Conjugation of Carboxylated Graphene Quantum Dots with Cecropin P1 for Bacterial Biosensing Applications

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Quantum dots have proven to be strong candidates for biosensing applications in recent years, due to their strong light emission properties and their ability to be modified with a variety of functional groups for the detection of different analytes. Here, we investigate the use of conjugated carboxylated graphene quantum dots (CGQDs) for the detection of *E. coli*, using a biosensing procedure that focuses on measuring changes in fluorescence quenching. Our CGQDs were conjugated with cecropin P1, a naturally-produced antibacterial peptide that facilitates the attachment of CGQDs to *E. coli* cells. We also confirm the structural modification of these conjugated CGQDs in addition to analyzing their optical characteristics. Our findings have the potential to be used in situations where rapid, reliable detection of bacteria in liquids, such as drinking water, is required, especially given our biosensor’s relatively low observed limit of detection (LOD).

INTRODUCTION

Biosensing assays involving quantum dots often rely on measuring changes in fluorescence when these quantum dots interact with a given analyte.\(^1,2\) By quantifying these changes in fluorescence, the amount of the analyte present can then be determined. These types of biosensing assays are important for various applications, such as the detection of disease-causing microorganisms in liquids and the determination of whether their concentrations are within acceptable limits.

In this study, we aim to create a simple biosensing assay for the detection of *E. coli* through the use of carboxylated graphene quantum dots (CGQDs) conjugated with the antimicrobial peptide cecropin P1.

Graphene quantum dots were chosen for this study because of their low biotoxicity\(^3\) and strong fluorescence,\(^4\) important characteristics to ensure the safety and efficacy of the assay, respectively. Meanwhile, cecropin P1 was chosen as the biorecognition component due to its ability to bind to *E. coli* cell walls as a part of its action mechanism,\(^5\) since ensuring CGQD-bacteria attachment is a key part of how our biosensing assay functions. Moreover, in low concentrations, cecropin P1 has been shown not only to retain its ability to bind to *E. coli* but also to avoid killing the bacteria.\(^6\) This is ideal because the primary goal of our assay is to detect, not kill, the bacteria.

The attachment of the CGQDs to the cecropin P1 is hypothesized to occur via the attachment of carboxyl groups on the CGQDs’ edge structure to the amine groups present on the cecropin. Such an attachment is possible in the presence of N-Hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which activate the carboxyl groups so that the conjugation reaction can take place. The solutions necessary for the biosensing assay can be prepared in relatively rapid, room-temperature reactions, an advantage for potential applications.

We hypothesize that the main factor responsible for the observed changes in fluorescence is the attachment of the bacteria to the conjugated quantum dots, which could lead to aggregation-caused quenching (ACQ). Such an interaction is suggested by the action mechanism of cecropin P1, namely its ability to attach to the cell walls of gram-negative bacteria.\(^4,5\) However, the precise mechanism by which this binding affects the fluorescence of the CGQD-bacteria unit is still unknown.

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EXPERIMENTAL METHODS

Graphene quantum dots functionalized with carboxyl edge groups were obtained from ACS Chemicals (Pasadena, CA) and used as received; the concentration of the CGQD solution was 1 mg/mL. Cecropin P1 Porcine was obtained from Bachem (Switzerland) and dissolved into sodium acetate buffer (pH 5.2, obtained from Sigma Aldrich) at a concentration of 1 mg/mL. N-Hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma Aldrich, and were dissolved into sodium acetate buffer at a concentration of 20 mmol/L.

To conjugate the CGQDs with the cecropin P1, 60 μL of GQDs, 60 μL of EDC, and 60 μL of NHS were allowed to react for 30 minutes at room temperature under mild stirring at 150-200 rpm. Thereafter, 120 μL of cecropin P1 solution was added, and the solution was allowed to react at room temperature under mild stirring at 150-200 rpm for another 2 hours. The resulting solution was then diluted to give a CGQD concentration of ~0.25 mg/mL and a cecropin P1 concentration of ~0.5 mg/mL in the final solution. The conjugated CGQDs were prepared fresh before each fluorescence test.

Figure 1: Image of the (a) fluorescence spectra for conjugated CGQDs (orange) and non-conjugated CGQDs (blue), as well as (b) the differences in fluorescence intensity between these two samples. All measurements taken with excitation wavelength = 400 nm. The quenching (potentially aggregation-caused quenching, or ACQ) following the conjugation indicates that the CGQDs have successfully been conjugated with cecropin P1.
The bacteria used in our experiment were cultured from competent K12 E. coli DH5-α cells. Cells with no antibiotic resistance, ampicillin resistance, and chloramphenicol resistance were tested. However, the cultures used in our experiments were given no genetic modification other than antibiotic resistance; this was done to minimize the interference of additional proteins with E. coli-CGQD binding.

We hypothesize that our conjugated CGQDs can also be used to detect virulent strains of bacteria, such as the Shiga toxin-producing E. coli O157:H7, due to cecropin P1’s ability to bind to various strains of gram-negative bacteria and the similarities in surface morphology between DH5-α and O157:H7. These virulent strains were not tested in our experiments due to the biosafety level of our laboratory.

To create the biosensing assay, we mixed our conjugated CGQDs and bacterial cultures in a black 96-well plate, and performed fluorescence measurements using a Tecan Spark plate reader. Into each well, we placed 100 uL of conjugated CGQDs and 20 uL of E. coli bacteria of varying concentrations (the original liquid culture concentration as well as 1:2, 1:4, 1:20, and 1:100 dilutions). A well with 100 uL of conjugated CGQDs mixed with 20 uL of sodium acetate buffer was also examined as a reference value. Upon loading all solutions, we then agitated the solutions in each well to ensure complete E. coli-CGQD binding before performing fluorescence measurements. Using these measurements, we analyzed the differences in fluorescence between CGQD/bacteria solutions with different concentrations of E. coli.

Additionally, a Vernier VSP-UV spectrophotometer was used to obtain absorbance spectra of our CGQDs to further confirm structural differences between conjugated and non-conjugated CGQDs.

**RESULTS AND DATA**

I. **Fluorescence Spectra for CGQDs and Cecropin P1-Conjugated CGQDs**

**Figure 1a** depicts the fluorescence spectra for non-conjugated CGQDs (blue) and CGQDs following cecropin P1 conjugation (orange). Both spectra were measured using an excitation wavelength of 400 nm.

As can be seen from the spectra in Figure 1a, CGQD conjugation with cecropin P1 results in significant fluorescence quenching. This quenching was quantified in Figure 1b, which shows the differences in fluorescence intensity between conjugated and non-conjugated CGQDs;

![Graph of the change in fluorescence intensity of the conjugated CGQD sample after mixing with various concentrations of E. coli cells with no antibiotic resistance. All measurements taken using excitation wavelength = 400 nm and emission wavelength = 670 nm.](image.png)
a negative difference indicates fluorescence quenching.

II. Change in Fluorescence versus Concentration of *E. coli*

*Figure 2* depicts the changes in fluorescence that result from mixing the conjugated CGQDs with liquid *E. coli* cultures of varying concentrations. A negative change indicates a decrease in fluorescence, and the fluorescence of the conjugated CGQDs without any *E. coli* added was taken as the reference value.

All measurements were performed using an excitation wavelength of 400 nm. Measurements taken at an emission wavelength of 670 nm demonstrated the most significant decreases in fluorescence following the addition of *E. coli* cells to the conjugated CGQDs, and were selected as a representative data set. Other emission wavelengths from 450 to 700 nm were also tested and demonstrated similar trends to that shown in *Figure 2* (full data available upon request). As can be seen in the graph, the fluorescence of the conjugated CGQDs decreases when they are mixed with low concentrations of *E. coli*. Unexpectedly, the fluorescence increases when the CGQDs are mixed with higher concentrations of bacteria (not shown in graph as this is not relevant per the Discussion section below).

Finally, we also tested our biosensors with *E. coli* cells resistant to ampicillin and chloramphenicol. The graphs of change in fluorescence vs. *E. coli* concentration are included in the Supporting Information section of this paper. Because these graphs (particularly for ampicillin-resistant *E. coli*, as shown in *Figure 4*) closely resemble *Figure 2*, they suggest that our biosensors can be used for the detection of bacteria with a variety of different plasmids. The slightly narrower range of concentrations at which chloramphenicol-resistant *E. coli* can be detected, as well as the smaller decreases in fluorescence for both ampicillin- and chloramphenicol-resistant bacteria compared to *Figure 2*, is likely attributed to additional surface proteins, such as efflux pumps, in these strains that could potentially interfere with *E. coli*-CGQD binding.8,9

III. Absorbance Spectra for CGQDs and Cecropin P1-Conjugated CGQDs

*Figure 3* depicts the absorbance spectra of CGQDs (yellow) and conjugated CGQDs (blue), at equal concentrations. As can be seen from the

![Figure 3: Graph of the absorbance spectra for non-conjugated CGQDs (yellow) and cecropin P1-conjugated CGQDs (blue), at equal concentrations. As can be seen in the spectra, conjugation with cecropin P1 results in a redshift of 10 nm and an increase in absorbance values.](image-url)
spectra, there is a redshift of 10 nm following cecropin conjugation, as well as a significant increase in absorbance values.

Furthermore, an absorbance spectrum of pure cecropin P1 solution confirmed that any residual cecropin from the conjugation procedure would have a negligible impact on absorbance values. Therefore, the observed increase in absorbance values results from cecropin conjugation rather than residual reactants from the conjugation procedure.

**DISCUSSION**

The quenching observed in Figure 1a following CGQD conjugation, as well as the increase in absorbance observed in Figure 3 following conjugation, are hypothesized to be due to aggregation-caused quenching (ACQ). In this case, ACQ could result from an increase in CGQD aggregation due to interactions between the cecropin P1 peptides attached to the CGQDs. Therefore, the observed quenching and increase in absorbance indicates that the CGQDs were successfully conjugated with cecropin P1.

Further suggesting successful conjugation is the 10-nm redshift in absorbance observed following conjugation in Figure 3. Indeed, shifts in absorbance have been found to result from the addition of functional groups, as well as other antimicrobial peptides, to quantum dots.2,10

The decrease in fluorescence for low concentrations of *E. coli* shown in Figure 2 is hypothesized to be due to interactions between the conjugated CGQDs and the surface of the *E. coli*, given cecropin P1’s proposed action mechanism of binding to the bacterial cell wall.4,5 Such interactions might include energy transfer between the CGQDs and *E. coli*, which has been described as a potential cause of blueshifts in past biosensing papers, as well as potential aggregation of the CGQDs on the bacterial cell wall.3

The increase in fluorescence for high concentrations of *E. coli* (not shown in Figure 2) is hypothesized to be due to the natural fluorescence of the *E. coli*. Even though non-fluorescent *E. coli* strains were used, bacteria still have some natural level of fluorescence due to their proteins. In high enough *E. coli* concentrations (especially those where the liquid cultures began to appear clouded and even opaque) this natural level of fluorescence could have hidden any quenching resulting from CGQD-*E. coli* attachment, leading to increases in fluorescence.

However, this is of little concern for practical applications of these CGQD biosensors, as such high *E. coli* concentrations are unlikely to appear in sample and/or necessitate a biosensing assay for detection, due to the cloudy appearance of the solution. Instead, it is the decrease in fluorescence corresponding to low *E. coli* concentrations that is of greatest interest for real-world use of these biosensors. Therefore, significant, easily detectable decreases in fluorescence corresponding to low-concentration *E. coli* cultures (as we observe in Figure 2 and the Supporting Information figures) signify great potential for developing robust, usable biosensors.

In fact, work by Gwimbi et al. shows that contaminated water sources in locations such as rural Lesotho have coliform concentrations as high as $10^5$ CFU/mL.11 This is within the range of *E. coli* concentrations where our biosensors have been observed to function, suggesting that the LOD of our biosensors is low enough to be used in real-world applications.

**CONCLUSION**

Through analysis of changes in fluorescence and absorbance intensity, we have demonstrated the conjugation of carboxylated graphene quantum dots with the protein cecropin P1, and have shown how these biosensors can be used to reliably detect the concentration of bacteria in a solution. Furthermore, the LOD of our biosensors appears to be low enough for real-world usability.

These findings hold great potential for fast-acting, practical applications, such as the quantification of bacterial levels in fluids like surface or drinking water. Our biosensing probes are made using nontoxic compounds, important for real-world safety, and facilitate rapid
detection of bacteria through a simple fluorescence assay (the time needed for fluorescence measurements at a given emission wavelength was well under 5 minutes, once the conjugated CGQDs were prepared and mixed with E. coli cells). We encourage further work to examine the structural properties of these conjugated CGQDs, to determine the precise LOD of our biosensors, as well as elucidate their precise interactions with E. coli and other bacteria.

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AUTHOR CONTRIBUTIONS
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COMPETING INTERESTS
The authors have declared that no competing interests exist.

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SUPPORTING INFORMATION

Figure 4: Graph of the change in fluorescence intensity of the conjugated CGQD sample after mixing with various concentrations of ampicillin-resistant E. coli liquid culture. All measurements taken using excitation wavelength = 400 nm and emission wavelength = 620 nm.
Figure 5: Graph of the change in fluorescence intensity of the conjugated CGQD sample after mixing with various concentrations of chloramphenicol-resistant *E. coli* liquid culture. All measurements taken using excitation wavelength = 400 nm and emission wavelength = 670 nm.