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Nanostructured biosensor for tear glucose detection based on bioluminescence resonance energy transfer (BRET) mechanism.

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A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Chemical and Biochemical Engineering

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Abstract

Bioluminescence Resonance Energy Transfer (BRET), a sensitive, non-destructive and self-illuminated method, has been now commonly used to test protein interactions. Here, we describe a BRET sensor for non-invasively detecting glucose molecules. The sensor is made by the bioconjugation of quantum dots and recombinant protein. The recombinant protein contains the bacterial glucose binding protein (GBP) and a bioluminescent protein, *Renilla* luciferase (Rluc), used as the donor with the emission peak at 470 nm, which is able to excite the acceptor of BRET sensor made of cadmium tellurium quantum dots (CdTe QDs) with the emission peak at 570 nm. The distance between the BRET pair depends on the shape of GBP. In the presence of glucose, the conformational change of GBP leads to the enhanced BRET phenomenon due to the short distance between the BRET pair. To optimal the BRET sensor, the ratios of the acceptor (QDs) to the donor (GBP-Rluc), the bioconjugation process, and the performance of recombinant protein have been thoroughly investigated. The results indicate a linear relationship between luminescence intensity ratio and the concentration of glucose in the two ranges, from 0mM to 0.1mM and from 0.2mM to 0.8mM, respectively. The limit of detection (LOD) is calculated as 15nM. Meanwhile, tear glucose of animal model were measured by this designed sensor. This study demonstrates a proof-of-concept of a non-invasive measurement system for monitoring glucose.

Keywords

Bioluminescence resonance energy transfer (BRET), Luciferase, Glucose binding protein, Quantum dots, Tear glucose.

Co-Authorship Statement

Chapter 1 (General introduction), Chapter 2 (Background and literature review) and Chapter 3 (Experimental) were written by Denghuang zhan with some suggestions given by Dr. Jin Zhang. Chapter 4 and Chapter 5 include the studies for GBP-Rluc, CdTe quantum dots and the construction for the biosensor, which have been published or under preparation. Dr. Jin Zhang proposed and guided the design of the BRET sensor. Denghuang Zhan prepared the elements, constructed, optimized the BRET sensor system and tested the sensor with glucose and tear samples. Longyan Chen helped with designing the sequence of GBP-Rluc. Andrew Tse helped with the cytotoxicity test for the quantum dots. Professor James Melling and Michelle Dotzert helped with providing diabetic animals and Longyi Chen helped with the collection of tear samples. Other invaluable help for my thesis are named in the acknowledge section.

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List of Abbreviations

BCA	-	Bicinchoninic acid
BRET	-	Bioluminescence resonance energy transfer
BSA	-	Bovine serum albumin
CTZ	-	Coelenterazine
DHLA	-	Dihydrolipoic acid
DSP	-	Dithiobis succinimidylpropionate
DTT	-	Dithiothreitol
<i>E. coli</i>	-	<i>Escherichia coli</i>
EDC	-	1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride
FRET	-	Förster (or Fluorescent) resonance energy transfer
FTIR	-	Fourier transform infrared spectroscopy
GBP	-	Bacterial glucose binding protein
LB agar	-	Liquid Broth agar medium
NPs	-	Nanoparticles
NCs	-	Nanocrystals
PBS	-	Phosphate buffered saline
PDMS	-	Polydimethylsiloxane
QDs	-	Quantum dots
Rluc	-	<i>Renilla luciferase</i>
SMCC	-	Succinimidyl 4-(N- maleimidomethyl)cyclohexane-1-carboxylate
TGA	-	Thioglycolic acid
TRIS	-	3-methacryloxypropyl tris (trimethylsiloxy) silane

UV - Ultraviolet

Chapter 1

1 General Introduction, Hypothesis and Objective

1.1 Background of glucose sensor for monitoring diabetes

According to the information and data provided by World Health Organization (WHO), the number of people with diabetes will increase to 366 million by 2030 and at that time, the diabetes will be the 7th leading cause of death among all other kinds of disease [1]. Diabetes can be divided into 3 main types: type 1 diabetes, type 2 diabetes and gestational diabetes. Type 1 is a metabolic disorder disease while type 2 is much more related to the habits of life [2]. Insulin is very important to help maintain the glucose level steady however for the patients, either their pancreas does not produce enough insulin or the body cannot use them effectively [3]. To control glucose concentration level is important for diabetic patients. This is particularly important for patients especially those with type 1 diabetes mellitus, where glucose monitoring can reduce the risk of hypoglycemia, of cardiovascular disease with microvascular and macrovascular problems and of neurological abnormalities [4,5]. Carefully controlling blood sugar also prevents patient death because of losing consciousness and heart failure[6]. Traditionally, glucose measurements are performed by skin punctures several times regularly throughout the day. However this method is invasive and results in pain and inconvenience to the patient. It is also identified that the finger-prick method affects patient compliance with glucose measurements[7].

Many efforts have been attempted on continuous glucose monitoring devices, which are normally designed to detect blood glucose levels in real-time based on the measurements of interstitial fluid glucose concentrations. These devices enabled some improvements in the self-management of diabetes with reduced hypoglycemia in some extent [8], however, these benefits are only evident in patients who have recently used an insulin pump, which can be a more efficient. More generally, the devices still present a number of limitations such as the poor accuracy and reliability[9]. Thus the devices cannot replace the finger-stick monitoring fully but are well suited to complement the shortage. Also, these systems

are still invasive as they involve the use of cannula inserted in the subcutaneous tissue of the abdomen.

Recently, a number of technologies and devices have been proposed and developed for noninvasively estimating blood glucose concentration. Basically, these technologies focus on the changes in the chemical and physical tissues properties which caused by the presence of glucose molecules. These changes can be detected based on optical, chemical or electrical phenomena, for example via Raman spectroscopy, fluorescence technology [10], optical coherence tomography [11] and so on. The challenge still lies in the accuracy as well as the portability of the devices, their safety and their cost [12]. For noninvasive detection, body fluids such as urine [13-15], sweat [16-19] and tear [20-23] have been widely used as testing samples. However, the glucose level in the body fluids are much lower than that in blood for both healthy and diabetic people. In order to accurately test the extremely low amount of the glucose in body fluids, more sensitive methods have been developed based on fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET).

1.2 Nanostructured Optical biosensors

Types of optical sensors and applications

The development of semiconductors in the 1940s and '50s laid the foundation for the fast development of optical sensors which lead to cheaper, compact and efficient light-sensing devices. Nowadays, the applications of optical sensors can be found everywhere in daily life. Photodetectors were widely used in street lights, camera light meters, and traffic counters. Fiber optics made the sensitive equipment possible to work in electrically noisy environments. Sensors packaged with tiny integrated circuits enabled detectors more convenient to use. Optical sensors have improved efficiency of energy consuming and reliability of control systems at a more reasonable cost. There are different kinds of optical sensors and the most common types which we have been using in our real world applications consists three types[24]. The first is photoconductive devices which can be used to measure the resistance through converting a change of incident light into a change

of resistance. The second is photovoltaic cell or solar cell which can convert incident light into an output voltage. The third type is photodiodes which convert an amount of incident light into an output current.

General background of BRET and constructing BRET system

A very important advantage of optical detection methods is their noninvasive properties. A successful and well-known example of building fluorescent sensor is based on the modulation of Fluorescence resonance energy transfer (FRET) by Campbell in 2009 [25]. In fact, fluorescence resonance energy transfer (FRET) is widely used in bio imaging since it was discovered in 1950s [26]. This is the phenomenon of energy transfer between two light-sensitive chromophores. In this system, the donor chromophore which initially in its electronic excited stage, may transfer its energy to a nearby acceptor chromophore through non-radiative dipole–dipole coupling [27]. Though the FRET can be very sensitive with the distance change, it has limitations. One biggest problem FRET is facing is that the system requires for external illumination to initiate the fluorescence transfer. This process may lead to background noise and photo bleaching to the original signal. In order to diminish the drawbacks, Bioluminescence Resonance Energy Transfer (or BRET) has been developed to make the assay more reliable [28,29]. This technique uses a bioluminescent luciferase to be the light source. As it is very sensitive to the distance change, BRET has been widely used in the area both *in vitro* and *in vivo* imaging and biosensor systems [30-32]. Fig.1.1 shows the mechanism of BRET.

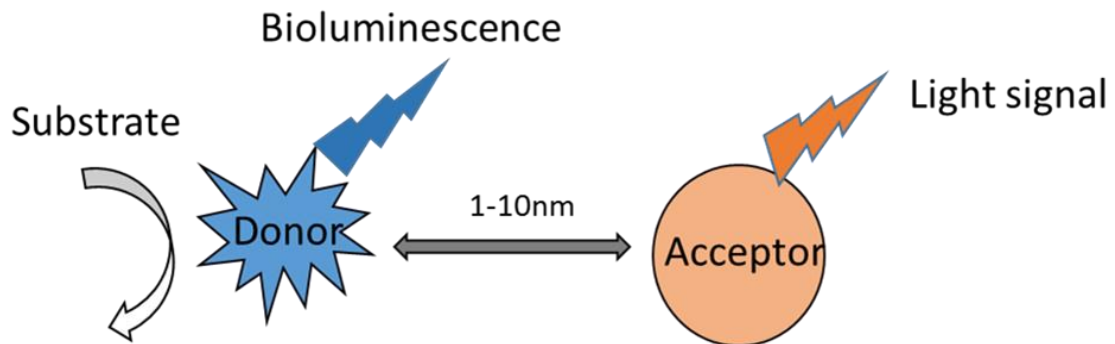


Figure 1. 1: The mechanism of BRET

FRET and BRET are both based on resonance energy transfer (RET). The efficiency E , is defined to represent the quantum yield of the energy transfer transition, which depends on 3 main physical parameters grouped as follows: The distance between the donor and the acceptor should be very close, generally within the range of 10 nm, the spectral of the donor emission spectrum should be able to cover the acceptor absorption spectrum and the donor emission dipole moment and the acceptor absorption dipole moment are relatively oriented [31]. The efficiency E can be calculated by using Equation 1.1.

$$E = \frac{R_0^6}{R_0^6 + r^6}, \quad \text{Eq. 1.1}$$

Where r is the separation donor-acceptor distance and R_0 is the Förster distance of this pair of donor and acceptor.

Furthermore, the Förster distance, R_0 , is given by equation 1.2:

$$R_0 = 0.21 [\kappa^2 Q_D n^{-4} J(\lambda)]^{1/6} \quad \text{Eq. 1.2}$$

Where $J(\lambda)$ is the spectral overlap between donor emission and acceptor absorption, Q_D the quantum yield of the donor, n the refractive index of the medium, and κ^2 an orientation factor, which is related to the relative orientation of the donor emission and acceptor absorption dipole moments. The orientation factor κ^2 is one of the most important effectors to the changes in energy transfer efficiency in both FRET- and BRET-sensors. However, unlike distance, it cannot be controlled easily. Energy transfer efficiency, E , according to equation 1.2, not only depends on the distance between the chromophores, but it is also dependent on the quantum yield of the donor (Q_D).

A key difference between fluorescence and bioluminescence is the number of photons that are generated. Thousands of photons per second can be generated when a single

fluorophore is excited. However, the light output of bioluminescence is much lower because the generation of each photon is caused by oxidation of a new substrate molecule. Most recently, bioluminescence has been used as a reporter gene predominantly, both in cell-based assays and in small animal *in vivo* imaging researches[33]. The recent genetic modification created brighter and more stable luciferases, as well as the concomitant improvement in luciferase substrates. This development has substantially decreased the sensitivity gap between fluorescence and bioluminescence. As a result, the use of BRET-sensors is no longer limited to intracellular population-based assays, the use for imaging of single living cells can also be applied, and even emerged as an attractive sensor format for point-of-care diagnostics[34,35].

An important consideration when designing a BRET-sensor is the choice of the donor luciferase. Until recently, the most popular luciferases were firefly luciferase and *Renilla* luciferase [36]. Luciferases have been found to be important research tools over the last two decades due to its ability to emit light. Typically, the luciferase from *Renilla reniformis* (Rluc) is widely employed in the research area such as molecular biology, using as a reporter gene in cell culture experiments as well as small animal imaging [37]. To achieve this bioluminescence, this 36-kDa enzyme Rluc catalyzes its substrate coelenterazine (CTZ) with the help of molecular oxygen, resulting in the product coelenteramide, carbon dioxide, and the desired wavelength of light. The catalytic process is shown in Figure 1.2.

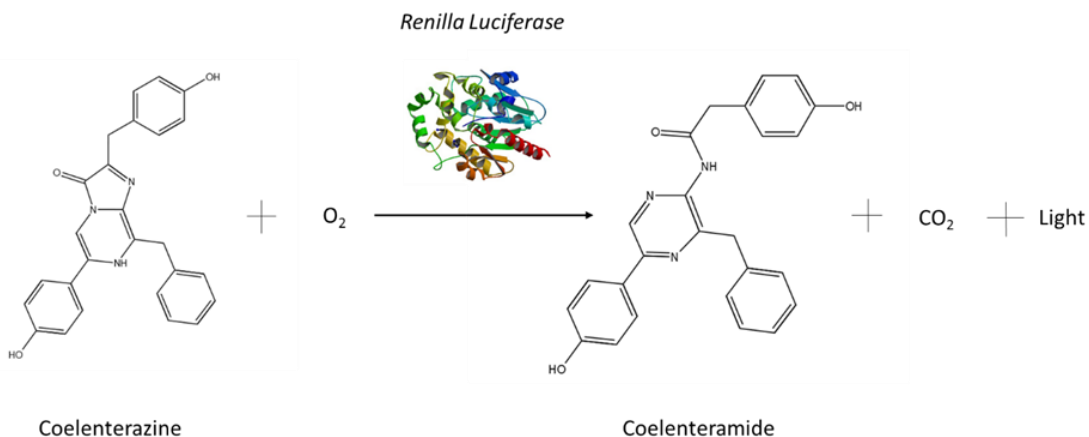


Figure 1. 2: *Renilla* Luciferase catalytic process

Since the successful cloning of the gene for Rluc, the gene of Rluc has been incorporated into a lot of applications of fusion reporter genes, and resonance energy transfer based sensors [38,39]. A lot of Work has been done using variants of *Renilla* luciferase to create novel imaging probes such as fusing the luciferase to engineered antibodies as well as to generate self-illuminating quantum dots by using the luciferase as a light source [40,41]. Until now, the mutants of Rluc have been widely accepted to replace the native Rluc. The mutants such as Rluc2 or Rluc8, using as luciferase donor, have achieved great improvements for luminescence intensity and stability of the assay [42]. However, it has also been found that the sensitivity of the native Rluc is better compared with Rluc2 or Rluc8 as the luciferase donor [43]. In this thesis and the following experiment, we thus choose the native Rluc for our experiment as the luminescence donor.

Limitations of traditional biosensors and the advantages of using nanomaterial

A biosensor is an analytical device to detect an analyte, which combines a biological component with a physicochemical detector. These analytes can be the original biological small molecules like DNAs of bacteria or viruses, or proteins generated from the immune system in infected or contaminated living organisms. The analytes can also be simple molecules like glucose when a biological receptor unit has the availability with particular specificity. One outstanding challenge in biosensor development is the signal capture efficiency of the biological recognition event. Such transducers are able to translate the interaction of the analyte with the biological element into different forms of signals like electrochemical, electro-chemiluminescent, magnetic, gravimetric, or optical ones. To increase the sensitivity as well as lower detection limits down to even single molecules level, nanomaterials are very promising candidates due to their possibility to immobilize an enhanced quantity of bioreceptor units at reduced volumes and even to act itself as transduction element [44]. Among such nanomaterials, gold nanoparticles [45], semiconductor quantum dots [23,31,46], magnetic nanoparticles [47], carbon nanotubes[48], and graphene[49] based biosensors are intensively studied in the recent years. The intelligent use of such nano-objects can lead to clearly enhanced performances and lowered detection limits with several orders of magnitudes. One common advantage of all

nanomaterials is the high specific surface which enable the packing of an enhanced amount of bioreceptor units [50].

Quantum dots (QDs), which is one important type of semiconductor nanocrystals (NCs), are under numerous investigation. One outstanding optical property is that the QDs have the broad absorption and narrow emission of which the peak positions are tunable by controlling their sizes and bandwidth [51]. Because of these unique properties, QDs have affected the area in bioimaging research much more than any other nanomaterial. In the early eighties, the relationship between band-gap and size of semiconductor materials was firstly described [52]. But the QDs was not established as a new kind of fluorophores in the area of biological research until 1998, when two articles published in Science indicated the advantages of using QDs for biosensing [53,54]. In the following years QDs received blooming research for their applications in biological macromolecule sensing, cell staining, bioconjugation and *in vivo* imaging [55-57].

CdTe is one types of the particular high quality QDs which consists of II/VI semiconductors. This nanocrystalline QD can be synthesized by capping different ligands and has great potential in the applications of biological probes and optoelectronic devices [58]. Wet –chemical synthesis of the nanocrystals (NCs) in colloidal solution is one of the most effective methods to obtain NCs with both high photoluminescence quantum yields and tunable shapes [59]. Three main approaches for producing QDs are: organic phase, water-phase, and two-phase. Compared to organic and two-phase approaches, the aqueous solution preparation method has gained much more research interest as it is simpler to make, has less toxicity, and more environmentally friendly. Furthermore, the products also have improved water-stability as well as biological compatibility [60].

1.3 Hypothesis

The detection of glucose level in tear has been long-timely studied and the main challenge still lies in the accuracy of the detection as the glucose level in tears which is much lower than that in blood. Most fluorescence sensor requires external light source which may be not suitable for *in vivo* test. Bioluminescence Resonance Energy Transfer (or BRET)

technique is a distance non-radiation process, and does not require external energy source could be applied for the non-invasive glucose sensor. BRET has been developed to make the assay more reliable [28,29]. This technique uses a bioluminescent luciferase, typically the luciferase from *Renilla reniformis* to be the light source [39,41,61]. In order to test the extremely low concentration of glucose from the tiny amount of the tear sample, and to minimize the drawbacks of fluorescence sensors, we are proposing a bioluminescence resonance energy transfer (BRET) sensor for non-invasively detecting glucose molecules. The sensor is assembled by conjugating quantum dots CdTe (emission wavelength, $\lambda_{em} \approx 565\text{nm}$), which is used as the acceptor, with a recombinant protein containing the bacterial glucose binding protein (GBP), at the N-terminal and a bioluminescent protein *Renilla* luciferase (Rluc), used as the donor, which is at the C-terminal. The BRET emission of the designed sensor is weak because the distance between the BRET pair is relatively far. In the presence of glucose, GBP binds glucose because of their strong interaction and leads to the conformational change of GBP, which results in the short distance between the Rluc and QDs, and, therefore, the emission intensity of the QDs increases due to the BRET mechanism. In my study, the recombinant protein was further expressed and purified from bacteria *Escherichia coli* BL21. Meanwhile, quantum dots used as the acceptor can be labelled on the N-terminal of the recombinant protein. Fig. 1.3 shows the illustration of the

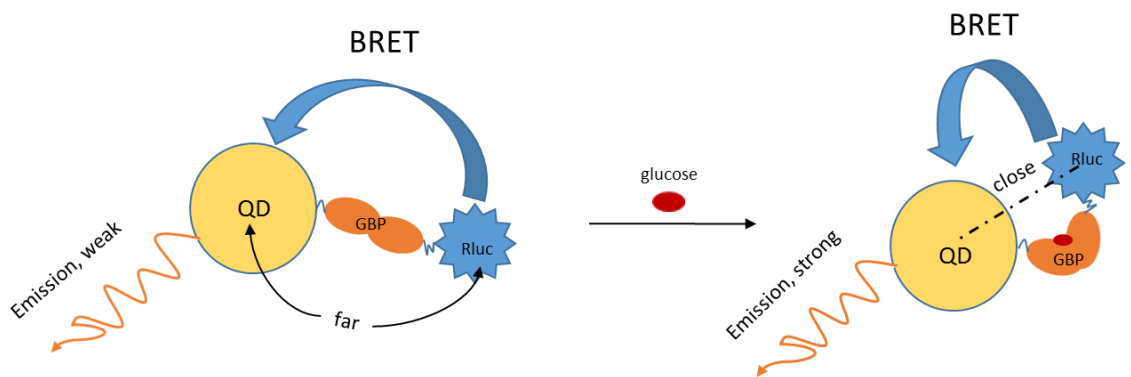


Figure 1. 3 : The scheme of the BRET biosensor. QDs were used as the BRET acceptor and Rluc was the donor. GBP was at the middle site and will fold in the presence of glucose molecules.

BRET sensor made of three parts, (1) quantum dots used as an acceptor in the BRET sensor (2) glucose sensitive protein GBP acting as the sensing element; (3) Rluc used as a donor in the sensor.

1.4 Thesis objectives

BRET is an inexpensive and very sensitive method for detecting the biomolecule interaction because of the correspondence of BRET intensity to the change of the distance between the biomolecules. Here, the goal of this thesis is to build a non-invasive and very sensitive biosensors by applying the BRET mechanism to test the level of glucose concentration; different body fluids including blood and tear are used to evaluate the performance of the designed sensor. A brief summary of the steps to achieve the goal is listed as follows:

- To synthesize and modify carboxylic groups coated CdTe QDs. Since the CdTe QDs will be used as the acceptor in the BRET pair, which will also be bioconjugated with hydrophilic protein, the quantum dots is prepared in aqueous to have the improved water-stability as well as biological compatibility.
- To construct the recombinant plasmid and purify the target protein, including design the recombinant DNA and purify the His-tag protein through His-trap column.
- To bioconjugate the recombinant protein onto the CdTe QDs and to optimal the assay for glucose sensing.
- Characterization of the CdTe QDs, the recombinant protein and the bioconjugation products.
- To evaluation of the performance of the designed BRET biosensor by using aqueous sample and animal model.

1.5 Thesis overview

An overview for the thesis is presented as follows:

Chapter 2 Literature review

This chapter reviews the current glucose detection methods, the methods of hydrophilization of QDs, the genetic engineering of luciferase and the derivations of the substrates, the bioconjugation methods of QDs with biomolecules in constructing biosensing systems.

Chapter 3 Experimental

This chapter describes the methods used to synthesize TGA-coated CdTe QDs in aqueous phase, the construction of recombinant DNA and the transformation of the recombinant plasmid, the expression and purification of the recombinant protein. In addition, various characterization techniques and methods which have been used in the experiment are briefly explained in this chapter such as FT-IR and UV-Vis. The SDS-PAGE test, photoluminescence test and bioluminescence test are also mentioned here.

Chapter 4 Development of the acceptor of the sensor made of CdTe QDs and recombinant protein of GBP-Rluc

This chapter focuses on the development of the CdTe QDs used as the acceptor of the sensor, the sensing element, GBP, for glucose and Rluc acting as, and the donor of the sensor. First the synthesis surface modification of suitable CdTe QDs were investigated and analyzed by FT-IR. Secondly, recombinant protein of GBP-Rluc was constructed. SDS-PAGE was carried out to test the recombinant protein. The properties especially the bioluminescence efficiency was also studied here to find out the optimal conditions (ratio, buffer and time) for the bioconjugation.

Chapter 5 BRET biosensor construction and evaluation

Chapter 5 focuses on the integration of the acceptor of QDs and the recombinant protein GBP-Rluc. This Chapter also provides the results on developing nanostructured BRET sensor. The performance of the designed BRET sensor were evaluated *in vitro*. The BRET donor, recombinant protein GBP-Rluc was conjugated on the BRET acceptor, TGA-coated CdTe QDs. The effect of the amount of EDC and the ratio of the recombinant protein to the acceptor and the bioconjugation processes on the glucose sensing performance were

studied. The BRET intensity ratio as a function of the concentrations of aqueous glucose were presented in this Chapter, In addition, the performance of the designed BRET biosensor was evaluated by the tear glucose of diabetic rats.

Chapter 6 Summary and future work

This chapter draws a conclusion of this study and addresses on the integration methods of three key parts of the sensor. Future work regarding the improvement of BRET biosensor for glucose detection are discussed.

1.6 Reference

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Chapter 2

2 Literature review

2.1 Quantum dots based biosensor for glucose detection

The accurate in situ detection of glucose has long been studied a lot due to its important role in the diagnosis of diabetes mellitus. Currently the commercialized glucose measurement device for diabetic patients are based on enzyme glucose oxidase [1,2]. However, the instability and high cost of such sensors are still serious problems. Accordingly, a lot of research has been done in developing synthetic reagents for monitoring glucose.

Quantum dots (QDs) have attracted a great deal of interests due to their high photostability, continuous absorption spectra, and size-controlled fluorescence properties [3-5]. Recently, some typical researches have been done in QDs coupled with boronic acid-based ligands for glucose detection in optical methods. In 2006, Singaram and his colleges were the first to utilize QDs for glucose sensing[6]. In their research, the fluorescence of CdTe/ZnS QDs was quenched by boronic acid-substituted viologen, while it could be recovered by introducing glucose to the quenched QDs solution. Later in 2009, Willner and coworkers developed a competitive assay for glucose by applying fluorescence resonance energy transfer (FRET) mechanism between phenylboronic acid-functionalized CdSe/ZnS QDs and fluorophore-labeled galactose molecules [7]. Zhou and coworkers prepared phenylboronic acid-modified CdTe/ZnTe/ZnS QDs for determining the intracellular glucose level in 2010 [8]. In their work, the QDs were found to be able to get self-assembled when glucose molecules were introduced, resulting in the fluorescence quenching of QDs. As boronic acid is widely used as ligands in constructing quantum dots based sensing system, the derivatives of boronic acid were also attracted attentions. As it is well known that both boronic acid and its derivatives are capable of forming reversible covalent complexes with 1,2- or 1,3-diols, such as glucose [9], glucose biosensor can be built with the resulting complexes based on fluorescent[8,10,11], holographic[12,13] and electrochemical methods [14]. Also by applying FRET quenching mechanism, a nanostructured biosensor was developed to detect glucose in tear by using CdSe/ZnS quantum dots (QDs)as FRET donor, and dextran-binding malachite green (MG-dextran)

as the acceptor, which was conjugated to an enzyme with specific affinity to glucose named concanavalin A (Con A) [15]. Compared to the semiconductor quantum dots, graphene QDs are also attracting considerable attention because they can be replaced the traditional QDs due to their chemical inertness, low toxicity biocompatibility. Graphene QDs can be obtained by effectively adjusting the band gap of graphene to convert the 2D graphene sheets into 0D. Graphene quantum dots broad the way in designing reagentless biosensors using direct electron transfer of immobilized enzymes on conducting substrates [16-18]. As graphene has the ability to promote electron transfer reactions, it has been widely used as an electrode material [19,20]. Thus, various redox proteins or enzymes can be coupled with graphene based materials to construct third generation electrochemical biosensors [21,22]. A typical example is to immobilize glucose oxidase on graphene QD modified carbon ceramic electrode to build a glucose biosensor [23]. The developed biosensor can respond to glucose presence over the concentration range 5–1270 μM with the detection limit at 1.73 μM and sensitivity at 0.085 $\mu\text{A } \mu\text{M}^{-1} \text{ cm}^{-2}$. Similarly, a fluorescent biosensor for blood glucose monitoring is also developed based on hemin-functionalized graphene quantum dots and glucose oxidase system with a detection limit at 0.1 μM [24].

2.2 Surface modification (hydrophilization) of QDs

Photoluminescence QDs are one of the most popular candidates for biomedical applications such as therapeutic labeling, bioimaging and biosensing. For these typical applications, the particles should meet some basic requirements, for example, QDs should be stable and maintain their optical properties in aqueous solutions with a tolerance in wide range of pH. Also, to label QDs with other biomolecules, it should have functional groups on the surface for conjugation. The traditional synthesis of QDs were generally resulted in very hydrophobic nanoparticles. In this case, the QDs will only be soluble in non-polar solvents and are limited in biomedical applications. The phase exchange of QD from organic solvents to water phase is usually accompanied with the decrease of the QY [25]. In order to improve the usage of QDs, the surface modification of QDs thus need to be explored [26]. There are mainly three strategies for transferring oil-phased QDs into water-phase: by ligand exchange, silanization, and encapsulation, as indicated in figure 2.1.

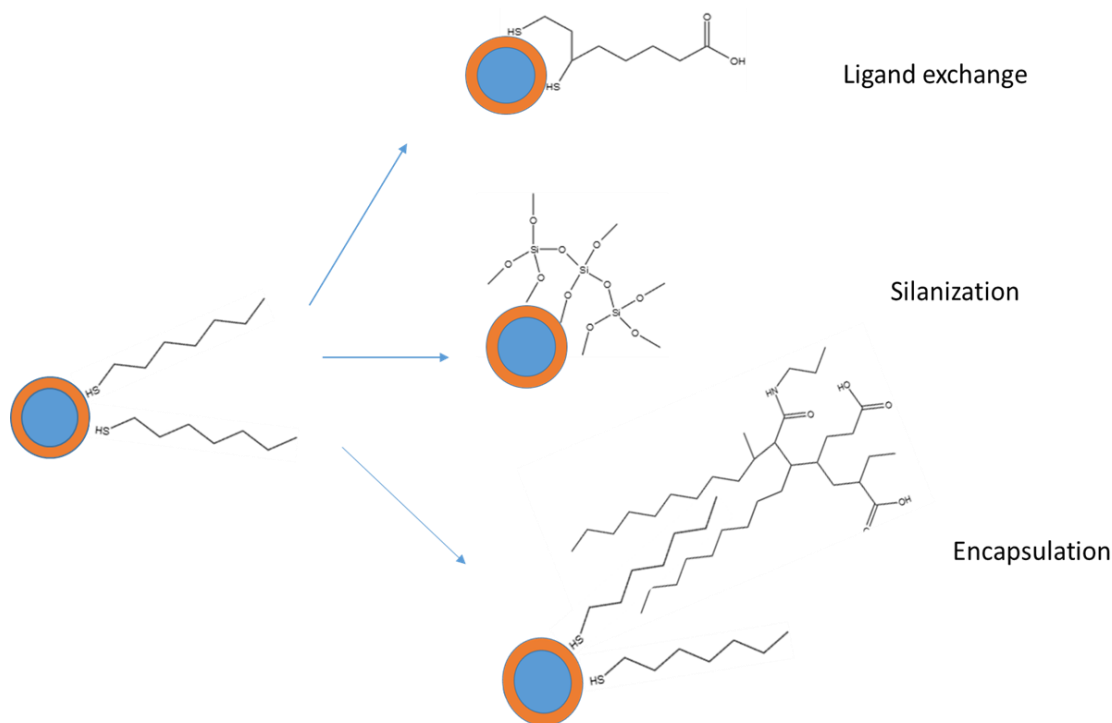


Figure 2. 1: Three main methods of hydrophilization of QDs

2.2.1 Ligand exchange and silanization

The first method involves ligand exchange, in which the original hydrophobic coating (e.g. trioctylphosphine oxide (TOPO)) is removed and replaced by water-soluble bifunctional molecules. The one end of the bifunctional molecules is attached to the QD surface and the other end is a hydrophilic tail thus makes the QDs available for further bioconjugation. The process is due to the surface group such as thiol, amine and carboxyl, which can passivate QDs more strongly than the original ligands[27]. Other examples of linker molecules such as dithiothreitol (DTT), 2-aminoethanethiol and dihydrolipoic acid (DHLA), can be mentioned [28]. The second method is called silanization, which actually can be seen as one form of ligand exchange strategy, but can be highlighted separately due to its prevalence[29]. The possibility of coating of a single QD in a silica shell is due to the non-toxic, chemically inert and optically transparent properties of the silica surface. The silica surface can not only protect the nanoparticles from been oxidized as silica matrix can

provide nanocrystals with an enhanced colloidal stability resist to the environment, it also protects the QD from leaching components (e.g. Cd). In addition, the silica based material is biocompatible thus can be functionalized for bioconjugation.[30,31].

2.2.2 Encapsulation

The third method is to encapsulate the hydrophobic nanoparticles into different carriers such as amphiphilic polymers [32], polymeric microbeads [33] or liposomes [34]. The hydrophobic shell of QDs have physical interactions with the original coating molecules, which makes the surface modification of QDs possible. Up to now, a number of amphiphilic copolymers and polyelectrolytes have been synthesized for modification [35-37]. The QDs can also be treated with surfactant, but because of the relatively weak anchoring interactions, the result product cannot be stable in biological environment for a long time. These problems can be solved by applying amphiphilic polymers into the system. Due to the multiple hydrophobic and hydrophilic units on the surface, it can have a strong interaction with the QD surface [38]. Liposomes can also be used in the encapsulation process as spherical lipid vesicles. Because of their porous spherical structure and high loading capacity, liposomes are very popular carriers for hydrophobic QDs [39]. The encapsulation of QDs into liposomes has some advantages, for example, it can help with the bioconjugation between QDs and the biomolecules such as proteins and enhance the analytical signal [40]. However, using liposomes as carrier vehicles has some drawbacks because it is not stable *in vivo* and easily be affected by the external environment such as the temperature and pH [41]. The best way to solve this problem is through coverage or templating them with polymer materials [42].

2.3 Bioconjugation of QDs with biomolecules

Covalent linking and non-covalent binding are the two main approaches to immobilize biomolecules on the QD surface. Non-covalent binding is mediated by the typical interactions between biomolecules and the QD surface, while covalent linking is obtained through the chemical reactions of the molecular surface groups [43,44]. The history of

bioconjugation of QDs can date back to 1998, when Bruchez and Chan were the first scientists who successfully conjugate QDs to biomolecules[45,46]. After that, the usage of QDs in imaging and other biomedical applications has been greatly studied and explored.

2.3.1 Non-covalent binding

Non-covalent attachment of biomolecules to the QD surface has two based types of QD surface-biomolecule interaction, which are electrostatic interaction between oppositely charged molecules and high affinity secondary interactions. Bioconjugation through electrostatic interaction is simplest and most popular non-covalent approach as there is no chemical reactions taken place. The mechanism of the electrostatic interaction is the attraction between oppositely charged species on the surface of QDs and the biomolecules for conjugation [43]. A popular method of electrostatic coupling of proteins with QDs is through interactions between the negatively charged surfaces of QD and the positive charged surface of a protein [47]. Another class of non-covalent binding is secondary interactions between functional groups on the surface of QD and the surface of biomolecules. Biotin-avidin interaction is most used for high affinity interactions such as DNA interactions or enzyme-substrate interactions. However, though the bioconjugation of QDs via biotin-avidin process is prevalence, the bioconjugated products are generally very large in size, which makes it less popular than EDC-mediated covalent conjugation chemistry. The smaller size of products can be more useful in targeted applications such as fluorescence resonance energy transfer (FRET) and cellular research [47,48]. The strong interaction between His-tagged biomolecules and Ni-NTA (NTA-Ni²⁺-His) is also a well-known kind of high affinity secondary interaction and is widely used for bioconjugation process [49]. This strategy has also been used to develop a QD-probe for performing Western blot analysis. Compared to the classical Western blot, this new approach has a shorter analysis time but a better sensitivity [50]. A less popular strategy is based on the barnase-barstar system designed for Ig multimerization but later developed to a scFv-(barnasebarnase) fusion protein, which allows binding of the scFv Ig to the QDs [51].

2.3.2 Covalent binding

Covalent conjugation between QDs and molecules involves the reaction of the functional groups between the QDs and the target biomolecules. The covalent binding strategy is determined by what kind of groups are on the surface of the QDs and the biomolecules. The surface modification of the target biomolecules is sometimes hard to achieve, while the surface modification of QDs is relatively easy as it can be determined by different hydrophilization strategies mentioned above. Covalent binding can be achieved by the usage of functional crosslinkers. Crosslinkers are different in the linker length thus they can be used for different design purpose.

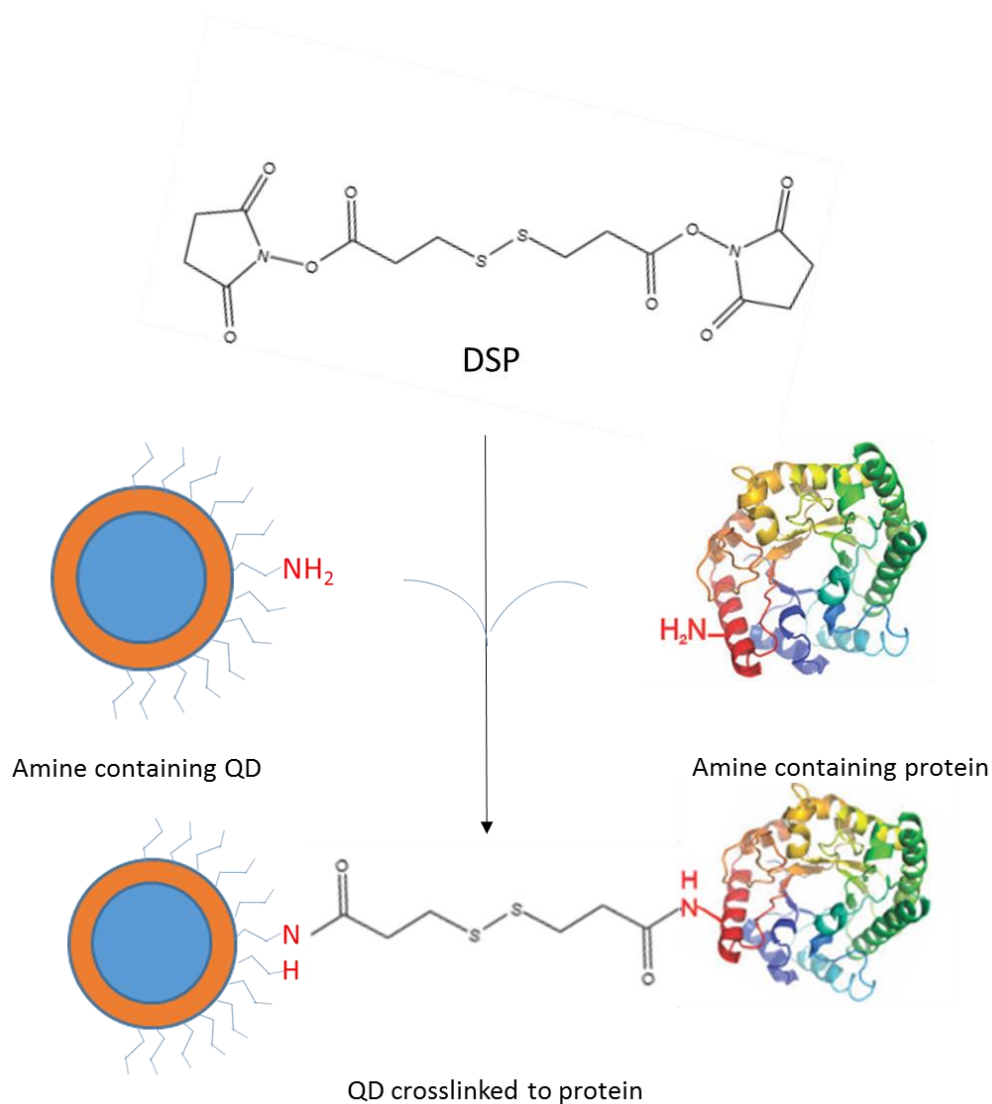


Figure 2. 2: DSP mediated conjugation of amine containing QD with protein

The smallest available crosslinkers for bioconjugation are so-called zero-length crosslinkers (e.g. EDC and N, N' -Dicyclohexyl carbodiimide (DCC)) because these compounds mediate the conjugation of two molecules directly by adding no additional atoms. Dithiobis succinimidylpropionate (DSP) is other popular homo-bifunctional crosslinking reagents used for bioconjugation. The mechanism is the DSP can connect two molecules with the same functional group on both ends of an alkyl spacer (Fig. 2.2). An obvious disadvantage is the potential of creating a range of conjugates as the reaction is non-specific thus these conjugates can further react with other functional groups, results in a mix conjugates. To avoid or reduce this problem, homo-bifunctional groups were used to develop two-step procedures [52].

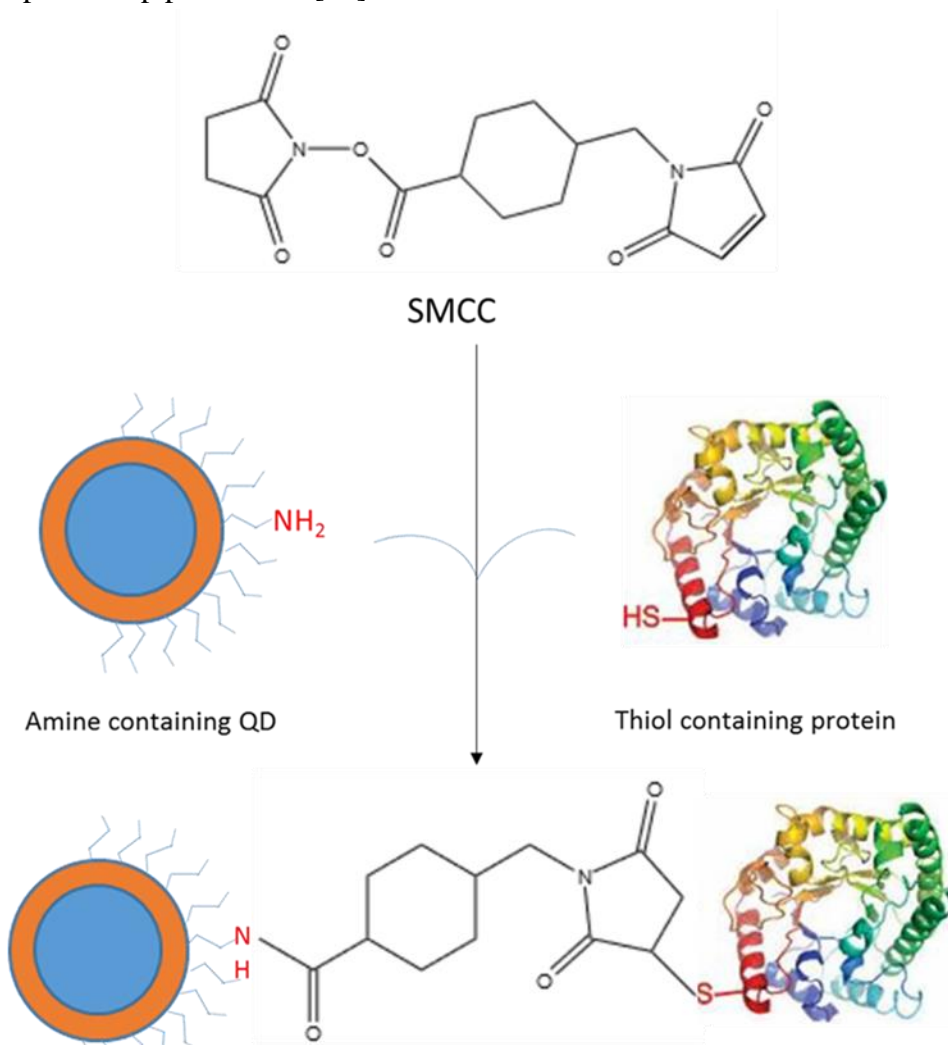


Figure 2. 3: SMCC mediated bioconjugation between amine containing QD and thiol containing protein

Heterobifunctional crosslinkers such as SMCC (succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate) contains two different reactive groups, allowing coupling of two different functional molecules. As shown in Fig. 2.3, the QDs part may contain an amine reactive group, while the target protein may consist of a thiol-reactive group. Carboxylic functionalized QDs are now the most popular commercial available ones. The carboxylic groups can be easily conjugated to free amine groups, which are quite common on the surface of proteins, peptides or Igs molecules [53]. In this method, no additional chemical modifications of the proteins are needed before conjugation, which makes the process easy and fast [44]. EDC is the most well-studied and easy-to-perform method for zero-length crosslinking the biomolecules onto QDs [54,55]. The terminal carboxyls on the QDs and amines on the biomolecule (e.g. proteins) directly form into amide bond by the mediation of EDC, although the reverse configuration may work equally in some cases [43]. Apart from carboxylic groups, amine groups are the second most popular functional groups for bioconjugation. A lot of heterobifunctional crosslinkers have been employed for bioconjugation of QDs- NH₂. Conjugation of QDs-NH₂ can be more selective if reacted with biomolecules containing thiol groups. As cysteine and methionine are the only two amino acids that contain thiol groups, other side reactions thus can be prevented [56].

2.4 Molecular bioluminescence in biomedical studies

2.4.1 Luciferases and their genetic mutants for constructing BRET system

Luciferases are mainly from the North American firefly *Photinus pyralis*, the click beetle *Pyrophorus plagiophthalmus*, the sea pansy *Renilla reniformis* and the marine copepod *Gaussia princeps*. Those are the most popular types of proteins used for bioluminescence tomography, however, their substrates are different due to the living environment. D-luciferin is the substrate for terrestrial organisms luciferases while coelenterazine analogs are used as substrates by marine organisms' luciferase, as summarized in Table 1 [7–23].

Table 2. 1: Luciferase reporters used for bioluminescence tomography and their specifications

Bioluminescent reporter	Emission peak	Size (kDa)	Substrate	Refs
<i>Luciola italica red</i> ;	~620 nm	64	D-Luciferin	[57]
<i>Photinus pyralis red</i> ;	618 nm	62	D-Luciferin	[58]
<i>Photinus pyralis</i> ; North American firefly luciferase	~600 nm	62	D-Luciferin	[59]
<i>Renilla reniformis</i> ; sea pansy luciferase	Rluc 480 nm	36	Coelenterazine	[60]
	Rluc8 480 nm	36	Coelenterazine	[61,62]
	Rluc8.6 535 nm	36	Coelenterazine	[61,62]
<i>Gaussia princeps</i> ; <i>Gaussia</i> luciferase	480 nm	20	Coelenterazine	[63]
<i>Oplophorus gracilirostris</i> ; NanoLuc luciferase	460 nm	19	Furimazine	[64]
<i>Cypridina noctiluca</i> ; <i>Cypridina</i> luciferase	~460 nm	62	Cyprinidid luciferin	[65]
<i>Vargula hilgendorffii</i> ; sea firefly luciferase	~460 nm	62	Cyprinidid luciferin	[66]
<i>Aequorea Victoria</i> ; aequorin photoprotein	~460 nm	62	Coelenterazine	[67,68]

Research on novel luciferase genes and their mutants has reached great improvement over the past decade. The new NanoLuc luciferase, which is only 19.1 kDa, attracts the most interests among the luciferases that use coelenterazine analogs. NanoLuc was isolated from a deep-sea shrimp and the substrate is a novel coelenterazine analog named furimazine.

The difference between coelenterazine and furimazine is the phenol group replaced by a furane, as indicated in Fig.2.4.

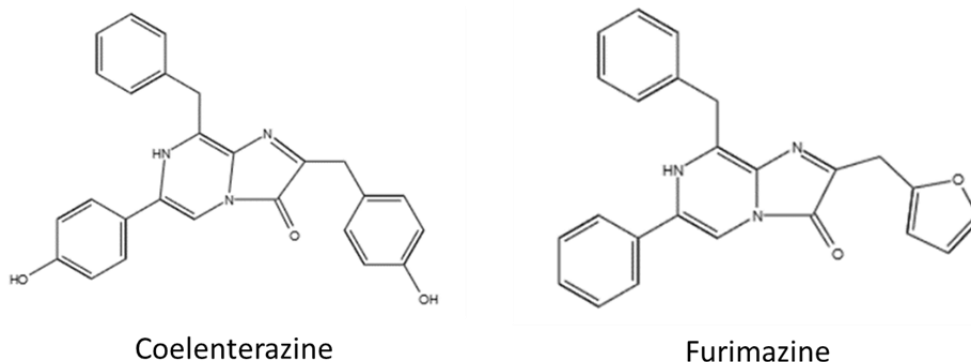


Figure 2. 4: Chemical structure of coelenterazine and its analog, furimazine.

This luciferase-substrate system has now also been successfully applied to *in vivo* bioluminescence imaging due to the high-intensity and glow-type luminescence it provides [64,69]. In addition to developing improved mutant genes alone, the luciferase gene can be designed to combine with other reporter genes. For example, intramolecular bioluminescence resonance energy transfer (BRET) systems can be established by coupling the luciferase with nanoparticles [70]. The fusion of enhanced *Renilla* luciferase and the fluorescent protein Venus is a very typical example of applying this technic into Nano-lantern applications. The novel Nano-lantern system can enhance the *in vivo* performance compared with using *Renilla* luciferase alone [71]. In addition, a more efficient near-infrared BRET systems for deep-tissue imaging can be developed by fusing *Renilla* with near-infrared fluorescent proteins (iRFPs) [72]. Another type of engineered probes using BRET phenomenon is bioluminescent quantum dots *in vivo* imaging. To construct the system, a bioluminescent protein is covalently attached with quantum dots but the distance may change in the presence of analyte. A sequential BRET-FRET energy transfer system can be built with multiple donors or acceptors by using this strategy. A typical example in recent study is combining firefly luciferase variants with near-infrared quantum dots, which resulted in high BRET efficiencies [73,74].

2.4.2 Luciferase substrates and the derivations

A recent study revealed that phenolate-luciferin-binding site interactions modulate bioluminescence colors by studying two different substrates, NH_2LH (amino luciferin) and its 5, 5- dimethyl analog $\text{Me}_2\text{NH}_2\text{LH}$ [75]. The modification of D-luciferin or amino-luciferin at the 6' position not only affect the spectral of the emission but also the emission intensity. Compared with unsubstituted 6'-aminoluciferin, the bioluminescence emission of analog N-alkylated 6'-aminoluciferin has been identified to be more efficient when catalyzed by both wild-type and mutant *P. pyralis* luciferase [76]. When replacing the D-luciferin sulfur atom at position 1 with selenium (Fig. 2.5), the emission will shift to red without changing luciferase types [77]. Furthermore, it has also been found that luciferin analogs that have conjugated double bonds between the thiazoline ring and the aromatic moiety tend to shift to red but the light intensity will drop in some extent [78]. Remarkably, the *P. pyralis* firefly luciferase can produce near-infrared light when using a dual-color firefly luciferin analog, infra-LH₂ (iLH₂), as the substrates [79]. Using the same approach, Aka-lumine hydrochloride (Aka-lumine-HCL) has been developed with a maximum emission at 675 nm (Fig. 2.5) [80].

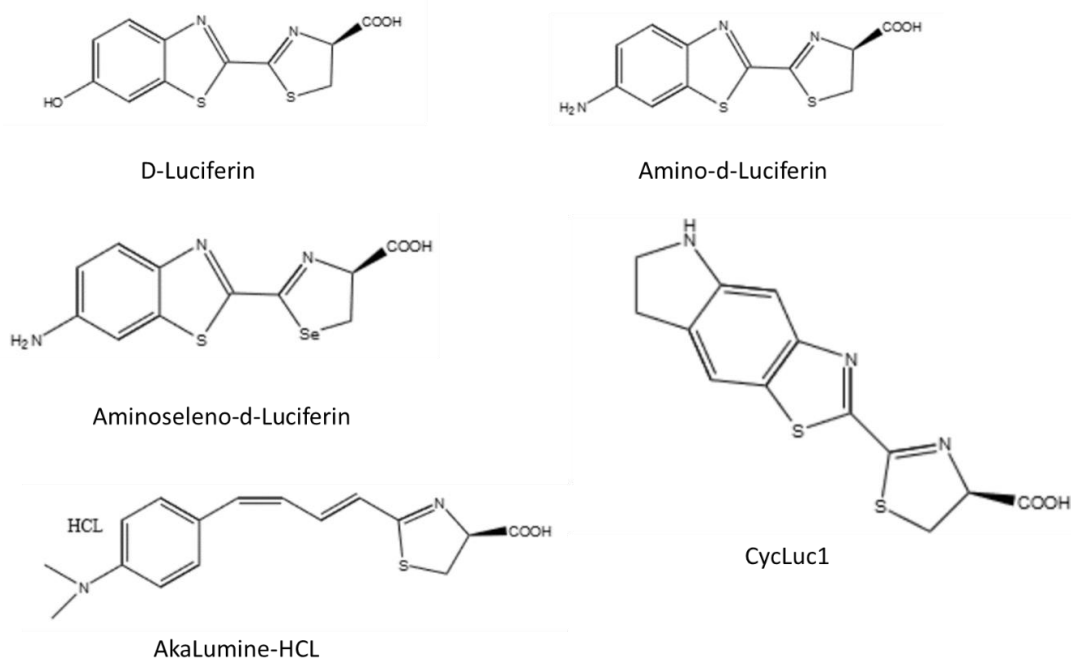


Figure 2. 5: Bioluminescent Substrates. Chemical structure of firefly luciferin and different analogs.

2.5 Reference

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Chapter 3

3 Experimental Methods

3.1 Preparation of thioglycolic acid (TGA) coated CdTe QDs

The colloidal CdTe QD solution was prepared based on the method described elsewhere. [1] Briefly, in a three-necked flask, 0.4mmol Cd(CH₃COO)₂·2H₂O was totally dissolved into 60 ml deionized water. 10 mins after the solution is clear, 36μl TGA was added into the solution. After stirring for 5 min, 0.08mmol Na₂TeO₃ which was dissolved in 50 ml deionized water was added into the above solution. Keep stirring for 3mins and then 160 mg of NaBH₄ was added into the precursor solution, the pH was adjusted with 1 M NaOH solution to 11. Then the three-necked flask was refluxed at 100 °C with a condenser attached under open-air conditions. The QDs with desired PL emission spectra can be obtained through controlling the reaction time.[2] The QDs nanoparticles were washed twice by isopropanol and finally dissolved in deionized water.

3.2 Plasmid construction for GBP-Rluc

The *mglb* gene originally from *E. coli* k-12 that encodes for wild-type GBP is used for plasmid construction. Bacterial glucose binding protein (GBP) was cloned *Rluc* gene was cloned from the plasmid pRL-null (Promega, Inc). A six amino acid linker was inserted to link the two proteins. Four primers were designed for construct the GBP-Rluc recombinant protein. For GBP, two primers were designed for Cloning (forward 5' TATACATATGAATAAGAAGGTGTTAACCTGTCTGC 3'; reverse 5' GCTGGATCCTTTCTTGCTGAATTCAGCCAGGTTG 3'). The forward and reversed primers were introduced restriction site Nde I and BamH I (restrict enzyme) respectively (underline). In order to separate the sequence of GBP from that of Rluc, a six amino acid linker SGGGS (bold underline) was inserted after BamH I site (underline) when designing the forward primer to amplify Rluc (5' AAAGGATCCAGCGGTGGTGGTGGTAGCATGACTTCGAAAGTTTATGATCCAG 3')

The reverse primer for Rluc (5'TGTGCTCGAGTTGTTTCATTTTT GAGAACTCGCTC 3') introduced restriction site Xho I (underline). The GBP is thus located at the upstream (N-terminal) of the fusion protein.

The above PCR products were further digested with relating restriction enzyme. The plasmid pET32a (Novagen, Inc) was used to clone and express the recombinant gene. The digested DNA insert were ligated into the relating MCS (multiple cloning) site at pET32a. A six histidine tail was introduced into the GBP-Rluc recombinant protein. The pET 32a-GBP-Rluc was transformed into *E. coli* BL21 cells. The DNA sequence of the recombinant plasmid was confirmed by DNA sequencing (Robarts Institute, Western University).[3]

3.3 His-tag recombinant protein purification

3.3.1 Bacterial culture

Choose the single colony on the agar plate which contains the bacterial cells with desired recombinant plasmid pET32a-GBP-Rluc with a loop. Insert the loop into 5ml of Luria Bertani (LB) borth containing 100 µg/mL ampicillin and let the bacterial grow overnight at 37 ° C. This culture was for the preparation of further inoculate 800 mL of broth containing 100 µg/ml ampicillin, which was also left to grow at 37° C. When the culture reached an OD600 of 0.375, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into the solution to 1 mM for the final concentration to induce the expression of GBP-Rluc. After adding the IPTG, the bacteria were left to grow for another 4 hrs at room temperature with shaking at 200rpm.

3.3.2 Harvest the protein

After 4 hours expression, the bacterial cells were harvested by centrifugation at 9,000 rpm for 5 min at 4° C. The supernatant was then discarded and the pellet was then re-suspended in a binding solution (BS), which contains 20 mM Tris/HCl, pH 7.4, 500 mM NaCl and 5 mM imidazole. Repeat the re-suspend steps at least once and then add Triton X-100 into the solution at a final volume of 0.5% (Sigma-Aldrich, Inc.). After gently shaking for 5mins,

10mg lysozyme which was pre-dissolved in Binding solution was added into the bacterial. The tube containing the above solution was then transferred to a 20ml beaker and sonicated on ice using 5-s bursts followed by 9-s rest for at least 50 cycles using a Mandel Scientific Q500 sonicator (Guelph, Canada).

3.3.3 Protein loading and elution

After the sonication, the suspension was centrifuged at 8,000 rpm at 4° C for 30 min to collect the supernatant from bacterial cell pellet. The protein solution was then transfer to a clean tube and filter the solution again by cellulose acetate membrane syringe filter (VWR, Inc.) which the pore size is at 0.45µm. The collected protein solution was purified via His-trap HP columns (GE lifescience, Inc.) by a syringe pump. The column was first prepared with Binding Solution to be equilibrated.

The supernatant containing the protein was loaded into the column at a low speed of 0.3ml/min and the column was washed with 10 column volumes of the Binding Solution (BS). To test the best concentration for washing out the target His-tag protein, the protein was eluted using BS with seven gradients of imidazole (20mM, 40mM, 60mM, 80mM, 100mM, 120mM and 150mM) over 10 column volumes at a mediate speed at 0.5ml/min, respectively. Each flowing through solution was collected and the fractions in each tube were around 8 milliliters. The column was then washed with 10 column volumes of DDW and then the same volumes of ethanol. Wash the column with DDW again before putting them into 4°C for storage.

3.3.4 Identification by sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE under reducing conditions and stained with Coomassie Blue) was used to verify the purity and the expression extend of each fractions containing the target protein.

For SDS-PAGE samples preparation, mix the protein from different fractions mentioned above in a ratio of 4:1 with the sample buffer and then heat the sample by boiling for 5-10 minutes in a water bath.

After solidification of the gel, the protein marker (Bio-rad, MW size range 10–250 kD) was added into the first well and the fractions of protein mentioned above were added into the wells from a low concentration of imidazole to higher range. The gel was running in SDS Running buffer at 100 V for 20 min and then increased to 150 V for 40mins in order to fully separate the recombinant protein. After 1hr, the gel was taken out and immersed into staining buffer in a box. The box containing the gel was anchored on a shaker with a speed of 60 rpm and incubated for 40mins.

After 40mins, the gel was gently taken out and then immersed into 1L destaining solution which was made up by mixing 100 mL methanol and 100 mL acetic acid in 800 mL DDW. After 4hrs shaking at a speed of 50 rpm, the gel was taken out gently for observation.

3.3.5 Dialysis of the recombinant protein GBP-Rluc

After getting the target fractions from different tubes, the fractions were collected into a new tube. Excess imidazole was removed from the combined fractions by buffer exchange with excess amount of 3 different buffers: 10 mM Tris/HCl, pH 7.4, 10mM PBS, pH 7.4, 10mM Borate, pH 8.0, respectively with adding the extra glycerol in a concentration of 0.5% in order to maintain the recombinant protein into different buffer for further testing.

3.3.6 Concentrate target protein

The above fractions were collected and concentrated using an Amicon Ultra centrifugal filter (ultra-15, MWCO 10 kDa, Millipore Inc). The resultant GBP-Rluc protein solution was stored at -20° C. The concentration of the protein was determined by Bicinchonici acid (BCA) protein assay (Thermo scientific Inc.)

3.4 Bioluminance performance of GBP-Rluc

To optimize the experimental conditions for the QD-GBP-Rluc assay, the best conditions for the bioluminescent performance of GBP-Rluc has to be tested. Here we tested the performance of GBP-Rluc dissolved in different buffers: 10 mM Tris/HCl, pH 7.4, 10mM PBS, pH 7.4 and 10mM Borate, pH 8.0, respectively. The ratio of GBP-Rluc and the substrate, native CTZ were tested by adding different amount of CTZ (3ul, 5ul, 7ul and 10ul in 1mg/ml) into 0.1mg protein dissolved in PBS solutions. The effect of protein concentration was also tested by adding 5 μ l CTZ (1mg/ml) into a gradient concentration of protein solution from 0.01mg to 0.4mg. As the bioluminescence is not very stable and decay with time [4], the performance of GBP-Rluc was tested by adding 5 μ l CTZ (1mg/ml) into protein solution to find the relationship between the intensity change and time. All of the bioluminescent emission spectra were collected by a Quanta Master™ 40 Spectrofluorometer (HORIBA-PTI Inc., London, ON).

3.5 Bioconjugation of TGA-CdTe QDs by GBP-Rluc protein and optimization of BRET sensor

The bioconjugation was mediated by 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC). In a typical experiment, TGA Stabilized CdTe (10 μ l at 2 μ mol/L) and EDC (10 μ L at 20mmol/L) were mixed in 80 μ l PBS. After the mixture were gently shaking for 10 min at room temperature, 50 μ l of 0.1mmol/L protein was added into the above solution and incubated for another 10min. Afterwards, 2 μ L of ethanolamine was added to stop the reaction. The particles were further purified by amicon ultra-0.5 filter (100kDa, MWCO, EMD MilliporeInc.) to remove free GBP-Rluc and other small molecules.

3.6 Characterization

The TGA-coated CdTe QDs were verified by using Fourier transform infrared (FTIR) spectrophotometer (BrukerFTIR-IFS55, Germany). The size and the absorbance peak of the nanoparticles were confirmed by UV-Visible Spectroscopy (UV-3600 Shimadzu,

Japan). Bioluminescent emission spectra were collected by a Quanta Master™ 40 Spectrofluorometer (HORIBA-PTI Inc., London, ON) with the step size at 0.1s.

3.6.1 Fourier transform infrared spectroscopy

FTIR is a technique based on the theory that each chemical group has characterized absorption infrared spectrum. In the measuring process, the FTIR instrument shines a beam with different frequencies of light at once, and then adsorbed by the sample. Next, the beam is modified to contain a different combination of frequencies, generating a second data point. This process will repeat. Afterwards, a computer analyze all these data and transfer what the absorption is at each wavelength by using a common algorithm called the Fourier transform. In this project, the chemical groups on the surface of quantum dots were characterized by FTIR.

3.6.2 Ultraviolet – Visible spectroscopy

Ultraviolet-Visible spectrophotometry (UV-Vis) refers to absorption spectroscopy and reflectance spectroscopy in the ultraviolet and visible spectral region. The light ranges from near-UV to near-infrared ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions.[5] UV-Vis was carried out to confirm the absorbance of selected quantum dots and to check the size of the synthesized CdTe QDs. UV-Vis is also used to calculate the concentration of the quantum dots solution.

3.7 Glucose assay

The above GBP-Rluc-QDs conjugate solution was diluted to 400µl by PBS and then treated with 3µl of aqueous glucose with different concentrations from 0mmol/L to 1mmol/L. The mixture was gently shaken for 2min at room temperature. The fluorescence emissions were then measured from 410nm to 640nm by the fluorometer immediately following addition

of 5 μ L of native CTZ (1mg/mL, dissolved in ethanol) to the above assay solutions. Each concentration of glucose was tested for three times.

3.8 Animal tear test

Rats with diabetes were used to get the tear sample and the process was reported elsewhere[6]. Briefly, Male Sprague-Dawley rats, eight-weeks of age, were obtained from Charles River Laboratories. Rats were housed two per cage at constant temperature and humidity on a 12-h dark/light cycle, and had access to water and standard chow ad libitum. Ethics approval was obtained from the University of Western Ontario Research Ethics Board, in accordance with Canadian Council on Animal Care guidelines. The experimental protocol followed the Principles of Laboratory Animal Care (US NH publication No83-85, revised 1985). Rats were housed for one week to familiarize with their surroundings prior to the initiation of experiments. Diabetes was induced with multiple low-dose Streptozotocin (STZ) injections. STZ (20 mg/kg; Sigma Aldrich, Oakville, ON, Canada) in citrate buffer was injected into the intraperitoneal cavity for five consecutive days (1). Diabetes was confirmed by two consecutive non-fasting blood glucose readings of ≥ 18.0 mmol/L. The blood glucose level of three male Sprague-Dawley rats were tested before collecting the tear. Tear fluid was collected from the ocular surface with a 1 μ L glass capillary tube (P1424 SIGMA). 1 μ L of rat tear sample were collected from each male Sprague-Dawley rats from the ocular surface with a 1 μ L glass capillary tube (P1424 SIGMA). The tear samples were diluted by PBS pH 7.4 to 10 μ L. To keep the same amount and the procedure of the BRET sensor measurement, 3 μ L of the diluted sample was used to measure the glucose level by BRET sensor with 3 independent tests. Ethics approval was obtained through the University of Western Ontario Research Ethics Board, in accordance with Canadian Council on Animal Care guidelines.

3.9 Reference

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Chapter 4

4 Development of the acceptor of the sensor made of QDs and recombinant protein of GBP-RLuc

QDs has been widely used in bioimaging and biosensing in the recent years as it has unique properties like strong fluorescence intensity, high stability and good biocompatibility [1-3]. In this chapter, the photoluminescence of CdTe was characterized and FTIR was used to identify the surface groups of CdTe. The successful purification of the recombinant protein was identified by SDS-PAGE. The bioluminescence property of GBP-RLuc was tested under different conditions including the ratio with substrate, buffer and time.

4.1 Introduction

Quantum dots are under heavy investigation due to their tunable emission peaks by controlling their sizes and bandwidth. The unique optical properties and good biocompatibility make it a very suitable kind of candidate in bioimaging and other biomedical fields [4,5]. CdTe QDs is a particular high-quality kind of QD consisting of II/VI semiconductors which has widespread applications in the biosensing field [6,7]. The wet-chemical preparation was one of the most successful methods to obtain nanocrystals with both high photoluminescence quantum yields (PL QYs) and tunable sizes and shapes [8,9].

Wet-chemical synthesis of QDs can be divided into water-phase, organic phase and two-phase approaches. Among them water-phase preparation attracts most interests as it is relatively simpler, less toxic, and more environmentally friendly. Furthermore, the products have enhanced water-stability and biological compatibility [10].

In this paper, we used a simple and economical one-pot method to prepare highly luminescent water soluble CdTe QDs which has been reported in 2012 [11]. The TGA-capped CdTe QDs which has an emission peak at 565nm was chosen for further experiment.

Currently, the most frequently used luciferases in bioimaging are from the firefly, *Photinus pyralis* (Fluc), and the sea pansy, *Renilla reniformis* (Rluc). Compared to Fluc, which is

ATP dependent, Rluc ATP-independent and is only 36 kDa. Rluc uses coelenterazine as the substrate, and emits at 480 nm. In addition, its smaller size compared with Fluc (62kDa) makes it more appropriate for application in bioluminescent system [12]. Currently, the mutation of native Rluc, Rluc2 or Rluc8, have been used as the luciferase donor to improve luminescence intensity and stability [5]. However, as the luciferase donor, the sensitivity of the native Rluc was found to be better than that of Rluc2 or Rluc8 [13]. Therefore, native Rluc was chosen for the construction of the BRET sensor. As the solution or pH may affect the efficiency of the catalysis process and the bioluminescence may decay in a short time [14], the fresh recombinant protein GBP-Rluc was purified and used to test for exploring the best conditions for generating stable and high intensity bioluminescence.

4.2 Results

4.2.1 Photoluminescence spectra of TGA coated CdTe QDs

It should be noted that NaBH₄ played two roles in this reaction. The first part is leading to a faster reduction of Te⁴⁺ to Te²⁻ to supply for the rapid nucleation of QDs. The second is to supply a protective environment to avoid the oxidation of Te²⁻ during QD growth [15].

Figure 4.1 showed the photoluminescence spectra of the TGA-stabilized CdTe QDs aqueous solution taken from the refluxing reaction mixture at different intervals of time. With the prolonging of reflux time, the photoluminescence emission spectra of the CdTe NCs shifted to longer wavelengths with increasing size of the CdTe NCs as a consequence of the quantum confinement [11]. The emission of 565 nm was chosen for following experiments of bioconjugation with protein as the peak is far from the donor peak, which is at around 470nm, but not too far for detection before decay. Water soluble CdTe NCs can be direct used for bioconjugation with protein to build potential implant biomaterial as the proved low toxicity and high biocompatibility. [9]

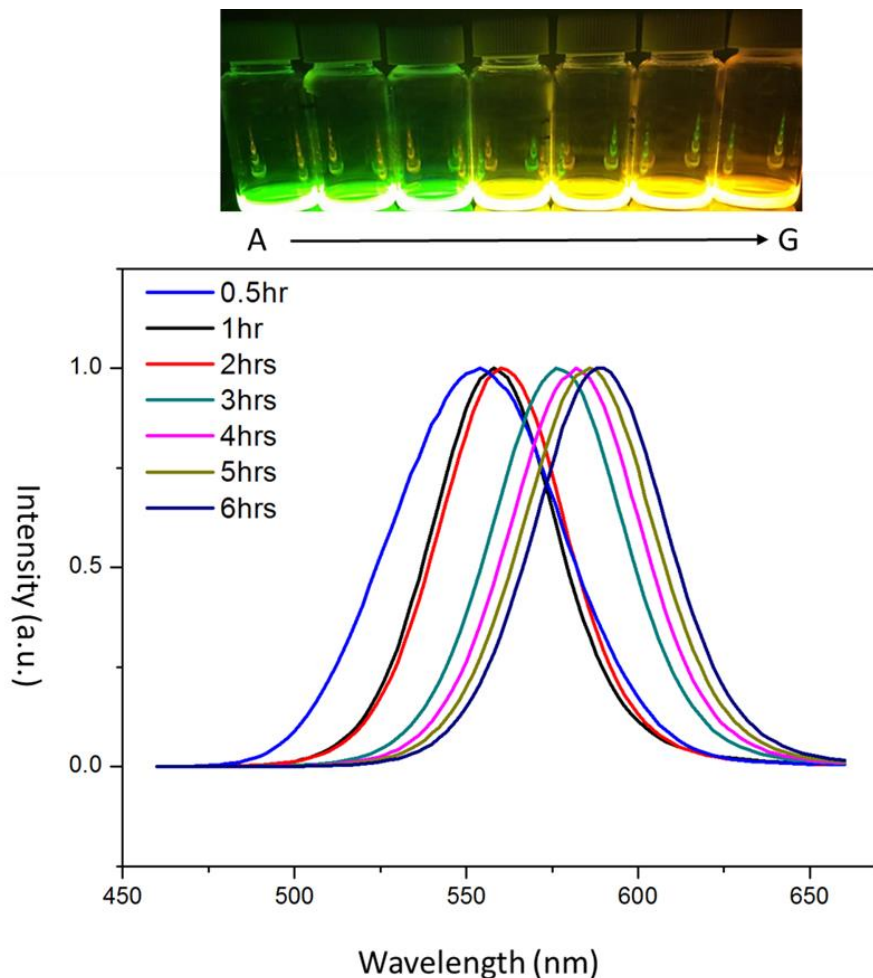


Figure 4. 1: The image of the TGA-coated CdTe QDs under an ultraviolet lamp (top) and the photoluminescence spectra (bottom). The photoluminescence was at (A) 0.5hr, (B) 1h, (C) 2h, (D) 3 h, (E) 4 h, (F) 5h, (G) 6h.

4.2.2 FTIR spectra of the TGA-stabilized CdTe

To confirm the surface groups of CdTe NCs are carboxylic groups. FTIR was used to analyse the sample. Fig.4.2 showed the FTIR spectra of the TGA-stabilized CdTe QDs. Asymmetric and symmetric stretching bands of -COO^- located at 1556 and 1378 cm^{-1} , respectively. The C-O stretch vibrational mode at 1225 cm^{-1} is also observed in the spectrum of TGA-QD [16].

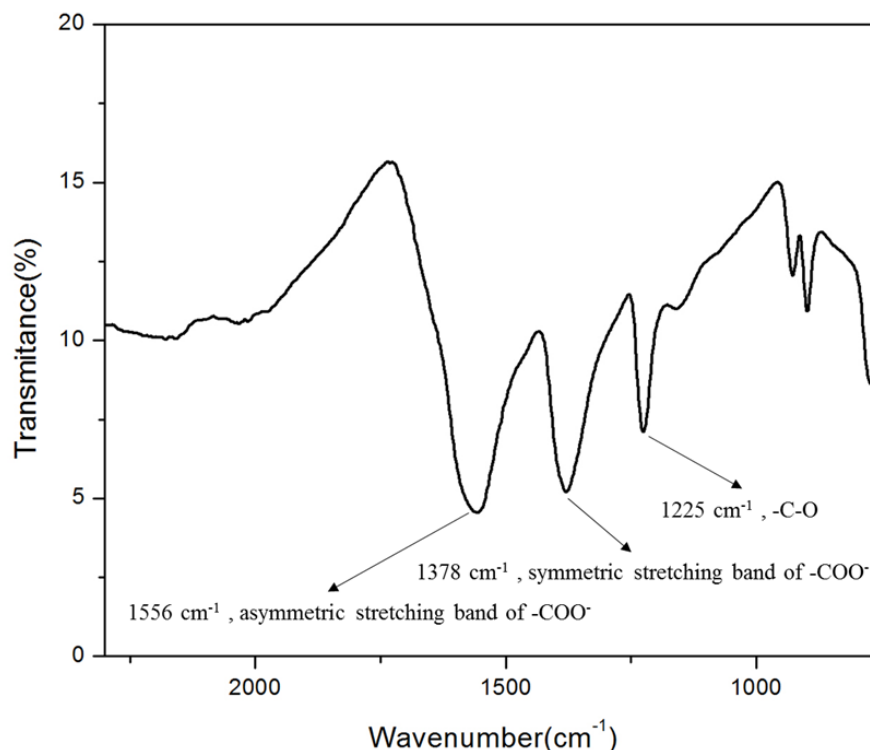


Figure 4. 2: FTIR spectra of TGA-coated CdTe

4.2.3 Protein expression and purification

The theoretical molecular sizes of Rluc and GBP were 36kDa and 33kDa, respectively. Taking the 6× His-tag, and linker into consideration, the molecular size of the fusion protein was about 71kDa. Purification of His-Tag proteins are based on the ability of six consecutive histidine residues on either the N-terminus or carboxyl terminus to bind to a resin containing nickel ions immobilized by covalently attached nitrilotriacetic acid (NTA). Based on this mechanism, after washing through the column, the fractions with different concentrations of imidazole (from 40mM to 120mM) elution buffer were collected. SDS-PAGE was carried out using the method of Laemmli on an acrylamide gel with 7.5% resolving gel and 4% stacking gel to identify the protein expression and purification. As indicated in Fig.4.3, The lanes from right to left were protein makers, fractions washed by 20mM imidazole solution, 40mM imidazole solution, 80mM imidazole solution, 120mM

imidazole solution and 160mM imidazole solution, respectively. From the SDS-PAGE, the recombinant protein GBP-Rluc was started to wash out from 80mM (Lane 3) while the purified protein was washed by 120mM imidazole elution solution.

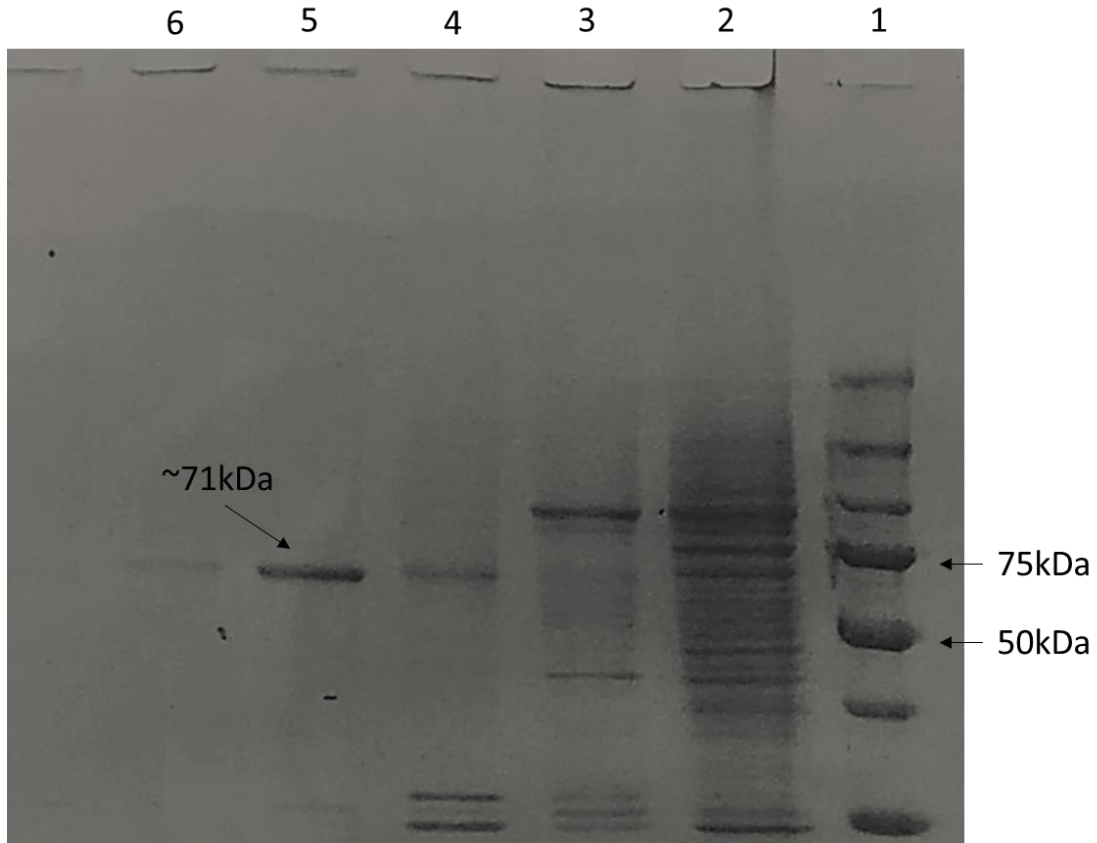


Figure 4. 3: SDS-PAGE characterization of recombinant protein GBP-Rluc washing by different concentrations of imidazole elution solution. From right to left: 1) Protein maker, 2) 20mM imidazole elution solution, 3) 40mM imidazole elution solution, 4) 80mM imidazole elution solution, 5) 120mM imidazole elution solution, 6) 160mM imidazole elution solution.

4.2.4 Optimization for bioluminescence of GBP-RLuc

In order to find the best performance of GBP-RLuc, different concentration of the protein, substrate and different buffer were tested. Fig. 4.4 showed the bioluminescence of 0.1mg protein dissolved in 400 μ L PBS with different amount of CTZ (1mg/ml). The intensity increased when CTZ increased from 3 to 5 μ L, while dropped when CTZ kept increasing to 10 μ L.

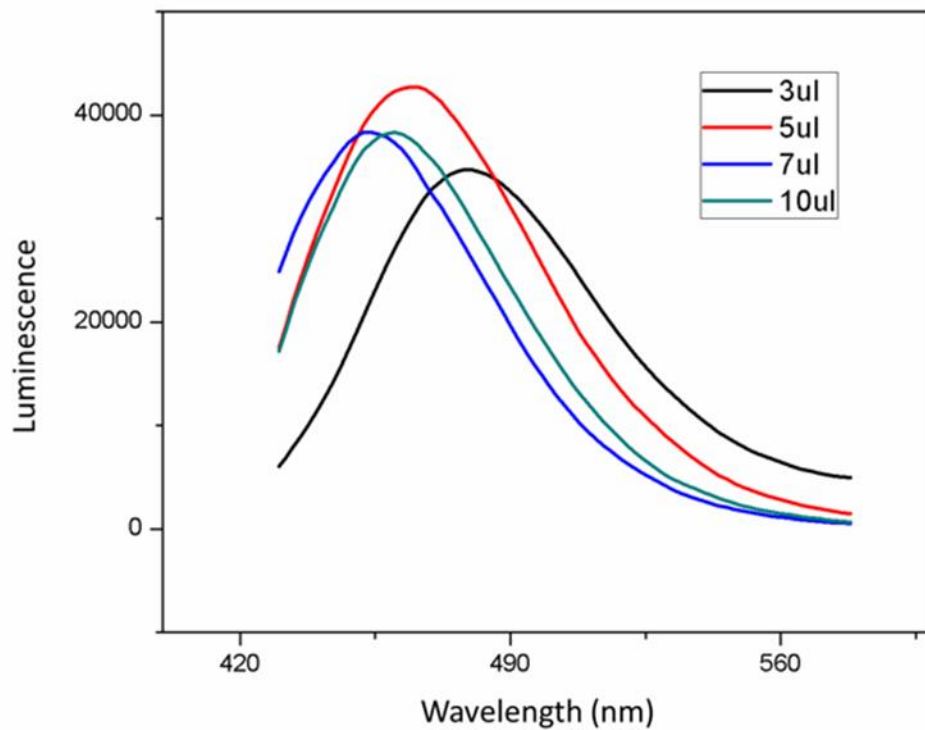


Figure 4. 4: Bioluminescence of 0.1mg protein dissolved in 400 μ L PBS with 3 μ L, 5 μ L, 7 μ L and 10 μ L CTZ (1mg/ml), respectively.

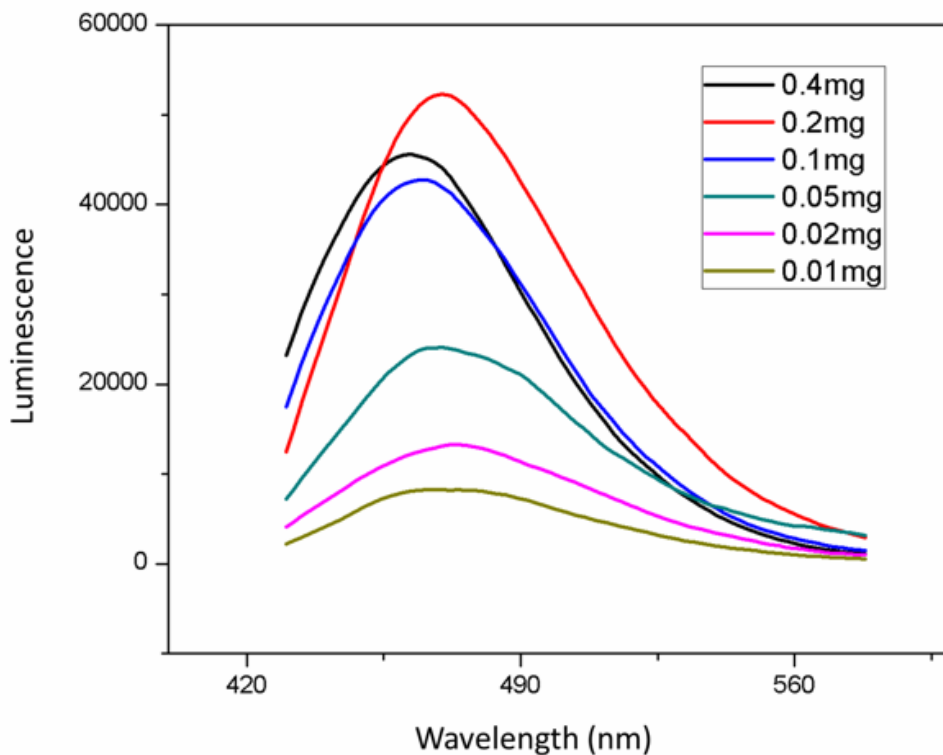


Figure 4. 5: The effect of protein amount to the bioluminescence performance. Different amount of protein (from 0.01mg to 0.4 mg, all dissolved in 400µl PBS) were tested with adding 5µl of CTZ (1mg/ml).

Fig.4.5 showed the effect of protein amount to the bioluminescence performance. The intensity were found to have a slight drop when protein mass increased over 0.2mg. The behaviour in different buffer: 10 mM Tris/HCl, pH 7.4, 10mM PBS, pH 7.4 and 10mM Borate, pH 8.0 were tested and the result was shown in Fig. 4.6, which indicates the 10mM PBS, pH 7.4 is best for the assay. The decay rate of the bioluminescence was measured with a time interval at 2s, with different amount of protein, as shown in Fig. 4.7. All measurements were repeated for 3 times.

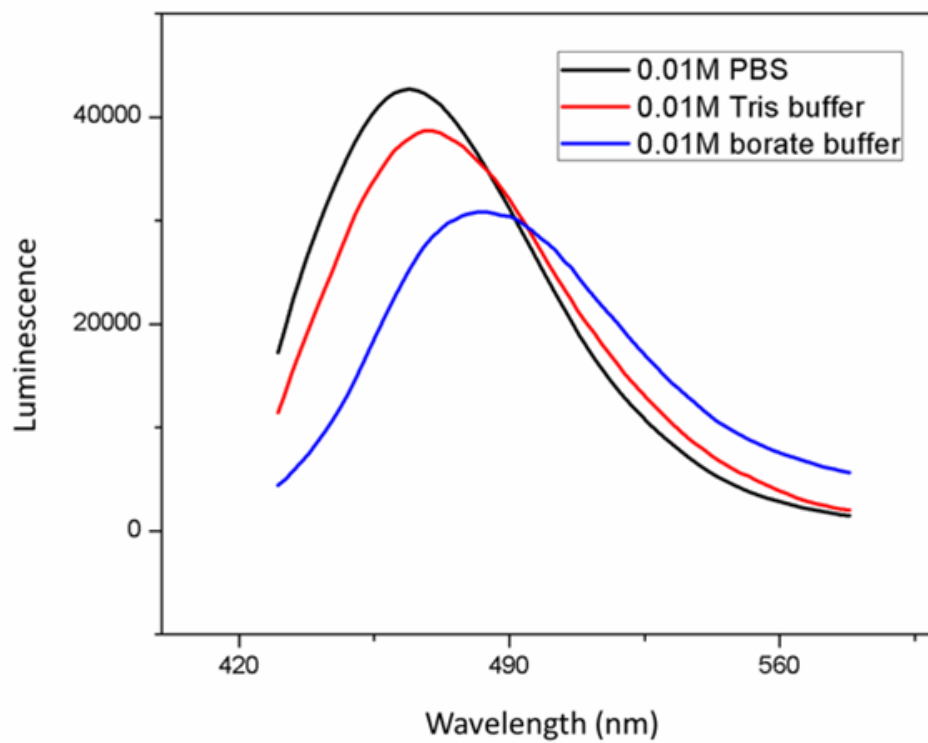


Figure 4. 6: Bioluminescence of GBP-Rluc in different buffers: 10 mM Tris/HCl, pH 7.4, 10mM PBS, pH 7.4 and 10mM Borate, pH 8.0.

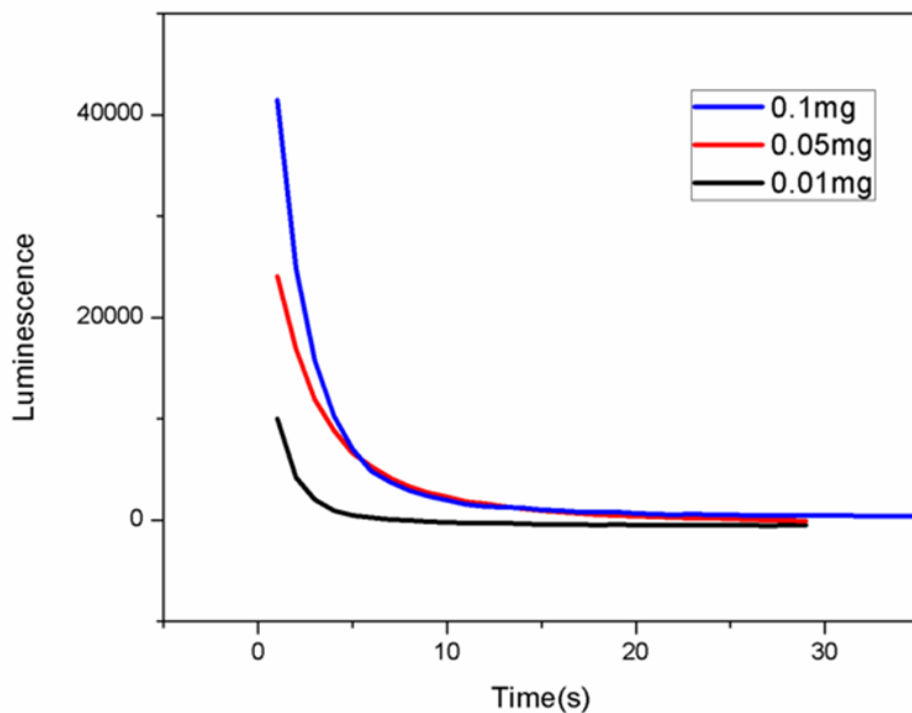


Figure 4. 7: Luminescence of 0.01 mg, 0.05mg and 0.1mg GBP-Rluc from 0 to 30s at intervals of 1s.

4.3 Conclusion

TGA-coated CdTe was synthesized by using a simple and economical one-pot method. The highly luminescent water soluble CdTe quantum dots were analyzed by FTIR to insure the carboxylic groups on the surface for further bioconjugation. GBP-Rluc was purified by his-trap column through low imidazole to high imidazole concentration. It was found the high imidazole elution solution (around 120mM) can wash the target his-tag protein efficiently. The best conditions for GBP-Rluc to catalyze native CTZ were tested through changing the ratio of the two components, the buffer and the decay rate with time. The best performance of GBP-Rluc for catalyzing was found to occur in 10mM PBS, pH 7.4 and mass ratio is 40 (GBP-Rluc):1 (native CTZ). By measuring the decay of bioluminescence

with time, it was found the bioluminescence decreased very quickly especially in the first 4 seconds after adding the native CTZ. This phenomenon indicates the detection should be started as soon as the substrate is added and mixed homogenously.

4.4 Reference

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Chapter 5

5 BRET biosensor construction and tear glucose test

TGA coated CdTe QDs with emission peak at 565nm was synthesized and the optimal performance conditions of GBP-Rluc was tested in chapter 4. In this chapter, the bioconjugation process of QDs (BRET acceptor) and GBP-Rluc (BRET donor) was optimized. A gradient concentrations of glucose solution were tested by the BRET biosensor and two linear relationship between the intensity ratio and the glucose concentrations were obtained. The rate tear were collected and diluted to certain range and tested by the BRET biosensor.

5.1 Introduction

As the numbers of diabetes patients increased so fast worldwide, the desire of constructing glucose sensor especially the non-invasive types have drawn numerous attentions[1]. Body fluids such as urine [2-4], sweat [5-8] and tear [9-12] thus have been widely used as testing samples. It has been found that tear glucose level is around 5 times higher in diabetes patients than in normal people and the tear glucose has been identified to have a positive correlation with the blood glucose based on previous research[13-15].

In order to test the extremely low concentration of glucose from the tiny amount of the tear sample, fluorescence resonance energy transfer (FRET) has been used in bioimaging and biosensing systems because of its high sensitivity for distance change [16-19]. However, one biggest problem FRET is facing is that the system requires for external illumination to initiate the fluorescence transfer. This process may lead to background noise and photo bleaching to the original signal [20]. In order to diminish the drawbacks, Bioluminescence Resonance Energy Transfer (or BRET) has been developed to make the assay more reliable [21,22].

Glucose/galactose-binding protein (GGBP) is a bacterial periplasmic-binding protein that exhibits a hinge motion and results of conformational change upon binding glucose or galactose molecules [23,24]. Though a lot of research has been done using

Glucose/galactose binding protein and its mutation to build biosensor for detecting glucose molecules [25-28], there is no report on the combination of GBP with BRET to our best knowledge.

The objective of our research is to build a novel glucose sensing system strategy which is based on the fluorescence changes of the probe as the protein undergoes a structural change on binding. The recombinant fusion protein consists of bacterial glucose binding protein (GBP), at the N-terminal and a bioluminescent protein *Renilla* luciferase (Rluc) at the C-terminal. Afterwards, quantum dots used as the acceptor can be labelled on the N-terminal of the recombinant protein.

5.2 Results

5.2.1 Optimization of the assay

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is a commonly used reagent in bioconjugation by forming amide bonds. In most cases, the coupling reaction will be better performed in the presence of EDC at pH between 6 and 8 [29]. However, the excess of EDC can lead to aggregation after the addition into the solution [30].

To optimal the sensor for glucose detection, Firstly the amount of EDC is fixed to be 20mM, where the aggregation and precipitation is hardly observed. The ratio between the protein and quantum dots were tested at 50:1, 150:1, 250:1 and 400:1, respectively and then treated with 3 μ L 0.1mmol/L of aqueous glucose. The BRET intensity ratio I_{BRET} and the intensity ratio change I_c is defined as follow:

$$I_{BRET}=I_A/I_D \quad \text{Eq.5.1}$$

$$I_c=(I_w-I_g)/I_g \quad \text{Eq.5.2}$$

Where I_A and I_D are the intensity of acceptor and donor, I_g and I_w are the intensity ratio of the acceptor and donor before and after treated with glucose solution. As EDC mediated all the amine groups on the surface of GBP-Rluc with the carboxylic groups on the surface

of CdTe, the bioconjugation process is non-site specific [26,31], the excess amount of either GBP-Rluc or QDs may affect the sensitivity of this sensor. Fig.5.1 showed the best sensitivity presents around the ratio at 250:1.

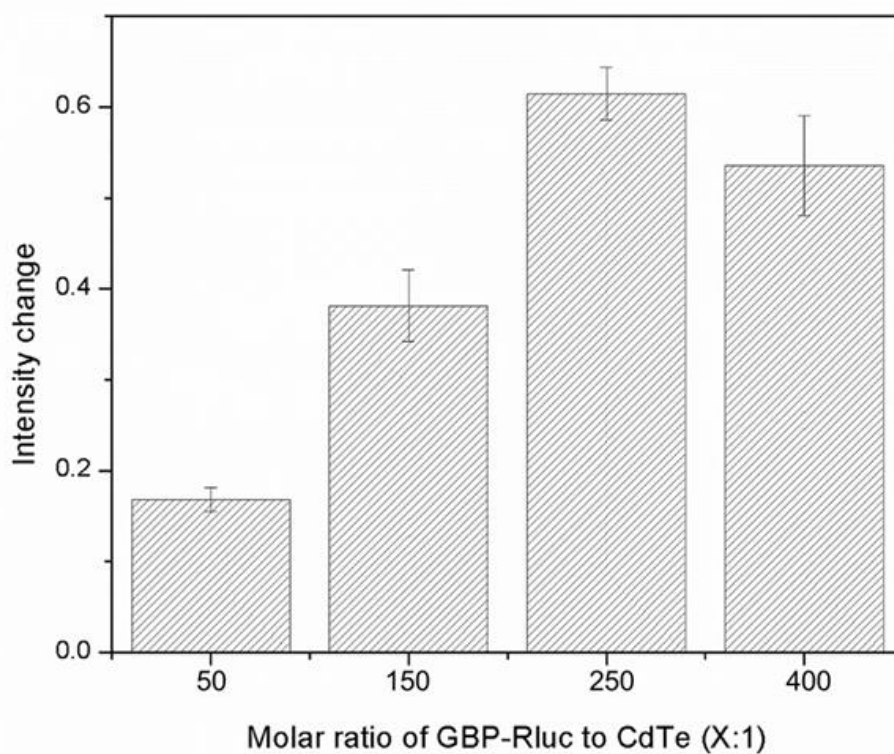


Figure 5. 1: Intensity change with different ratio of GBP-Rluc to QDs. All the test solutions were treated with 0.1mM glucose.

Also, the molar ratio of EDC to QDs was also tested at 1000:1, 2500:1, 5000:1, 10000:1 and 20000:1. The ratio between GBP-Rluc and QDs is mixed at 250:1 and 3 μ L of 0.1mmol/L aqueous glucose was added for the test. Though the amount of EDC is key to mediate the reaction, high concentration of EDC could cause the self-crosslink of proteins and irreversible precipitation of the products which result in low signal response, as shown in Fig.5.2. The best ratio between the EDC and the QDs is found to be at 10000:1.

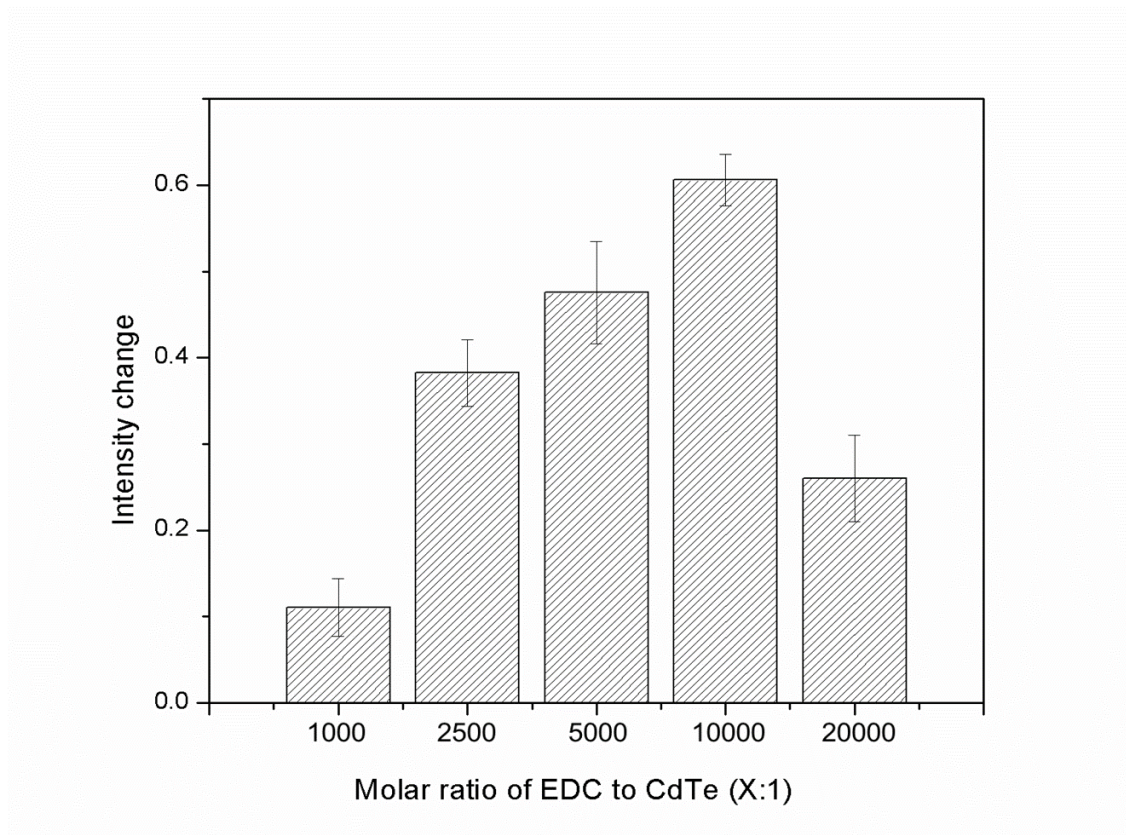


Figure 5. 2: Intensity change with different ratio of EDC to QDs. All the test solutions were treated with 0.1mM glucose.

5.2.2 Glucose assay and animal tear test

The capability of the nanostructured BRET sensor to measure the concentration of glucose is evaluated under the above optimized condition. Fig.5.4 showed the BRET spectra with different concentration of glucose. The increase of glucose concentration results in an increasing of the BL emission intensity.

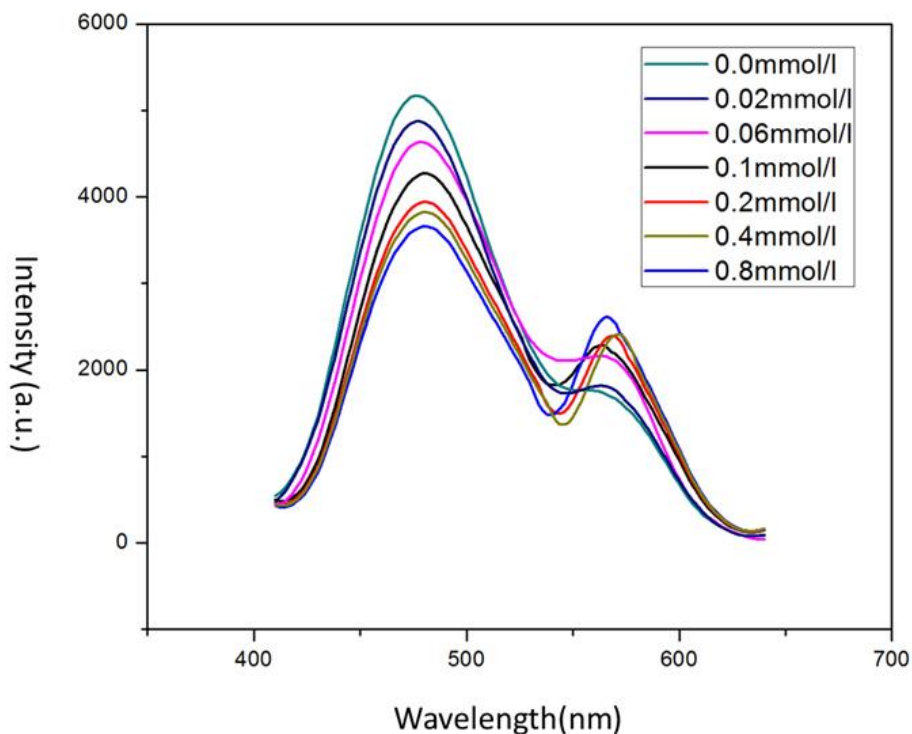


Figure 5. 3: The BRET spectrum with different concentrations of glucose.

According to Eq.5.1, The BL intensity were calculated and the linear relationships between glucose concentration and the BL intensity are found in the ranges of 0– 0.1mM and 0.2mM -0.8mM, respectively as shown in Fig.5.4. The limit of detection (LOD) for this assay is secured to 0.03mM based on 3σ from three independent measurements. The relationship between the concentration of glucose and the intensity ratio in the two ranges mentioned above can be expressed as the following equations, respectively.

$$Y=1.7692X+0.34648 \quad \text{Eq. 5.3}$$

$$Y=0.21064X+0.55406 \quad \text{Eq. 5.4}$$

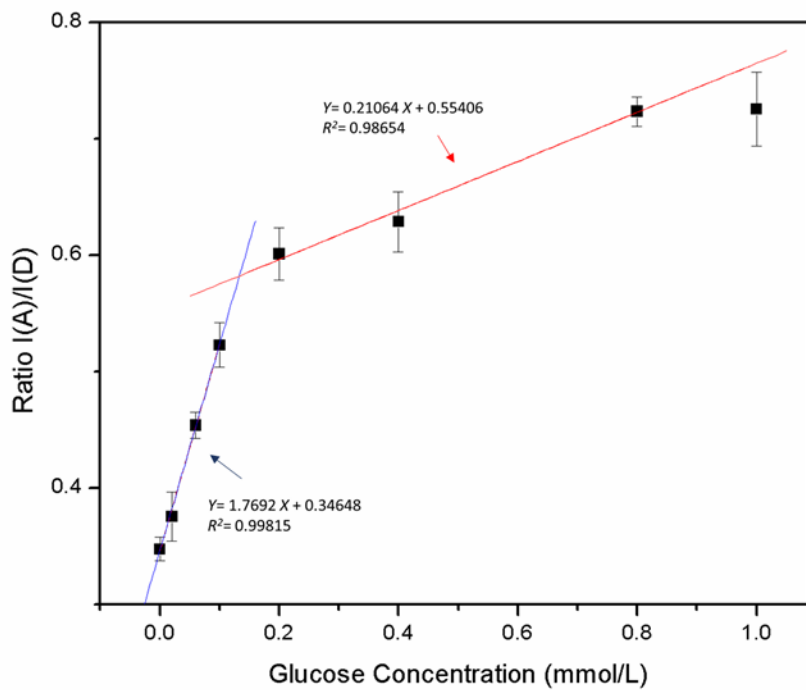


Figure 5. 4: Linear relationship between BRET intensity ratio of the designed sensor and the concentration of glucose.

1 μ L tear samples from 3 rats with different blood glucose levels were diluted to 10 μ L by 10mM PBS, pH 7.4. The detected signal were calculated by Eq. 5.4 to convert to glucose level. Table 5.1 shows the tear glucose level measured by BRET sensor and the corresponding blood glucose level in the three rats.

Table 5. 1 Glucose level in rats' tear samples measured through BRET biosensor

Blood glucose (mmol/L)	Tear glucose (mmol/L)
5.6	0.38±0.04
6.3	0.52±0.04
22.1	1.62±0.05

5.3 Conclusion

A novel BRET biosensor for monitoring the tear glucose was developed. CdTe QDs was used as acceptor and luciferase Rluc was used as the bioluminescence donor. The distance between the donor and the acceptor is mediated by the folding of GBP, which is at the upstream of Rluc, with existence of glucose molecules. The ratio of the acceptor (QDs) and the donor (Rluc) has been observed to increase with the increasing of the glucose concentration and a similar linear relationship is found both in the range from 0mM to 0.1mM and from 0.2mM to 0.8mM. The limit of detection is as low as 0.03mmol/L. Further, three rats tear samples with different level of blood glucose were tested by the BRET sensor. The tear glucose level of normal rat (blood glucose around 5.6mmol/L) was found to be 0.38±0.04mmol/L, while the tear glucose level of diabetes rat (blood glucose at 22.1mmol/L) was found at 1.62± 0.05mmol/L, which is much higher than the normal one by the measurement of our BRET sensor. Consequently, our designed nanostructured BRET glucose biosensor could be a promising tool for non-invasively and rapidly detecting tear glucose.

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Chapter 6

6 Summary and future work

6.1 Summary

As the numbers of diabetes patients increased so fast worldwide, the desire of constructing glucose sensor especially the non-invasive types have drawn numerous attentions. Body fluids such as urine sweat and tear thus have been widely used as testing samples. It has been found that tear glucose level is around 5 times higher in diabetes patients than in normal people and the tear glucose has been identified to have a positive correlation with the blood glucose based on previous research. In order to test the extremely low concentration of glucose from the tiny amount of the tear sample, fluorescence resonance energy transfer (FRET) has been used in bioimaging and biosensing systems because of its high sensitivity for distance change. However, one biggest problem FRET is facing is that the system requires for external illumination to initiate the fluorescence transfer. This process may lead to background noise and photo bleaching to the original signal. In order to minimize the drawbacks, Bioluminescence Resonance Energy Transfer (or BRET) has been developed to make the assay more reliable. This technique uses a bioluminescent luciferase, typically the luciferase from *Renilla reniformis* to be the light source. Glucose/galactose-binding protein (GGBP or GBP) is a bacterial periplasmic-binding protein that exhibits a hinge motion and results of conformational change upon binding glucose or galactose molecules. Though a lot of research has been done using Glucose/galactose binding protein and its mutation to build biosensor for detecting glucose molecules, there is no report on the combination of GBP with BRET to our best knowledge. We successfully synthesized a GBP based BRET sensor for non-invasively detecting glucose molecules. The sensor is made by the bioconjugation of quantum dots and recombinant protein. The recombinant protein contains the GBP and a bioluminescent protein, *Renilla* luciferase (Rluc), used as the donor with the emission peak at 470 nm, which is able to excite the acceptor of BRET sensor made of cadmium tellurium quantum dots (CdTe QDs) with the emission peak at 570 nm. The distance between the BRET pair depends on the shape of GBP. In the presence of glucose, the conformational change of GBP leads to the enhanced BRET phenomenon due to the short distance between the BRET

pair. The quantum dots were chosen as the BRET acceptor due to their tunable emission peaks by controlling their sizes and bandwidth. The unique optical properties and good biocompatibility make the QDs very suitable candidates in bioimaging and other biomedical fields.

In this thesis, TGA-coated CdTe QDs was synthesized by using a simple and economical one-pot method. The highly luminescent water soluble CdTe QDs were analyzed by FTIR to insure the carboxylic groups on the surface for further bioconjugation. GBP-Rluc was purified by his-trap column through low imidazole to high imidazole concentration. It was found the high imidazole elution solution (around 120mM) can wash the target his-tag protein efficiently. The best conditions for GBP-Rluc to catalyze native CTZ were tested through changing the ratio of the two components, the buffer and the decay rate with time. The best performance of GBP-Rluc for catalyzing was found to occur in 10mM PBS, pH 7.4 and mass ratio is 40 (GBP-Rluc):1 (native CTZ). By measuring the decay of bioluminescence with time, it was found the bioluminescence decreased very quickly especially in the first 4 seconds after adding the native CTZ. This phenomenon indicates the detection should be started as soon as the substrate is added and mixed homogenously

A novel BRET biosensor for monitoring the tear glucose was developed. CdTe QDs was used as acceptor and luciferase Rluc was used as the bioluminescence donor. The distance between the donor and the acceptor is mediated by the folding of GBP, which is at the upstream of Rluc, with existence of glucose molecules. The ratio of the acceptor (QDs) and the donor (Rluc) has been observed to increase with the increasing of the glucose concentration and a similar linear relationship is found both in the range from 0M to 0.1 mM and from 0.2mM to 0.8mM. The limit of detection is as low as 0.03mmol/L. Further, three rats tear samples with different level of blood glucose were tested by the BRET sensor. The tear glucose level of normal rat (blood glucose around 5.6mmol/L) was found to be 0.38 ± 0.04 mmol/L, while the tear glucose level of diabetes rat (blood glucose at 22.1mmol/L) was found at 1.62 ± 0.05 mmol/L, which is much higher than the normal one by the measurement of our BRET sensor. Consequently, our designed nanostructured BRET glucose biosensor could be a promising tool for non-invasively detecting tear glucose.

6.2 Future work

In this thesis, we constructed the BRET system by bioconjugation the CdTe QDs with GBP-Rluc, where QDs serve as acceptor, Rluc as donor and GBP as the sensing element through conformational change. As mentioned the Chapter 2, there are a lot of other candidates qualified to use as BRET donors. For example, based on the recent development of NanoLuc luciferase (Nluc), a small (19 kDa), highly stable, ATP independent, bioluminescent protein, an extremely robust and especially high sensitivity screening system has been developed whereby primary hits of therapeutic antibodies and antibody fragments could be characterized and quantified without purification. The smaller size of Nluc provides unique advantages during bioconjugation process. The sensing element in the thesis, GBP can be replaced by other glucose affinity proteins like Con.A etc. Also, as the bioconjugation process is non-specific thus makes the bioconjugation process hard to maintain. A lot of site-specific methods can be applied to make the system more stable.

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