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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 chosen biomarker for activation. This provides sensitive and specific detection of biomarkers suitable for continuous readout. Although pandemic related delays and a death of one of the chief investigators significantly impacted the project, substantial outputs were completed. Functional biosensor electrodes were developed first by entrapping developed biosensors through dialysis membrane entrapment, and subsequently by employing orientated immobilization and performance evaluation of the PQQ-GDH functionalized electrodes, including in biofluids. A modified sensor architecture using caged peptide and a switchable luminescent/fluorescent readout was developed. Finally, the rate of GDH biosensor activation was identified as the critical parameter for sensor suitability for continuous monitoring, and addressed via systematic optimization of the core molecular switch.

15. SUBJECT TERMS
Biosensors, Continuous monitoring, Glucose dehydrogenase

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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 (glucose dehydrogenase; 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 is the final report for the grant (0-18 months, + 12 months no cost extension). 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).**

Summary of major activities and accomplishments.

Note: For completed grant work that has resulted in a published paper or completed report, a summary and/or abstract is provided in this main document, with the full paper or document provided as an uploaded appendix.

Significant delays and problems with this work occurred due to the Covid-19 pandemic, and the death of main Clarkson grant participant Artem Melman in November 2021. Additionally, a main SOW goal (creation of a Cystatin C biosensor) proved not to be possible within the project, due to the inability to find suitable Cystatin C binding elements using the existing pipeline at **QUT**.

Although this was initially delayed by Covid-19 based closures of the laboratory at **QUT**, the subsequent 12-month extension did not allow this target to be achieved.

Despite these challenges and delays, the project achieved significant success in adapting the GDH-PQQ biosensor platform technology toward continuous monitoring applications. Because the Cystatin C sensor was not available, this work used existing versions of the sensor that sensed methotrexate, Cyclosporin C or calmodulin binding peptide. The work led to four significant published papers, and several other completed reports as detailed in this document and attached as appendices. Although extending the PQQ-GDH biosensor to new sensing ligands such as Cystatin is difficult without suitable binders, a significant outcome of the grant was development of novel techniques for GDH-PQQ biosensor construction that was tested on therapeutic drug methotrexate, and formed the basis of one of the four primary grant publication outputs (*Nat Commun 12, 7137, 2021*). Ongoing work at the **QUT** site aims to streamline the process of binder development using techniques such as mRNA display, building on work done during this project.

Our experiments identified a need for GDH-PQQ variants with faster activation kinetics that would be suitable for continuous monitoring, as the rate of biosensor response needs to be significantly faster than the measured event **(QUT)**. This was achieved via successful optimization of the core protein switch, and published in *J Mol Biol. 2022 Sep 15;434(17):167678*.

Work at **Clarkson** was focused on electrochemical investigation of GDH based biosensors using cyclic voltammetry, either using freely diffusing system entrapped by dialysis membrane or covalently attached to various electrodes. Further electrochemical investigation using cyclic voltammetry of GDH based biosensors, including development of improved electrodes for GDH-PQQ biosensor conjugation was completed. A significant outcome of this work was an alternative approach for fluorometric detection for the GDH-PQQ based biosensors (*ACS Sens. 2021 Oct 22;6(10):3596-3603*). Additionally, orientated enzyme immobilization techniques were tested and demonstrated the utility of the biosensor for quantifying biomarkers in *Angewandte Chemie - International Edition 61 (6)*.

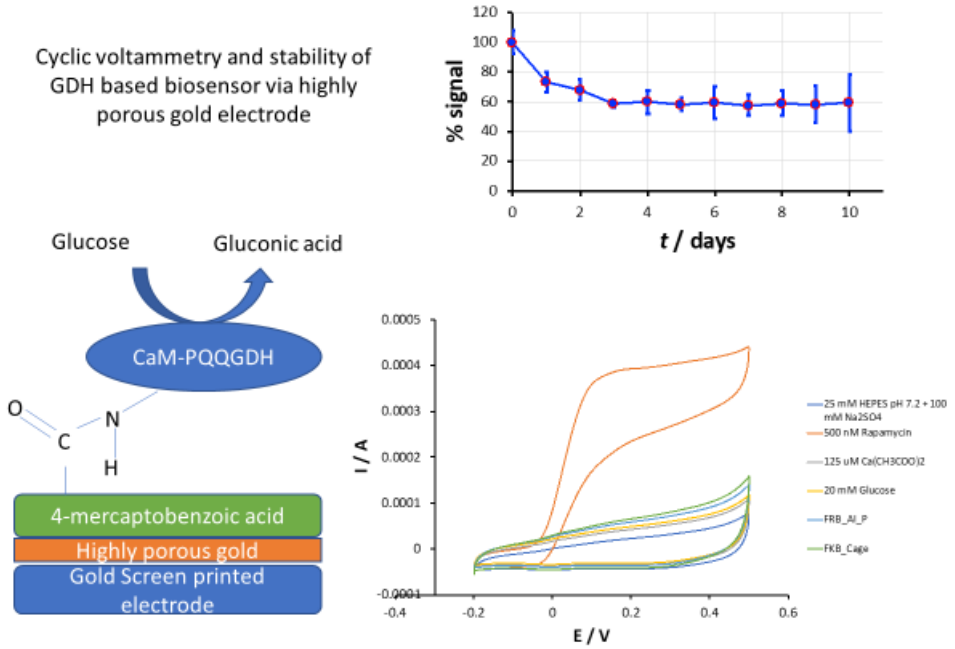
Specific objectives

- *GDH biosensor bioconjugation/testing on porous gold electrodes*
- *GDH biosensor validation/reuse in biofluids via both dialysis membrane entrapment and directed enzyme immobilization*
- *Development of modified sensor architecture using caged peptide and switchable luminescent/fluorescent readout suitable for continuous monitoring (as an alternative to electrochemical readout of biosensor activity).*
- *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 GDH-PQQ biosensors via chemical dimerization.*
- *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).

GDH biosensor bioconjugation/testing on porous gold electrodes

Initial grant work (Year 1) required demonstration of functional bioconjugation of the GDH-PQQ biosensor chassis to electrodes. This bioconjugation work used an existing rapamycin GDH sensor conjugated to highly porous gold electrode. (**Clarkson 1.1, 1.3**)



Prelim data information cyclic voltammetry: Buffer: 25 mM HEPES buffer pH 7.2 + 100 mM Na_2SO_4.
FRB-AI-P: 2500 nM
FKB-Cage: 2500 nM
Rapamycin: 500 nM
Glucose: 20 mM
Blue curve: plain buffer
Grey curve: 125 µM $Ca(CH_3COO)_2$ addition (CV recorded after 20 mins incubation)
Yellow line: 125 µM $Ca(CH_3COO)_2$ (CV recorded after 20 mins incubation adding 20 mM Glucose)
Cyan curve: 125 µM $Ca(CH_3COO)_2$ + 2500 nM FRB-AI-P (CV recorded after 20 mins incubation adding 20 mM Glucose)
Green Curve: 125 µM $Ca(CH_3COO)_2$ + 2500 nM FKB-Cage (CV recorded after 20 mins incubation adding 20 mM Glucose)
Orange curve: 125 µM $Ca(CH_3COO)_2$ + 2500 nM FRB-AI-P + 2500 FKB-Cage + 500 nM Rapamycin (CV recorded after 20 mins incubation adding 20 mM Glucose)
When repeating the switch ON-OFF (experiment in the inset), the electrode was thoroughly rinsed with the working buffer 25 mM HEPES buffer pH 7.2 + 100 mM Na_2SO_4.
The reversibility is not about the proteolytic cleavage but about the activation of the reporter.

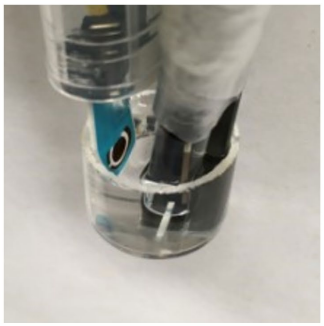
Prelim data stability test: Buffer: 25 mM HEPES buffer pH 7.2 + 125 µM $Ca(CH_3COO)_2$ + 100 mM Na_2SO_4.
FRB-AI-P: 2500 nM
FKB-Cage: 2500 nM

Rapamycin: concentration variated between 1000 nM
Glucose: 20 mM
The experiment is performed incubating the electrode in buffer containing Rapamycin, FKB-Cage and FRB-AI-P for 20 minutes. The CVs were performed adding Glucose after 20 minutes. The catalytic current is derived from the subtraction between the CV recorded in the presence of Rapamycin (glucose added after 20 mins) and the CV in the absence of rapamycin (containing FRB-AI-P, FKB-cage, calcium and glucose added after 20 mins).
Lately, the % of signal is calculated based on the catalytic current obtained in the day 0.
The electrode was rinsed and stored in the working buffer 25 mM HEPES buffer pH 7.2 + 125 µM $Ca(CH_3COO)_2$ + 100 mM Na_2SO_4. The electrode was stored in the refrigerator at +4ºC.

GDH biosensor performance testing in biofluids via both dialysis membrane entrapment and directed enzyme immobilization.

Preliminary work at **Clarkson** demonstrated non-specific immobilization of the GDH-PQQ sensor for methotrexate using a disposable electrode (**Clarkson**, **1.3**). Electrode re-use in biofluid (critical for continuous monitoring), was also demonstrated (**Clarkson**, **2.1**). This work validated the concept of reusable/continuous monitoring allowing further work on direct enzyme immobilization.

Experimental setup (Site 2 Clarkson, MTX GDH sensor, sensitivity and re-use testing)



Subsequent work involved validating orientated enzyme immobilization techniques (as opposed to simple entrapment under dialysis membrane), in order to evaluate sensor activity and function in biofluids (**Clarkson 1.1, 1.2**). The biofluid use is also relevant to (**Clarkson 2.1**).

This work was published as *Guo, Zhong, Smutok, Oleh, Johnston, Wayne A., Ayva, Cagla Ergun, Walden, Patricia, McWhinney, Brett, Ungerer, Jacobus P. J., Melman, Artem, Katz, Evgeny, and Alexandrov, Kirill (2022). Circular permutated PQQ-glucose dehydrogenase as an ultrasensitive*

electrochemical biosensor. Angewandte Chemie - International Edition 61 (6). https://doi.org/10.1002/anie.202109005, attached and listed as **Appendix 1** to this report.

The abstract of this published work is as follows: *Protein biosensors play an increasingly important role as reporters for research and clinical applications. Here we present an approach for the construction of fully integrated but modular electrochemical biosensors based on the principal component of glucose monitors PQQ-glucose dehydrogenase (PQQ-GDH). We designed allosterically regulated circular permutated variants of PQQ-GDH that show large (>10-fold) changes in enzymatic activity following intramolecular scaffolding of the newly generated N- and C termini by ligand binding domain/ligand complexes. The developed biosensors demonstrated sub-nanomolar affinities for small molecules and proteins in colorimetric and electrochemical assays. For instance, the concentration of Cyclosporine A could be measured in 1 mL of undiluted blood with the same accuracy as the leading diagnostic technique that uses 50 times more sample. We further used this biosensor to construct highly porous gold bioelectrodes capable of robustly detecting concentrations of Cyclosporine A as low as 20 pM and retained functionality in samples containing at least 60% human serum.*

Development of modified sensor architecture using caged peptide and switchable luminescent/fluorescent readout suitable for continuous monitoring

A modified sensor architecture using caged peptide and a switchable electrochemical/ luminescent/fluorescent readout was successfully developed. As for the immobilized enzyme work using the GDH biosensor chassis, this work suggests that these techniques suitable for continuous monitoring will port to new GDH-PQQ biosensors.

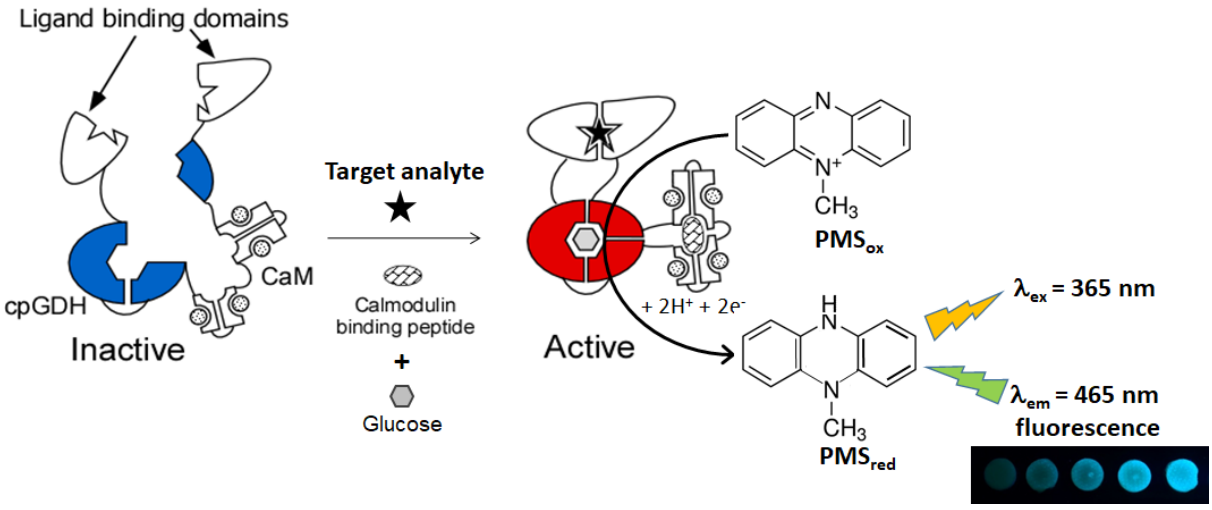
This work was published as *Bollella P, Edwardraja S, Guo Z, Vickers CE, Whitfield J, Walden P, Melman A, Alexandrov K, Katz E. Connecting Artificial Proteolytic and Electrochemical Signaling Systems with Caged Messenger Peptides. ACS Sens. 2021 Oct 22;6(10):3596-3603. doi: 10.1021/acssensors.1c00845. Epub 2021 Oct 12. PMID: 34637274*, included as **Appendix 2** to this report.

The abstract of this work is as follows: *Enzymatic polypeptide proteolysis is a widespread and powerful biological control mechanism. Over the last few years, substantial progress has been made in creating artificial proteolytic systems where an input of choice modulates the protease activity and thereby the activity of its substrates. However, all proteolytic systems developed so far have relied on the direct proteolytic cleavage of their effectors. Here, we propose a new concept where protease biosensors with a tunable input uncage a signaling peptide, which can then transmit a signal to an allosteric protein reporter. We demonstrate that both the cage and the regulatory domain of the reporter can be constructed from the same peptide-binding domain, such as calmodulin. To demonstrate this concept, we constructed a proteolytic rapamycin biosensor and demonstrated its quantitative actuation on fluorescent, luminescent, and electrochemical reporters. Using the latter, we constructed sensitive bioelectrodes that detect the messenger peptide release and quantitatively convert the recognition event into electric current. We discuss the application of such systems for the construction of in vitro sensory arrays and in vivo signaling circuits.*

Alternate fluorometric readout of activity from GDH-PQQ sensor chassis

This completed work (**Clarkson 1.3, 2.1)** interfaced 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 completed work was attached as Appendix 1 to Year 1 annual report ("Nanostructured Interface Loaded with Chimeric Enzymes for Fluorimetric Quantification of Cyclosporine A and FK506"). The abstract and schematic figure following is derived from this report (not included in this final report due to 6xfile limit)

Advances in protein engineering resulted in increased efforts to create protein biosensors that can replace instrumentation-heavy analytical and diagnostic methods. Sensitivity, amenability to multiplexing and manufacturability remain to be among key issues preventing broad utilization of protein biosensors. Here, we attempt to address these by constructing arrays utilizing protein biosensors based on the artificial allosteric variant of PQQ-glucose dehydrogenase (GDH). We demonstrated that silica nanoparticle-immobilized GDH protein could be deposited on fiberglass sheets without loss of activity. Particle-associated GDH activity could be monitored using changes in fluorescence of commonly used electron mediator phenazine methosulfate. Constructed biosensor arrays of macrocyclic immunosuppressant drugs Cyclosporine A and FK-506 displayed very low background and a remarkable dynamic range exceeding 300-fold that resulted in a limit of detection of 2 pM for both analytes. This enabled us to quantify both drugs in human blood, serum, urine, and saliva. The arrays could be stored in dry form and quantitatively imaged using a smartphone camera demonstrating the method's suitability for field and point-of-care applications. The developed approach provides a generalizable platform for biosensor array development that is compatible with inexpensive and potentially scalable manufacturing.



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

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

As stated previously, we consider this work and its subsequent publication as the main achievement at site **QUT** in the absence of a working Cystatin biosensor. Fast and reversible biosensor response is critical for continuous biosensor monitoring suitable for wearable application. 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 GDH-PQQ sensor chassis was not successful, this sensor optimization work was the main grant thrust at **QUT** during the second year of the grant **(1.4, 2.2).**

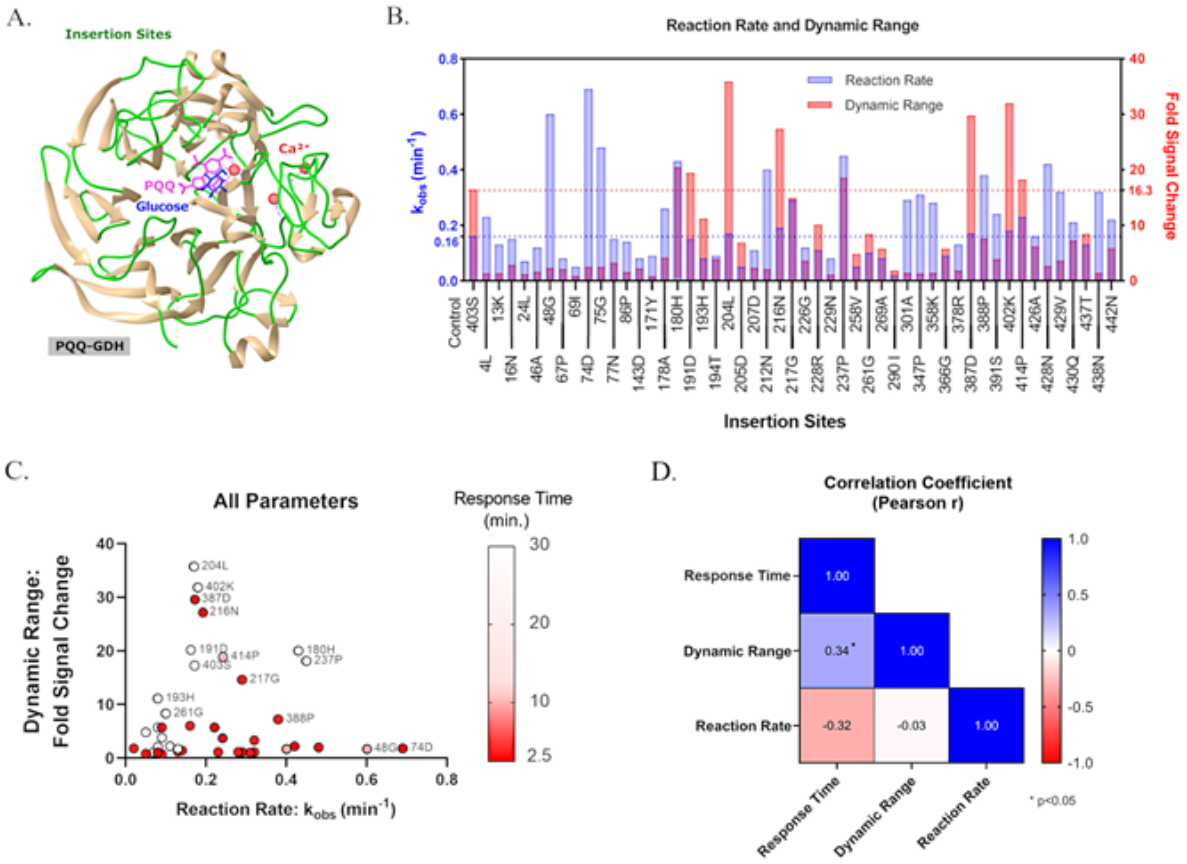
To improve sensor response time for the GDH biosensor chassis, we developed an *in vitro* expression-based platform for the analysis of chimeric protein libraries and screened a focused library of chimeras between PQQ-glucose dehydrogenase and calmodulin. Using this approach, we identified 50 chimeras 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 GDH-PQQ 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 Sep 15;434(17):167678. doi: 10.1016/j.jmb.2022.167678. Epub 2022 Jun 14. PMID: 35709893.
(**Appendix 3**).

The abstract of this work is as follows: *Biological information processing networks rely on allosteric protein switches that dynamically interconvert biological signals. Construction of their artificial analogues is a central goal of synthetic biology and bioengineering. Receptor domain insertion is one of the leading methods for constructing chimeric protein switches. Here we present an in vitro expression-based platform for the analysis of chimeric protein libraries for which traditional cell survival or cytometric high throughput assays are not applicable. We utilise this platform to screen a focused library of chimeras between PQQ-glucose dehydrogenase and calmodulin. Using this approach, we identified 50 chimeras (approximately 23% of the library) that were activated by calmodulin-binding peptides. We analysed 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. In order to*

understand the extent of structural changes in the reporter protein induced by the receptor domains, we have analysed one of the chimeric molecules by CD spectroscopy and hydrogen–deuterium exchange mass spectrometry. We concluded that subtle ligand-induced changes in the receptor domain propagated into the GDH domain and affected residues important for substrate and cofactor binding. Finally, we used one of the identified chimeras to construct a two-component rapamycin biosensor and demonstrated that core switch optimisation translated into improved biosensor performance.

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



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 Ca2+ 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, kobs (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.

Development of GDH-PQQ biosensors via chemical dimerization.

Although it was not possible to develop a Cystatin C biosensor within the grant duration, work on integration of sensing elements to the GDH-PQQ chassis at both sites **QUT** and **Clarkson** allowed development of a parallel biosensor for the drug methotrexate. This work is documented in a high-impact published work where the optimal strategy for developing chemical dimerization systems was mapped (i.e. to bind sensing elements together via the sensed ligand, thus restoring enzymatic activity of the biosensor). This work demonstrated activity of the sensor in serum biofluid (**Clarkson 2.1).**

The work was published as: Guo Z, Smutok O, Johnston WA, Walden P, Ungerer JPJ, Peat TS, Newman J, Parker J, Nebl T, Hepburn C, Melman A, Suderman RJ, Katz E, Alexandrov K. *Design of a methotrexate-controlled chemical dimerization system and its use in bio-electronic devices. Nat Commun. 2021 Dec 8;12(1):7137. doi: 10.1038/s41467-021-27184-w. PMID: 34880210; PMCID: PMC8654847.* Federal support acknowledged (**Appendix 4**).

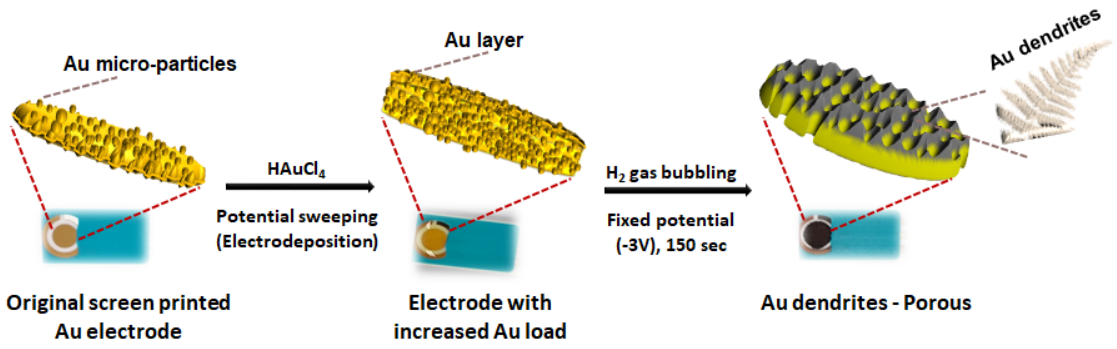
The abstract of this work is as follows: *Natural evolution produced polypeptides that selectively recognize chemical entities and their polymers, ranging from ions to proteins and nucleic acids. Such selective interactions serve as entry points to biological signaling and metabolic pathways. The ability to engineer artificial versions of such entry points is a key goal of synthetic biology, bioengineering and bioelectronics. We set out to map the optimal strategy for developing artificial small molecule: protein complexes that function as chemically induced dimerization (CID) systems. Using several starting points, we evolved CID systems controlled by a therapeutic drug methotrexate. Biophysical and structural analysis of methotrexate-controlled CID system reveals the critical role played by drug-induced conformational change in ligand-controlled protein complex assembly. We demonstrate utility of the developed CID by constructing electrochemical biosensors of methotrexate that enable quantification of methotrexate in human serum. Furthermore, using the methotrexate and functionally related biosensor of rapamycin we developed a multiplexed bioelectronic system that can perform repeated measurements of multiple analytes. The presented results open the door for construction of genetically encoded signaling systems for use in bioelectronics and diagnostics, as well as metabolic and signaling network engineering.*

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

For the GDH-PQQ sensors to be incorporated into 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 H_2 bubble based created porosity on the electrode surface. This technique was effective and is being further trialed for the interfacing of the GDH-PQQ chassis with electrochemical readout at **Clarkson**. A report on this work is included as Appendix 3 in the Year 1 report (not attached to this final report due to 6xfile limit).

The abstract of this completed work is as follows: *Gold screen printed electrodes (Au-SPEs) were treated electrochemically to produce a micro-rough pattern increasing the real electrode surface. The procedure based on the Dynamic Hydrogen Bubble Template (DHBT) method included electrochemical deposition of Au layers onto the surface of the Au-SPEs, followed by a reductive process at -3 V (vs. Ag/AgCl) leading to formation of H2 bubbles, which produced pores in the Au multilayer. The morphology of the micro-porous Au electrode was characterized by scanning electron microscopy (SEM), surface mapping, surface profilometry, and confocal microscopy. The electrode surface morphology was controlled by the time of the electrode reductive treatment (H2 evolution) and the optimized condition resulting in the best surface structuring was found. Notably, the surface roughness leading to the highest electrode surface area was significantly increased compared to previously reported results with Au-SPEs.*

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 was concluded 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 in this final report as it built on work at **Clarkson** during the first annual period.

These works were included as Appendix 4 in the Year 1 report (not attached to this report due to 6xfile limit): *Stimulation/Inhibition of Protein Release from Alginate Hydrogel Using Electrochemically Generated Local pH Changes* and *Multifunctional Hybrid Nanocomposite Hydrogel Releasing Different Biomolecular Species Triggered with Different Biochemical Signals Processed by Orthogonal Biocatalytic Reactions.*

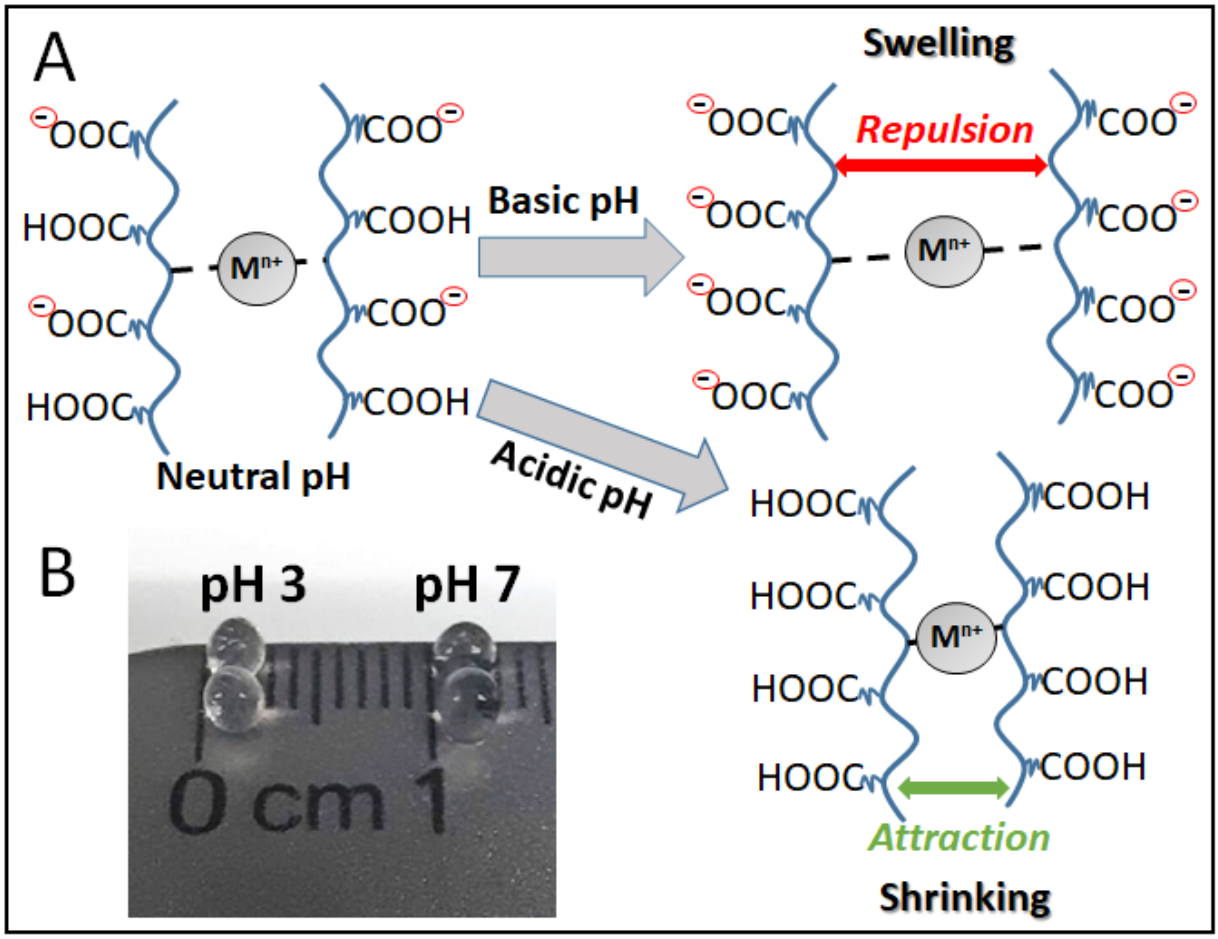
Abstracts of these completed works are as follows:

Electrochemically controlled release of proteins was studied from Ca2+-cross-linked alginate hydrogel deposited on an electrode surface. Electrochemical oxidation of ascorbate or reduction of O2 was achieved upon applying electrical potentials +0.6 V or -0.8 V (vs. Ag/AgCl/KCl 3M), respectively, then resulting in decreasing or increasing pH locally near an electrode surface. The obtained local acidic solution resulted in protonation of carboxylic groups in the alginate hydrogel and as the result formation of a hydrophobic shrunk hydrogel film. Conversely, the produced alkaline local environment resulted in a hydrophilic swollen hydrogel film. The release of the proteins was effectively inhibited from the shrunk hydrogel and activated from the swollen hydrogel film. Overall, the electrochemically produced local pH changes allowed control over the biomolecule release process. While the release inhibition by applying +0.6 V was always effective and can be maintained as long as the positive potential applied, the release activation was different depending on the protein molecular size, being more effective for smaller species, and molecule charge, being more effective for negatively charged species. The repetitive change from the inhibited to stimulated state of the biomolecule release process was obtained upon cyclic application of oxidative and reductive potentials (+0.6 V □ -0.8 V). The alginate hydrogel film shrinking/swelling as well as the protein release process were studied and visualized with a confocal fluorescent microscope. In order to be observed, an external surface of the alginate film and the loaded protein molecules were labeled with different fluorescent dyes, then producing colored fluorescent images using a confocal microscope.

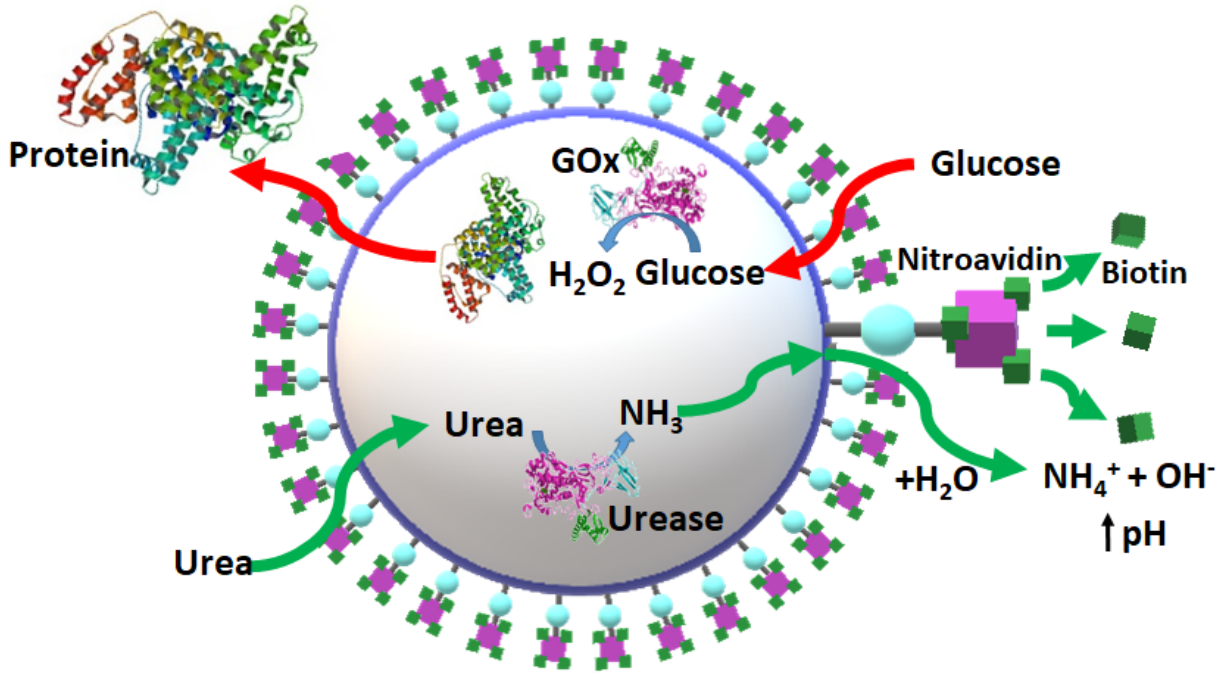
A micro/nano-shaped system composed of alginate microspheres (microgels) decorated with silica oxide nanoparticles functionalized with nitroavidin was used for on-demand biomolecule release stimulated by different input signals. Enzymes preloaded in the microgels processed the applied signals producing either basic pH locally near the microspheres or generating H2O2 inside the hydrogel, or both simultaneously. The pH increase resulted in cleavage of the affinity bonds between nitroavidin and biotin, then releasing the later. The H2O2 produced resulted in oxidative cleavage of cross-linking bonds in the alginate matrix, then opening pores and releasing a loaded model protein (bovine serum albumin). Overall, the orthogonal biochemical processes allowed the dual release of different biomolecule species triggered by different input-signals. The developed

material could result in a universal multidrug delivery platform given the wide range of enzymes that induce a pH change or produce H2O2 in their catalytic reactions

Sample figures illustrating the concepts of these works are included below.



(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 (Mn+) might be different cations, particularly in the present study they were Ca2+ 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: Release of proteins from Alginates via electrochemically induced pH change

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

The need to improve sensor response time allowed significant work by a PhD student at **QUT,** Cagla Ergun. Under supervision by Zhong Guo and Kirill Alexandrov, this opportunity led to one of the primary research outputs/publications of the grant.

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

How were the results disseminated to communities of interest?

The article "Design of a methotrexate-controlled chemical dimerization system and its use in bio-electronic devices. Guo Z., Smutok O., Johnston WA, Walden P., Ungerer J., Peat TS, Newman J, Parker J, Nebl N., Hepburn C., Melman A., Suderman R, Katz E. and Alexandrov K. Nature Communications 2021, 12 (1), 1-13" (**Appendix 4**) that resulted from this (work was subject of a press releases by QUT, CSIRO, and Clarkson University, as well as the US-based "Diagnostic World News".

What do you plan to do during the next reporting period to accomplish the goals?
Grant is completed and no further work is occurring

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 sensors. Specifically, to be 'wearable' such sensors must be fast acting in order to capture data from biosensors intended for continuous monitoring. This work showed that modification of an existing biosensor type derived commercial glucometers could be made faster and more suitable for this application.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

Changes in approach and reasons for change

Actual or anticipated problems or delays and actions or plans to resolve them
The onset of the Covid-19 pandemic led to significant delays due to hiring delays at **Clarkson,** along with laboratory shutdowns and disrupted supply chain of scientific products to **QUT**. A significant delay occurred 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.

Changes that had a significant impact on expenditures

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.

Guo, Zhong, Smutok, Oleh, Johnston, Wayne A., Ayva, Cagla Ergun, Walden, Patricia, McWhinney, Brett, Ungerer, Jacobus P. J., Melman, Artem, Katz, Evgeny, and Alexandrov, Kirill (2022). *Circular permutated PQQ-glucose dehydrogenase as an ultrasensitive electrochemical biosensor. Angewandte Chemie - International Edition 61 (6).* Federal support acknowledged. *https://doi.org/10.1002/anie.202109005*. This is from work primarily at Site 1 **QUT** but in collaboration with Site 2 **Clarkson** project participants (**Appendix 1**)

Bollella P, Edwardraja S, Guo Z, Vickers CE, Whitfield J, Walden P, Melman A, Alexandrov K, Katz E. Connecting Artificial Proteolytic and Electrochemical Signaling Systems with Caged Messenger Peptides. *ACS Sens. 2021 Oct 22;6(10):3596-3603. doi: 10.1021/acssensors.1c00845. Epub 2021 Oct 12. PMID: 34637274.* Federal support acknowledged (**Appendix 2**).

Guo Z, Smutok O, Johnston WA, Walden P, Ungerer JPJ, Peat TS, Newman J, Parker J, Nebl T, Hepburn C, Melman A, Suderman RJ, Katz E, Alexandrov K. *Design of a methotrexate-controlled chemical dimerization system and its use in bio-electronic devices. Nat Commun. 2021 Dec 8;12(1):7137. doi: 10.1038/s41467-021-27184-w. PMID: 34880210; PMCID: PMC8654847.* Federal support acknowledged (**Appendix 4**).

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 3)**

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**

During the reporting period we conducted pilot manufacturing of methotrexate test strips at the facilities of Australian company Universal Biosensors. We are rectifying some of the design issues identified in the process.

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Yearly contributions similar over grant (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, leading to a significant ongoing impact on the grant.

What other organizations were involved as partners?

Collaboration occured 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. *Guo, Zhong, Smutok, Oleh, Johnston, Wayne A., Ayva, Cagla Ergun, Walden, Patricia, McWhinney, Brett, Ungerer, Jacobus P. J., Melman, Artem, Katz, Evgeny, and Alexandrov, Kirill (2022). Circular permutated PQQ-glucose dehydrogenase as an ultrasensitive electrochemical biosensor. Angewandte Chemie - International Edition 61.*

Appendix 2. *Bollella P, Edwardraja S, Guo Z, Vickers CE, Whitfield J, Walden P, Melman A, Alexandrov K, Katz E. Connecting Artificial Proteolytic and Electrochemical Signaling Systems with Caged Messenger Peptides. ACS Sens. 2021 Oct 22;6(10):3596-3603. doi: 10.1021/acssensors.1c00845. Epub 2021 Oct 12. PMID: 34637274.*

Appendix 3. *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 4. *Guo, Z., Smutok, O., Johnston, W.A. et al. Design of a methotrexate-controlled chemical dimerization system and its use in bio-electronic devices. Nat Commun* ***12,*** *7137 (2021). https://doi.org/10.1038/s41467-021-27184-w.*