

Quantum Dot Mediated Bead-Based Assays for Continuous Biomarker Monitoring in Diabetes
and Ex Vivo Lung Perfusion Systems

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Sanjana Srikant is the sole author of chapters 1, 2, 3, and 4 of this thesis under the supervision of Dr. Mahla Poudineh. This thesis consists in part of manuscripts written for publication. Exceptions to sole authorship of material are as follows:

Research presented in chapter 2 is collaborative between Hesam Abouali (affiliated with Dr. Mahla Poudineh's group, University of Waterloo), Md Fahim Al Fattah (affiliated with Dr. Dayan Ban's group, University of Waterloo), and Dr. Nicole G. Barra (affiliated with Dr. Jonathan D. Schertzer's group, McMaster University)

H.A designed and fabricated all moulds and devices for QIRT-ELISA project and performed device evaluations. H.A also developed an app for QIRT-ELISA data analysis, and performed data analysis for in vitro continuous experiments, and in vivo GTT experiments. F.F developed and validated optofluidic detection components for use in all QIRT-ELISA experiments. S.S designed and validated all assays presented in chapters 2 on the benchtop, and in modular and integrated QIRT-ELISA devices. H.A, S.S and N.B performed all GTT experiments together. This manuscript has been submitted for reviewing.

Research presented in chapter 3 is collaborative between Hesam Abouali (affiliated with Dr. Mahla Poudineh's group, University of Waterloo), Md Fahim Al Fattah (affiliated with Dr. Dayan Ban's group, University of Waterloo), and Dr. Andrew T. Sage (Toronto Lung Transplant Program, University of Toronto)

QIRT-ELISA platform previously reported in chapter 2 developed by H.A and F.F with data analysis app developed by H.A were used to validate assays for glucose and lactate in perfusate. Ex vivo lung perfusate was provided by A.S. S.S completed all assay validation on the plate reader, benchtop and on-chip, and performed data analysis. This manuscript is under preparation.

Abstract

The continuous monitoring of biomolecules such as hormones, polypeptides, and monosaccharides can provide crucial information about biological systems, leading to a better understanding of human physiology and an improved ability to diagnose and treat complex, multisystemic illnesses.

There is a lack of technological advancements that can measure a diverse range of biomarkers with the necessary sensitivity, specificity, and temporal resolution at physiologically relevant concentrations, in a multiplexed manner. Traditionally, enzyme-linked immunosorbent assays (ELISAs) have been used to detect protein biomarkers, while small carbohydrate biomarkers like glucose and lactate levels are measured enzymatically. However, these methods are time-consuming, do not offer real-time or multiplexed detection, and require substantial amounts of reagents, sophisticated detection instrumentation, and trained personnel to perform.

This study focuses on developing assays for use in two diseases with significant clinical impact: diabetes, where the detection of hormones insulin and glucagon is demonstrated, and ex vivo lung perfusion, where the detection of small molecules lactate and glucose is shown. The system utilizes Quantum Dot mediated Bead-Based Assays (BQA) with microfluidic modules to create a real-time ELISA (QIRT-ELISA) platform for continuous and multiplexed detection of diverse biomarkers in heterogeneous biological matrices. This system surpasses traditional ELISAs by significantly improving sensitivity, specificity, and measurement times, while also enabling multiplexing through the use of quantum dots and their unique optical properties to extract multiple discrete emission spectra signals from targets of interest with a single UV excitation laser.

Validation experiments demonstrate that QIRT-ELISA can detect insulin and glucagon in the low picomolar range in whole blood, and lactate and glucose in the low millimolar range in ex vivo lung perfusate. Continuous measurements of insulin and glucagon in vivo in rats undergoing glucose tolerance tests further validate the platform, showing comparable results to conventional ELISA. Thus, the platform presents unique advancements in continuous monitoring technology that can be applied clinically to improve personalized precision medicine and enhance patient outcomes. We believe that the platform can be further developed to encompass a wider range of biomarkers, making it universally applicable across various clinical settings.

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I would like to give special thanks to Dr. Grant W. Brown, at the University of Toronto, for being the first person to take a chance on me, and introduce me to healthcare research, and his continued support throughout my academic journey.

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Dedication

This work is dedicated to W.Z, A.Z, G.S, and S.S, and courageous, selfless people in the healthcare space whose lives may benefit from improvements to continuous monitoring technology. You are my strength and inspiration every day.

Table of Contents

Author's Declaration.....	ii
Statement of Contributions.....	iii
Abstract.....	iv
Acknowledgements.....	v
Dedication.....	vi
List of Figures	ix
List of Tables	x
Chapter 1 Introduction.....	1
1.1 Importance of Continuous Monitoring in Healthcare	1
1.2 Biosensing Mechanisms	1
1.3 Challenges with Current Microfluidic Technologies.....	2
1.4 Role of Microfluidics in Biosensing.....	3
1.5 QIRT-ELISA for Continuous Monitoring in Diabetes.....	4
1.6 QIRT-ELISA for Continuous Monitoring in Ex Vivo Lung Perfusion.....	5
Chapter 2 QIRT- ELISA for Continuous Monitoring in Diabetes.....	7
2.1 Abstract.....	7
2.2 Introduction.....	7
2.3 Results and Discussion.....	10
2.3.1 Bead-Based Quantum Dot Mediated Immunoassay (BQI) Validation.....	10
2.3.2 In Vitro Continuous Multiplexed Measurement of Insulin and Glucagon.....	13
2.3.4 In Vivo Multiplexed Measurement of Insulin and Glucagon.....	17
2.3.5 In Vivo Continuous Multiplexed Measurement of Insulin and Glucagon.....	20
2.4 Conclusions and Future Directions.....	23
2.5 Materials and Methods.....	24
2.6 Supplementary Information.....	27
Chapter 3 QIRT-ELISA for Continuous Monitoring in Ex Vivo Lung Perfusion.....	28
3.1 Abstract.....	28
3.2 Introduction.....	28
3.3 Results and Discussion.....	29

3.3.1 Bead-Based Quantum Dot Mediated Immunoassay (BQI) Validation.....	30
3.4 Conclusions and Future Directions.....	34
3.5 Materials and Methods.....	36
3.6 Supplementary Information.....	38
Chapter 4 Conclusions and Future Directions.....	41
References.....	42

List of Figures

Chapter 2 QIRT-ELISA for Continuous Monitoring in Diabetes

Figure 1. Insulin and Glucagon Dynamics for Glucose Homeostasis with Conventional Monitoring Approaches and QIRT-ELISA for Real-Time Continuous Monitoring.....	9
Figure 2. Validation of Bead-Based Quantum Dot Mediated Immunoassay (BQI) Sensing Mechanism on Micromixer Module.....	12
Figure 3. In Vitro Continuous Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform.....	16
Figure 4. In Vivo Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform.....	19
Figure 5. In Vivo Continuous, Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform.....	22
Figure S1. Benchtop Validation of Insulin and Glucagon BQI in Buffer.....	27
Figure S2. Benchtop Validation of Insulin and Glucagon BQI in Whole Blood.....	27

Chapter 3 QIRT-ELISA for Continuous Monitoring in Ex Vivo Lung Perfusion

Figure 6. Toronto EVLP Platform with QIRT-ELISA for Real-Time Continuous Monitoring of Glucose and Lactate.....	30
Figure 7. Validation of Bead-Based Quantum Dot Mediated Assays (BQA) for Lactate and Glucose Monitoring.....	33
Figure S1. Plate Reader Validation of Aptamer and Displacement Strand Functionality.....	38
Figure S2. Flow Cytometry Optimization of Ratios of Aptamer to Displacement Strand for Bead-Based Assays.....	39
Figure S3. Glucose and Lactate BQA Benchtop Validation in Perfusate.....	40

List of Tables

Chapter 1 Introduction

Table 1. Bioconjugation Strategies Used for Continuous Biomarker Monitoring in QIRT-ELISA Systems in Diabetes and Ex Vivo Lung Perfusion.....	6
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Chapter 2 QIRT-ELISA for Continuous Monitoring in Diabetes

Table 2. Statistical Analyses of Benchtop and On-Chip BQI.....	13
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Chapter 3 QIRT-ELISA for Continuous Monitoring in Ex Vivo Lung Perfusion

Table 3. Statistical Analyses of Benchtop and On-Chip BQA.....	34
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Chapter 1

Introduction

1.1 Importance of Continuous Monitoring in Healthcare

Continuously monitoring of biomolecules like hormones, polypeptides, and monosaccharides can help us obtain crucial information about biological systems, further our understanding of human physiology, and enhance our ability to diagnose and treat complex, multisystemic illnesses^{1–10}. Precision medicine is an evolving discipline of healthcare that relies on continuous monitoring technologies to adapt clinical management to individuals. This type of care strategy currently involves sampling steps at timed-intervals and subsequent lab-based measurements to determine concentrations of biomarkers of interest^{10–13}.

The current gold standard practices for biosensing in a clinical setting are enzyme-linked immunosorbent assays (ELISAs) and high-performance liquid chromatography-tandem mass spectrometry (HPLC). These techniques are highly sensitive, selective, and can measure several targets of interest simultaneously, however, they require sophisticated and expensive reagents and equipment, several sample processing steps, and trained personnel to perform^{11–14}. This results in measurements being available only after hours to days, preventing physicians from having a complete picture of the physiological processes in their patients, and is especially problematic in acute clinical settings where expedited treatment responses are required.

Due to the vast diversity of individual metabolic responses, delays in monitoring biomarkers can lead to inefficient treatment, incorrect dosing, increased occurrences of side effects, and prolonged recovery, all of which, leads to further complications in the clinical management of diseases, increasing healthcare costs, and negative outcomes for patients. Continuous monitoring platforms can mitigate these instances by allowing monitoring of recovery, facilitating timely indication of advancement of disease, promoting earlier and more accurate diagnoses, and even preventing the onset of symptoms by facilitating earlier intervention.

1.2 Biosensing Mechanisms

Proteins and nucleic acids are often the core components in biosensors used in clinical monitoring. Proteins, usually enzymes or immunoglobulins, and nucleic acids, such as DNA or RNA-based aptamers, specifically and rapidly recognize and bind to targets of interest, causing binding event-led transduction into measurable signals. These signals can then be correlated back to target concentration, thus culminating in a comprehensive biosensing platform^{12,14}.

Enzymatic biosensors are limited in their applicability because of the limited number and type of substrates with which they can be used, moreover, they lack long-term stability, and require very specific cofactors, and ranges of temperature and pH for optimal activity. Antibody-based or immunoaffinity based biosensors overcome this issue, because antibodies can be raised against a wide range of proteinaceous biomarker antigen of interest in a host species, and are more stable, however, they do not work with non-protein biomarkers or metabolites^{12,14}.

The importance of continuous monitoring extends beyond proteinaceous biomarkers such as

hormones, cytokines, and immunoglobulins, and includes cells, therapeutic drugs, and small molecular metabolites such as glucose and lactate^{5,7-9,15-18}. Nucleic acids solve this problem, with the development of aptamers that can be custom designed to recognize any type of molecular target of interest, and are highly stable, and functional within large ranges of temperature and pH¹⁹.

Biorecognition elements are only one piece of the biosensing puzzle – the second component is a reporter or transducer element which gives a measurable signal in response to the binding of molecular targets to biorecognition elements. Some assays use fluorescence reporters like FITC, Cy5, or Phycoerythrin^{2,6,9,18,20,21}, among others, and some others, use cascading signal amplification methods. Electrochemical assays rely on redox reporters, or catalytic activity, while some assays such as surface plasmon resonance, rely on refraction of incident wavelengths^{2,15,22-34}.

ELISAs, are based on the principal of immunoaffinity biorecognition, and use a colorimetric detection strategy based on chemically induced colour changes that reflect target concentration. Since their development in 1971, ELISAs have been used in clinical diagnosis, food, and environmental monitoring, to name a few applications¹⁴. The advantages of ELISAs are their high selectivity and specificity, low limits of detection, and potential for high throughput multiplexed detection of targets of interest. Research is always being done to adapt ELISAs for use in different applications and to improve their ease of operation by custom designing antibodies, incorporating nanomaterials into the assays with unique chemical, optical, electrical, biosensing, or catalytic properties, and using detection modalities beyond colorimetric detection^{13,14}.

ELISAs are the focus in the following chapters – particularly, the development of ELISA-based platforms for continuous monitoring of biomarkers. The development of ELISA-based assays discussed in the upcoming chapters focus on applications in diabetes and lung transplantation, both of which affect large populations of individuals globally, are only growing in incidence, and will benefit from advancement in continuous monitoring technology.

1.3 Challenges With Current Measurement Technologies

The challenges associated with ELISAs are multifold. To begin with, they lack temporal resolution, because of the nature of incubation, washing, and detection steps required to complete the assay. This is not an issue in the case of monitoring biomarkers where a single timepoint measurement is sufficient, however, when biomarkers that undergo metabolic or circadian fluctuations need to be monitored, this type of assay is ineffective^{11,12}.

Secondly, they require large volumes of expensive reagents and sophisticated equipment to perform. This is due to the nature of the assay requiring sample processing, incubation, removal of unbound antigens, and chemically induced development of detection signal. For the same reason, they also require trained personnel to perform. In the context of continuous biomarker monitoring, real-time measurements, and automated processes are needed to achieve meaningful temporal resolution. ELISAs are also limited in that they cannot be used to measure multiple molecular targets of interest within the same heterogeneous sample matrix. In physiology, rarely are molecular targets working in isolation. Molecular reactions usually occur in tandem with each other, with complex, multistep, afferent and efferent mechanisms, and trends in their levels are much more diagnostically valuable in conjunction, rather than individually and in a single

timepoint^{3,5, 6,15,22,26,27,31,35-39}. Take together, continuous monitoring of biomarkers, requires not only improved temporal resolution, but also multiplexed measurement capabilities.

The evolution of bead-based immunoaffinity assays has improved upon some of the drawbacks associated with conventional liquid-phase assays⁴⁰. The surface-to-volume ratio of beads is much higher compared to traditional well-plate methods used for immobilization in conventional ELISAs, meaning that a larger concentration of biorecognition elements can be immobilized on the surface of beads. This also offers a larger surface area over which target capture and detection can occur, conferring faster incubation times and the need for smaller volumes of reagents. The issue of temporal resolution, however, still remains, because incubation and wash steps, though far simplified, are still not sufficiently low enough to make real-time measurements possible.

The use of colorimetric and fluorescence-based detection in ELISAs is another drawback. Colorimetric detection is based on catalytically induced colour changes that occur in accordance with target concentration and are detected via absorbance. These changes in colour take several minutes to develop and can be inconsistent from batch to batch of reagents. Fluorescence-based methods rely on organic fluorophores which are highly prone to photobleaching and do not lend themselves well to multiplexing as they each have their own respective excitation and emission spectra that may interfere when multiple measurements need to be taken simultaneously.

1.4 Role of Microfluidics in Biosensing

The incorporation of microfluidics with a bead-based sandwich ELISA has successfully led to the development of a real-time ELISA with continuous monitoring capabilities⁷. Microfluidics is a diverse discipline that allows the development of novel technologies for various applications in fundamental sciences like chemistry, physics, and biology, materials science, environmental^{40,41}, and energy engineering^{11-13,42,43}. In biology particularly, microfluidics has facilitated the development of rapid diagnostic platforms, targeted drug delivery systems, lab-on-a-chip, and organ-on-a-chip platforms^{2,4,6,9,11-13,21-34}. The unique properties of fluidic manipulation that microfluidics offers are particularly useful in conjunction with immunoaffinity and enzymatic assay-based platforms to improve upon setbacks such as long incubation times, reagent usage, and lack of automation¹²⁻¹⁴.

The design of a real-time ELISA (RT-ELISA) platform was facilitated by microfluidics with the design, development and validation of three microfluidic modules for incubation, washing, and detection respectively⁷. This work was done by Dr. Poudineh and collaborators at Stanford University and was further developed in the Poudineh group to improve upon limitations in the first-generation version of the platform. The first RT-ELISA platform was used to continuously detect insulin and glucose in live, conscious rats. The assays used in the platform were bead-based, and used organic fluorescence detection reporters which were monitored in the detection module using a high speed CMOS camera. The first module, called the micromixer, was developed to bring incubation times for conventional immunoaffinity- and aptamer-based biorecognition down from hours to sub-minute timescales. This was achieved by incorporating the bead-based assays in serpentine channels embedded with herringbone structures to facilitate chaotic mixing in addition to increasing surface area for molecular interaction to take place.

The second module, based on deterministic lateral displacement (DLD), known as the DLD module, was employed for real-time and simplified washing out of blood components and removal

of unbound assay reagents. The module was predesigned to allow particles with a critical diameter above 15 μm to flow into the detection module, and smaller particles to flow out into a waste outlet. Components of hematocrit were thus removed, doing away with any long, complex preprocessing steps required prior to running the assay to remove cellular components, thus reducing overall assay run-time and automation of steps that normally require sophisticated lab equipment and trained personnel to run. The final module was a detection module, where completed assay beads were interrogated for fluorescence intensity, as they were flown through, allowing real-time, continuous measurements to be recorded.

The incorporation of bead-based assays with these microfluidic modules also ensured that the kinetics of the assays would not reach saturation or a state where binding events and dissociation events were in equilibrium. This further improved the sensitivity of the assays as increased their dynamic ranges, which is especially important, as these were validated in whole blood, with insulin and glucose, which are targets that undergo rapid fluctuations in short timespans and are in picomolar and millimolar concentrations in mammalian physiology. The only drawback of the system were the use of organic fluorophores as detection reporters, and subsequent need for a bulky high-speed CMOS camera for fluorescence detection. In the second generation of the RT-ELISA system, as developed in the Poudineh lab, the development of an optofluidic module, and the use of quantum dots as detection reporters overcomes this issue.

1.5 QIRT-ELISA For Continuous Monitoring in Diabetes

Insulin and glucagon are hormones involved in maintain blood glucose levels and are crucial biomarkers to monitor in the clinical management of diabetes^{38,49}. Diabetes is characterized by dysregulated glucose homeostasis, and is a multisystemic, metabolically complex disease, with highly variate individual responses to treatment^{38,49–51}. Continuous glucose monitoring platforms and exogenous insulin replacement therapy have vastly improved the treatment of diabetes, however, the current gold standard of monitoring progression of disease and remission of symptoms of diabetes through measurements of insulin and glucagon is conventional ELISA.

This method, as described above, is time-consuming, requiring lengthy protocols involving sample preparation steps, target incubation, multiple wash steps, and catalytically induced colorimetric detection. The physiological changes in the levels of insulin and glucagon, their dynamics in relation to one another, and in the context of glucose homeostasis cannot be meaningfully studied with single timepoint measurements that traditional ELISA offers. These issues were first tackled with the development of the RT-ELISA system, offering continuous, multiplexed monitoring of insulin and glucose in live, conscious rats, however, the platform used organic fluorescence reporters, and needed sophisticated equipment for detection. The second generation of the platform incorporates an optofluidic module in the place of the detection module, which far simplifies the high-speed CMOS camera used in the first generation platform, and incorporates quantum dots as fluorescence reporters in the assays.

Insulin and glucagon are measured through novel bead-based quantum dot mediated assays (BQA) in the second generation of the platform, which is being termed, QIRT-ELISA, from Quantum Dot Integrated Real-Time ELISA. Quantum dots are ideal candidates for incorporation in biosensing platforms due to their unique optical properties^{24,45,46,52–56}. They are extremely photostable, and much brighter compared to their organic counterparts, and are universally excited by ultraviolet wavelengths of light. This confers a particular advantage to them in

developing platforms for multiplexing, as multiple quantum dots can be used for detection and excited by a single wavelength of ultraviolet light, while emitting very discrete fluorescence spectra. The bioconjugation strategies used in the development of the assays discussed herein are summarized in Table 1.

Chapter 2 will discuss the development and validation of two such assays, for insulin and glucagon continuous monitoring, starting with in vitro validation, and demonstrating in vivo validation in a mammalian small rodent model by tracking insulin and glucagon levels in healthy rats in response to a glucose load.

1.6 QIRT-ELISA for Continuous Monitoring in Ex Vivo Lung Perfusion

To broaden the universality of applications possible with the QIRT-ELISA system, assays have been developed for the monitoring small molecular biomarkers that are implicated in inflammation occurring in ex vivo lungs being assessed prior to lung transplantation. Organs donated for transplantation post-circulatory death, and brain death, are a crucial therapeutic modality for patients in late-stage pulmonary deficiency, however, around 80% of lungs obtained this way are disqualified from transplantation due to injury during transportation, ex vivo perfusion, and paucity of gas exchange, causing a chronic and severe shortage of lungs available for transplantation.⁵⁷⁻⁶⁰

The assessment of lungs prior to transplantation currently involves standard biochemical and inflammatory workups of oxygen, pH, glucose, lactate, IL-6, and IL-8 levels, and stratification of organs based on these criteria into four levels that predict their success during and post-transplantation^{61,62}. This is known as the Toronto Lung Score. The development of this score, is an important technological advancement in preventing the disposal of potentially viable lungs, by noting trends in biomarkers that may point out injury and inflammation, which can then be addressed. Biomarkers glucose and lactate are particularly important in this assessment, because their levels rapidly fluctuate in short timespans when lung injury is taking place. Current time intervals of monitoring during ex vivo lung perfusion are as short as 15 minutes, however, these tests are enzymatic, and require costly lab-based equipment to perform⁶¹⁻⁶⁶.

The opportunity, therefore, is crucial for the development of a continuous monitoring platform that can assess the levels of lactate and glucose in lungs during ex vivo perfusion and allow even more timely intervention when injury, inflammation, or infection are forewarned, thereby preventing their disqualification, and promoting positive outcomes in patients post-transplant. The development of quantum dot mediated aptamer-based bead-based assays for the continuous, multiplexed measurement of glucose and lactate will be discussed in chapter 3. The bioconjugation strategies used in the development of the assays discussed herein are summarized in Table 1. Validations of both assays individually, and their multiplexing capability, on the integrated QIRT-ELISA platform will demonstrate the successful development of the platform and its readiness to be deployed in-line in ex vivo lung perfusion systems.

Biomarker	Assay Format	Capture Strategy	Detection Strategy	Rationale
Insulin	Bead-Based Sandwich ELISA Signal-On Assay	Carboxylate-modified beads functionalized with capture antibodies through EDC/NHS mediated covalent conjugation	Click chemistry conjugation of detection antibodies through DIBO-modified Quantum dots and azide-modified antibodies	Insulin capture and detection antibodies are best when stored at 4C, and are stable for the duration of click chemistry conjugation at room temperature. Quantum dots cannot be frozen, so the temperature stability of the insulin detection antibodies lent themselves well to this conjugation strategy.
Glucagon	Bead-Based Sandwich ELISA Signal-On Assay	Carboxylate-modified beads functionalized with capture antibodies through EDC/NHS mediated covalent conjugation	Streptavidin-Quantum dots with biotinylated detection antibodies	Glucagon capture and detection antibodies are highly temperature-sensitive and need to be stored at – 20C. Since quantum dots cannot be frozen, detection antibodies were biotinylated so they could be frozen to ensure maximum viability. These were then conjugated with Streptavidin-Quantum dots for 30 minutes prior to use in assay experiments.
Glucose	Aptamer-Based Signal-Off Assay	Aptamer-Displacement strand-Quantum dot complex covalently conjugated to Streptavidin-modified beads through biotin group on 5'- end of aptamer	Displacement strand modified with amino group and carboxylate-modified Quantum dots were conjugated together using EDC/NHS mediated covalent coupling	Small molecule biomarkers are detected through an aptamer-based assay to improve upon traditional enzymatic approaches. The use of quantum dots facilitates multiplexing and offers advantages of photostability and brightness. The signal-off format was chosen because quantum dots do not quench, unlike organic fluorophores, so traditional signal-on fluorescence formats cannot be used.
Lactate	Aptamer-Based Signal-Off Assay	Aptamer-Displacement strand-Quantum dot complex covalently conjugated to Streptavidin-modified beads through biotin group on 5'- end of aptamer	Displacement strand modified with amino group and carboxylate-modified Quantum dots were conjugated together using EDC/NHS mediated covalent coupling	Small molecule biomarkers are detected through an aptamer-based assay to improve upon traditional enzymatic approaches. The use of quantum dots facilitates multiplexing and offers advantages of photostability and brightness. The signal-off format was chosen because quantum dots do not quench, unlike organic fluorophores, so traditional signal-on fluorescence formats cannot be used.

Table 1. Bioconjugation Strategies Used for Continuous Biomarker Monitoring in QIRT-ELISA Systems in Diabetes and Ex Vivo Lung Perfusion.

Chapter 2

QIRT-ELISA For Continuous Monitoring in Diabetes

2.1 Abstract

Analyzing diabetes-related hormones like insulin and glucagon has traditionally relied on enzyme-linked immunosorbent assays (ELISAs), which are considered the gold standard. However, ELISAs involve lengthy sample processing times, including the preparation of blood serum or plasma, and are limited by the number of measurement time points they can handle. This restricts the ability to assess hormone dynamics and their correlation with blood glucose levels. In response to these limitations, we present the development and validation of a Quantum Dot Integrated Real-Time (QIRT-ELISA) system, a multi-module microfluidic platform that employs a bead-based quantum dot-mediated immunoassay (BQI) for multiplexed, continuous, real-time detection of insulin and glucagon. The QIRT-ELISA system eliminates the need for large and costly equipment, and significantly improves sensitivity, specificity, and temporal resolution of measured analytes. With Dr. Jonathan D. Schertzer and collaborators at McMaster University, we further validated the platform through discrete and continuous glucose tolerance tests in rat models and confirmed its accuracy in simultaneously measuring endogenous hormone levels within the physiological range.

2.2 Introduction

Advancements in diabetes management particularly with continuous glucose monitoring, and insulin replacement therapy, have significantly improved the lives of diabetes patients⁴⁹. However, variances in individual glucose metabolism, and the relationship between the hormones involved in glucose homeostasis, are still poorly understood^{5,7,38,49}.

The synergy between insulin and glucagon is particularly important to understand. These hormones are the cornerstone of glucose homeostasis and their profiles individually and in cohesion, not only provide diagnostic value, but changes in their profiles are also known to be implicated in the development and progression of diabetes^{16,22,36,37}. Understanding the diversity of these profiles, and the metabolic processes leading up to progression of disease, could have a role in developing personalized therapies against diabetes⁵⁰.

An example of this is in the clinical management of diabetes and patient outcomes prior to and post-exogenous insulin therapy. Insulin dosing is a key part of assuring recovered blood glucose levels. However, the dysregulation of insulin and glucagon, as seen in diabetes pathophysiology, often causes devastating downstream physiological effects, including preventable negative outcomes like fatal hypoglycaemia from inaccurate insulin dosing^{7,8,49}. Having a method to continuously monitor the physiological concentrations of these hormones, in conjunction with glucose, will allow for further insights to be drawn about their relation to one another and their roles in glucose homeostasis.

Currently, there is a lack of technologies that can measure these biomarkers at a sufficiently time-resolved manner, and we cannot map trends in their levels at the timescales at which their concentrations change in accordance with individual physiological metabolic syndromes^{2,3,4-7,10-15}. This continues to negatively impact clinical management of this disease. Having a method to continuously monitor the physiological levels of these hormones, in conjunction with glucose, will

give us a much more accurate picture of diabetes pathophysiology and vastly improve the daily management and outcomes of patients with diabetes.

Historically, enzyme-linked immunosorbent assays (ELISAs) have been used to detect proteinaceous biomarkers in heterogeneous biological matrices, while small carbohydrate biomarkers like glucose and lactate are measured enzymatically^{3,16,19,36}. These methods require several hours for completion, do not afford real-time or multiplexed detection capability, and require large amounts of reagents, sophisticated detection instrumentation, and trained personnel to perform. Most problematic, is the lack of temporal resolution in determining target concentrations, and lack of multiplexing capability. Temporal resolution is achieved when real-time measurements are possible, which, given the timescales of incubation in immunoaffinity-based methods, this is rendered unfeasible. With enzymatic assays, the need for cofactors to promote enzyme activity, the risk of denaturation, and mostly colorimetric detection mechanisms, further prevents real-time and multiplexed measurements of proteinaceous and small molecule biomarkers^{16,17,19}.

Extending the work done by Dr. Poudineh and collaborators at the Soh lab at Stanford University to develop the RT-ELISA system⁷ – a bead-based microfluidic-enabled continuous monitoring system for insulin and glucose in vivo – which featured multiplexed detection of insulin and glucose in live, conscious rats, using organic fluorophores as detection reporters, and a CMOS high-speed camera to record fluorescence emission intensity, we have developed a platform for continuous monitoring of insulin and glucagon using quantum dots as detection reporters to facilitate easier multiplexing and simplify the optical detection equipment into a miniaturized system more suited to clinical and bedside monitoring applications.

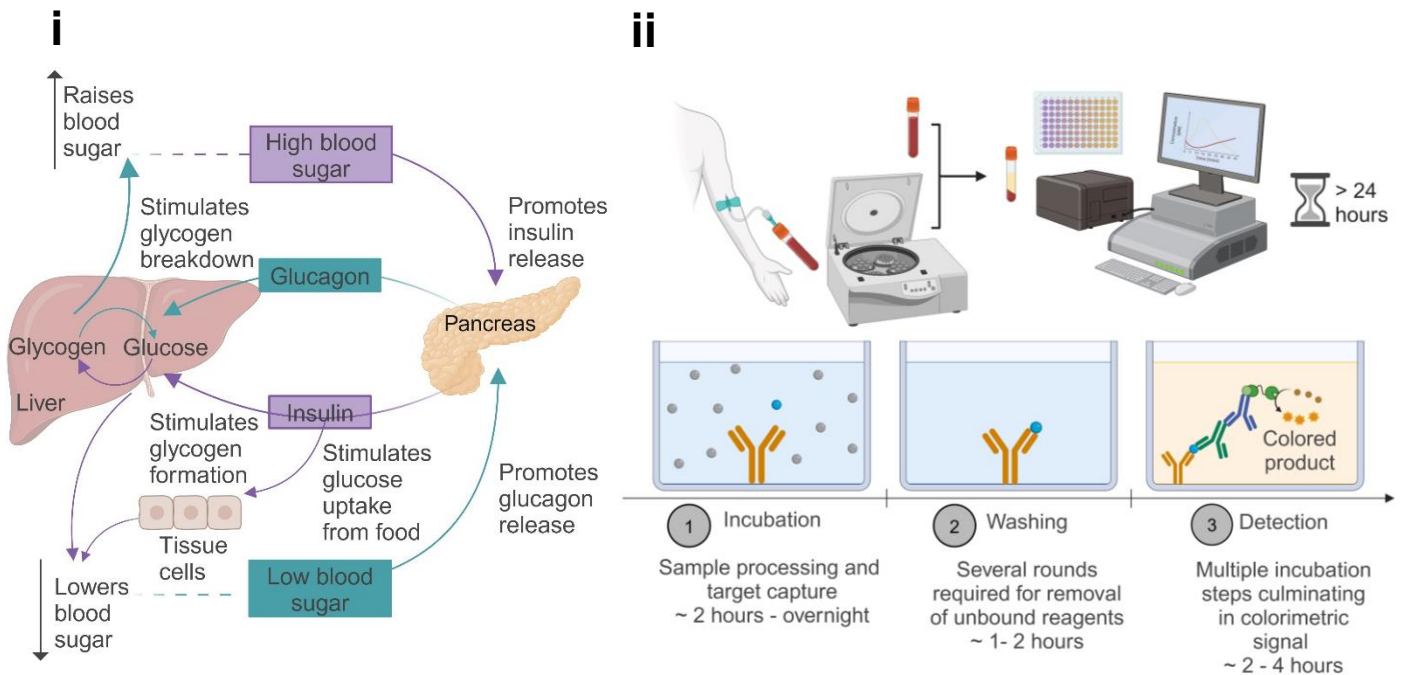
To improve upon plate-reader-based liquid-phase assays, bead-based immunoaffinity, and aptamer-displacement strand capture assays were designed, which outperform their liquid-phase counterparts due to higher surface-to-volume ratio and area for molecular interaction⁷. The incorporation of microfluidic modules specifically designed to ameliorate temporal-resolution issues outlined above in conventional ELISA, such as long incubation times, multiple wash steps, and further challenges with multi-step colorimetric detection, was validated with the successful measurement of insulin and glucose in live, conscious rats⁷.

The microfluidic modules designed were as follows - A mixing module with serpentine channels embedded with herringbone structures to facilitate chaotic mixing for increased molecular diffusion, thereby cutting down assay incubation times from hours to seconds for completion, a depletion module based on deterministic lateral displacement facilitating removal of blood components and unbound reagents, and a detection module for capturing emitted fluorescence from beads⁷. The same overall design was maintained, with changes to the assay concept, including using quantum dots as detection reporters, and an optofluidic module with UV excitation and collection of emitted fluorescence signals from beads, to develop the next generation of the system, known as Quantum-Dot Integrated Real-Time ELISA (QIRT-ELISA).

Figure 1. a. depicts a schematic of the metabolism of glucose by the liver and pancreas with hormones insulin and glucagon that lower and increase blood glucose respectively. The gold standard for blood insulin and glucagon monitoring is the conventional ELISA consisting of lengthy sample preprocessing, incubation, washing, and detection steps, requiring sophisticated equipment, expensive reagents, and trained personnel to perform. Figure 1. b. depicts the QIRT-ELISA platform with a schematic of the assays taking place within the system from initial

incubation to detection, requiring ~ 60 seconds to complete. This chapter will discuss the validation of the Quantum Dot Mediated Bead-Based Assays, in the microfluidic system, and their validation in vitro and in vivo in a mammalian model of healthy glucose homeostasis.

a



b

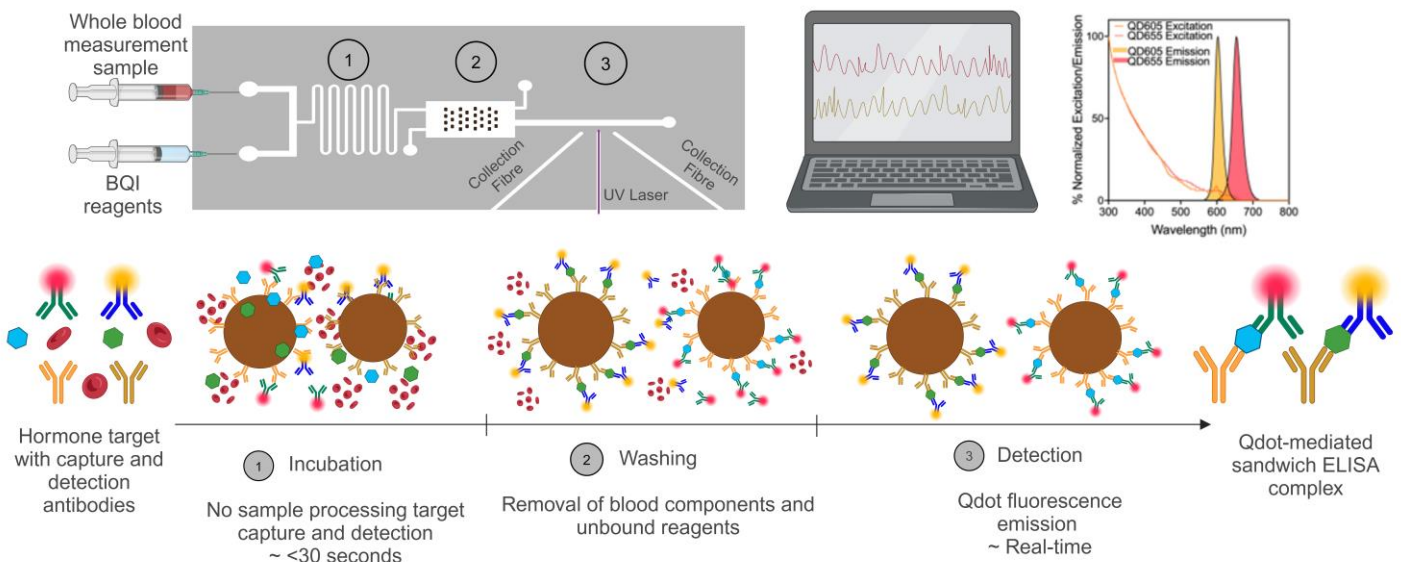


Figure 1. Insulin and Glucagon Dynamics for Glucose Homeostasis with Conventional Monitoring Approaches and QIRT-ELISA for Real-Time Continuous Monitoring.

a) i Schematic of glucose homeostasis conducted by the liver and pancreas with hormones insulin and glucagon affecting the absorption and release of glucose into the bloodstream respectively. a) ii Schematic of current gold standard method of measuring insulin and glucagon requiring complex sample preprocessing, incubation, washing, and detection steps. b) Depicts the microfluidic modules comprising the QIRT-ELISA system and their individual roles, alongside the assay schematic showing multiplexed detection of two biomarkers, and their progression through each module of the microfluidic workflow with <60 seconds time-to-completion.

2.3 Results and Discussion

2.3.1 Bead-based Quantum Dot-Mediated Immunoassay (BQI) Validation

Similar to the original RT-ELISA, bead-based immunoassays were employed for target protein detection. Capture and detection antibodies against insulin and glucagon were deployed in a sandwich ELISA format to improve selectivity and quantum dots were used as detection probes for their numerous benefits including high optical stability, and brightness^{23,24,52,54–56}.

The bead-based sandwich immunoassay schematic is depicted in Figure 2. a. i. 15 μm magnetic beads were functionalized with anti-rat antibodies specific to insulin or glucagon while the detection was achieved using detection antibodies conjugated to specific quantum dots as fluorescence probes. QDot 655 and QDot 605 were employed for detection of insulin and glucagon, respectively. The use of quantum dots enables multiplexing due to their unique optical properties of being excited universally by wavelengths in the ultraviolet (405 nm) range and having discrete emission spectra^{24,44,45,52–56}. The quantum dots used in the assay are depicted with their respective excitation and emission spectra in Figure 2. a. ii.

First, individual bead-based immunoassays (BQIs) for insulin and glucagon were validated in buffer on the benchtop (Figure S1 i and ii) followed by validation in whole blood (Figure S2 i and ii) on the benchtop to determine the performance of the assay in a complex biological matrix with cellular components and plasma proteins. Concentrations were selected for insulin and glucagon based on their physiologically relevant ranges. Insulin was tested between 100 and 1000 pM and glucagon was tested between 10 and 100 pM. Large ranges were chosen to reflect the levels of these hormones in blood in the euglycemic and hypo/hyper glycaemic ranges.

Briefly, beads functionalized with capture antibodies and detection antibodies conjugated with QDots were incubated with their respective targets in buffer or blood (Figure S1 and S2 i and ii) on the benchtop for 90 minutes, and after washing they were imaged using flow cytometry. Next, the formation of the insulin-BQI and glucagon-BQI were tested within the micromixer module of QIRT-ELISA which mimics the incubation step. The micromixer module is a serpentine channel with embedded herringbone structures that enables chaotic and rapid mixing⁷. Blood samples spiked with different concentrations of target analytes were injected from one inlet while the functionalized beads and detection antibodies tagged with QDots were injected from another inlet of the device at an optimized flow rate of 15 $\mu\text{L}/\text{min}$ ^{7,40,41}. Upon mixing for ~ 30 seconds, the bead-target complexes were collected from the device outlet and imaged using flow cytometry. Figure 2 b i and 2 c i depict individual calibration curves for insulin and glucagon respectively.

The micromixer module outperforms the conventional assay in terms of incubation time, requiring ~30 seconds, as opposed to the 6 or 36 hours needed for the conventional insulin and glucagon ELISAs, respectively. The improvements offered by incorporating the assays in the microfluidic platform are not only in terms of temporal resolution, but also dynamic range. The comparisons of signal gains calculated as $F1 - F0 / F0$ where F1 refers to the median fluorescence intensity emitted by beads that are exposed to target analytes, and F0 is the median fluorescence intensity emitted by beads in the absence of target analytes, also demonstrates that larger signal gains, and therefore, better dynamic range and sensitivity are achieved when the assay is used in a microfluidic format. This comparison is shown in Figure 2. b iii and c iii for insulin and glucagon in their individual assay formats respectively.

Statistical analyses, including R-squared values, limits of detection (LOD) and limits of quantitation (LOQ) values calculated for benchtop and on-chip assays individually in whole blood are shown in Table 2. These also reflect improvements to the assays conferred from using them in conjunction with the microfluidic platform.

Figure 2. b ii and c ii demonstrate the specificity of each assay. Target capture and detection reagents were incubated with the highest concentration of non-target analyte spiked in whole blood on-chip, and signal gains were compared to the lowest concentration of target analyte. These were additionally compared to signals observed when no analytes are present. This experiment was performed to ensure no cross-reactivity in the assays, as this would impede their multiplexing capability. As demonstrated, insignificant cross-reactivity was observed, and assays were further validated in a multiplexed manner in whole blood on-chip.

Multiplexed detection of insulin and glucagon is important because these hormones work in opposing mechanisms to ensure glucose homeostasis^{5,6,36,37,49}. Insulin levels are highest when blood glucose is high, as it promotes the uptake of glucose by tissue cells and the conversion of glucose into glycogen in the liver. Glucagon levels, in contrast, are highest when blood glucose levels are low. They promote the breakdown of stored glycogen into glucose and its subsequent release back in the bloodstream. To ensure the QIRT-ELISA system could measure the dynamics of both these hormones simultaneously, three different combinations of insulin and glucagon concentrations were spiked into whole blood and tested with the micromixer module. The combinations used were as follows – 1000 pM insulin with 10 pM glucagon, 400 pM insulin with 40 pM glucagon, and 100 pM insulin with 100 pM glucagon. After target capture within the micromixer module, insulin-BQI and glucagon-BQI complexes were collected and analyzed using flow cytometry. Figure 2. d. depicts the results of this experiment. The collected measurements confirm that multiplexed detection of insulin and glucagon is achievable using the micromixer module.

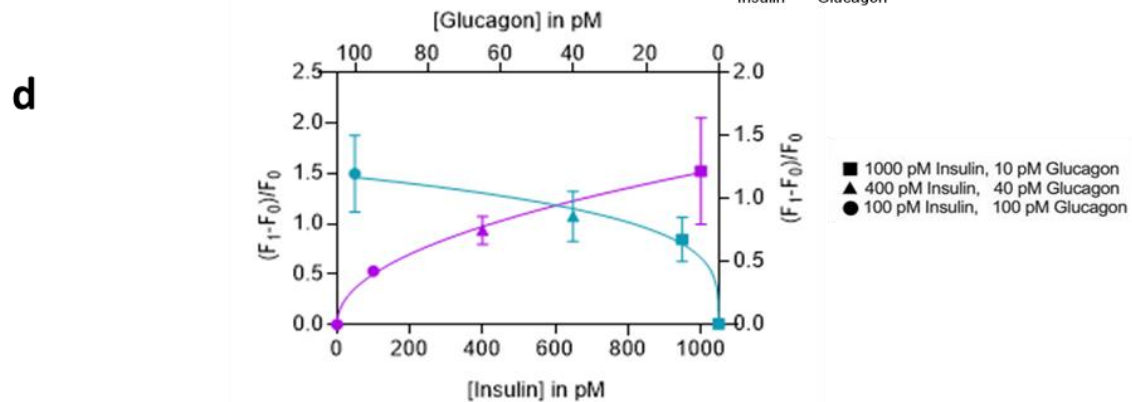
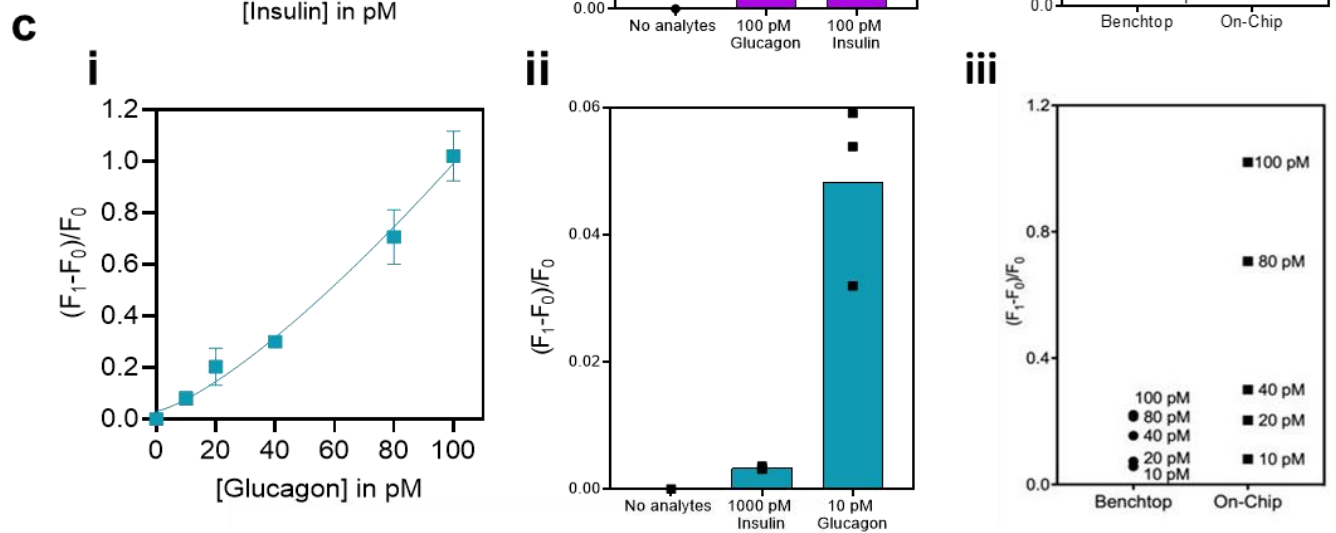
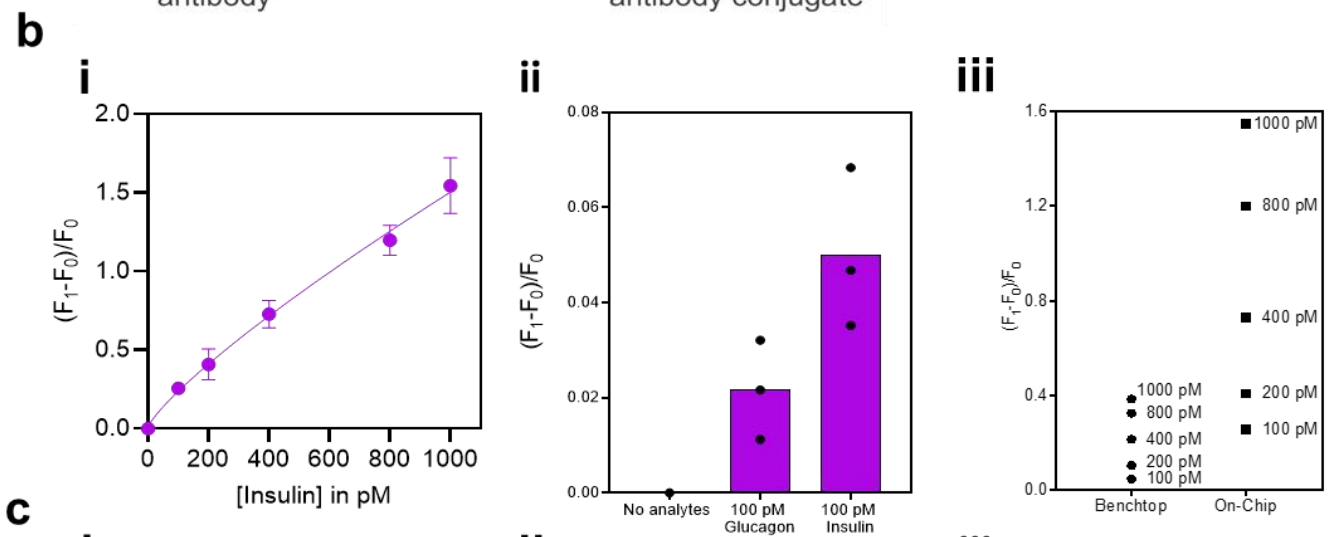
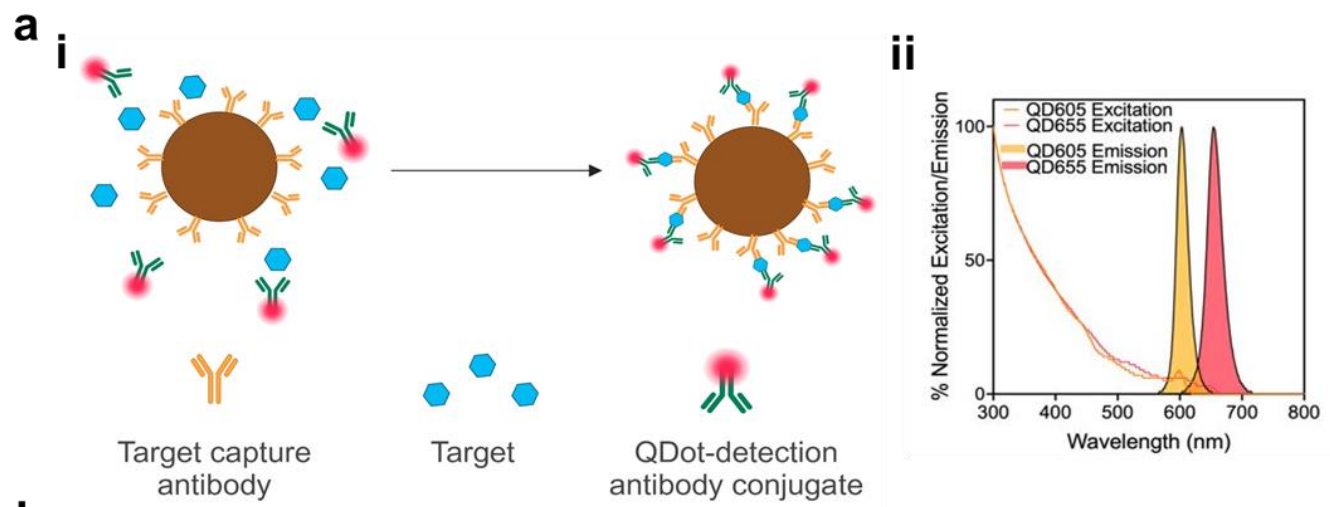


Figure 2. Validation of Bead-based Quantum Dot-Mediated Immunoassay (BQI) Sensing Mechanism on Micromixer Module.

a) i. Assay schematic employing beads functionalized with capture antibodies along with target analytes, and detection antibodies tagged with QDots to form a sandwich immunoassay. a) ii. Excitation and emission spectra for QDots used in BQIs and QIRT-ELISA platform. QDot 655 and QDot 605 were used to detect insulin and glucagon, respectively. The excitation spectrum for both QDots is in the 405 nm range. b) i. Individual calibration curve of insulin depicting normalized fluorescence intensity as a function of increasing target concentration. Target concentrations range from 100 – 1000 pM. b) ii. Specificity of individual insulin assay. Lowest target signal gain is compared to no analyte signal, and signal gain from incubation with highest concentration of non-target analyte. b) iii. Signal gains comparison between benchtop and on-chip assay for insulin depicting improved dynamic range when used on-chip. c) i. Individual calibration curve of glucagon depicting normalized fluorescence intensity as a function of increasing target concentration. Target concentrations range from 10 – 100 pM. c) ii. Specificity of individual glucagon assay. Lowest target signal gain is compared to no analyte signal, and signal gain from incubation with highest concentration of non-target analyte. c) iii. Signal gains comparison between benchtop and on-chip assay for glucagon depicting improved dynamic range when used on-chip. d) Multiplexed measurement of insulin and glucagon. Whole blood samples were spiked with insulin and glucagon concentrations that mimic their physiological trends - the lowest concentration of insulin (100 pM) and the highest of glucagon (100 pM) were spiked together, followed by medium level concentrations of both targets (insulin = 400 pM and glucagon = 40 pM), and finally the highest concentration of insulin (1000 pM) and the lowest concentration of glucagon (10 pM). F0 is the fluorescent signal for the sample with no target analytes, and F1 is the fluorescent signal for each sample. The data shows the mean $\pm$ standard deviation (SD) of three replicates. All figures presented here are contributed by S.S

Benchtop BQA Statistical Analyses				On-Chip BQA Statistical Analyses			
Analyte	R-square	LOD [pM]	LOQ [pM]	Analyte	R-square	LOD [pM]	LOQ [pM]
Insulin	0.98	23.7	71.8	Insulin	0.99	9.8	29.6
Glucagon	0.99	4.8	14.7	Glucagon	0.97	1.4	4.2

Table 2. Statistical Analyses of Benchtop and On-Chip BQI.

2.3.2 In Vitro Continuous Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform

Having validated the performance of the QIRT-ELISA system with both analytes individually and having demonstrated that insulin and glucagon can be measured simultaneously in the micromixer module, the next challenge was to perform continuous, multiplexed measurements of insulin and glucagon concentrations from spiked whole blood samples. To achieve this, whole

blood samples spiked with both insulin and glucagon at different concentrations were injected into the system such that the platform measured simultaneous signals from both targets.

Whole blood samples were spiked with physiological concentrations of insulin and glucagon, as described during initial multiplexing experiments - 1000 pM insulin and 10 pM glucagon, 400 pM insulin and 40 pM glucagon, a blank sample (without spiked analytes), 100 pM insulin and 100 pM glucagon, and lastly 200 pM insulin and 80 pM glucagon. Between samples with different concentrations, a wash sample was run (whole blood without any insulin or glucagon being added) to capture the change in the fluorescence readout of the system. To ensure the continuity of this experiment, the reagents syringe, and the QIRT-ELISA device was kept constant throughout the duration of the experiment, with only the sample syringe being switched in and out.

Data collection in this experiment, and in all subsequent experiments was performed with the optofluidic module, and fluorescence intensity was measured as a function of area under the curve derived from peak amplitude current. As each bead with a completed assay passed through the optofluidic module, it was interrogated with a 405 nm UV laser, allowing the quantum dots on the surface of the beads to emit their specific emission wavelength, which was measured as sharp increase in peak current. This was recorded for each bead passing through the optofluidic module as scans lasting for a duration of 60 seconds. On average, 30 peaks within a 30 second time frame were observed in continuous measurement experiments. The fluorescence signal measurements were then used to derive calibration curves for multiplexed detection of insulin and glucagon. Data collected from the optofluidic scans were analyzed with an app developed internally within our group, to calculate area under the peaks, and provide derived fluorescence intensity measurements.

Figure 3. a. depicts a schematic of multiplexed detection of insulin and glucagon in whole blood with steps describing each stage of the development of the completed sandwich ELISA complex on beads through each microfluidic module. Module 1 depicts the micromixer, where incubation occurs, module 2 depicts the removal of unbound hematocrit and reagents, and module 3 depicts the optofluidic detection module where real-time detection of fluorescence signal from quantum dots takes place.

Figure 3. b. depicts the area under the curve and individual peaks from beads showing fluorescence signal measurements from optical scans of insulin and their fluctuations over time as samples were switched in and out. Figure 3. c. depicts the same for glucagon. The variation in the number of peaks among different samples primarily results from the settling of beads in the reagents syringe during measurements. A scan is considered successful if a minimum of 10 peaks, indicative of beads entering the detection channel, are observed within a 30-second time frame.

Figure 3. d. i and ii depict calibration curves of normalized signal gains vs concentrations derived for insulin and glucagon respectively using the integrated QIRT-ELISA platform, with their respective R-squared values showing further improvements of the assay. The fully integrated QIRT-ELISA system achieved an LOD of 27.4 pM for insulin and 1 pM for glucagon measurements in blood. Compared to the micromixer-only module or benchtop assays (Figure 2) that relied on manual washing, incorporating the DLD module for automated washing in the integrated QIRT-ELISA system enhances the washing process⁷, thereby improving the

LOD. The previously published, original RT-ELISA reported an insulin LOD of 109 pM⁷. Hence, the new QIRT-ELISA has a higher sensitivity. This improvement is primarily attributed to the utilization of quantum dots as the fluorescence reporter and the implementation of highly sensitive fiber optic excitation and collection methods.

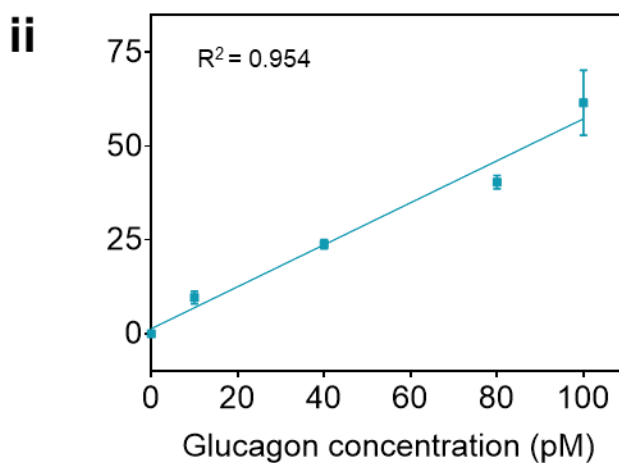
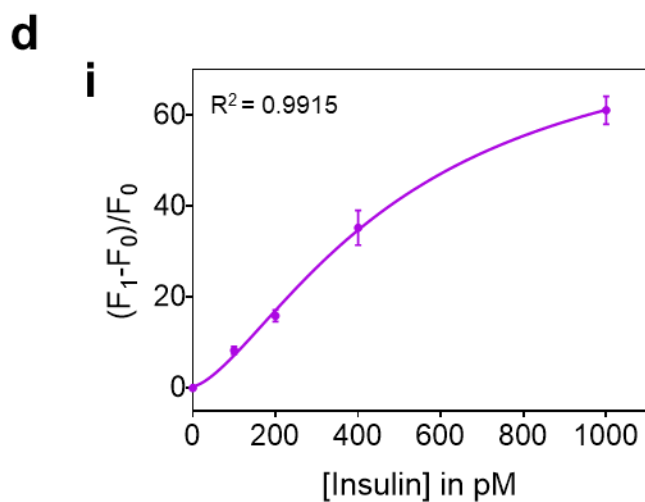
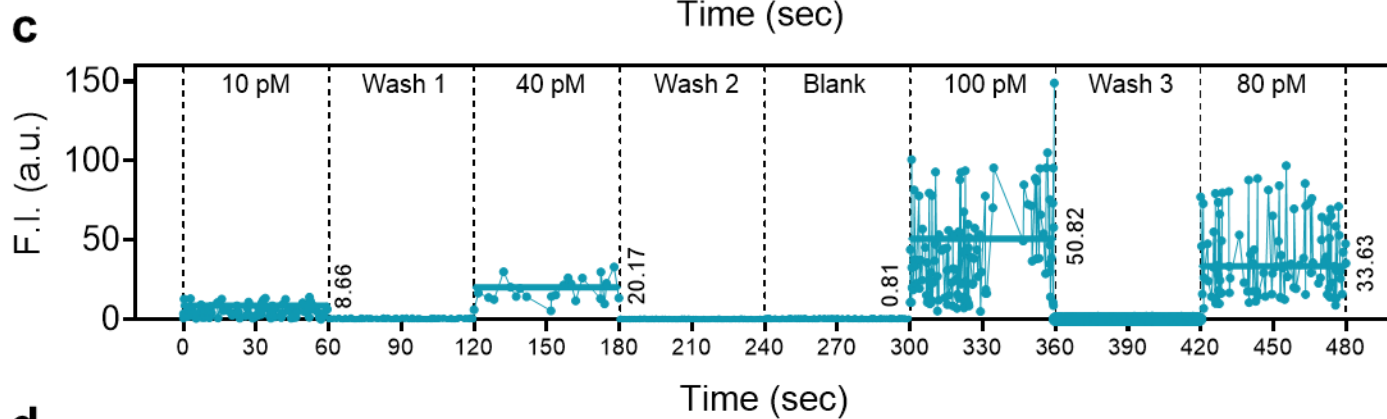
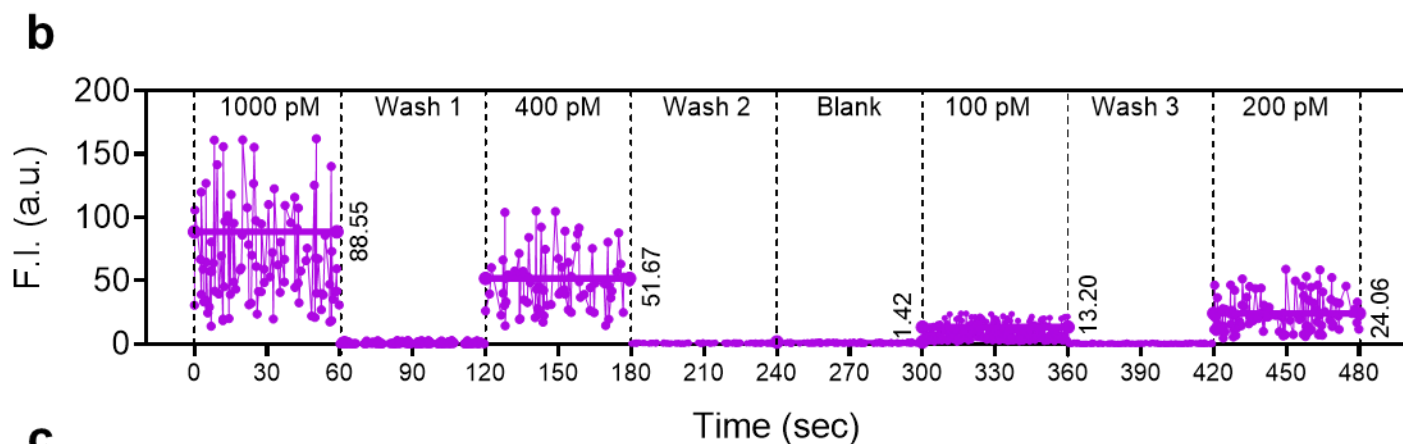
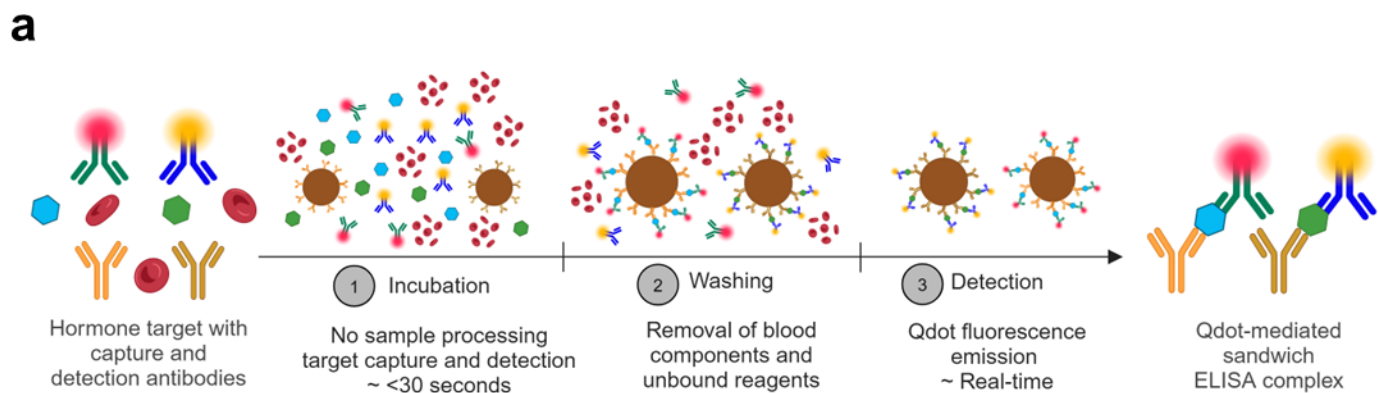


Figure 3. In Vitro Continuous Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform.

a) Schematic of multiplexed detection of insulin and glucagon in the fully integrated QIRT-ELISA platform with each microfluidic module fulfilling a certain role in the progression of the assay from incubation to removal of unbound hematocrit and reagents and detection. b) and c) Area under the peak measurements of the collected fluorescence signals for different concentrations of insulin and glucagon spiked in whole blood samples. Changes in fluorescence intensity were shown over time as samples were changed in-line. Scans cover durations of 60 seconds for each sample. d) i and ii depict derived insulin and glucagon calibration curves from the continuous, multiplexed measurements. F0 is the measured fluorescence signal for the sample with no spiked target analytes and F1 is the measured fluorescence signal for each sample spiked with a predetermined concentration of target analyte. Fluorescence intensity was measured as a function of area under the curve from peak amplitude, and the data shows mean $\pm$ SD of three scans for each sample with three replicates. S.S and H.A performed continuous monitoring experiments together. F.F designed and validated optofluidic modules being used. H.A designed app for data analysis and performed data analysis leading to generation of timescale figures and calibration curves for insulin and glucagon.

2.3.3 In Vivo Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform

The glucose tolerance test (GTT) is a particularly useful preclinical and clinical assessment of blood glucose control. Briefly, male Wistar rats were fasted overnight, followed by an oral administration of D-glucose. Blood draws were then performed at three different time points, once before oral glucose administration, then at 15 minutes and 60 minutes post-glucose administration. The collected blood samples were then tested with QIRT-ELISA and conventional ELISA to cross-validate QIRT-ELISA results with the current gold standard method of measuring insulin and glucagon in vivo.

A schematic of the GTT procedure is shown in Figure 4. a. Two sets of GTTs were performed, and each was confirmed with both conventional ELISA and QIRT-ELISA. Fasted rats were administered an oral glucose dose, which causes a spike in insulin 15 minutes post administration. Prior to glucose administration, blood insulin levels are low, and a gradual increase is seen leading up to the peak at 15 minutes, followed by gradual plateau and decline to fasting levels of insulin around the 60-minute mark. Blood glucagon levels were not altered significantly during these tests. These trends were observed by QIRT-ELISA measurements as shown in Figure 4. b. i and ii and were further confirmed by ELISA results. Here, measurements on QIRT-ELISA were multiplexed and real-time, however, they were not continuous in vivo measurements.

Figure 4. b. i and ii show a comparison of trends observed by both methods of measuring insulin and glucagon, along with insulin and glucagon concentrations obtained from conventional ELISA over time. Signal gain values for QIRT-ELISA were calculated by using F0 as the fluorescence intensity measured at T=0 prior to glucose administration. The data from both sets of GTTs was then combined, to show a correlation between QIRT-ELISA signal gains and conventional ELISA concentration measurements.

Figure 4. c. i and ii show the correlation between insulin and glucagon measurements as obtained by conventional ELISA with their respective QIRT-ELISA signal gains. This experiment offers

further confirmation that the pace of obtaining concentration measurements is significantly improved when using the QIRT-ELISA platform, as individual ELISAs for these targets required separate ELISA kits, and several hours to perform, whereas, data obtained from QIRT-ELISA could be analyzed in real-time, and showed good correlation with ELISA in monitoring trends of insulin and glucagon post-gavage.

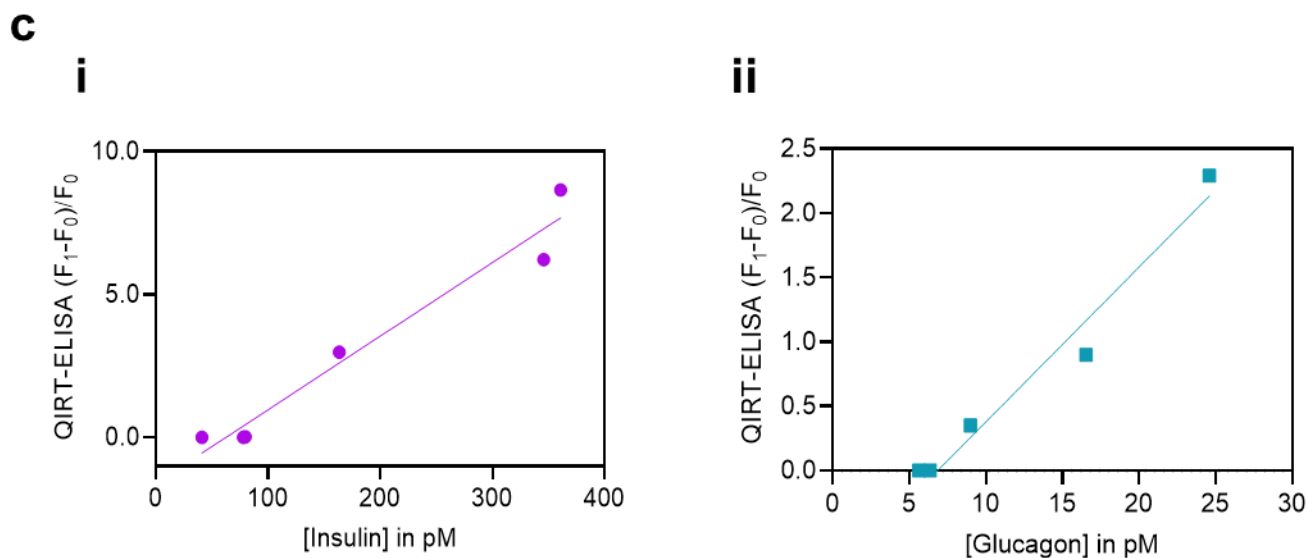
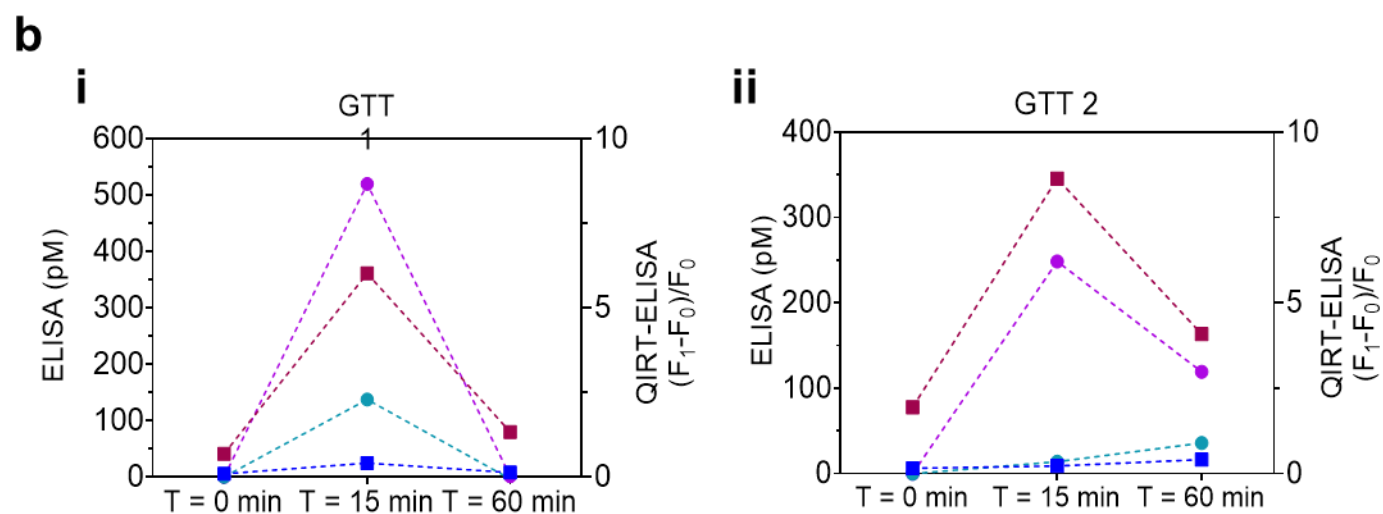
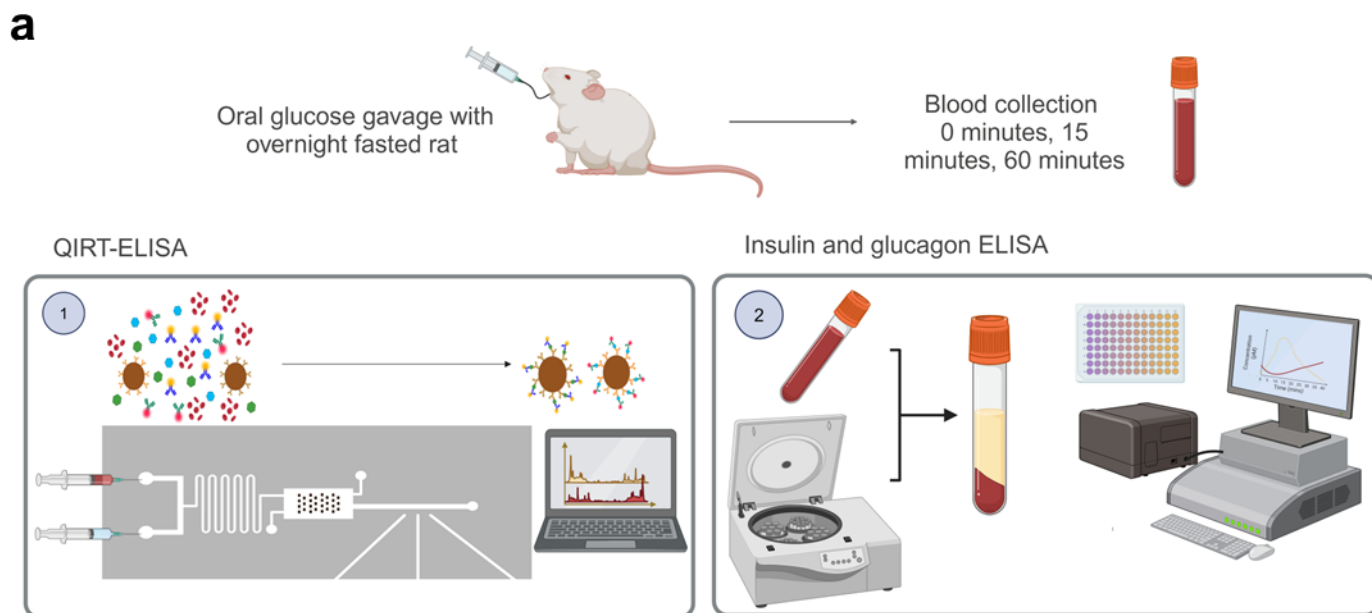


Figure 4. In Vivo Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform.

a) Schematic of GTT in rats followed by measurements of insulin and glucagon through conventional ELISA and QIRT-ELISA. b) i and ii depict two consecutive rounds of GTT showing trends of insulin and glucagon empirical concentrations obtained by conventional ELISA along with corresponding fluorescence signal gain reading from QIRT-ELISA across three time points following glucose administration. The fluorescence signal measured at T = 0 min was chosen as F0 for normalization. All measurements of insulin and glucagon from blood draws at each time point were performed in a multiplexed manner with the QIRT-ELISA platform, while measurements for glucagon and insulin were performed using two separate conventional ELISA kits and the targets were individually analyzed. The correlation between QIRT-ELISA measurements and ELISA for insulin and glucagon using the collected data of the two GTT experiments are shown in c) i and ii. S.S and H.A ran QIRT-ELISA experiments on collected blood, N.B performed glucose tolerance tests, collected blood, and ran conventional ELISAs. H.A performed data analysis using app.

2.3.4 In Vivo Continuous, Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform

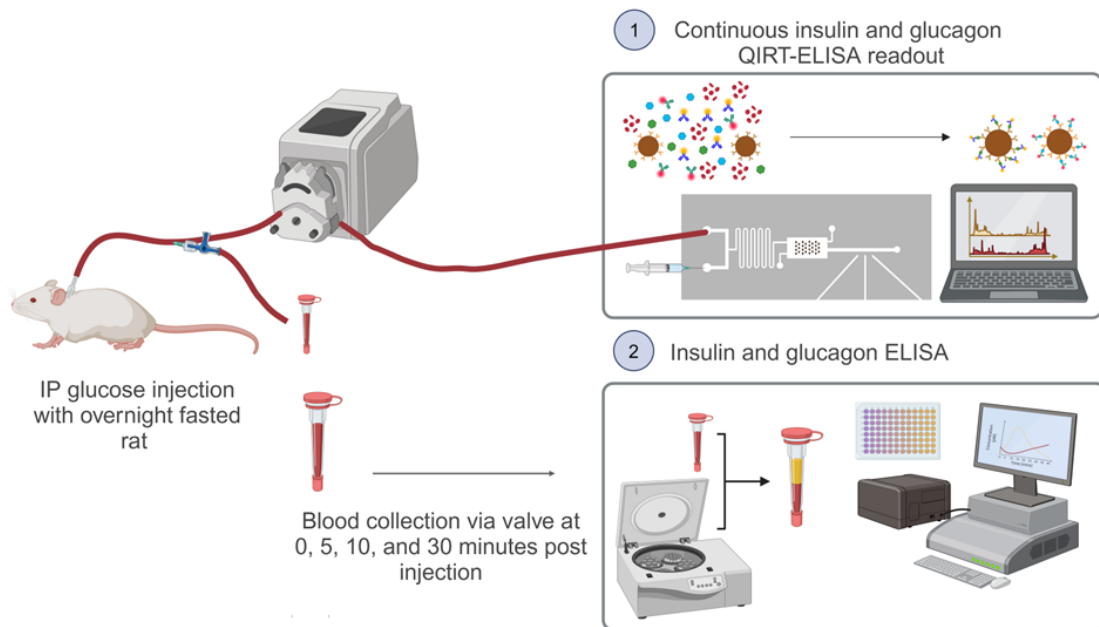
The QIRT-ELISA system was evaluated for improved time resolution in the measurement of insulin and glucagon in the in vivo models. This time, continuous monitoring of insulin and glucagon was performed by running the QIRT-ELISA platform on a rat while undergoing a GTT, as opposed to obtaining discrete timepoints and measuring only those levels of insulin and glucagon. The continuous GTT experiments were also cross-validated by conventional ELISA measurements taken at discrete timepoints to establish a trend in insulin and glucagon levels over time with the measured QIRT-ELISA signal gains.

These sets of experiments were performed thrice – twice on rats that were under anesthesia, and once on a conscious rat. All rats were fasted overnight prior to an intraperitoneal injection of glucose to start the GTT. On the day of the experiment, the rat under test was connected to a peristaltic pump, and blood was withdrawn from a jugular catheter at a flow rate of 15 μ l/min and injected into the QIRT-ELISA device as shown in Figure 5. a. The reagents were injected into the reagent inlet of the micromixer module at the same time using a syringe pump. The optical system analysed the levels of insulin and glucagon in whole blood for a duration of 30 minutes. Blood samples for conventional ELISA were collected from the same tubing as the jugular catheter through a valve at discrete timepoints, namely, zero, 5, 10, and 30-minutes post-injection.

Once again, concentration results for insulin and glucagon measured by conventional ELISA show good correlation with fluorescence measurements obtained from the QIRT-ELISA platform. Figure 5. b. i and ii show concentration measurements from conventional ELISA and fluorescence measurements from QIRT-ELISA over time for a period of 30 minutes for the two GTT experiments that were performed on rats under anesthesia. Figure 5. c. shows the same for the continuous experiment performed on the conscious rat.

The results show that the QIRT-ELISA system detects the levels of both targets in the blood samples in good correlation with conventional ELISA. The QIRT-ELISA system offers enhanced time resolution compared to the conventional ELISA by providing insulin and glucose levels in 60 to 80-second time intervals during the experimental time. The QIRT-ELISA provided at least 22 measurements while the conventional ELISA measurements were limited to 3 data points in a duration of 30 minutes experimental time.

These rapid real-time concentration measurements cannot be done by ELISA since not only the sampling can be restrictive, but also conventional ELISA is time-consuming and labour-intensive. Additionally, the correlation between ELISA results and QIRT-ELISA trends show physiologically relevant phenomena taking place in glucose homeostasis, as reflected in the differences between insulin and glucagon dynamic profiles over time between anesthetized rats and the conscious rat.

a**b**

■ ELISA-Insulin ■ ELISA-Glucagon
● QIRT ELISA - Insulin ● QIRT ELISA - Glucagon

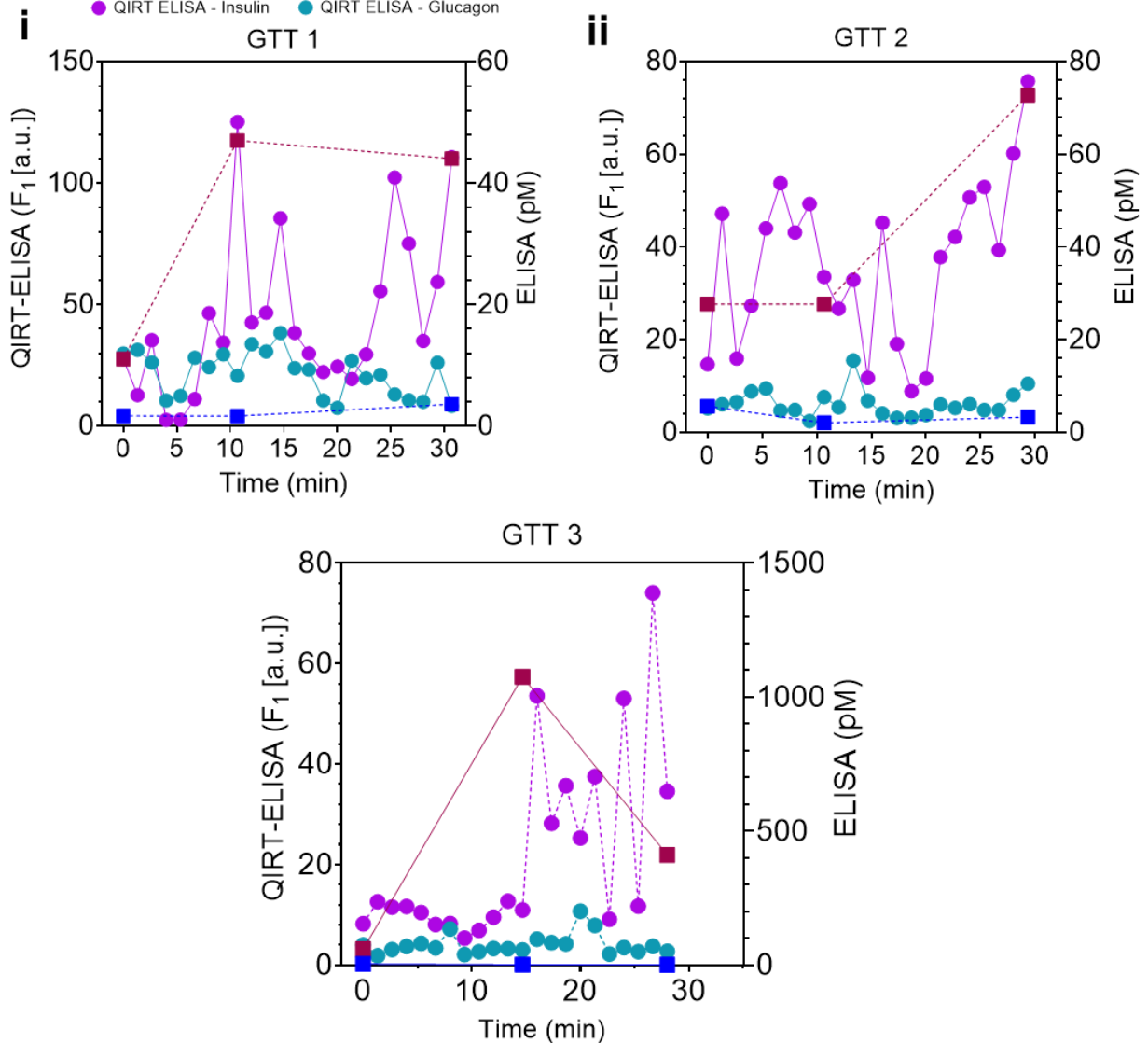
**c**

Figure 5. In Vivo Continuous, Multiplexed Measurement of Insulin and Glucagon on Fully Integrated QIRT-ELISA Platform.

a) Schematic of continuous GTT experimental procedure showing peristaltic pump withdrawing blood continuously from the jugular catheter of rat undergoing GTT. Rats were fasted overnight prior to intraperitoneal injection with glucose. Blood from the jugular catheter was injected into QIRT-ELISA device continuously, while reagents were injected simultaneously with a syringe pump. Blood samples for conventional ELISA were collected from a valve on the same jugular catheter line at relevant time points (0, 50, 10, and 30-minutes post-intraperitoneal injection). Each GTT experiment ran for a duration of 30 minutes. Fluorescence signals for insulin and glucagon were collected by the optofluidic system continuously and compared to the three main time points sampled for the conventional ELISA. Each data point in the QIRT-ELISA graphs show the average F1 (area under the curve) values for a scan with a duration of 60-80 seconds. b) i and ii depict GTT 1 and 2 experiment done with rats under anesthesia. c) depicts GTT 3 performed on a conscious rat under the same experimental conditions. S.S, H.A, and N.B performed all experiments together. H.A analysed collected data using app, and N.B performed conventional ELISA on blood collected at timepoints.

2.4 Conclusions and Future Directions

This is the first report of a quantum-dot mediated real-time immunoassay microfluidic platform for continuous monitoring of insulin and glucagon in vivo and in a multiplexed manner. The incorporation of quantum dots in the bead-based sensing mechanism facilitates multiplexed monitoring, allows further system miniaturization, ease-of-use, and potential automation of real-time and continuous measurements of insulin and glucagon in whole blood.

The enhanced sensitivity of the assays conferred again by the use of quantum dots as detection reporters, means that the system is capable of detecting these biomarkers at sub-pico molar concentrations which are reflected in euglycemic and hypoglycemic states – LODs of 27.4 pM and 1 pM are reported for insulin and glucagon respectively. Furthermore, the assessment of the system in vivo with both discrete and continuous monitoring of insulin and glucagon in healthy rats undergoing a glucose tolerance test, and subsequent correlation of measurements with a conventional ELISA confirms the functional validity of the system and its improved performance – most notably, its temporal resolution, providing signals for fluctuating insulin and glucagon levels in sub-minute timescales.

This significant decrease in assay time is particularly important during in vivo measurements, as physiological changes in insulin and glucagon during a glucose load as such in the glucose tolerance test occur in timescales that conventional ELISA sampling methods cannot match. Traditional blood collection and ELISA protocols take too long to provide meaningful insights into the dynamics of these hormones, pre-and post-prandially, in relation to one another and blood glucose. The QIRT-ELISA system offers continuous, real-time measurements, thereby improving the time resolution of hormone measurements in vivo, and allowing further studies to be done on the kinetics of hormonal glucose homeostasis in response to direct glucose stimuli, which could lead to the identification of novel indicators of metabolic diseases and physiological control mechanisms.

Future directions involve adapting the platform to monitor insulin, C-peptide^{3,5,6,26,38,49}, glucagon, and glucose in a real-time, continuous, multiplexed manner. This will be followed by further in vivo validations in conscious preclinical rat models of obesity, prediabetes, and diabetes, facilitating deeper understanding of hormone dynamics during the progression of diabetes and insight into personalized clinical management and prevention.

2.5 Materials and Methods

Materials

Chemicals and kits were purchased from Sigma Aldrich and Thermofisher respectively, unless otherwise mentioned. Carboxylate functionalized magnetic 15 μ m microspheres were purchased from Spherotech Inc. Matched pair antibodies against insulin and glucagon were purchased from Mercodia. Insulin antigen was obtained from Toronto Biosciences, and glucagon antigen was purchased from Prospec Bio. The ELISA kits for insulin and glucagon were purchased from Mercodia.

Microbead Functionalization

Carboxylate modified beads were based on the manufacturer's protocol for functionalizing 15 μ m beads with immunoglobulins. Briefly, beads were washed on a magnetic rack and incubated for 30 minutes in MES buffer (pH 5.2) with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to activate carboxylate groups on the surface. Beads were then incubated with capture antibodies for 4 hours in pH 7.5 MES buffer, before being quenched for 30 minutes in a 30mM glycine solution in BSA to terminate coupling reaction. Beads were finally washed with 1X PBS + 0.05% Tween-20 (1X PBST) and resuspended in 1X PBS + 0.01% Tween-20 + 0.05% BSA (1X PBSBT). Glucagon capture beads were aliquoted and stored at -20 C and insulin capture beads were stored at 4 C.

Benchtop BQI

Detection antibodies against glucagon were biotinylated as per protocol outlined in the Pierce Antibody Biotinylation kit for immunoprecipitation, and detection antibodies against insulin were conjugated with QD655 with a SiteClick kit. Biotinylated glucagon detection antibodies were aliquoted and stored in -20 C and Qdot conjugated insulin detection antibodies were stored in 4 C. Prior to their use in assay experiment, glucagon detection antibodies were incubated with 50 nM Streptavidin-Qdot 605 to form a detection antibody-Qdot complex for glucagon detection. To complete the immunoassay complex, and validate the performance of the respective matched pair antibodies, capture beads and detection antibodies were incubated on the benchtop with increasing concentrations of target antigen for 90 minutes, before being washed on the mag rack with 1X PBST and analyzed by a flow cytometer (CytoFlex, Beckman Coulter). Calibration curves for each target were generated after benchtop incubations with capture beads and detection antibodies, both in 1X PBSBT buffer and in whole blood.

On-chip BQI

Once these were established, the immunoassay was incorporated into the microfluidic micromixer module to validate the assay's performance on chip. Devices were fabricated and degassed as outlined in the device fabrication protocol. A syringe filled with assay reagents (capture beads and detection antibodies) and another filled with whole blood spiked with target antigen to a final volume of 200 μ L were fitted into a syringe pump and flown through the degassed microfluidic micromixer module to perform the previously described benchtop immunoassay in minutes. A flow rate of 15 μ L/min was used and the solution collected in the outlet of the micromixer module was then washed with 1X PBST before subjecting it to flow cytometry analysis (CytoFlex, Beckman-Coulter).

QIRT-ELISA Device Fabrication

First, the microchannels were designed on AutoCAD and KLayout software. The masks needed for the two-layer photolithography of the micromixer and the detection module were printed

(CAD/Art Services). An SU-8 3050 (MicroChem) layer of 45 μ m was coated on a Silicon wafer (University Wafer) for the first layer, which was the main channel of the micromixer. Then the same height of SU-8 3050 was coated on the developed first layer to pattern the herringbone structures. Both steps were fabricated using a mask aligner with a UV light (MA6, SUSS MicroTec). The process for the master mold of the washing (DLD) module followed a one-layer fabrication protocol. The channel height for the devices (also including the micropost) was 50 μ m, and it was carried out using a UV Direct Write photolithography (MLA 150, Heidelberg Instruments). The detection module was fabricated through the conventional photolithography method as well. The first layer, which was for the main channel and has a thickness of 250 μ m, was done using SU-8 2100 (MicroChem). The second layer, which included the fabrication of optical fiber grooves, followed a similar approach. The microfluidic devices were fabricated following a standard polydimethylsiloxane (PDMS)-based method. The casted PDMS on the master molds were peeled off and bonded to glass slides (Electron Microscopy Sciences) using a plasma etcher (Tergeo Plasma Cleaner, Pie Scientific) with the following setting. (15W, 30sec, 10sccm, air). Prior to the experiment, all the devices were conditioned and degassed with a non-ionic surfactant, Pluronic F108 (Sigma Aldrich) overnight.

Continuous In Vitro Measurement of Insulin and Glucagon

QIRT-ELISA devices were fabricated and degassed as outlined in the device fabrication protocol. A syringe filled with a larger volume (~1 ml) of assay reagents (capture beads and detection antibodies) and another (sample syringe) filled with whole blood spiked with target antigens to a final volume of 200 μ L were fitted into a syringe pump and flown through the degassed QIRT-ELISA platform. Each sample syringe contained a distinct test condition. Test conditions were as follows: 1000 pM insulin with 10 pM glucagon, 400 pM insulin with 40 pM glucagon, 200 pM insulin with 80 pM glucagon, and 100 pM insulin with 100 pM glucagon. Between each concentration, a wash sample, i.e. a whole blood sample without any spiked insulin or glucagon was processed. Sample syringes were each run until enough volume to obtain signals for three to four minutes. The sample syringe was then switched out to the next consecutive sample, while keeping the reagents syringe unchanged. A flow rate of 15 μ L/min was used for injecting reagents and samples into the micromixer module, and a flow rate of 75 μ L/min was used as the DLD sheath flow rate. Samples were run until sufficient peaks were obtained for both targets.

Glucose Tolerance Test

All animal procedures were approved by the Animal Research Ethics Board of McMaster University. Male Wistar rats (Charles River) at 9-12 weeks of age were fasted overnight for 16 hours. The next morning, blood was drawn before (i.e., time = 0) or 15 minutes or 60 minutes after oral gavage of 8g/kg D-glucose (Sigma). For blood collection, rats were anesthetized using isoflurane, and blood was drawn from the aorta and placed into EDTA-coated tubes (BD). For insulin and glucagon ELISAs (Mercodia: insulin 10-1250-01, glucagon 10-1281-01), a portion of blood was centrifuged at 10,000 g for 10 minutes at 4 degrees to separate and collect plasma, and then subsequently stored at -80 degrees until needed.

Discrete GTT Experiments

QIRT-ELISA devices were fabricated and degassed as outlined in the device fabrication protocol. A syringe filled with assay reagents (capture beads and detection antibodies) and another filled with collected whole blood samples from rats undergoing GTT were attached to a syringe pump and connected to QIRT-ELISA devices. The DLD sheath flow was prepared on a second syringe pump. Sample syringes were each run until enough volume to obtain signals for three to four minutes. The sample syringe was then switched out to the next consecutive sample, while keeping

the reagents syringe unchanged. A flow rate of 15 $\mu\text{L}/\text{min}$ was used for injecting reagents and samples into the micromixer module, and a flow rate of 75 $\mu\text{L}/\text{min}$ was used as the DLD sheath flow rate. Samples were run until sufficient peaks were obtained for both targets.

Continuous GTT Experiments

QIRT-ELISA devices were fabricated and degassed as outlined in the device fabrication protocol. A syringe filled with a larger volume (~ 1 ml) of assay reagents (capture beads and detection antibodies) was prepared on its own syringe pump. Rats were fasted overnight prior to receiving hydration and an intraperitoneal glucose injection at 5 g/kg to begin the GTT. Rats with jugular catheters were employed for use in continuous monitoring experiments. Blood was withdrawn from each rat through the jugular catheter using a peristaltic pump at a flow rate of 15 $\mu\text{L}/\text{min}$ and injected into the QIRT-ELISA platform. Assay reagents were injected from their syringe pump at the same flow rate, and DLD sheath was run at a flow rate of 75 $\mu\text{L}/\text{min}$. Samples for conventional ELISA were collected from a valve on the peristaltic pump tubing at discrete timepoints (0, 5, 10, and 30-minutes post injection). Rats were either under ketamine anesthesia or conscious depending on the experimental condition. Optofluidic scans were collected every 60 – 80 seconds for a duration of 30 minutes for each GTT experiment.

App for Continuous Experiment Data Analysis

To facilitate the automated and rapid data analysis for the continuous experiment, a custom app was developed. In this app, the data files from the oscilloscope are exported to the computer and imported to the app. In the first step, the app processes the data files, and extracts information for both insulin and glucagon. In the following step, the app can smooth the signal for either QDot 605 or QDot 655 channels using the Savitzky-Golay filter. Next, a package that utilizes an iterative polynomial regression algorithm is used to estimate and remove the undesired baseline from the signals, making it possible to calculate the area under the peaks. In the next stage, the app finds the peaks in the signals if the condition of exceeding 10% of the maximum peak amplitude is satisfied in the signal peaks and integrates the area under each peak. Finally, the average area under the peaks for each scan is translated into the normalized signal readout, and using the derived calibration curves, the app calculates the concentration from each scan. The developed code in the app can be found online at <https://github.com/hesam-abouali/2B-QIRT-ELISA> or the app can directly be used from the Streamlit web apps repository at <https://2b-qirt-elisa.streamlit.app>.

Statistical Analysis

All the statistical analysis was conducted using GraphPad Prism 10. Each experiment was carried out in three parallel replicates. All data are demonstrated as mean $\pm$ SD of three experimental replicates or three spectral scans. The calibration curves were calculated using the sigmoidal 4PL nonlinear regression model. The LOD is defined as the minimum target concentration that can be detected by every benchtop or on-chip BQI and calculated by $\text{LOD} = 3.3 \times \text{SD} / S$ where SD is the standard deviation and S is the slope of the calibration curve. LOQs were calculated by interpolating the mean fluorescence signal of blank plus ten times SD.

2.6 Supplementary Information

Figure S1. Benchtop Validation of Insulin and Glucagon BQI in Buffer

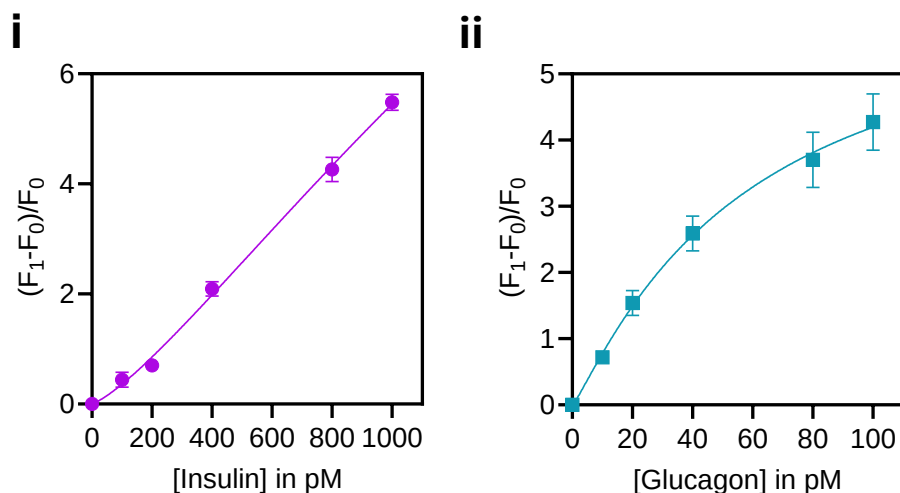


Figure S1. Benchtop BQIs in buffer. i shows the results for the insulin BQI in the buffer and ii shows the results for the glucagon BQI in the buffer. The purpose of these singular-analyte BQIs in the buffer was to confirm that the capture and detection antibodies are suitable for assay development prior to BQIs in whole blood. The data shows the mean $\pm$ standard deviation (SD) of three replicates. F0 is the fluorescent signal for the samples with no target analyte and F1 is the fluorescent signal for each sample.

Figure S2. Benchtop Validation of Insulin and Glucagon BQI in Whole Blood

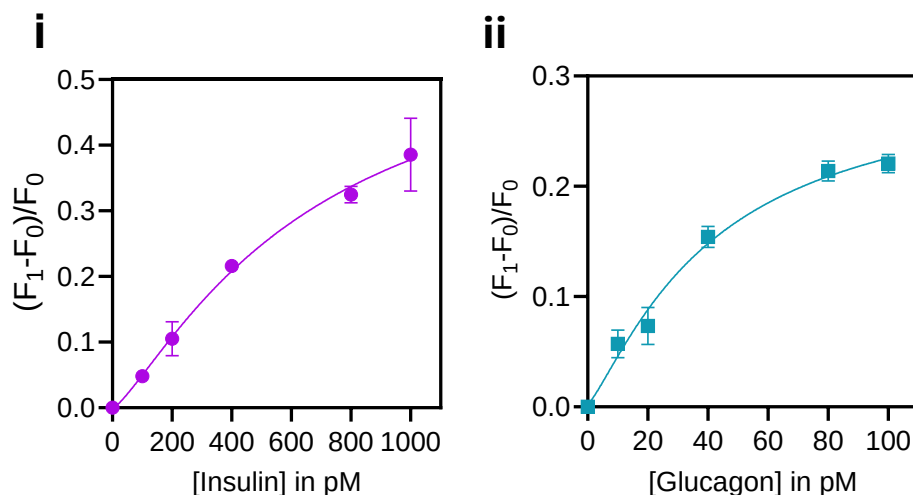


Figure S2. Benchtop BQIs in whole blood. i shows the results for the insulin BQI in the whole blood and ii shows the results for the glucagon BQI in whole blood. The purpose of these singular-analyte BQIs was to confirm that the capture and detection antibodies are suitable for assay development in complex biological matrices. The data shows the mean $\pm$ standard deviation (SD) of three replicates. F0 is the fluorescent signal for the samples with no target analyte and F1 is the fluorescent signal for each sample.

Chapter 3

QIRT-ELISA For Continuous Monitoring in Ex Vivo Lung Perfusion

3.1 Abstract

Continuous monitoring of biomarkers like glucose and lactate, particularly in the context of lung transplantation, is crucial as these metabolites serve as critical indicators of organ health, especially during Ex Vivo Lung Perfusion (EVLP). EVLP, employing the Toronto protocol, maintains lung viability through precise biochemical and inflammatory monitoring, however, these readings are only obtained on an hourly basis, and cannot fully capture the rapid changes in biochemistry of these organs when they undergo physiological decline. In collaboration Dr. Andrew. T. Sage, at the Toronto Transplant Program at the University Health Network, we address this, with the development of a bead-based quantum dot mediated aptamer-derived assay integrated with microfluidic modules developed to reduce assay incubation time, washing, and optical analysis, which facilitates continuous, multiplexed, and real-time monitoring of glucose and lactate. In addition to the Toronto Lung Score, this can significantly improve outcomes of patients post-transplantation by enabling timely interventions to address organ distress and reduce the risk of post-graft dysfunction (PGD).

3.2 Introduction

Lung transplantation stands as a crucial therapeutic modality, offering a lifeline to patients with end-stage pulmonary diseases^{67–69}. While lung transplantation has significantly advanced since its inception, one persistent challenge is a stark shortage of viable donor organs for transplantation. Organs donated post-circulatory death, and after brain death, are lifesaving treatments for patients requiring organ transplantations, however, particularly with lungs, nearly 80% of donor organs are disqualified for transplantation due to injury in the respiratory tract, or vasculature, and paucity of gas exchange^{60,67}.

Glucose is a monosaccharide that is an important source of energy for cells, and lactate is a monosaccharide that is produced as a byproduct of anaerobic cellular metabolism and is prevalent in inflammatory physiological conditions^{1,70–73}. These small molecules are particularly important as biomarkers of lung injury and viability during Ex Vivo Lung Perfusion (EVLP)^{10,62,64,66,67,69,74,75}.

EVLP emerged as a modality of increasing the donor pool, by assessing donor lungs and reviving high-risk donor lungs on the margin of viability to being acceptable in transplant criteria. EVLP also has the potential to treat damaged lungs in isolation ex vivo and improve their survival post-transplantation^{10,69,76,77}. The Toronto protocol of EVLP is a normothermic protocol that maintains lung homeostasis via low tidal volume ventilation, microcirculation, and perfusion using an acellular lung perfusate solution with optimized colloidal pressure and nutrients flown through the organ at a low flow rate using a centrifuge pump^{10,76}. This is depicted in Figure 6. a. The protocol combined with tools for monitoring organ biochemistry and inflammatory profiles has led to the development of the Toronto Lung Score which further assists in categorizing lungs according to transplant inclusion criteria as well as their potential in leading to post-graft dysfunction (PGD)^{62,63}.

The Toronto Lung Score is a profound improvement on EVLP in improving patients' outcomes post-transplantation, particularly with the inclusion of inflammatory profile determination focusing on cytokines IL-6 and IL-8⁶². In conjunction with biochemical workups (pH, glucose, and lactate) on perfusate, IL-6 and IL-8 levels are assessed using conventional immunoaffinity-based methods on lung perfusate samples that are collected hourly for the whole duration of an EVLP run of up to 6 hours^{62,63,66,74}.

The assessment of lung perfusate leading to further categorization of lungs based on their preparedness for transplant and potential for PGD has shown that metabolic profiles of organs change as they undergo EVLP, and anti-inflammatory, or immunomodulatory therapies – particularly reflected in lactate accumulation and glucose consumption as they undergo these treatments⁶². The monitoring of glucose and lactate levels in lungs is demonstrably important, as it is evident that rapidly changing biochemical profiles indicate distress on the organ that can be rectified and allow the organ to recover to a desirable biochemical state with lower chances of developing PGD⁶².

Current timescales of biochemical monitoring of glucose and lactate are as short as 15 minutes, however, monitoring these analytes requires costly lab-based biochemical workup involving enzymatic-oxidase-based detection assays. Furthermore, measuring these analytes in real-time and continuously will allow far quicker response time to those organs that are imminently descending in quality and require immediate intervention, to bring them back to physiological equilibrium.

The critical shortage of donor organs underscores the urgency for innovative strategies in expanding the donor pool and optimizing organ utilization. PGD necessitates finely tuned treatment regimens to balance graft survival and mitigate adverse patient outcomes. Additionally, infectious complications pose significant risks, necessitating vigilant monitoring and prophylactic measures.

Timelines associated with lung transplantation are influenced by a myriad of factors, including organ availability, and recipient status. While urgency dictates expedited evaluation and transplantation for critically ill candidates, expediting initial evaluation to post-operative surveillance, is crucial in maximizing the likelihood of successful transplantation and long-term graft function. Thus, the development of a continuous monitoring platform for glucose and lactate, is therefore, a key technological advancement that would greatly benefit current EVLP protocols and provide far-reaching physiological insights into the clinical management of organ prior to and post transplant.

Extending the RT-ELISA platform developed for continuous glucose and insulin monitoring in live rats⁷, and the next generation QIRT-ELISA platform for quantum-dot mediated in vivo continuous monitoring of insulin and glucagon, to include aptamer-based detection of small molecules is a necessary and crucial next step. The development of the individual assays for lactate and glucose respectively, along with assessments of the specificities of each assay, and the multiplexing capability of the platform will be discussed in the following chapter.

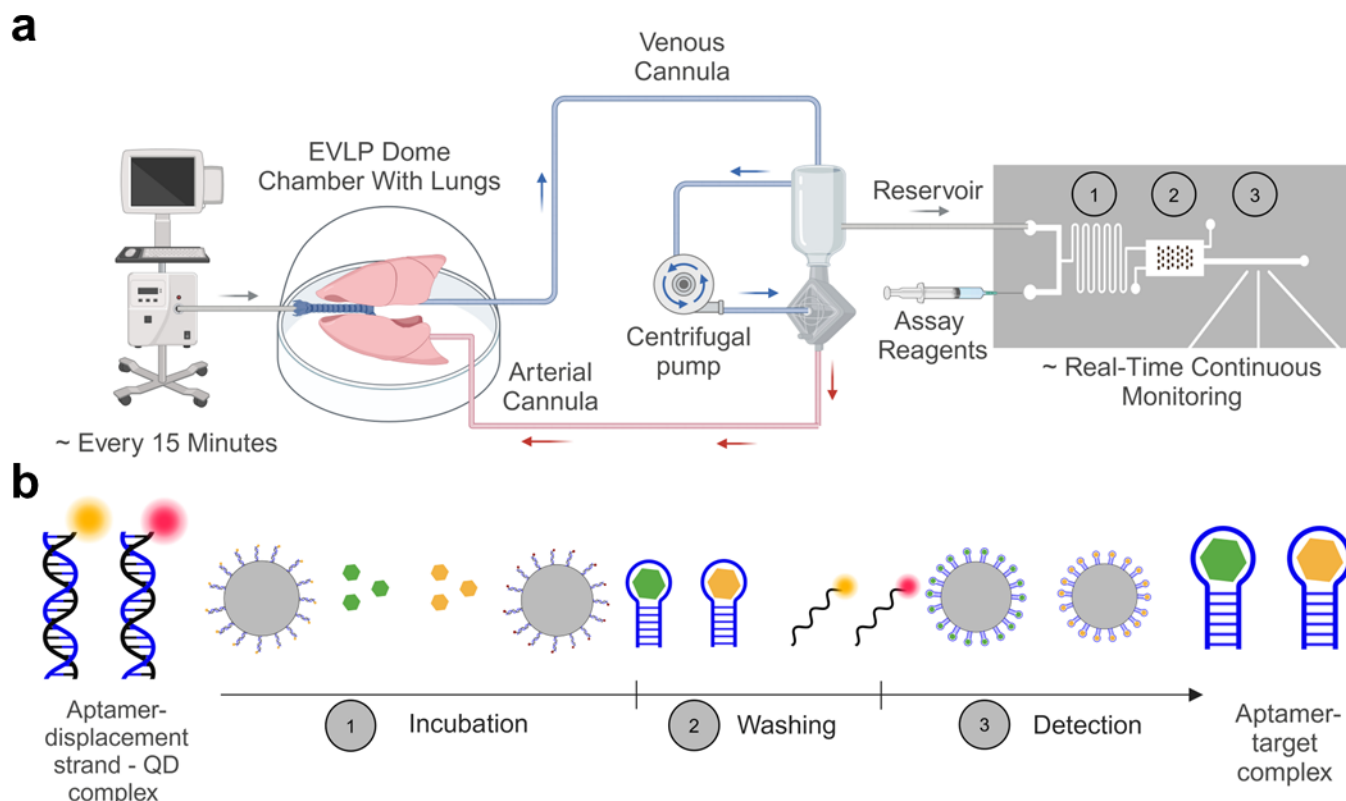


Figure 6. Toronto EVLP platform with QIRT-ELISA for Real-Time Continuous Monitoring of Glucose and Lactate.

a) Schematic of Toronto protocol for EVLP showing lungs in dome chamber with acellular perfusion, with the current gold-standard of measurements to determine the Toronto Lung Score (pH, glucose, lactate, IL-6, and IL-8) as well as a reservoir for collecting lung perfusate for continuous monitoring of glucose and lactate using the QIRT-ELISA platform. b) Depicts the signal-off aptamer-based quantum dot-mediated assay deployed for lactate and glucose monitoring, with demarcations of each step of the assay and its corresponding step in the microfluidic module workflow.

3.3 Results and Discussion

3.3.1 Bead-Based Quantum Dot Mediated Assays (BQA) for Glucose and Lactate Monitoring

Aptamers were used as biorecognition elements in the development of assays for glucose and lactate monitoring¹⁹. Glucose and lactate are small molecules, specifically monosaccharide metabolites used as cellular fuel during aerobic respiration and produced during anaerobic respiration respectively¹⁹. The measurements of glucose and lactate are conventionally performed enzymatically using glucose oxidase, lactate oxidase, and lactate dehydrogenase, respectively¹⁹. These methods are reliable, and provide concentration information in real-time, and have been developed to include continuous monitoring capabilities, however, the sensing mechanisms here typically involve interfering signals from hydrogen peroxide produced as a byproduct in these enzymatic reactions, which do not lend themselves as good candidates for multiplexing the detection of glucose and lactate. Aptamers ameliorate this issue as they are highly specific biorecognition probes that work on the basis of structural modifications in the presence or absence of their targets.

Aptamers designed and validated for the capture and detection of glucose and lactate were used to develop individual assays for these targets^{7,19}. Signal-off assays were conceptualized for the monitoring of glucose and lactate using quantum dots as detection beacons. The use of quantum dots further improved the multiplexing capability of the platform due to the unique optical and electrical properties of quantum dots whereby they can be excited by a single UV excitation laser and emit two discrete emission spectra.

Each aptamer in this assay concept was designed with its own displacement strand – the displacement strand was conjugated to a designated quantum dot based on the aptamer's specific target, and the combined aptamer-displacement strand-quantum dot complex was functionalized on the surface of microspheres coated with Streptavidin using Biotin-Streptavidin coupling. Beads, therefore, would emit a fluorescence wavelength corresponding to the quantum dot deployed on their surface, in the absence of target analyte. Incubation with target analytes would cause the removal of the displacement strand, and a subsequent loss of fluorescence signal directly proportional to target concentration. Figure 7. a. depicts this assay within the context of Ex Vivo Lung Perfusion.

The initial validation of each set of aptamer and displacement strand was performed using aptamers with an organic fluorescence probe and a displacement strand with a quencher. This initial testing was performed for both targets, to ensure functional integrity of the aptamers and displacement strands being employed, using conventional liquid-phase fluorescence intensity analysis using a plate reader. In this initial validation, optimized ratios of aptamer to displacement strand were used¹⁹, and a simple quantitative test of increasing emitted fluorescence intensity vs increasing target concentration was performed (Figure S1. i and ii)

Following this successful validation, the same sets of aptamers with their displacement strands were functionalized on beads and tested using traditional flow cytometry. This time, aptamer to displacement strand ratios were tuned to determine the best ratios for use in a solid-phase bead-based assay. The optimized ratios are as follows: 4:1 aptamer: displacement strand for lactate, and 1:1 aptamer: displacement strand for glucose (Figure S2. i and ii). Finally, these optimized ratios were employed in all following validation experiments using the quantum-dot mediated bead-based system.

Displacement strands functionalized with Qdots were hybridized with their respective aptamers before immobilization on the surface of beads. Qdot 525 was designated for use with glucose, and Qdot 605 was designated for use with lactate. These functionally charged beads were then incubated with lung perfusate spiked with their respective targets individually on the benchtop to derive individual benchtop calibration curves for each target, solidifying the functional integrity of the assay (Figure S3.i and ii).

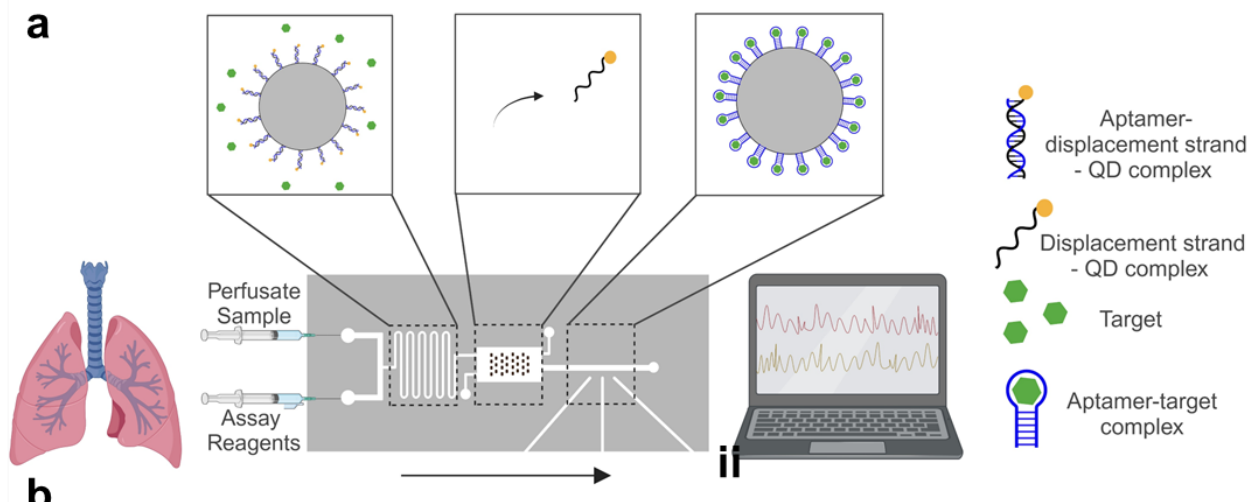
Figure 7. b i and ii depict calibration curves derived for lactate and glucose respectively using the bead-based quantum dot mediated assay system along with the integrated QIRT-ELISA platform. The mixing module depicted as module 1 in Figure 6 a. is where the assay incubation takes place, module 2, is where the washing of unbound displacement strands takes place, and module 3 is the optofluidic module where the final completed assay is interrogated with a UV laser to determine emitted fluorescence intensity from the remaining Qdot-displacement strands still hybridized to the surface of the beads.

Fluorescence intensity was measured as a function of peak amplitude current derived by measuring the area under the curve for a set of scans of beads passing through the optofluidic module. Fluorescence intensity of beads incubated with targets was normalized against the fluorescence intensity obtained for beads incubated with lung perfusate without any spiked targets. Absolute values of the normalized loss of signal upon target binding was reported as a

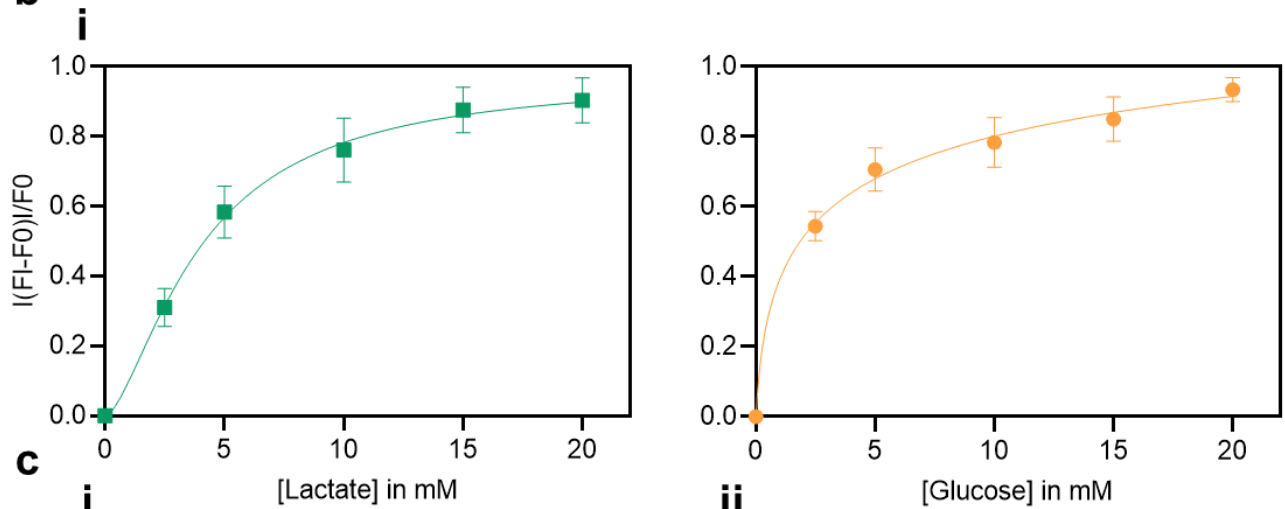
signal gain, and plotted vs increasing concentrations of target analyte to derive calibration curves for both targets. The range of concentrations used for both glucose and lactate was 2.5 mM to 20 mM, allowing for a large dynamic physiological range. R-squared values for individual targets along with their LODs and LOQs are depicted in Table 3 and are compared to the same derived for benchtop versions of the same assays showing an improvement in the performance of the assay when integrated with microfluidic modules.

Figure 2. c i and ii depict specificity experiments performed for each assay. These were done to ensure low-to-no cross reactivity between aptamers for their non-target analyte. In a similar fashion to the calibration curve experiments, the reagents for a particular target analyte were incubated with the highest concentration of the non-target analyte in the integrated QIRT-ELISA platform, and normalized signal gain absolute values were compared to the lowest condition of appropriate target analyte to determine if any cross-reactivity was observed. Almost no cross-reactivity is observed when target reagents are incubated with their non-target analytes in the integrated QIRT-ELISA system.

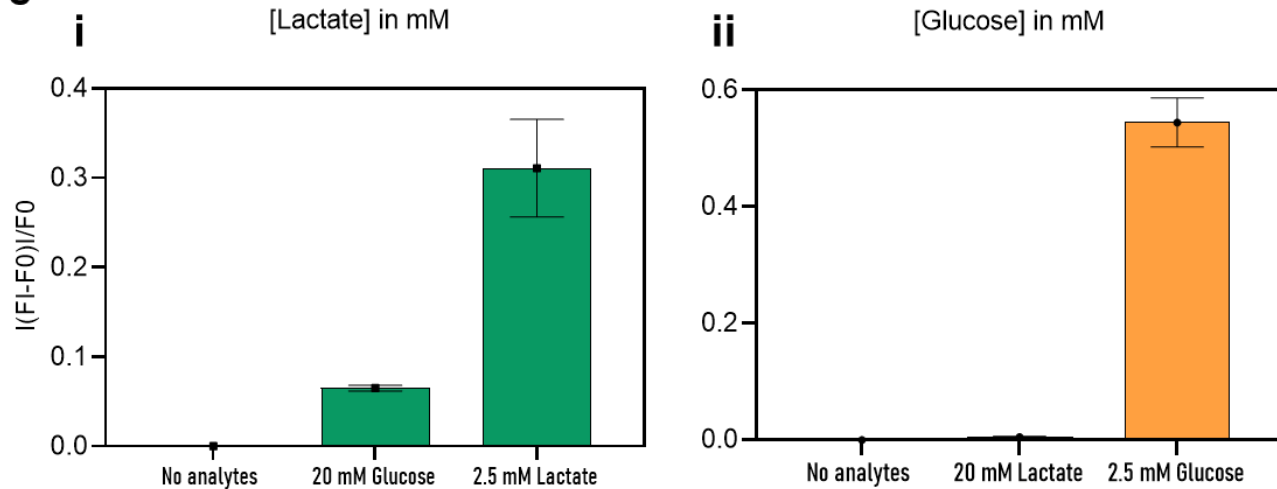
Figure 2 d depicts multiplexed measurements of lactate and glucose spiked in perfusate in four different combinations that correspond to physiologically relevant trends observed during EVLP. The trend of glucose consumption and lactate accumulation that occurs when organs are undergoing distress during EVLP shows glucose concentrations dropping rapidly, and lactate concentrations climbing before the two start to plateau. These were tested by combining varying concentrations of glucose and lactate and incubating them with their reagents simultaneously in a single QIRT-ELISA device and measuring peak amplitudes for both targets. The multiplexed measurements of both targets correlate well with their individual values demonstrated in the calibration curves and provide further confirmation of the reliability of the platform in measuring these targets in a complex biological matrix in a highly specific, sensitive, and temporally resolved manner.



b



c



d

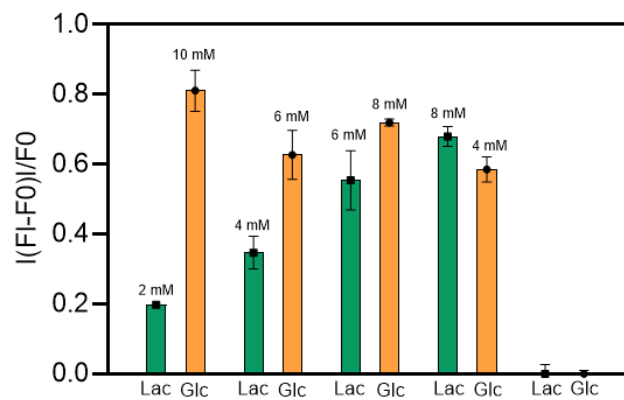


Figure 7. Validation of Bead-Based Quantum Dot Mediated Assays (BQA) for Lactate and Glucose Monitoring.

a) Assay schematic depicting ex vivo lung perfusion monitoring using beads functionalized with aptamer-displacement strand-quantum dot complexes for target capture and detection. Aptamers are hybridized with quantum dot modified displacement strands. These are then immobilized on the surface of microspheres via biotin-streptavidin coupling. Target incubation causes the removal of displacement strand and a subsequent loss of fluorescence signal from quantum dots which is measured as a loss of peak amplitude. Target concentration is thus measured as a function of loss of signal. b) i and ii are calibration curves of lactate and glucose derived from normalized signal gain from the absolute value of reduction of peak amplitude vs target concentration. Individual calibration curves were derived by spiking perfusate with known concentrations of target and measuring corresponding loss of fluorescence signal as reduction in peak amplitude. Concentrations of glucose and lactate range from 2.5 mM to 20 mM. Qdot 525 was designated to measure glucose concentrations and Qdot605 was used to measure lactate. Fluorescence intensity (FI) emitted by the quantum dots was determined by measuring the area under the curve in pA for each set of concentrations, and normalized to the fluorescence intensity measured when perfusate was not spiked with target solution (F0). Normalized values were plotted as signal gain by using their absolute values ($(FI-F0)/F0$). c) i and ii examine the specificity of lactate and glucose assays respectively. The normalized absolute value signal gain of highest non-target analyte concentration is compared to the lowest target analyte concentration, and zero analytes condition. d) Demonstrates the multiplexed measurements of glucose and lactate in physiologically relevant combinations of concentrations. Perfusate samples were spiked with four different combinations of glucose and lactate, namely, 10 mM and 2 mM, 8 mM and 6 mM, 4 mM and 8 mM, and 6 mM and 4 mM, and measured on the integrated QIRT-ELISA setup with discrete devices and measurements for each condition, using reagents for both analytes simultaneously. Normalized signal gain absolute values are plotted for each condition and a no analytes condition. Normalized signal gain absolute values are plotted in all graphs as a mean of three replicates $\pm$ their standard deviation. H.A and F.F developed and validated device and optofluidic components for QIRT-ELISA, and H.A developed app for data analysis. S.S designed and validated all assay experiments on QIRT-ELISA setup and performed all data analysis using app.

Benchtop BQA Statistical Analyses				On-Chip BQA Statistical Analyses			
Analyte	R-square	LOD [mM]	LOQ [mM]	Analyte	R-square	LOD [mM]	LOQ [mM]
Glucose	0.94	0.39	1.18	Glucose	0.97	0.05	0.15
Lactate	0.94	0.94	2.85	Lactate	0.97	0.43	1.33

Table 3. Statistical Analyses of Benchtop and On-Chip BQA.

3.4 Conclusions and Future Directions

This is the first report of a quantum-dot mediated aptamer-based microfluidic biosensing platform developed for monitoring of glucose and lactate in real-time and in a multiplexed manner. The incorporation of quantum dots in the bead-based sensing mechanism facilitates multiplexed

monitoring, allows further system miniaturization, ease-of-use, and potential automation of real-time and continuous measurements of glucose and lactate in-line in ex vivo lung perfusion systems.

The enhanced sensitivity of the assays conferred again by the use of quantum dots as detection reporters, means that the system is capable of detecting these biomarkers at sub-milli molar concentrations. Although the levels of these metabolites do not fall as low as the LODs achieved by the system, we report a large dynamic range of concentrations for glucose and lactate, which provides opportunities for broader applications of the assays in clinical monitoring settings.

Furthermore, the assessment of the system in vitro demonstrated the functional caliber of the individual assays, as evidenced by their individual calibration curves and the LODs obtained thereafter. The LODs achieved for glucose and lactate are 0.05 mM and 0.43 mM respectively, which are well below the ranges observed for lungs undergoing ex vivo perfusion^{61–66}. The high specificity observed for each assay further confirms their capability to selectively measure their respective targets and allows more sensitive measurements during multiplexing.

The initial validation of multiplexing also demonstrated that the assays were selective and sensitive enough to measure four different combinations of glucose and lactate showing small and large fluctuations that reflect physiological changes in lungs undergoing ex vivo perfusion when they begin to become unviable – notably, the assay was able to measure large fluctuations in lactate concentration – increasing from 2 mM to 6 mM, then 8 mM and plateauing to 4 mM, while following a reducing trend in glucose concentrations from 10 mM, to 8 mM, 4 mM, and back up to 6 mM. The temporal resolution of the system already having been established, all of these measurements further confirmed this, as they were recorded in sub-minute timescales. This is particularly useful in ex vivo perfusion monitoring as changes in the health status of ex vivo lungs can decline rapidly, causing lactate levels to rise sharply, and glucose to fall just as sharply. Even with frequent sampling, as frequent as 15-minute intervals, this time resolution is still insufficient in determining the exact timepoints at which lungs start to decline. The real-time, continuous measurements of the QIRT-ELISA system will be crucial in tackling this hurdle.

The future directions for this platform involve validating it further with clinical samples pulled from lungs undergoing ex vivo perfusion to confirm that the concentrations measured by the system correlate well with biochemical workup already performed on the samples. This will further validate the readiness of the system to be used in-line in ex vivo perfusion systems for continuous monitoring.

Following the validation of the platform with discrete clinical samples, the platform will be adapted for use in-line, and tested in a simulation system without lungs, but with spiked perfusate samples to ensure it's proper functioning in a clinical setup. Once this is confirmed, the system will be used with ex vivo human lungs undergoing perfusion for real-time continuous monitoring of glucose and lactate.

Similar to bead-based quantum dot mediated immunoassays (BQI) designed for insulin and glucagon, there is potential for incorporation of antibody-based assays for IL-6 and IL-8 monitoring in the same QIRT-ELISA platform that is monitoring glucose and lactate to allow inflammatory cytokines to be monitored alongside cellular metabolites during lung perfusion. This will facilitate further studies into the potential causes of decline in functional integrity of lungs during ex vivo perfusion and could help to derive treatment regimens for the prevention of such decline, or the recovery of declining lungs to restore functional integrity and prevent them being disqualified for use in transplantation.

3.5 Materials and Methods

Materials

All chemicals for preparation of buffers were purchased from Sigma Aldrich, Qdot conjugation ITK carboxyl kits were purchased from Thermofisher, aptamers were purchased from Integrated DNA Technologies., Streptavidin-coated 15 μ m polystyrene microspheres were purchased from Bangs Laboratories Inc. Streptavidin coated 10 μ m magnetic beads were purchased from SpheroTech Inc. Ex Vivo Lung Perfusate was donated as a gift from Dr. Andrew T. Sage. Aptamer and displacement strand sequences for lactate for initial plate reader and flow cytometry validations are as follows - /5BiosG/i6-FAMK/TTTTTTTCTCTCGACGACGAGTAGCGCGTATGAATGCTTTTCTATGGAGTCGTC and CGTCGTCGAGAG/3IABkFQ/. Aptamer and displacement strand sequences for glucose for initial plate reader and flow cytometry validations are as follows - /5BiosG/iCy3/CTCTCGGGACGACCGTGTGTGTTGCTCTGTAAACAGTGTCCATTGTCGTCCC and GGTCGTCCCAGAG/3DAb/. Aptamer and displacement strand sequences for lactate signal off Qdot assays are as follows - /5BiosG/TTTTTTTCTCTCGACGACGAGTAGCGCGTATGAATGCTTTTCTATGGAGTCGTC and CGTCGTCGAGAG/3AmMO/. Aptamer and displacement strand sequences for glucose signal off Qdot assays are as follows - /5BiosG/CTCTCGGGACGACCGTGTGTGTTGCTCTGTAAACAGTGTCCATTGTCGTCCC and GGTCGTCCCAGAG/3AmMO/. Lactate SELEX buffer recipe is as follows – 500 mM NaCl, 50 mM HEPES buffer, 10 mM MgCl₂, 10 mM KCl. Glucose SELEX buffer recipe is as follows – 1 M NaCl, 20 mM HEPES buffer, 20 mM MgCl₂, 20 mM KCl.

Plate Reader Validation of Aptamer and Displacement Strand Functionality

Aptamer, displacement strand, and SELEX buffer were added into wells at a 100 nM concentration using a final volume of 100 μ L, at a molar ratio of 1:10 for glucose and 1:4 for lactate. They were incubated at room temperature for 15 minutes to ensure hybridization and quenching, following which they were incubated with increasing concentrations of respective target analytes for 40 minutes. Wells were then analysed for emitted fluorescence intensity using a plate reader (BioTek, Agilent Technologies.) and appropriate wavelengths of excitation and emission for FAM (lactate) and Cy3 (glucose)

Flow Cytometry Optimization of Ratios of Aptamer to Displacement Strand for Bead-Based Assays

10 μ m Streptavidin-coated magnetic beads were incubated with varying ratios of hybridized aptamer to displacement strand at room temperature for 2 hours before washing and incubation with increasing concentrations of target analytes for 40 minutes. Target incubation was followed by final wash steps before analysis on a flow cytometer (NovoCyte, Agilent Technologies.).

Benchtop Bead-Based Quantum Dot Mediated Assays

Amino-modified displacement strands were conjugated with carboxyl-modified quantum dots as per protocol outlined in Thermofisher ITK carboxyl quantum dot kits. Displacement strands were then hybridized with their respective aptamers in optimized molar ratios with their respective SELEX buffer for 20 minutes at room temperature, before incubation with 15 μ m streptavidin coated polystyrene microspheres for 30 minutes at room temperature. Beads were washed and resuspended in SELEX buffer prior to target incubation. 2E4 beads were incubated with perfusate spiked with increasing concentrations of target analytes individually at room temperature for 40

minutes, followed by final washes and fluorescence emission measurement using flow cytometry (CytoFlex, Beckman Coulter).

QIRT-ELISA Device Fabrication

First, the microchannels were designed on AutoCAD and KLayout software. The masks needed for the two-layer photolithography of the micromixer and the detection module were printed (CAD/Art Services). An SU-8 3050 (MicroChem) layer of 45 μ m was coated on a Silicon wafer (University Wafer) for the first layer, which was the main channel of the micromixer. Then the same height of SU-8 3050 was coated on the developed first layer to pattern the herringbone structures. Both steps were fabricated using a mask aligner with a UV light (MA6, SUSS MicroTec). The process for the master mold of the washing (DLD) module followed a one-layer fabrication protocol. The channel height for the devices (also including the micropost) was 50 μ m, and it was carried out using a UV Direct Write photolithography (MLA 150, Heidelberg Instruments). The detection module was fabricated through the conventional photolithography method as well. The first layer, which was for the main channel and has a thickness of 250 μ m, was done using SU-8 2100 (MicroChem). The second layer, which included the fabrication of optical fiber grooves, followed a similar approach. The microfluidic devices were fabricated following a standard polydimethylsiloxane (PDMS)-based method. The casted PDMS on the master molds were peeled off and bonded to glass slides (Electron Microscopy Sciences) using a plasma etcher (Tergeo Plasma Cleaner, Pie Scientific) with the following setting. (15W, 30sec, 10sccm, air). Prior to the experiment, all the devices were conditioned and degassed with a non-ionic surfactant, Pluronic F108 (Sigma Aldrich) overnight.

On-Chip BQA – Individual Calibration Curves, Specificity, and Multiplexing

Beads were prepared as described as outlined in the benchtop section but resuspended in SELEX buffer + 20% sucrose solution as a device running buffer. Perfusate samples were spiked with known concentrations of target analytes. Microfluidic devices were prepared and degassed as outlined in the device fabrication protocol. A syringe was filled with ~600 μ L of reagents (~ 2E4 target capture beads) and running buffer (SELEX + 20% sucrose) and a second syringe (sample syringe) was filled with spiked perfusate corresponding to each experimental case. These were connected to a syringe pump and flown through an integrated QIRT-ELISA device at a flow rate of 15 μ L/min and a DLD sheath flow rate of 75 μ L/min. Each sample was run until enough scans had been obtained for data analysis of peak amplitude. Each scan had a duration of ~ 60 seconds and each sample was run for a minimum of 3 scans. For specificity experiments, reagent syringes were maintained but sample syringes were switched to those containing 20 mM of a non-target analyte, and the same data collection procedure was followed. For multiplexed experiments, reagents syringes contained capture beads for both targets (~1E4 beads each for a total of 2E4 beads) and sample syringes contained perfusate spiked with specific combinations of glucose and lactate corresponding to four different physiological cases. Data collection procedures were the same as described for individual calibration curves.

Data Analysis and Statistical Analysis

To facilitate the automated and rapid data analysis for the continuous experiment, a custom app was developed. In this app, the data files from the optofluidic scans are exported to the computer and imported to the app. In the first step, the app processes the data files, and extracts information for both lactate and glucose. In the following step, the app can smooth the signal for both quantum dot channels using the Savitzky-Golay filter. Next, a package that utilizes an iterative polynomial regression algorithm is used to estimate and remove the undesired baseline from the signals, making it possible to calculate the area under the peaks. In the next stage, the app finds the peaks in the signals if the condition of exceeding 10% of the maximum peak amplitude is satisfied in the

signal peaks and integrates the area under each peak. The developed code in the app can be found online at <https://github.com/hesam-abouali/2B-QIRT-ELISA> or the app can directly be used from the Streamlit web apps repository at <https://2b-qirt-elisa.streamlit.app>. All the statistical analysis was conducted using GraphPad Prism 10. Each experiment was carried out in three parallel replicates. All data are demonstrated as mean $\pm$ SD of three experimental replicates or three spectral scans. The calibration curves were calculated using the sigmoidal 4PL nonlinear regression model. The LOD is defined as the minimum target concentration that can be detected by every benchtop or on-chip BQI and calculated by $LOD = 3.3 \times SD / S$ where SD is the standard deviation and S is the slope of the calibration curve. LOQs were calculated by interpolating the mean fluorescence signal of blank plus ten times SD.

3.6 Supplementary Information

Figure S1. Plate Reader Validation of Aptamer and Displacement Strand Functionality

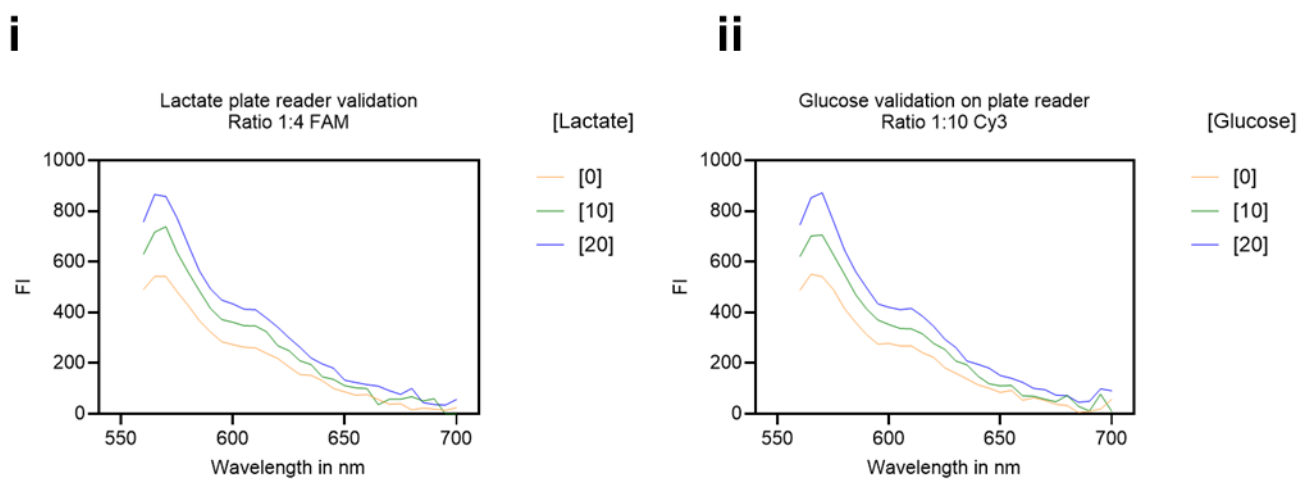


Figure S1.i and ii depict initial plate reader validation of aptamer and displacement strands' functional integrity for lactate and glucose respectively. Aptamer and displacement strands were incubated at their optimized ratios for fluorophore quenching and target binding. Spectral scanning was performed to determine the most appropriate excitation and emission spectra for FAM (lactate) and Cy3 (glucose) fluorophores. Fluorescence intensity values are plotted as a function of increasing target concentration across wavelengths from 500 to 700 nm for optimized ratios of aptamer to displacement strand.

Figure S2. Flow Cytometry Optimization of Ratios of Aptamer to Displacement Strand for Bead-Based Assays

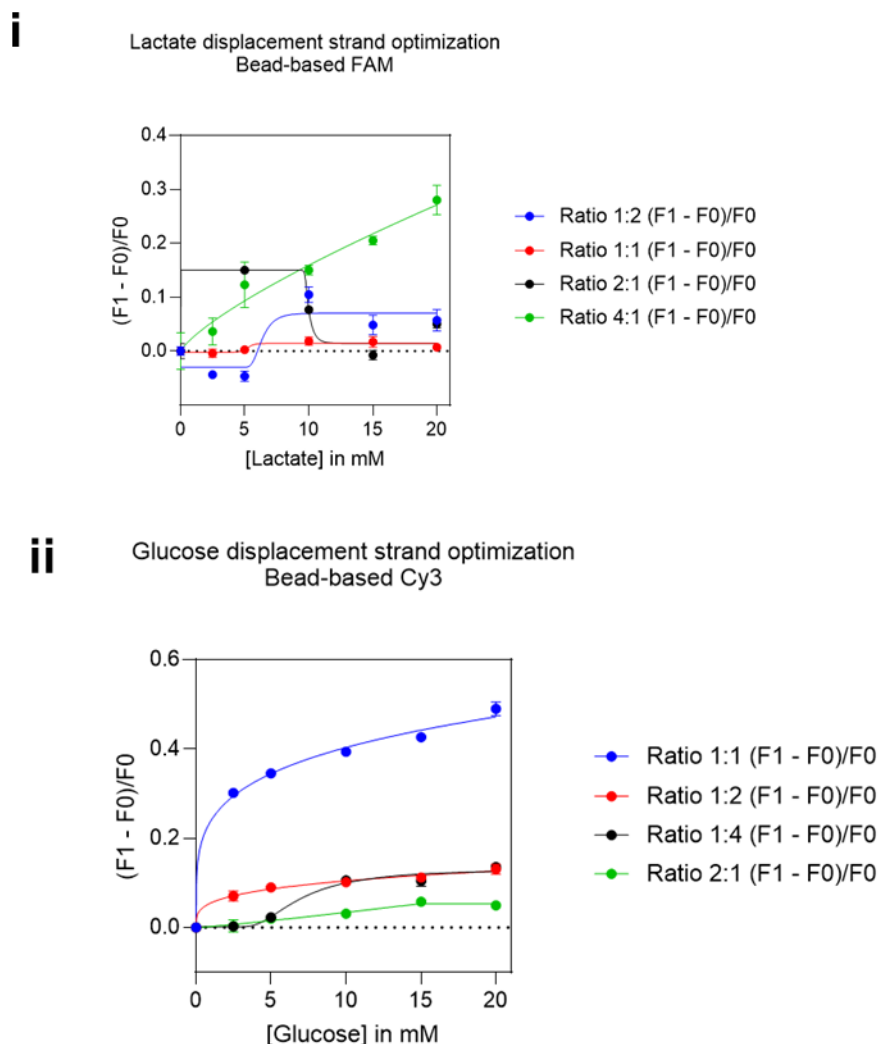


Figure S2.i and ii depict benchtop bead-based ratio optimizations of aptamer to displacement strand for lactate and glucose respectively showing normalized absolute value signal gains as a function of increasing target concentration. F1 is the fluorescence intensity from flow cytometry, and F0 is the fluorescence intensity of beads incubated with perfusate without any target analyte present. Data represented is the mean of three replicates along with their standard deviations.

Figure S3. Glucose and Lactate BQA Benchtop Validation in Perfusate

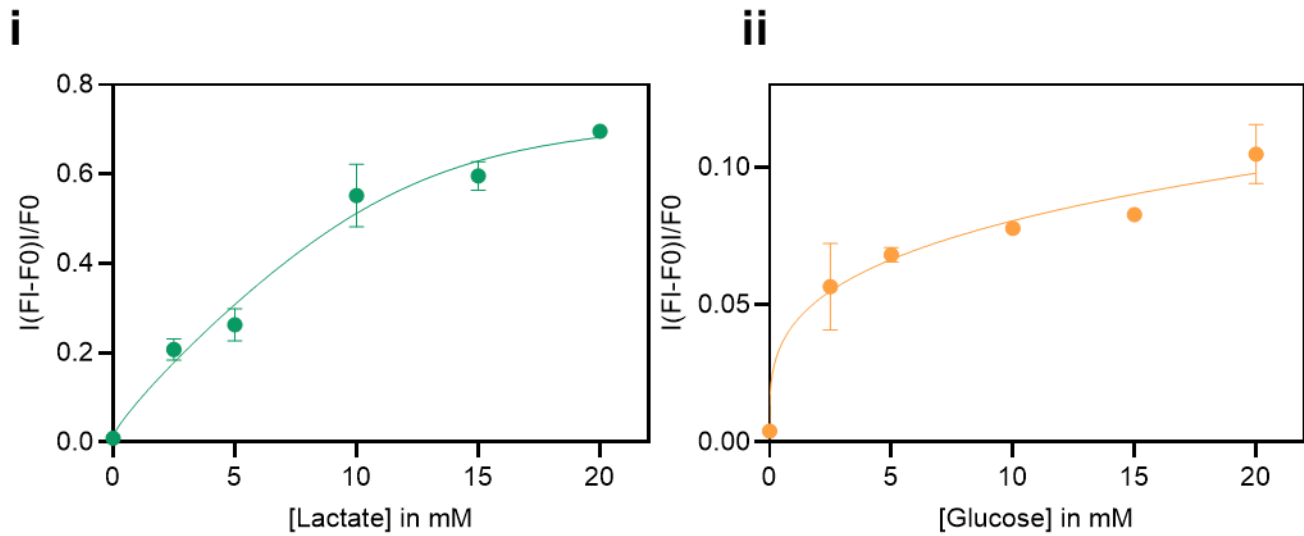


Figure S3.i and ii depict benchtop calibration curves derived for lactate and glucose respectively showing normalized absolute value signal gains as a function of increasing target concentration. FI is the fluorescence intensity from flow cytometry, and F0 is the fluorescence intensity of beads incubated with perfusate without any target analyte present. Data represented is the mean of three replicates along with their standard deviations.

Chapter 4

Conclusions and Future Directions

Continuous biomarker monitoring is pivotal in advancing precision medicine and improving patient outcomes through real-time insights into physiological and pathological states. Current methods for biomarker measurement such as enzyme-linked immunosorbent assays (ELISAs) although effective in detecting biomarkers, are limited by their inability to provide real-time data and require extensive sample processing. This delay impedes the availability of temporally resolved biochemical data, and the assessment of dynamics of hormones and metabolites and their function in homeostasis.

Here, we demonstrate the development of a platform for continuous biomarker monitoring with quantum dot mediated bead-based immunoaffinity and aptamer-based assays (BQI/A) incorporated in a real-time ELISA (RT-ELISA) platform, named Quantum Dot Integrated Real-Time ELISA (QIRT-ELISA) for continuous multiplexed monitoring of hormones insulin and glucagon, and small molecules, lactate, and glucose in heterogenous biological matrices.

The use of bead-based quantum dot mediated immunoassays (BQI) to enhance sensitivity, specificity, and temporal resolution has been validated by demonstrating continuous monitoring of insulin and glucagon in vivo in glucose tolerance tests in live rats in correlation with conventional ELISA measurements. The QIRT-ELISA platform could lead to critical insights being drawn about the dynamics of these hormones during glucose tolerance tests, which cannot be captured by conventional measures. These rapid real-time concentration measurements cannot be done by conventional ELISA – the volumes of sample required for ELISA for each timepoint would render real-time measurements unfeasible, especially since the sampling process itself involves drawing blood, which if performed at the frequencies needed for real-time measurements would be time-consuming and labour-intensive, stressful, and may not meet ethical criteria.

It is widely known that insulin responses to blood glucose occur in a biphasic manner⁷⁸, with increasing levels of glucagon-like-peptide-1^{79–81}, however, very little is known about the dynamics of insulin and glucagon during glucose tolerance tests, and this is largely due to limitations in technologies able to measure these hormones with sufficient sensitivity and temporal resolution. QIRT-ELISA will allow for tests to be performed in vivo that can help draw conclusions about the dynamics of insulin and glucagon, and their downstream consequences on glucose homeostasis in physiological and pathological states.

Individual and multiplexed measurements of glucose and lactate in ex vivo lung perfusate further validate the universal applicability of the system. Initial in vitro assessments of quantum dot mediated bead-based assays for glucose and lactate in the QIRT-ELISA platform are promising and demonstrate the readiness of the system for deployment in ex vivo lung perfusion, and potentially broader clinical utilities.

This system has shown promise in continuous monitoring of insulin and glucagon in diabetes management and small molecular biomarkers in ex vivo lung perfusion for transplantation assessment. By improving the temporal resolution and enabling real-time data acquisition, QIRT-ELISA addresses critical gaps in current monitoring technologies and holds potential for broad applications in clinical diagnostics and personalized medicine

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