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Fluorescence Resonance Energy Transfer-Based Biosensor between Graphene Quantum Dots and Gold Nanoparticles for Optical Diagnostic of Staphylococcus Aureus Oligonucleotide

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Author: Li Hui Jun (1309)

Interdisciplinary Division of Biomedical Engineering,
The Hong Kong Polytechnic University

Chief Supervisor: Dr. Mo YANG
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ABSTRACT

Staphylococcus aureus (S. aureus) as the most common bacteria both in the skin infection and food-poisoning, requires a sensitive and specific diagnosis strategy for the daily direct detection. An efficient optical detection strategy for S. aureus, involving binary probes in a sandwich FRET between GQDs and AuNPs, was designed in this study. Fluorescence resonance energy transfer (FRET), referring to the effect of transferring energy from fluorescent donor to quenching acceptor, is distant-depended and required spectrum overlapping between donor and acceptor. GQDs are excellent emitter with strong fluorescence, in addition to AuNPs are well-known peachy fluorescence quencher due to the wide range absorption spectrum, making them as partners in the fulfillment of spectral requirement of FRET, and thus have been chosen to be as donor and acceptor respectively in this sandwich structure FRET system. Without labeling target directly on the GQDs or AuNPs as probe, amine-modified and thiol-modified complementary single-strain oligonucleotide were synthesized for the conjugation of DNA immobilized GQDs and DNA immobilized AuNPs to form capture probe and reporter probe. In the process, capture probe hybridized with target cDNA with complementary sequence, then the reporter probe subsequently hybridized with the remaining unbinding sequence of target, bringing GQDs and AuNPs into proximity, leading the energy transfer from GQDs to AuNPs under excitation.
The quenching efficiency of oligonucleotide immobilized AuNPs was found to increase with the increasing target concentration, and it reached a plateau with the beginning concentration with 100nM and tend to be stable at 200nM with approximately 80% efficiency. The linear working range of detection was from 100pM to 100nM with a detection limit of 10nM. The strategy also possesses a high selectivity when comparing to the single-base and double-base mismatch oligonucleotides, with a trend that increasing number of mismatch base increasingly decreases the quenching efficiency.
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<td>Gold nanoparticle</td>
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<td>BPs</td>
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<td>DI water</td>
<td>Deionized water</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>EDC</td>
<td>1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbent assay</td>
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<tr>
<td>$F_0$</td>
<td>Fluorescent emission without quenching</td>
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<td>FI</td>
<td>Fluorescence intensity</td>
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<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<td>$F_q$</td>
<td>Fluorescent emission after quenching by AuNP</td>
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<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<td>GQDs</td>
<td>Graphene quantum dot</td>
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<td>GQDs-ssDNA</td>
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<td>HAuCl$_4$</td>
<td>Chloroaauric acid</td>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HNO$_3$</td>
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<td>IDT Inc.</td>
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<td>IMS</td>
<td>Immunomagnetic separation</td>
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<td>LOD</td>
<td>Limit of detection</td>
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<td>MBs</td>
<td>Molecular beacons</td>
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<tr>
<td>NP</td>
<td>Nanoparticle</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>RT-PCR</td>
<td>Reverse-transcription Polymerase chain reaction</td>
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<td>S. aureus</td>
<td>Staphylococcus aureus</td>
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<td>Staphylococcus enterotoxins</td>
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<td>SPR</td>
<td>surface plasmon resonance</td>
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<td>ssDNA</td>
<td>Single-strand DNA</td>
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1. Introduction

Staphylococcus aureus (S. aureus) as the most common bacteria both in the skin infection and food-poisoning, requires a sensitive and specific diagnosis strategy for the daily direct detection. Fluorescence resonance energy transfer (FRET), referring to the effect of transferring energy from fluorescent donor to quenching acceptor, is distant-dependent and required spectrum overlapping between donor and acceptor (Ming Li et al, 2011). FRET, considered as the burgeoning and constantly improving biosensor, could be designed differently according to the requirements of study. Basically, FRET has a rule of occurrence that a limited detachable distance of less than 10nm is required which would affect the rate of the energy transfer (Walter, Russell, Algar., 2010). Otherwise, the overlap spectrum between donor emission and acceptor absorption would be as a quantitative measurement of the probability of resonant transition and the effectiveness of donor emission quenching by the acceptor to some extent would represent the efficiency of FRET (Walter, Russell, Algar., 2010). Graphene quantum dots (GQDs) and gold nanoparticles (AuNPs), both as nanoparticles, are considered as materials with high potential value. Both of them usually are selected to be as probes carrier and materials for diagnosis due to the outstanding characteristics. Comparing to the general pair conjugated FRET effect, a sandwich hybridized FRET exists an extra cDNA to link the capture probe and report probe. Despite of the additional cDNA, in the sandwich hybridized FRET effect, the
separated distance of donor- GQDs and acceptor- AuNPs is mainly controlled by the
probes (including the capture probe on the donor and reporter probe on the acceptor) length
(Ming Li et al, 2011).

In this study, GQDs (about 3nm, blue fluorescence) conjugated with a 10 base ssDNA-a as
a capture probe and AuNPs (15nm) conjugated with 10 base ssDNA-b as a reporter probe
will be designed in a sandwich hybridized FRET to detect a 20 base target cDNA of S.
aureas. Beside, considering the emission (relaxation) spectrum peak of blue fluorescence
GQDs is 420nm with a larger spectral range from 400nm to 600nm (James M., 2013) and
the absorption spectrum peak of 15nm AuNPs is 520nm, there are spectral overlap between
3nm blue fluorescence GQDs and 15nm AuNPs that it satisfies the basic requirement for
the FRET occurrence. Apart from the consideration of excellent properties of GQDs as a
FRET donor and AuNPs as an FRET acceptor, the sensitivity of sandwich hybridized
FRET effect would be considered as well. Therefore, a calibration of concentration of
target S. aureas cDNA which would affect the efficiency of detection will be set up in order
to explore the limit of detection (LOD).

The objectives of this study:

(i) To design an efficient FRET-based sensor for S. aureus diagnosis;
(ii) To characterize the concentration effect of target DNA on quenching efficiency;
(iii) To characterize the detection limit (LOD) of the system;
(iv) To characterize the specificity of the system.
1.1 Introduction of Staphylococcus aureus (S.aureas)

Staphylococcus aureus (S. aureus), as the most virulent species (Archer, 1998) among the 38 species of Staphylococcus (Kleeman et al, 1993; Kloos and Bannerman, 1994), may cause significant number of patients’ infections (Archer, 1998) and it can widely exists in the environment (T. G. Miao et al, 2011), such as in food, most commonly, and on the fester surface, due to its ability to grow in conditions with a wide range of temperature (M. Schnitt et al, 1990), pH (M. S. Bergdoll, 1989) and sodium chloride (Y. Le Loir et al, 2003) concentration. S. aureus also leads to a worldwide pandemic infection and only in the United States (Spellbery, B. et al, 2008), there are approximately 19 thousands deaths per year due to S. aureus, which surpassing those due to HIV-AIDS (Klevens, R. et al, 2007; Peterson, J. F. and D. J. Diekema, 2010). The infection of S. aureus could cause the increase of patient morbidity and mortality (Wolk, D. M. et al, 2009). In the Tacconelli’s study at hospital admission, the true community-acquired MRSA (Methicillin-resistant Staphylococcus aureus) infected patients were not timely diagnosed in the first 24h though they had symptoms of central venous catheter and skin ulcers (E. Tacconelli et al, 2004).

Though the clinical symptoms (nausea, vomiting, abdominal cramps and diarrhea ) of infections and food poisoning by S. aureus could alert clinician and patient the situation of S. aureus infection and assist in targeting exact strategy and therapy to prevent the cross-transmission, they could hardly provide an immediately and accurate information for
a best and timely prescription. Thus, S. aureus, as the most frequently identified bacteria in
blood cultures (Poppert et al., 2010), hungers for a faster and more efficient diagnostic
strategy to prevent the deterioration and provide the best and accurate treatment to the
infected patients (Ruimy et al. 2008; Weinstein, 2003).

1.2 Existing Detection Methods of Staphylococcus aureus (S.aureas)

According to the data of U.S. medical centers in 2009, there is no longer clear distinction
between hospital-acquired and community-acquired MRSA and community-acquired
MRSA is the most common now (Richter S. et al, 2011). Molecular methods for the S.
aureus detection were adopted more than 50% and the majority of molecular detection
based on the nares screening, according to the Clinmicronet survey of 70 laboratories of S.
aureus.

According to the comparison of two standardization methods in real-time quantitative
RT-PCR to quantify S.aureus by Heidy Eleaume and Said Jabbouri (2004), the gene
expression were studied during the incubation of clinical isolated S. aureus and they also
suggested 16S rRNA of S. aureus as internal standard in the real-time quantitative RT-PCR
quantification. This real-time quantitative RT-PCR strategy is based on the principle that it
required a range of dilutions of the target sequences with a known concentration and
therefore, after the RT-PCR, the results are expressed relatively by dividing the number of
cDNA copies by the number of colony forming units from the enumeration of the bacterial
culture (Vandecasteele et al, 2001). Though the real-time quantitative RT-PCR is of better
reliability and sensitivity, the results of absolute quantification would vary differently
depending on different selected standardization strategies.

In the study and survey of food-poisoning via Staphylococcus enterotoxins (SEs) from
Staphylococcus aureus (S. aureus) by Yu-Cheng Chiang et al (2008) in Taiwan, they use
PCR to detect the Staphylococcal food-poisoning. As described, SEs are superantigenic
toxins including five major classical types. For the PCR detection, Yu et al also synthesis
specific PCR primers for the target gene sequences. Comparing to the unavailable
immunoassay kit for the new types of SEs, DNA probes, PCR primers and RT-PCR are
used to detect these kind of superantigenic toxin. Similarly, Tereza T. et al (2009) used
selective enrichment and real-time PCR targeting a gene marker to detect S. aureus in food.
They also designed a specific PCR primers and TaqMan probe to target specific S. aureus
gene. Otherwise, available standard strategy to detect the S. aureus in food was based on
the selective enrichment and subsequent isolation of colonies, which would be identified
and morphologically characterized by the confirmations based on microbiology and
biochemistry, which is time-consuming and labor-intensive. Moreover, it is not a
sufficiently reliable identification for S. aureus detection when there is a bacteria with similar morphological and biochemical characteristics. Comparing to the bacterial identification method, polymerase chain reaction (PCR)-based strategies satisfies the criteria of high specificity and sensitivity, which is considered as a reliable alternative to the conventional methods (Nugen and Baeumner, 2008).

When dealing with the clinical features identification and diagnosis of Staphylococcal skin and soft tissue infection, strategies vary based on the patients’ situation: (i) Primary pyodermas including folliculitis, furuncles and carbuncles were diagnosed via clinical grounds alone, but is the patient fails to respond to initial therapy, microbiological investigations are needed. (ii) Soft tissue infection including cellulitis and erysipelas were detected by microbiological diagnosis strategy using the skin biopsies, blood cultures and aspirates, but the microbiological yield from blood cultures alone is low (Perl B. et al, 1999; Bisno A.L. et al, 1996). Also, ultrasonography, CT scans or magnetic resonance imaging (MRI) are also alternative reliable strategies for the evaluation of a cute soft tissue infection including the Staphylococcal infection (S. Roberts and S. Chambers, 2005). (iii) Infection associated with injectable drug use mostly due to the skin and soft tissue infection in the hospital admission. When it only appears as cellulitis, it is not difficult to diagnosis, however, when developing into abscesses, it would be hard to distinguish from
heamatoma, cellulitis, pseudoaneurysms, phlegmon or thrombosed veins. In this situation, ultrasonography, CT scans and MRI imaging could help to clarify the extent of abscesses and neighboring structures involved, as well as the blood cultures.

Comparing to the conventional strategy based on the bacteria isolation, bio-molecular diagnosis strategies based on enzymes and radioactive molecules of S. aureus would be more sensitive and specific relying on the indicated information of labeled target DNA/RNA. Immunoassay, such as ELISA (Enzyme-Linked Immunoabsorbent Assay), IMS (Immunomagnetic Separation) and immunosensors, is a common applied strategy in the detection of food-borne pathogens including Staphylococcal food-poisoning. However, enzyme-based detection method, relating to the antibodies, is susceptible to temperature, and the signals also depend on the background signal and applied devices, it also significantly depends on the situation of target binding the primes binding (Thaxton et al., 2006). Though conventional ELISA strategy is rapid and easy operated, the sensitivity is limit due to the limit binding sites for the analytes on the fixed solid supports, as well as the variational background signals and low sensitivity of colorimetric detection devices (Grund et al, 2013).

Diagnosis strategy based on the DNA hybridization assay has numerous ways to achieve the purples of sequences detection, such as fluorescence-based sensor,
electroluminescence-based sensor, and nanostructure-based methods. The fluorescence in situ hybridization (FISH) strategy, including both the DNA sequence hybridization and peptide-nucleic acid hybridization, plays important role in detecting S. aureus. Fluorescence signal intensity depend on the remaining fluorescence DNA probes conjugated with the complementary target DNA (Jansen et al., 2000; Oliveira, 2002) because the fluorescence probes without conjugation with complementary sequences would be required to be separated ((Southern et al., 1999; Taton et al., 2000). Therefore, if there is no target complementary DNA localized on the solid substrate, all the fluorescence probes would be washed out and there would be no fluorescence signal.

Biosensors based on hybridization between target DNA and DNA probe are direct, sensitive and rapid diagnosis strategies of bacterial DNA without the requirement of DNA amplification (Pang et al., 2013). The fluorescence intensity could also be enhanced by the relative signal expressed by the reporter probe (usually the acceptor), which do not need to amplified DNA quantity to enhance the sensitivity of the diagnosis system as the conventional strategy (e.g. PCR). Recently, there are numerous approach to achieve diagnosis including S. aureus via fluorescence-based biosensors, DNA complementary-based sensors, and majority of these kind burgeoning biosensors are still constantly improving and developing.
1.3 Introduction of Fluorescence Resonance Energy Transfer (FRET)

1.3.1 Principle of FRET

The fluorescence Resonance Energy Transfer refers to the radiationless transfer of energy from the excited donor fluorophore to a suitable acceptor fluorophore subject to the distances and spectrum overlap [S. A. Hussain et al]. Comparing to the radiative energy transfer, which requires emission and reabsorption of a photon, FRET involves a donor fluorophore in an excited electronic state which may transfer its excitation energy to a nearby acceptor chromophore, based on the spectrum overlap between the donor and acceptor, in a radiationless fashion by long-range dipole-dipole interaction. This mechanism of supporting energy transfer bases on the theory of treating an excited donor as an oscillating dipole to undergo an energy exchange with a second dipole with a similar resonance frequency.

The FRET as non-radiative resonance energy transfer can yield a critically significant amount of structural information considering the donor-acceptor pair. Comparing to those are sensitive to the solvent environment of the fluorophore, the resonance energy transfer is not so sensitive to the surrounding solvent shell of fluorophore that it reveals molecular information by the solvent-dependent events of fluorescence quenching, excited-state reactions, solvent relaxation, or anisotropic measurements, which major due to the effect
on spectral properties of donor and acceptor. The distance between donor and acceptor is a primarily dependent aspect of the efficacy of resonance energy transfer for radiationless energy transfer takes place over much longer distances than short-range solvent effects. The phenomenon of FRET referring to as a donor-acceptor pair is not mediated by photon emission and does not require a fluorescent acceptor.

In the process of FRET, a donor fluorophore would absorb the energy due to the excitation of incident light and the nearby acceptor chromophore would absorb the emission from donor fluorescence to transfer the excitation energy as shown in Figure 1. The donor can transfer its excited energy to the acceptor directly in the presence of a suitable acceptor. By FRET, the fluorescence intensity of acceptor increase by decreasing or quenching the donor fluorescence and reducing the lifetime of excited state of donor. As shown in figure 1, during the process of FRET, the coupled transitions occurs between the donor emission and acceptor absorption.

Nowadays, the strongly distance-dependent FRET can be measured by techniques in sophisticated and accurate ways that a wide range of applications are born [Jares E. et al, 2003]. Structure measuring (Haas E. et al, 1975; Lakowicz J.R. et al, 1990; Chapman E.R. et al, 1992), molecular interactions (Hink M.A. et al, 2002; Parsons M.B. et al, 2004) and
biochemical indication (Bunt G. et al, 2004) can be all realized by donor-acceptor pair of FRET effect.

However, to support the FRET occurrence, there are few criteria: (i) the overlap between the fluorescence emission spectrum of donor and the absorption or excitation spectrum of the acceptor (figure 1); (ii) the close enough distance between donor and acceptor, typically 1-10 nanometer); (iii) a long lifetime of donor fluorescence to supply the sufficient duration for the allowance of FRET occurrence.

**Figure 1**

Based on the precondition of suitable distances and spectrum overlap, the acceptor fluorescence intensity increases by quenching the excited energy of donor. The Jablonski diagram and the spectrum map both illustrate that the FRET process involves in the energy transfer from the donor emission to the acceptor absorption based on the spectrum overlap between fluorescence spectrum of donor and absorption spectrum of acceptor. The more resonance transition there are, the larger the efficiency of energy transfer (Algar, W. R., 2011).
1.3.2 DNA FRET Probe

FRET is widely used as a reporter method especially for the real-time monitoring of biochemical reactions (Vladimir V. Didenko, 2001). The FRET acceptor chromophore becomes excited after the fluorescence of FRET donor fluorophore is quenched because excitation is transferred from the donor to the acceptor by dipole-dipole interaction. Meanwhile, the intensity of FRET donor fluorescence, lifetime, quantum efficiency decrease.

Based on the above FRET theory, oligonucleotide labeled with chromophore can form a FRET system to be as a probe for DNA detection according to the hybridization based on the DNA bases complementary. The FRET probe can signal the occurrence of target oligonucleotide via the changes in the fluorescence energy transfer. The FRET donor conjugated with a capture oligonucleotide which is complementary to target oligonucleotide can serve as a capture probe, while the acceptor would serve as the reporter probe to release the signal of target.

DNA-based FRET probes are used as monitors for various types of DNA and RNA reactions, such as PCR, hybridization, ligation, cleavage, recombination and synthesis, and these probes are also applied into sequencing, mutation detection, and biosensor for metal ion, DNA/RNA and protein.
The hybridization reaction is one of the sensitive strategies in the molecular biology arsenal that the FRET-based hybridization probes improve the hybridization technology in two aspects: (i) real-time observation of the hybridization reaction is directly supported by the quenched signal from the unreacted probes; (ii) the multiple formats of FRET hybridization probes are developed based on the high speed of hybridization in solution and the unique detected signal of hybridization probe makes it possible to perform in vivo. Molecular beacons are one of the latest development in the DNA FRET probes based on hybridization [Tyagi S. et al, 1996]. In the presence of complementary target hybridization sequence, it release the probe’s fluorescence for information. In these strategy, the donor’s fluorescence is quenched with a non-complementary target sequence because the donor and the acceptor are brought into a close distance, while the probe forms a stronger hybrids with a complementary target, the fluorescence signal from donor is released. Multiple molecular beacons are under developing for detection of multiple molecules target at the same time [Tyagi S. et al, 1998].

Another one of the FRET-based DNA probe strategy is double-stranded hybridization probes formed by two complementary oligonucleotide. To form a double-stranded hybridization probe, the donor conjugated with the 5’-end of one of the oligonucleotide, and the other complementary oligonucleotide labeled with the acceptor by the 3’-end. In
this system, with the target complementary sequence, the hybridization of the labeled
donor and labeled acceptor is stopped by the hybridization of target and one of the labeled
molecules (donor or acceptor), then it release the quenched fluorescence.

FRET-based DNA probes are advantageous that they are not only considered as a potential
monitor of live cells, but also used in the quickly developing field of biosensors. And based
on the simple and constant rationale of resonance energy transfer, there are various
strategies of detection under designing.

1.4 Definition and Properties of Graphene Quantum Dots (GQDs)

Graphene quantum dots (GQDs), with outstanding optical and electronic properties, are
edge-bound nanometer-size graphene sheets. GQDs is of unique characteristics due to the
structures of graphene confined with the quantum size and the edge effects of specific
functional groups (Sun, H. et al, 2013). Based on the unique features usually serves as
excellent material for biomedical applications, such as the carriers of drugs, contrast agent
in functional imaging and especially the application of GQDs-based sensor.

1.4.1 Introduction of GQDs

As defined, graphene quantum dots (GQDs), with outstanding optical and electronic
properties, are edge-bound nanometer-size graphene fragments. Generally, the sizes of most GQDs has a range from 3nm to 20nm with an almost circular shape and it possesses less than 5 layers of graphene sheets comparing to the large graphene sheet with a large range of dimension from 1.5nm to 100nm and height from 0.5 to 5nm. GQDs is of unique characteristics due to the structures of graphene exciton confinement with the quantum size and the edge effects of different functional chemical groups (Sun, H. et al, 2013). This kind of 0D material compared with the organic dyes and semiconductive quantum dots (QDs) are superior due to the excellent properties of biocompatibility, low toxicity and high stability, and it also unique due to the possession of grapheme structure inside the dots (Sun, H. et al, 2013). Based on the similar components of C,O and H, and the similar surface group of carbonyl, carboxyl, hydroxyl and epoxy groups, GQDs possesses similar nature characteristic with graphene. Though GQDs is known as an excellent material in sensor applications, it is really recent before GQDs material is applied in the analytical field and tremendous effort was devoted in the preparation methods of GQDs and features exploring. GQDs-based sensors perform highly eminent relying on their novel and specific characteristics.

Top-down and bottom up are two categories to prepare GQDs: top-down approach generates small pieces of graphene sheet by cutting down the large stock of graphene sheet,
carbon nanotubes and fibers or graphite based on the effects of nanolithography technique, acidic oxidation, hydrothermal or solvothermal microwave assisted, chemical exfoliation, etc; bottom-up approach builds up the GQDs from different small molecules as starting materials, and different starting materials base on the disparate effects during preparations (Sun, H. et al, 2013). Otherwise, GQDs functionalization could potentially alter the properties of photoluminescence, biocompatibility and electricity, and it could also improve the optical features and quantum yield by the reduction of non-radiative recombination. For example, both biocompatibility and fluorescence quantum yield could be improve during the glutathione functionalization (Bacon, M. et al 2013). The tunable properties based on size and modification make GQDs as preference of biosensors.

1.4.2 GQDs Based Biosensor

1.4.2.1 Photoluminescence (PL) Sensors

The blue and green PL of GQDs are relatively common among the deep UV, blue, green, yellow and red PL of GQDs. GQDs generate PL with various colors based on different preparation approaches. Generally, the intrinsic state emission, induced by quantum size effect or zigzag edge sites or recombination of localized electron-hole pairs, and the defect state emission originated from energy traps both contribute to the PL of GQDs. However, the PL mechanism of GQDs remains controversial probably due to the large range of
factors dominating the PL of GQDs such as size, shape, excitation wavelength, PH, concentration, surface oxidation degree, surface functionalization, N-doping and S-doping.

GQDs-based PL sensors could strongly interact with some organic molecules with aromatic structures based on the electrostatic interaction, $\pi$-$\pi$ stacking or hydrogen bonding, leading to energy transfer or luminescence resonance energy transfer (LRET) causing quenching effect by these organic molecules. In some cases, sensitive TNT detection system was generated based on the quenching effect by $\pi$-$\pi$ stacking between TNT and GQDs, and the PL of GQDs-based sensors decreased with the increase of pyrocatechol due to the electrostatic interaction of oxygen-containing group in GQDs, $\pi$-$\pi$ stacking or hydrogen bonding interaction between GQDs and pyrocatechol. Both of the GQDs-based sensor of TNT and pyrocatechol detection lead to the energy transfer and finally lead to a PL quenching of the GQDs.

1.4.2.2 Electrochemical Sensors

GQDs are widely used as a novel electrochemical sensors due to the advantage of possessing similar characteristics of graphene. Thanks to the tunable size effect, GQDs modified with specific single-strain DNA (ssDNA) serve as probes in the electrochemical platform with multivalent redox properties. It is simple but smart to functionalize the
GQDs with specific ssDNA as probes because it could easily immobilize ssDNA onto GQDs based on \(\pi-\pi\) stacking and the specific ssDNA would add a unique property of selectivity for GQDs sensors. For example, the ET effect between the electro-active species \([\text{Fe(CN)}_6]^{3-}/4^+\) and electrode by electro-static repulsion would be forbidden by the ssDNA functionalization and leads to a electrochemical signal decrease of GQDs, while peak currents signal would reappear once the target molecules complementary with the immobilized ssDNA removes the immobilized ssDNA and electrostatic repulsion of electro-active species \([\text{Fe(CN)}_6]^{3-}/4^+\), then release the GQDs (Sun, H. et al, 2013 and Bacon, M. et al, 2013). Beside the ssDNA, proteins could also be as the specific functional molecules to cause a similar effect. For example, in a case of enzyme immobilization, the electrochemistry of glucose oxidase (GOx) was immediately realized once the GQDs coated with carbon ceramic electrode immobilized with GOx (Sun, H. et al, 2013 and Bacon, M. et al 2013). It could selectively, accurately and sensitively determine the concentration of GOx. Due to a larger surface-to-volume ratio and outstanding biocompatibility of GQDs, and the hydrophilic edge and hydrophobic plane of GQDs which enhance the enzyme absorption onto the electrode surface (Sun, H. et al, 2013), GQDs is usually superior material using as electrochemical sensors.
1.4.2.3 Electrochemiluminescence Sensors

Considered as electrogenerated chemiluminescence, electrochemiluminescence (ECL) combines both the chemiluminescence and electrochemistry, and ECL sensors become increasingly recognized in analytical chemistry because of the advantage of easily set-up, naturally label-free, high signal-to-noise-ratio, high sensitivity (Sun, H. et al, 2013). Generally, QDs-based ECL has been widely used as analytical applications, but the inherent toxicity caused by most of QDs may raise serious health and environmental problem. Fortunately, ECL emission is observed from GQDs as well as other known nanocrystals. Therefore, GQDs with properties of considerable biocompatibility and low toxicity could be explored as a benign nanomaterials with good ECL effects to show the promise for the construction of ECL biosensors (Li, L., L. et al, 2012). GQDs-based ECL sensors also rely on the electron transfer effect. For example, $S_2O_8^{2-}$, as coreactant, and GQDs would produce strongly oxidizing $SO_4^{2-}$ radicals and GQDs$^-$ anion radicals respectively, and it shows a peak of electrooxidation, resulting in electron-transfer annihilation between $SO_4^{2-}$ and GQDs$^-$, and finally generate GQDs with excited state to emit light. In another case, when $H_2O_2$ serves as coreactant, the electrooxidation of GQDs would create cation radicals of GQD$^+$ and show a relatively different electrooxidation peak. Though possessing a different electrooxidation peak between different coreactant, they lead to the formation of excited GQDs and could finally emit light for detection. There is
also a novel GQDs-based ECL sensor for Cd$^{2+}$ detection according to the competitive coordination between cysteine and GQDs. As define before, ECL based on H$_2$O$_2$ could actually develop an ECL aptamer sensor for ATP measuring. Comparing to traditional sensor, ECL aptamer sensor is highly sensitive, exactly precise, acceptably stable and reproducible. Moreover, ECL signal could be amplified by SiO$_2$/GQD composites which could improve the sensor sensitivity. Due to the low toxicity, excellent solubility and ease of labelling, GQDs-based ECL sensors are expected to be explored as novel biosensor.

1.4.2.4 Electronic Sensors

GQDs based electronic sensors are charge sensors based on single electron transistor (SET) which refers to a novel type of shifting device to amplify the current by controlling electron tunneling. Building up a GQDs-based SET system, it required the pattern-guarded large graphene flakes with electron beam lithography carving or oxygen reactive ion etching to form desired geometric GQDs as an island where the electrons could not entre when both the gate and bias voltages are zero due to not enough energy and the current could not flow. It can detect the realizable charge when the bias voltage between the source and drain increase which helps an electron pass through the island and generates current. GQDs-based electronic sensors could be also used in the detection of humidity and pressure relying on the tunneling-barrier distances between GQDs caused by the pressure
and humidity to modulate the properties of conductivity. GQDs-network devices generate obviously higher current modulation than traditional graphene-based sensors and GQDs-based electronic sensors are of higher sensitivity for a lower humidity range.

1.4.3 GQDs as a Donor of Fluorescence Resonance Energy Transfer (FRET)

Graphene Quantum Dots (GQDs) which is referred to the less than 20nm intrinsic fluorescent Graphene fragments, are nanometer-sized objects that has unique property of blue photoluminescence emission wavelength peak of 420nm (F. Liu et al, 2010; Bacon M. et al, 2013; F. Liu et al, 2013), and it is because its oxygen-free structure and intrinsic exciton on the bipole surface that the spectrum emission range is quite certain and the lifetime of fluorescence is quite stable and long (F. Liu et al, 2013). GQDs are easy to be handled due to the molecule-like characteristic and desirable spectrum properties of quantum dots (QDs) (Bacon M. et al, 2013). Being as a QD, the relaxation or emission of GQD is narrow and symmetric (Algar, W. R.,2011) and GQDs is developed into a high sensitive and multiplexing capable molecular sensor that it could provide a quantitative detection of the target molecules, especially the nucleic acid. As well as its high brightness at the narrow and symmetric emission fluorescent spectrum, a longer lifetime and a better stability of fluorescence are also the ascendant performances of GQDs, which lead GQDs to be as a valued donor of fluorescence resonance energy transfer (FRET). The FRET
donor-acceptor overlap spectrum and FRET efficiency are ensured to be at a high level due to the narrow and symmetric emission spectrum of GQDs, avoiding the bleed-through of donor spectrum. GQDs could also provide a larger surface to adsorb molecules so that more acceptors could be bonded on to a single GQD, which also could improve the efficiency and detection sensitivity of FRET effect (Yi, Zhang et al, 2012). Another aspect considered is that GQDs have the ability of conjugating small nucleic acid, prompting GQDs to be as platform with fluorescent probes for target special nucleic acid detection. In view of the excellent characteristic of GQD including the high brightness and intrinsic emission fluorescent spectrum, the ability of conjugating small molecules and quite stable and long lifetime of the fluorescent emission, GQD could be as an outstanding FRET donor.

1.5 Definition and Properties of Gold Nanoparticles (AuNps)

As nanomaterials, gold nanoparticles (AuNPs) feature unique physicochemical properties. AuNPs are of distinct physical and chemical natures leading them excellent scaffolds to fabricate novel chemical and biological sensors (Biosselier et al, 2009; Daniel et al, 2004; Haick et al, 2007; Zayats et al, 2005; Zhao W. et al, 2008; Bunz et al, 2010; Sperling et al, 2008; Radwan et al; 2009; Zeng S. W. et al, 2011), which is also of great utility in building up sensors in novel recognition and transduction processes, as well as improving the signal
to noise (S/N) ration via miniaturization of the sensor elements (Sheehan, P. E. et al, 2005).

Among numerous excellent attributes of nanomaterials, AuNPs are highly stable though they are synthesized in a straightforward manner and are of unique optoelectronic properties. Also, as well as providing high surface- to- volume ratio with an excellent biocompatibility as long as using appropriate ligands (Daniel et al, 2004), properties of AuNPs readily vary depending on their sizes, shapes and the chemical solution environment. Each of these properties leads AuNPs allowing researchers to constantly improve and develop innovative sensing strategies with progressive stability, sensitivity and selectivity. In the past decades, AuNPs provided us the applications for the detection of small molecules, nucleic acids, proteins, metal ions, malignant cells with a record-breaking efficient manner.

1.5.1 Introduction of Gold Nanoparticles (AuNPs)

As introduce above, AuNPs, as nanomaterials, are of distinct physical and chemical natures leading them excellent scaffolds to fabricate novel chemical and biological sensors, which made a great flutter in academic community. High stability, unique optoelectronic properties, high surface- to- volume ratio with excellent biocompatibility and size-dependent flexible properties make AuNPs excellent and preferred material for sensors. AuNPs offer a feasible support for multi-functionalization with different ligands
(including organic and biological) for selected detection of molecules and targets (Daniel et al, 2004; Haick et al, 2007; Zayats et al, 2005; Radwan et al, 2009) because binding event as the specific sites between the recognition element and the analyte can vary physicochemical features of transducer AuNPs (conductivity, plasmon resonance absorption, redox behavior, etc), which leads to the generation of detectable response signal (e.g. fluorescence quenching).

It is multipath to synthesize and surface-functionalize AuNPs. Generally, “top-down” (referring to physical manipulation) and “bottom-up” (referring to chemical transformation) are the well-known approaches to prepare AuNPs (Daniel et al, 2004). It is worth noting that the processes of AuNPs preparation is implicated in the attributes of AuNPs, such as the size, shape, solubility, stability and functionality. Term colloid, more than 10nm, and cluster, smaller than 10nm, are frequently interchangeable during the synthesis. The Brust-Schiffrin Method for Thiol protected AuNPs, during the two-phase synthesis, reduce gold salts in present of external thiol ligands to stabilize and protect AuNPs (Krishnendu S. et al, 2012), making AuNPs easiliy handled, characterized and functionalized. To form mixed monlayer AuNPs, place exchange interchanging the initially anchored thiol ligands by free thiol ligands, also can introduce two or more functional ligands during exchanging, which provides optimization of the interaction with analytes (Boal A. K. et al, 2000). Polymers,
identically, have the ability to stabilize AuNPs, such as PVP (Meguro K. et al, 1988; Carotenuto G. et al, 2001; Seoudi R. et al, 2010), PEG (Hayat et al, 1989; Oh E. et al, 2010; Susumu et al, 2009; Tracy et al, 2007), PVA (Pucci et al, 2006), PVME (Bhattacharjee et al, 2006), PEI (Sun X. P. et al, 2004), PDDA (Gole A. et al, 2005), PMMA (Mandal et al, 2002; Yilmaz et al, 2010), etc. Otherwise, other sulfur-containing ligands, including disulfides (Shelley et al, 2002; Hasan et al, 2002), di- (Resch et al, 1999) and trithiols (Li A. J. et al, 2002; Tan Y.W. et al, 2002), thioethers (Maye et al, 2002; Li X.M. et al, 2001), xanthates (Tzhayik et al, 2002) have been used to passivate AuNPs and functionalize AuNPs via oxidation and decomposition of thiol-stabilized AuNPs (Sun L. et al, 2001). Studies of stabilizing ligands for AuNPs using phosphine (Weare et al, 2000; Moores et al, 2004), carboxylate ligands (Yamamoto et al, 2003; Sarkeri et al, 2010; Sarkar et al, 2010; Wang W.X. et al, 2007), lactic acid (Yin X.Y. et al, 2010) and hydroquinone (Sirajuddin et al, 2010) have also been documented.

Size-tunable AuNPs are of size-dependent electronic and optoelectronic properties. Quantum size effect lead AuNPs to discrete electron transition energy levels. Moreover, AuNPs, behaving as quantized capacitance charging, can be modified by superficial ligands, electrolyte ions and magnetic fields to potentially apply in electronchemical labels and electronic devices (Schmid et al, 2005; Subramaniam et al, 2005). As well as redox
reactions, surface plasmon resonance (SPR) embellish the features of AuNPs (Jain P.K. et al, 2006). The intense colors of AuNPs attribute to the resonance condition with satisfaction at visible wavelengths (Halas et al, 2011; Jain P. K. et al, 2008; Link S. et al, 2000). Size, solvent, ligands, interparticle distance and temperature are impression factors to influence SPR. Solvent refractive index changes can lead to the spectral shift (Templeton et al, 2000). Moreover, surface plasmon band, influenced by the core charge, can cause shifts to higher energy, with excess electronic charge, or to a lower one, with electron deficiency (Templeton et al, 2000; Link et al, 1999; Yan B.H. et al, 2003; Rechberger et al, 2003). The proximity between AuNPs and other nanoparticles also contributes to sensitivity of the SPR frequency, resulting in a significant red-shift and broadening in the surface plasmon band via the aggregation of nanoparticles and the color change from red solution to blue one due to the interparticle plasmon coupling (Su K.H. et al, 2003; Srivastava et al, 2005). Flexible surface plasmon band energy status and shiftable wavelength make AuNPs an attractive candidate for colorimetric sensors.

1.5.2 AuNPs for Detection

A visible color change from red to blue at nanomolar (nM) concentration is induced by interparticale surface plasmon coupling due to the aggregation of AuNPs of appropriate sizes (Srivastava et al, 2005). Therefore, absorption-based colorimetric sensing strategies can
apply the color change as practical platform to detect target analyte via direct or indirect trigger of aggregation and redispersion of AuNPs. For example, the present of analyte metal ion, requiring an incorporation of chelating agent onto the surface of AuNPs-based colorimetric sensor, leads the AuNPs aggregation via generating multidentate interparticle compound with a chelating agent (ligand). MUA-functionalied 13nm AuNPs have been reported by Hupp et al. to detect aqueous heavy metal ion, such as Pb$^{2+}$, Cd$^{2+}$ and Hg$^{2+}$. Numerous efforts of AuNPs-based sensors for detection of anions, small organic molecules, oligonucleotides, proteins have been also reported.

1.5.3 Gold Nanoparticles as Quenchers of Fluorescence Resonance Energy Transfer (FRET)

For FRET-based assays, AuNPs are considered as excellent fluorescence quenchers (Sapsford K.E. et al, 2006). It mainly due to the broad energy bandwidth and extraordinary high molar extinction coefficients (Jain P.K. et al, 2007). FRET-based AuNPs strategies for detection also utilize quantum dots for improving efficiency and stability (Medintz et al, 2003). AuNPs being as a probe of molecular detection is not something new to researchers, but it can’t be denied that AuNPs is of peachy characteristics in being a molecular detective tool. It improves the level of sensitivity and selectivity in an assay due to its high stability, cooperative binding, catalytic activity. And its certain properties vary differently based on
the critical size of AuNPs mainly reflecting in the spectrum wavelengths and colors (C., Shad et al, 2010; Kamaladasan Kalidasan et al, 2013). DNA and ligands can be conjugated on to the surface of AuNPs via chemical modification for the purpose of molecular detections. AuNPs are qualified tool for detections. Compared to the double stranded DNA (dsDNA), single stranded DNA (ssDNA) has a prominent ability to be adsorbed on to the surface of AuNPs. Otherwise, the surface area of AuNP is large as 7.07E+02 nm² for more molecular probes conjugated onto the surface which would increase the efficiency of the detection. AuNPs have persuasive reasons to be FRET acceptors including the large surface for DNA conjugated, certain and wide range absorption spectrum of corresponding sizes of the particles, and the brilliant ability of stably conjugating the DNA and ligands.

1.6 Introduction of Project Design

In this study, GQDs and AuNPs are selected as the energy donor-acceptor pair for the effective FRET detection strategy. GQDs are labeled with a 10-base single-strand DNAa (ssDNAa) to form structure of GQDs-ssDNAa, while AuNPs are labeled with 10-base single-strain DNAb (ssDNAb) to form structure of AuNPs-ssDNAb. In the energy donor-acceptor pair of FRET, GQDs serves as energy donor and AuNPs serves as energy acceptor (quencher). In the FRET detection system, GQDs-ssDNAa plays a role as capture probe while AuNPs-ssDNAb acts as reporter probe. The 10-base ssDNAa and ssDNAb are
complementary respectively to adjacent region of the 20-base target S. aureus cDNA.

According to Figure 2, in the present of 20-base target cDNA, the two oligonucleotide strands on GQDs and AuNPs respectively are brought together and then it forces the two fluorophores (GQDs-ssDNAa and AuNPs-ssDNAb) into a close proximity. As the excitation of energy donor (GQDs-ssDNAa), energy is transferred via AuNPs energy absorption from GQDs energy relaxation based on the FRET mechanism, which results in significant reduction in fluorescence intensity (or fluorescence quenching). To ensure a positive signal, ssDNAa and ssDNAb must be close enough to each other and in a correct orientation to hybridize with target cDNA. Intermolecular FRET between two randomly distributed probes is negligible due to the adoptive concentration of probes are low. Hence, the possibility of capture probe and reporter probe both binding to the same nonspecific target is extremely low. Therefore, the design of binary probes (BPs) in the FRET system can efficiently prevent the occurrence of false-positive signals via nonspecific binding between probes and target, and they ensure the reliability of diagnosis via binary probes.
2. Methodology

2.1 Materials and Facilities

I. Oligonucleotides including 10-base Amino-modified ssDNAa (to conjugate onto GQDs), 10-base Thiol-modified ssDNAb (to conjugate onto AuNPs), 20-base cDNA of S.aureus (a fragment of S.aureus gene sequence), single-base and double-base mismatch oligonucleotide (to use as contrast group to verify the selectivity of system) were synthesized by Integrated DNA Technologies (IDT) Inc. (Coralville, IA, U.S.):
i. Sequence of 10-base Amino-modified ssDNAa:

5’/-5ANMC6/(amino)AAT GAC GCT A -3’

ii. Sequence of 10-base Thiol-modified ssDNAb:

5’-TGA TCC CAA T/3ThioMC-D/(Thiol)-3’

iii. Sequence of 20-base cDNA of S. aureus (a fragment of S.aureus gene sequence):

5’- ATT GGG ATC ATA GCG TCA TT -3’

iv. Sequence of single-base mismatch cDNA (contrast group for selectivity verification):

5’- ATT GGG ATG ATA GCG TCA TT -3’

v. Sequence of double-base mismatch cDNA (contrast group for selectivity verification):

5’- ATT GGG ATG ATA GCC TCA TT-3’

II. Solution of 10× Phosphate buffered saline (PBS), Dithiothreitol (DTT), 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) purchased from Sigma Aldrich (St. Louis, MO, U.S.) were prepared for the conjugation of AuNPs-ssDNAb and GQDs-ssDNAa.

III. Devices of gel-columns (illustra Microspin G-25 columns, GE Healthcare, UK) were prepared for isolating and purifying the modified ssDNAb for AuNPs conjugation.

IV. UV-visible spectrophotometer (Ultrospec. 2100 pro.) was applied for verification of ssDNAb concentration during the preparation of AuNPs-ssDNAb and drawing map of absorption spectrum of AuNPs and AuNPs-ssDNAb.
V. Edinburgh FLSP920 spectrophotometer with 450 W steady-state xenon lamp at room temperature was applied for the verification of emission spectrum of GQDs and GQDs- ssDNAa.

VI. Tecan Infinite F200 microplate reader was applied for the fluorescence intensity measurements.

### 2.2 Preparation of Gold Nanoparticles (AuNPs)

Briefly, HAuCl₄ (3 μL, 14.3 wt%) and DI water (10 mL) were firstly mixed to transfer into a clean beaker which was firstly washed with aqua regia, a mixture of HCl and HNO₃ at a ratio of 3:1, and then with DI water. Secondly, sodium citrate solution (1 mL, 1 wt%) was added to the boiling solution within one second upon vigorous stirring. When the solution change color from pale yellow into wine red, in a few minutes, then continue to boil for another 15 minutes. At last, let the wine red solution cool down to room temperature by continuous stirring. (Grabar et al, 1995)

### 2.3 Preparation of Capture Probe (GQDs-ssDNAa conjugation)

I. Firstly, GQDs (1mg/ml, 60 μL) was reacted with EDC (3.3 M, 0.5 μL), for 15-20 minutes, in order to activate the functional group on the surface of GQDs.

II. Secondly, Amine-modified ssDNAa (100mM, 3μL) was added to conjugate onto the
surface of GQDs, for 30-40 minutes incubation. (Figure 3)

III. GQDs-ssDNAa was generated via forming amide linkage.

2.4 Preparation of Reporter Probe (AuNPs-ssDNAb conjugation)

I. Thiol-modified oligonucleotide ssDNAb (100μM, 19.2μL) was treated with deionized water (DI water) (4.8μL), 10× Phosphate buffered saline (PBS) (3μL) and Dithiothreitol (DTT) (0.1M, pH 8.2, 3μL), for incubation of 40mins at room temperature, in order to cleave disulphur linkage for the enhancement of efficiency during oligonucleotide immobilization on AuNPs subsequently.

II. Using gel-column (G25) to purify the treated thiol-modified oligonucleotide (ssDNAb) via centrifugation at 3000rpm for 2mins.
III. Using UV-visible spectrophotometer to verify the concentration of purified treated ssDNA, and then calculate the required amount of AuNPs (7nM, prepared in section 2.2) with the ssDNA: AuNPs ratio at 300:1.

IV. Adding AuNPs with the required amount (e.g. 500μL, 7nM AuNPs for about 30μL, 20nM ssDNA) into purified treated ssDNA, for incubation of 24 hours at room temperature, in order to form the sulf-linkage between AuNPs and ssDNA to generate stable AuNPs-ssDNA complex. (Figure 4)

V. Adding sodium chloride solution (e.g. 5M, 10μL for 500μL AuNPs solution) and sodium dihydrogen phosphate (e.g. 2M, 3μL for 500μL AuNPs solution) to neutralize the remaining negative charge on the surface of AuNPs which is not binding to ssDNA to solidify the sulf-linkage of AuNPs-ssDNA. This step needs incubation for 16 hours.

VI. To centrifuge complex solution at 13,200 rpm for 30mins in order to precipitate AuNPs-ssDNA at bottom, separating from the unreacted reagents, and it needs washing by DI water for 3 times by repeating centrifuge at some condition.

![Figure 4](image.png)

**Figure 4**
Formation of Au-S linkage between AuNPs and thiol-modified ssDNA to generate AuNPs-ssDNA complex.
2.5 Quenching Efficiency and Specificity Characterization

I. GQDs-ssDNAa (50μL) prepared in section 2.3 was incubated with target cDNA of S.aureus with different concentration (e.g. 100pM, 1nM, 5nM, 10nM, 50nM, 100nM, 200nM, 300nM, 400nM, 500nM), for 2 hours at room temperature to allow DNA hybridization, under dark condition. (Figure 5)

II. Each of the cDNA hybridized capture probe (GQDs-ssDNAa) (50μL), with different cDNA concentration, were incubated with AuNPs-ssDNAab (50μL, prepared in section 2.4), for 24 hours at room temperature to hybridize the remaining unhybridized part of cDNA with ssDNAab labeled by AuNPs, under dark condition. Otherwise, another identical set of solution I were prepared as control group and were treated with DI water (50μL). (Figure 6)
III. Using microplate reader to measure the fluorescence intensity (both the experiment group and control group) (100μL for each sample). Setting the result of control group (without quenching effect) as $F_0$, while experiment group (quenching by AuNPs) as $F_q$.

IV. Calculate the quenching efficiency via the formula $(F_0 - F_q)/F_0 \times 100\%$. Identify the linear region of the detection and manipulate the fluorescence quenching signal $(F_0 - F_q)$ with the linear range in order to establish a quadratic equation relating fluorescence quenching signal $(F_0 - F_q)$ to the concentration of target cDNA in logarithmic scale. Calculate the limit of detection (LOD) via the change value of

*Figure 6*

The process of reporter probe hybridizing with cDNA hybridized capture probe to stimulate the FRET effect.
control signal plus three times of the background signal (standard deviation).

Otherwise, the contrast system of single-base and double-base mismatch were evaluated for verification of the selectivity of the system of experiment group (S.aureus cDNA), at same condition with same concentration, and finally used to compare the quenching efficiency with experiment group.

**Figure 7**

Flow chart of the investigation of the effect of target cDNA concentrations on quenching efficiency.
3. Results

3.1 Spectra of GQDs and AuNPs

Figure 8 Spectrum of GQDs and AuNPs
(a) The emission spectrum of GQDs and GQDs-ssDNAa complex with 360nm excitation.
(b) The absorption spectrum of AuNPs and AuNPs-ssDNAab from 400nm to 700nm.
(c) The overlapping spectrum of GQDs-ssDNAa emission and AuNPs-ssDNAab absorption.
3.2 EDC-enhanced GQDs Fluorescence Intensity

![Graph showing fluorescence intensity of EDC enhanced GQDs and GQDs without EDC treating.](image1)

**Figure 9**
Fluorescence intensity of EDC enhanced GQDs and GQDs without EDC treating. Fluorescence intensity of EDC enhanced GQDs have strongly and regularly increased with the increasing GQDs concentration.

![Graph showing fluorescence intensity of GQDs with increasing EDC concentration.](image2)

**Figure 10**
Fluorescence intensity of GQDs with increasing EDC concentration.
3.3 Quenching Efficiency of AuNPs

![Graph showing the relationship between target cDNA concentration and quenching efficiency.](image)

**Figure 11**
The effect of target cDNA concentration on AuNPs quenching efficiency.

\[ y = 9.0074 \ln(x) + 21.749 \]  
\[ R^2 = 0.7769 \]

3.4 Limit of Detection (LOD)

![Bar graph showing fluorescence signal quenching value versus target cDNA concentration.](image)

**Figure 12**
Fluorescence signal quenching value (F0-Fq) versus target cDNA of S. aureus concentration from 5nM to 200nM. The red dotted line shows the sum of control signal plus 3 times its standard deviation to calculate the limit of detection (LOD) which is obtained approximately at 10nM.
3.5 Specificity of The System

![Quenching efficiency graph](image)

**Figure 13**
Quenching efficiency at the presence of complementary target cDNA of S. aureus, single-base mismatch DNA and double-base mismatch DNA, with the same concentration of...

![Fluorescence scanning images](image)

**Figure 14**
Fluorescence scanning images of solution (a) with no quenching (F0), and with quenching effect at the presence of (b) double-base mismatch DNA, (c) single-base mismatch DNA, and (d) complementary target cDNA of S. aureus.
4 Discussion

4.1 Spectrum Measurement of GQDs and AuNPs

The emission spectrum of GQDs and GQDs-ssDNAa was obtained in section 3.1 (Figure 8-a). There was no significant difference between the emission peak of GQDs and GQDs-ssDNAa. After the immobilization of oligonucleotide (ssDNAa), there was a slight red shift of the emission peak of GQDs at 465nm.

The absorption spectrum of AuNPs and AuNPs-ssDNAAb were also shown in section 3.1 (Figure 8). The absorption spectrum after immobilization of oligonucleotide on AuNPs was almost the same as the absorption peak of AuNPs at 512nm.

Section 3.1 (Figure 8-c) showed overlap between the emission spectrum of oligonucleotide immobilized GQDs and the absorption spectrum of oligonucleotide immobilized AuNPs. According to the Figure 8 (c), there was a great extent overlap between GQDs-ssDNAa emission and AuNPs-ssDNAab absorption, allowing a feasible and effective FRET between GQDs and AuNPs after immobilization of oligonucleotide.

4.2 Fluorescence of EDC-enhanced GQDs

According to section 3.2 (Figure 9 and Figure 10), differences of fluorescence intensity
between raw material of GQDs and EDC treated GQDs were obtained. Obviously, based on the result showing in figure 9, EDC-treated GQDs had strong fluorescence intensity, while comparing to that of raw material of GQDs, and it also showed the stable and increasing curve with the increasing GQDs concentration. Generally, EDC was used to activate the function group on the GQDs surface, during or before the oligonucleotide immobilization onto GQDs. However, according to the comparison between fluorescence intensity of GQDs before and after EDC treated, EDC is of potential ability to enhance the fluorescence of GQDs via activating the function group.

4.3 Quenching Effect

The fluorescence intensity (emission fluorescence) of the solution of target cDNA hybridized GQDs-ssDNAa with the addition of AuNPs-ssDNAb or DI water were respectively measured, in order to compare the quenching effect of AuNPs with the different target cDNA concentrations (from 100pM to 500nM). The relationship of quenching efficiency and target cDNA concentration was obtained in section 3.3 (Figure 11). It showed that the increasing target cDNA concentrations within the range from 100pM to 100nM were following with the rapidly increasing quenching efficiency of AuNPs, and finally reached a plateau of approximately 80% at the range from 100nM to 400nM. A linear response range was under the concentration of 100nM, and a logarithm
curve fits the trend well.

The fluorescence quenching signal value (F0-Fq) caused by FRET with the concentration range from 5nM to 200nM was showed in Figure 12. Increasing target cDNA concentration increased the fluorescence quenching signal value (F0-Fq). The limit of detection (LOD), calculated by attenuation signal of GQDs fluorescence intensity of control group plus three times of its standard deviation, was labeled in Figure 12, displayed as a red dotted line, indicating that the estimated detection limit of target cDNA concentration is 10nM.

4.4 S. aureus Oligonucleotide Detection

An efficient optical detection strategy for S. aureus, involving binary probes in a sandwich FRET between GQDs and AuNPs, was designed in this study. It was designed to solve the problems of conventional detection methods with complexity, expensive-cost and time-consuming. Figure 2 in the section 1.6 was illustrating the principle of designed detection system of sandwich FRET. Briefly, FRET required spectrum overlap between donor emission and acceptor absorption, together with the requirement of their physical proximity. GQDs are excellent emitter with strong fluorescence, in addition to AuNPs are well-known peachy fluorescence quencher due to the wide range absorption spectrum,
making them as partners in the fulfillment of spectral requirement of FRET (section 4.1).

In the designed FRET system, GQDs are used as donor and the subsequent oligonucleotide immobilized GQDs (GQDs-ssDNAa) become as capture probes, while AuNPs are used as energy acceptor making the oligonucleotide immobilized AuNPs (AuNPs-ssDNAb) becoming as reporter probes. Without labeling target directly on the GQDs or AuNPs as probe, it satisfied the detection requirement of simplicity and cost efficiency. Oligonucleotides used to conjugate with GQDs and AuNPs are respectively complementary of 20-base cDNA of S. aureus (segment of S.aureus gene). According to the requirement of sequence orientation and reaction group used to conjugation, oligonucleotide for GQDs conjugation (ssDNAa) was modified at 5’end with amino group, while that for AuNPs (ssDNAb) was modified at 3’ end with sulfide group, thus making the 5’ end to 3’ end of cDNA complementary from 5’end of ssDNAa to 3’end of ssDNAb through the 3’ end of ssDNAa and 5’ end of ssDNAb. After the preparation of conjugations of GQDs and amine-modified ssDNAa, AuNPs and Thiol-modified ssDNAb, the designed detection strategy only involved two further operations, including the hybridization between target cDNA and GQDs-ssDNAa and a subsequent hybridization of AuNPs-ssDNAb and cDNA hybridized GQDs-ssDNAa. In the process, capture probe (GQDs-ssDNAa) hybridized with 20-base target cDNA and complementary to the first 10 base from 5’ end of target, then the reporter probe (AuNPs-ssDNAb) subsequently
hybridized with the remaining 10 base from the middle to the 3’ end of target, bringing GQDs and AuNPs into proximity. In the contrast, in the absence of target cDNA, the two probes are distributed randomly leading them separated by a larger distance in the solution system, and resulting in a relative strong fluorescence intensity of GQDs-ssDNAa. To measure the fluorescence intensity, in order to identify the present of target, microplate reader was applied. Therefore, in the designed detection strategy, the present of target oligonucleotide leads to a physical proximity of capture probe and reporter probe by the complementary sequence, and under the appropriate excitation, the energy is transferred from the GQDs to AuNPs.

4.5 Selectivity and Specificity of FRET Based Biosensor

Single-base mismatch and double-base mismatch DNA were used to verify the selectivity and specificity of complementary target cDNA of S. aureus of the system. Respective quenching efficiencies of both the mismatch DNA and target cDNA, with the concentration of 200nM, were obtained in Figure 13, indicating a decreasing trend with increasing number of mismatch bases. The quenching efficiency of single-base and double-base mismatch DNA hybridized system were dropped to 37% and 22% respectively, while the quenching efficiency of that of complementary target cDNA was approximately 80%, indicating that the DNA detection system is of high selectivity and specificity, similar to
the reports of Tao et al.(2012) and Li et al.(2011). Fluorescence intensity images were showed in the section 3.5 (Figure 14), verifying the distinguishing quenching effect of target cDNA of S. aureus and groups of mismatch DNA.
5 Conclusion

A rapid and sensitive strategy for early S. aureus DNA detection, involving binary probes in a sandwich structure based FRET biosensor between GQDs and AuNPs, has been developed. The quenching efficiency of oligonucleotide immobilized AuNPs was found to increase with the increasing target concentrations, and it reached a plateau with the beginning concentration with 100nM and tend to be stable at 200nM with approximately 80% quenching efficiency. The linear working range of detection was from 100pM to 100nM with a detection limit of 10nM which is acceptable comparing to existing detection strategies. The strategy also possesses a high selectivity when comparing to the single-base and double-base mismatch oligonucleotides, with a trend that increasing number of mismatch base increasingly decreases the quenching efficiency. Moreover, the design is also of simplicity, low cost as well as high specificity due to the operation with no need for conjugation of target as probe. However, our study bears some limitations in sensitivity. Further studies are needed to be conducted for further improvement of better LOD and also cut down the incubation time during the hybridization between reporter probe and target-hybridized capture probe.
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