension.*

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Significant changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications. Work on faster responding sensor chassis variants at **QUT** was published: Ergun Ayva
C, Fiorito MM, Guo Z, Edwardraja S, Kaczmarski JA, Gagoski D, Walden P, Johnston WA, Jackson CJ, Nebl T, Alexandrov K. Exploring Performance Parameters of Artificial Allosteric Protein Switches. J Mol Biol. 2022 Jun 14:167678. doi: 10.1016/j.jmb.2022.167678. Epub ahead of print. PMID: 35709893. Federal support acknowledged **(Appendix 2 to this report)**

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Conference attendance was unavailable during the funded portion of the grant due to the Covid19 pandemic.

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Contributions similar over year 1 for Q5-Q6 (end of funded portion prior to no-cost extension)

Name: Dr. Zhong Guo
Project Role: Research officer Site 1 (QUT)
Research identifier:
Nearest person month worked: 6
Contribution to project: Protein Engineering

Name: Dr. Wayne Johnston
Project Role: Research officer Site 1 (QUT)
Research identifier:
Nearest person month worked: 6
Contribution to project: Protein Engineering, Bioelectrochemistry

Name: Dr. Oleh Smutok
Project Role: Research officer Site 2 (Clarkson)
Research identifier:
Nearest person month worked: 9
Contribution to project: Bioelectrochemistry

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

As reported previously, key member at **Clarkson** (Artem Melman) was unfortunately deceased in November 2021. Although this occurred at the end of the previous reporting period, it has had a significant ongoing impact on the grant.

What other organizations were involved as partners?

Collaboration has continued Q1-Q4 as per original agreed granting split:

1) Queensland University of Technology (QUT), Australia (Originating)
2) Clarkson, USA (Collaborating)

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Appendix 1. Nanostructured Interface Loaded with Chimeric Enzymes for Fluorimetric Quantification of Cyclosporine A and FK506

Appendix 2. Ergun Ayva C, Fiorito MM, Guo Z, Edwardraja S, Kaczmarski JA, Gagoski D, Walden P, Johnston WA, Jackson CJ, Nebl T, Alexandrov K. Exploring Performance Parameters of Artificial Allosteric Protein Switches. J Mol Biol. 2022 Jun 14:167678. doi: 10.1016/j.jmb.2022.167678

Appendix 3. Othman, Ali & Bilan, Hubert & Katz, Evgeny & Smutok, Oleh, Highly Porous Gold Electrodes – Preparation and Characterization

Appendix 4 Stimulation/Inhibition of Protein Release from Alginate Hydrogel Using Electrochemically Generated Local pH Changes

Appendix 5 Multifunctional Hybrid Nanocomposite Hydrogel Releasing Different Biomolecular Species Triggered with Different Biochemical Signals Processed by Orthogonal Biocatalytic Reactions.