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Application of Carbon Nanoparticles as DNA Detection Probe and Fluorescent Ink

Luckio Frank Owuocha
Missouri State University, Owuocha094@live.missouristate.edu

APPLICATION OF CARBON NANOPARTICLES AS DNA DETECTION PROBE AND FLUORESCENT INK

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Chemistry

By

Luckio Frank Owuocha

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ABSTRACT

There is a significant interest in developing a sensitive, selective, efficient, and inexpensive method for rapid molecular diagnostic tests. This research aims to develop an inexpensive nucleic acid detection method by using DNA-conjugated carbon nanoparticles that exhibit fluorescence in the visible region. Carbon nanoparticles of this class can be detected without specialized equipment and have great promise toward the development of analytical methods that can be used in resource-limited environments with a lack of access to proper diagnostic and healthcare. We employed EDC-NHS (two-step) and EDC (one-step) coupling techniques to prepare DNA-conjugated carbon nanoparticles. The dot blotting method was adapted as a format in evaluating the applicability of the prepared nanoparticles in DNA detection. Along with the pursuit of nucleic acid detection method development, we found that the carbon nanoparticles bind to filter papers that are made of cellulose. By capitalizing on this property of carbon nanoparticles, the potential utility of carbon nanoparticles as a new fluorescent ink has also been investigated.

KEYWORDS: carbon nanoparticle, deoxyribonucleic acid detection, dot blotting, nitrocellulose membrane, fluorescent ink

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Approved by:

Keiichi Yoshimatsu, Ph.D., Thesis Committee Chair

Adam Wanekaya, Ph.D., Committee Member

Gary Meints, Ph.D., Committee Member

Kartik Ghosh, Ph.D., Committee Member

Julie Matterson, Ph.D., Dean of the Graduate College

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I dedicate this thesis to my dad, Francis Owuocha to whom I owe my education and proper upbringing through his constant discipline, love, support, and care. You made me who I am today. I celebrate you in your absence, may your soul rest in perfect peace with the angels.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1 Carbon Nanoparticles

Carbon nanomaterials (CNM) are a class of compounds that have a diameter of less than 100 nm. CNM are derivatives of carbon, an element in the periodic table whose electronic configuration is [He] $2s^2$ $2p^2$. Carbon is one of the most plentiful elements in the universe with a molar mass of 12.01 g/mol. Carbon nanomaterials exist in a variety of forms as illustrated in Figure 1.[1] A range of different techniques has been developed to synthesis different forms of CNMs that have been used in various applications. Examples previously synthesized CNMs include graphene, fullerene, carbon nanotubes, carbon nanodots, and carbon nanoparticles.[1]

Each class of carbon nanomaterials exhibits unique physical and chemical properties, even though they share common characteristics. In some cases, they can be interconverted when subjected to certain experimental conditions.[1] Historically carbon particles had been considered to be of low solubility in an aqueous solvent with little fluorescent characteristics.[2] It was not until nanomaterial became a matter of interest in the scientific society that carbon nanoparticles (CNPs) started attracting broad attention due to their interesting properties including good suspension stability in aqueous solutions, ease of functionalization, low toxicity, chemical inertness, biocompatibility, and photostability.[3-11]

It has previously been reported that the photoluminescence properties of CNPs are dependent on their size, edge shape, surface ligands, and defects.[12] One of the particularly interesting photoluminescence properties of CNPs is their excitation wavelength-dependent shift of emission wavelength in a wide range of spectrum from UV to near-infrared region.[4, 13, 14] Meanwhile, excitation wavelength-independent photoluminescence of CNP has also been

reported for the CNPs that exhibit a uniform surface state.[15-18] These unique properties of CNPs present opportunities for new applications.

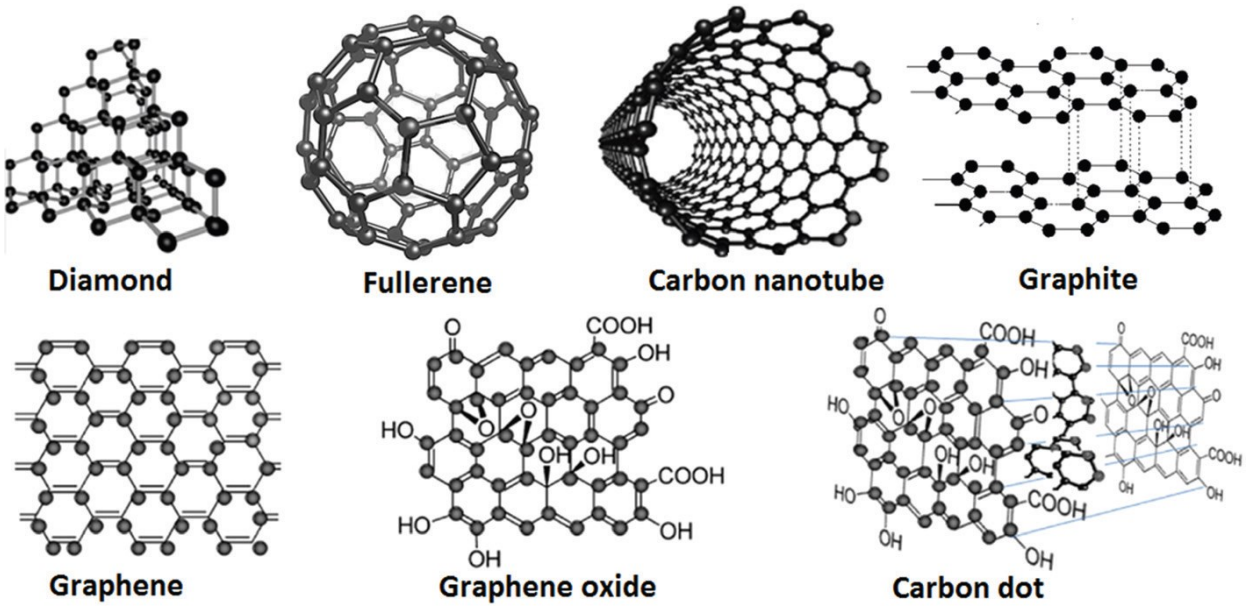


Figure 1. Examples of carbon nanomaterials. Reprinted from Ref 1, which was published by The Royal Society of Chemistry, and licensed under a Creative Commons Attribution 3.0 Unported Licence.

Advantages of carbon nanoparticles over other fluorescent compounds. CNPs typically contain conjugated pi-systems. In addition, they may also contain many other functional groups including ether, epoxy, carbonyl, hydroxyl, amine, amide, and carboxylic acid (Figure 2).[16] The diversity of functional groups that can be introduced onto the CNP surface provides several routes to conjugate them with various polymeric, inorganic, organic and biological compounds.[16-19] In addition, the modification of surfaces with hydrophilic functional groups imparts good suspension stability in water.

Another advantage is the simplicity of the preparation procedures. Furthermore, CNPs often exhibit high photostability[18] and a longer shelf-life compared to many organic fluorescent

molecules. These properties make CNPs desirable in application areas such as point-of-care diagnostics. Also, CNPs are more resistant and exhibit photostability.[18]

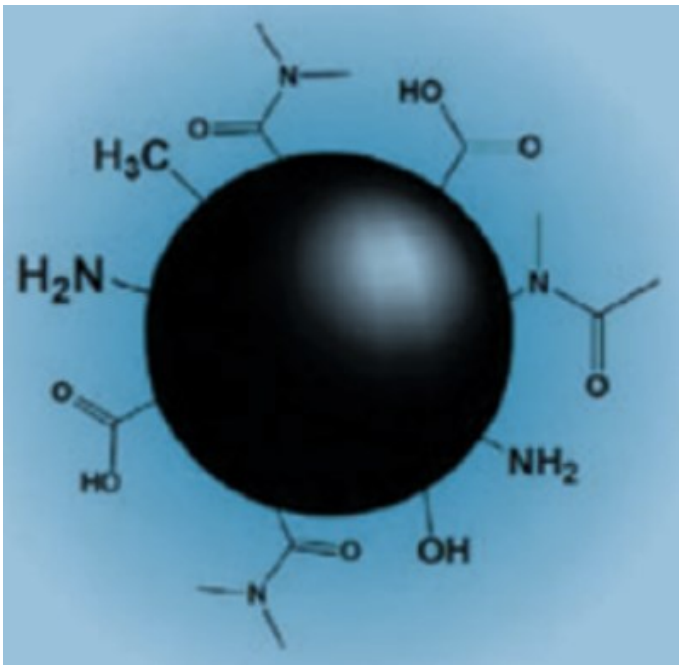


Figure 2. Functional groups on the surface of N-doped carbon nanoparticles. Reprinted from Ref 25 with permission from The Royal Society of Chemistry.

Synthesis of carbon nanoparticles. Preparation of CNPs can be categorized into top-down or bottom-up approaches.[3, 20, 21] In top-down approaches, precursors such as graphite powder are typically subjected to harsh physical and chemical conditions such as laser ablation. On the other hand, in the bottom down approaches, low-molecular-weight precursors such as glucose are treated by microwave pyrolysis, ultrasonication and autoclave.[20-24] Preparation of CNPs by these methods can often be achieved with inexpensive precursors such as ground coffee,[25] used tea,[26] candle soot [13] and grass.[12]

Figure 3 shows two schemes for introducing different functional groups onto CNPs using different techniques. The functional groups introduced in the outer shell may be utilized in the

conjugation of CNP to different biorecognition agents[27] for several applications in biological, material science, communication, and medicine.

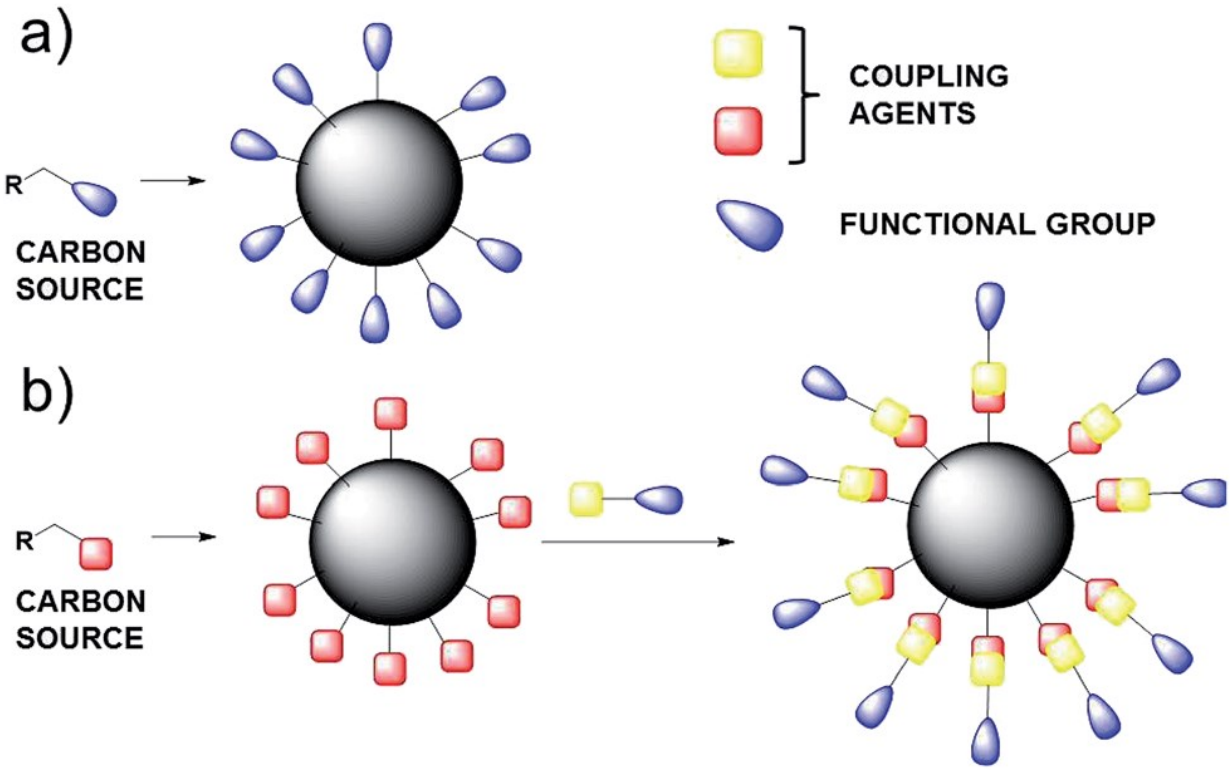


Figure 3. (a) One-pot synthesis of CNPs (b) covalent functionalization of the outer shell. Reprinted from Ref 27, which was published by The Royal Society of Chemistry, and lisensed Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

Optical properties. Many CNPs exhibit fluorescence or phosphorescence as shown in Figure 4. Electrons in their ground state (S_0) are excited by a source of energy electromagnetic radiation) to a higher energy level (S_1). When the electrons eventually fall back to their ground state, fluorescence and phosphorescence are observed. When the electrons fall back to the ground state in the same system, the phenomenon is referred to as fluorescence. However, if the electron undergoes an intersystem transition to the triplet state (T_1) before falling back to the ground state, the phenomenon is referred to as phosphorescence (Figure 4).[28] Generally, fluorescence occurs within a short time while phosphorescence occurs over longer time scale.

Crystallization or embedding in the solid matrix such as polyvinyl alcohol has been reported to stabilize intercrossing and induce phosphorescence.[29-31]

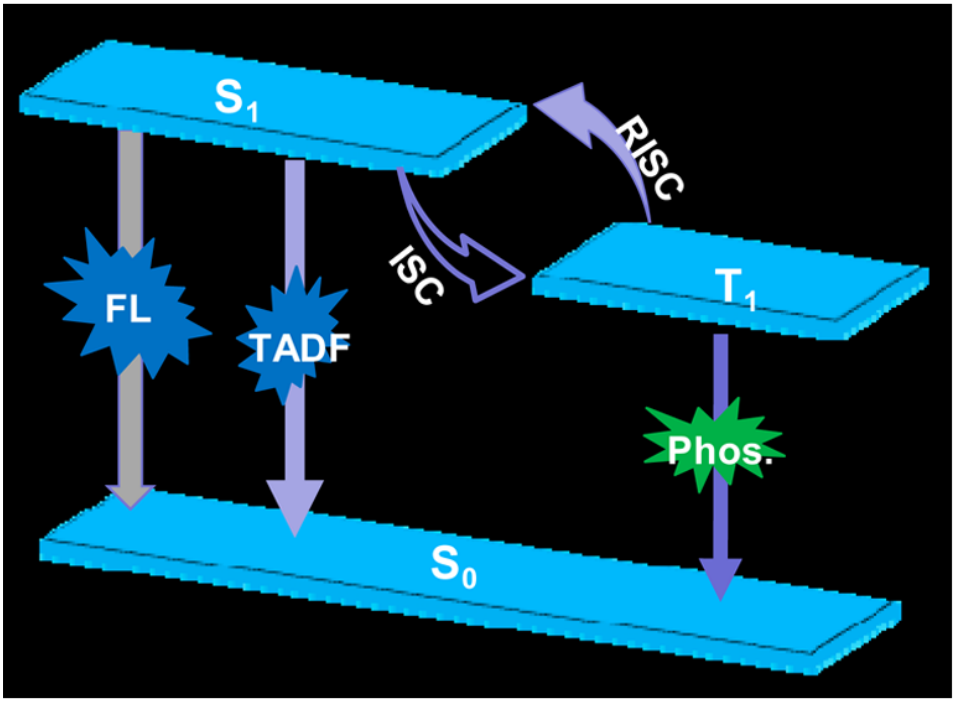


Figure 4: Energy diagram of fluorescence and phosphorescence phenomena. Reprinted from Ref 28, which was published by The Royal Society of Chemistry, and lisensed Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

The origin of the optical characteristics of CNP is not fully understood, however, it can be attributed to the pi-conjugated nature of CNPs and doping. Doping can be done through either direct synthesis or post-treatment technique.[32-35] In a direct synthesis, a doping agent is included alongside the CNPs preparation precursors hence this is a one-pot synthesis and the end product is a conjugated system with some substituted carbon atoms. In post-treatment, the synthesized CNP is treated with a doping agent. Both techniques are effective in doping CNPs, however, the direct synthetic technique is prevalent because it uses simpler and readily available precursors and lower temperatures.

1.2 Applications of Carbon Nanoparticles in Chemical/Biochemical Analysis

As discussed earlier, CNPs have several advantages that make them suitable candidates for many chemical and biochemical applications. Applications of CNPs have been demonstrated in several scenarios as a photocatalyst,[27] biosensing,[36] bioimaging,[37] theranostics,[38] and molecular communications.[39, 40] In the era of a larger population with a lack of access to proper diagnostic and healthcare especially in developing countries, there is a significant need for developing a sensitive, selective, efficient, and yet inexpensive method for rapid molecular diagnostic tests. Since papers are inexpensive, readily available, and portable, paper-based analytical tools offer several advantages over other analytical techniques. This elaborates why the paper has been a central matrix for a huge portion of work that has been published on CNP.

Application of CNPs in paper-based analytical devices. Lateral flow assay is an analytical method that can be performed with an inexpensive and portable device. Figure 5 shows a schematic of a device for lateral flow assay.[41] Lateral flow test strips are based on the principle of chromatography that utilizes capillary action to interact target analytes with a reagent for different signals to be realized. The matrix used is typically the nitrocellulose membrane.[42] The four major components are sample pad, conjugate pad, membrane, and absorbent pad. Samples are introduced at the sample pad. There are two possible ways of sample introduction; first, the sample can be dispensed by an applicator directly onto the sample pad Second, the kit can be immersed into the sample solution, also referred to as dipstick. In both cases, the sample moves via capillary action.

The samples then interact with different reagents on the conjugate pad producing a signal on the membrane. A signal on both the control and test line indicates positive results, while a

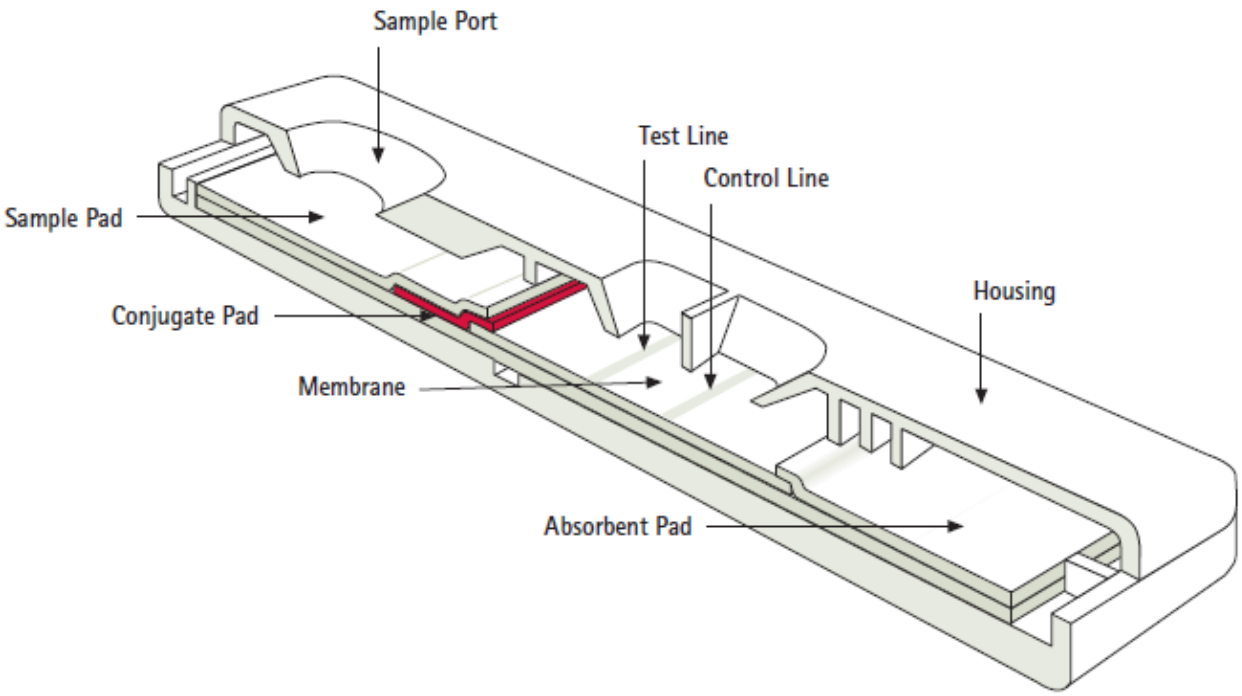


Figure 5. Examples of devices for lateral flow assay. Adapted from Ref 41.

signal on the control line alone indicates negative results. However, a signal on the test line alone is an indication of a faulty or expired kit. The remaining reagents and samples that pass through the membrane are wicked at the absorbent pad. Guodong Liu and coworkers have recently reported a fabricated lateral flow biosensor that uses DNA that is labeled with UV-excitable fluorescent CNPs to capture target DNA using biotinylated-DNA on immobilized streptavidin on nitrocellulose.[42]

Dot blotting. Dot blotting is another assay format that can be performed with simplified experimental setup. In this method, reagents are directly spotted onto the nitrocellulose membrane and exposed to subsequent reagent by direct soaking. This increases the probability of target DNA to interact with the reagents, as opposed to lateral flow assay where the different reagents and samples are transported by capillary action and might be exposed to evaporation.

Chemistry for conjugation of CNPs with amine-modified DNA. Activation of CNPs and their conjugation to DNA can be performed by using a one-step protocol that employs EDC or a two-step protocol that employs both EDC and NHS. As shown in Figure 6, in the two-step coupling protocol, carboxylates can be coupled with NHS in the presence of a carbodiimide such as EDC, yielding a semi-stable NHS ester, which provides a good leaving group. The NHS ester can then be reacted with an amine to form an amide bond. In the case of the one-step coupling protocol, NHS is omitted, and an amine directly reacts with the *o*-acylisourea ester intermediate to form an amide product. The activation of carboxylates with EDC and NHS is typically carried out at pH 4.5-7.2. MES buffer is often used as it effectively works as a buffer of choice at pH 4.7-6.0. The reaction of NHS-activated carboxylic acids with the amine-containing molecules are typically carried out at pH 7-8. Phosphate-buffers at pH 7.2-7.5 are frequently used.[43] Excess EDC is often quenched with thiols such as 2-mercaptoethanol.

Visualization of the fluorescence signal. One of the key steps in the chemical and biochemical analysis is the detection of signals. In a previously reported work by Guodong Liu and coworkers, a quant reader, a specialized device for readout of fluorescence signals from CNPs on lateral flow devices, was used to detect the fluorescence signal on the lateral flow device.[42] After lateral flow assay, the nitrocellulose membrane was excited using UV light and the fluorescence observed and captured by the quant reader as shown in Figure 7. However, it is imperative to note that signal readout without specialized equipment would be an added advantage to such a technique. Once such a device has been engineered and approved for the market, the user would not need any expertise to determine the result. This would make the device accessible to remote areas with no specialized expertise needed. It would also make the device more affordable.

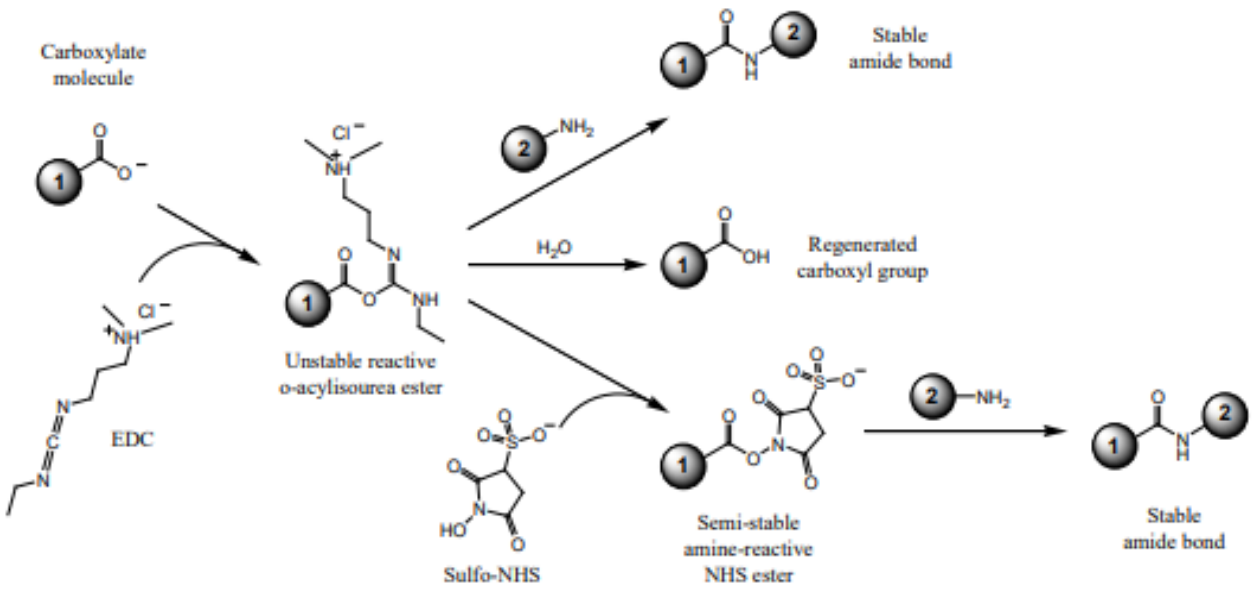


Figure 6. One step (EDC) coupling and two steps (EDC/NHS) coupling. Adapted from Ref 43.

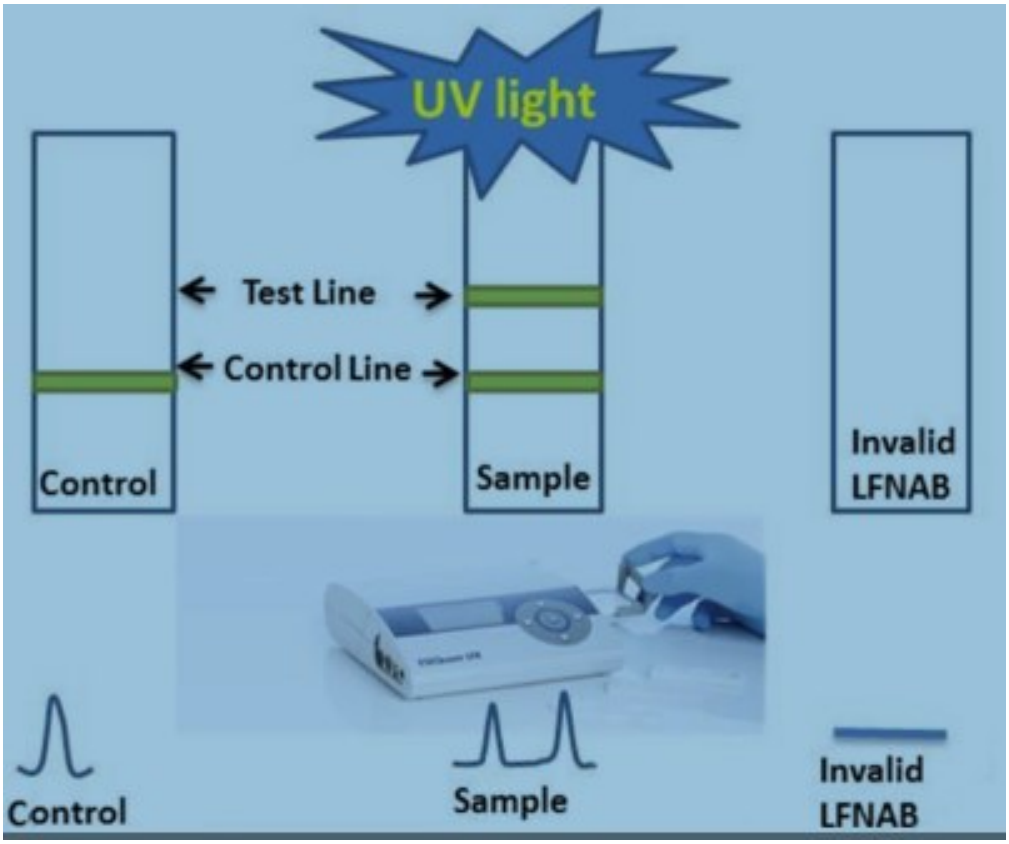


Figure 7. Qualitative and quantitative detection of fluorescence using an ESE-quant reader. Reprinted from Ref 42, with permission from Elsevier.

Application of CNPs as fluorescent ink. The potential utility of fluorescent and phosphorescent CNPs in encryption of data have previously been investigated.[44] Furthermore, complex optical properties such as excitation wavelength-dependent emission peak shift, up-conversion, and phosphorescence open paths for the application of CNPs as a special ink.[44] Qu *et al*. have recently reported the potential utility of CNPs as excitation wavelength-dependent biocompatible ink. CNPs exhibited both fluorescence and biocompatibility with minimal to no cytotoxicity.[45] Toxicity assays with plants and animals confirmed the lack of toxicity of CNPs to biological organisms. Bean sprouts grown on an aqueous dispersion of CNPs (1.5 mg/ml) exhibited fluorescence (Figure 8), while this did not inhibit the growth of the plants, indicating the biocompatibility of CNPs. Mice were also fed with an aqueous dispersion of CNPs (0.7 mg/ml) and the fate of CNPs was analyzed for five weeks. The excitation wavelength-dependent fluorescence peak shift was observed in the urine excreted. The administration of CNPs did not cause any pathological symptoms. It was inferred that non-biological substances had a quenching effect on the fluorescence of the CNP.[45]

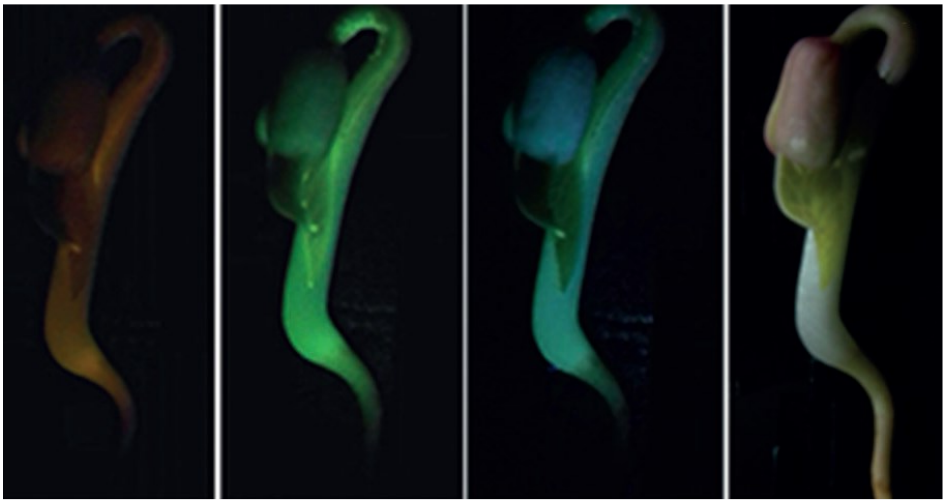


Figure 8. From left to right, optical and fluorescent images of a bean sprout grown with C-dot aqueous solution (1.5 mg/ml) under daylight, 340 nm excitation (395 nm), 420 nm excitation (450 nm), and 500 nm excitation (550 nm). Reprinted from Ref 45 with permission from John Wiley & Sons, Inc.

1.3 Objectives

As described in the previous section, CNPs hold a great promise in broad application areas including the use as probes for biochemical analysis. However, the CNPs that were utilized in the previously reported DNA detection required UV light as an excitation light source. Therefore, it was necessary to use specialized equipment for reading out the test results. We hypothesized that the use of visible light excitable CNPs allows for the readout of the result by eyes. The objective of this work was to develop a dot blotting-based assay method with CNPs that can be excited with visible light for DNA detection.

In addition, during the development of the dot blotting-based assay method, it was observed that CNPs can be stably adsorbed on cellulose paper. Building upon this finding, I have expanded the scope of objectives to include the potential applicability of CNPs as a fluorescent ink.

2. MATERIALS

2.1 Reagents and Chemicals

Citric acid (99.5%), ethylenediamine (99.9%), and formamide (99.5%) used for preparing carbon nanoparticles were purchased from Aldrich chemical, Millipore-Sigma, and Sigma Aldrich respectively. Chemically modified oligo DNAs (FITC-DNA, biotinylated DNAs, and amine-modified DNA were purchased from Integrated DNA Technologies. The properties of those oligo DNAs are summarized in Table 1. Phosphate buffered saline (PBS), 4'-hydroxyazobenzene-2-carboxylic acid (HABA), streptavidin (biotechnology grade), biotin (99.9%) and bovine serum albumin (BSA, biotechnology grade) used for dot blotting assays were purchased from Sigma (St. Louis, U.S.A.). *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC). and 2-mercaptoethanol used for coupling reactions were purchased from VWR chemicals, LLC.SYBR safe dye, agarose (99.9%), loading used for running gel electrophoresis were purchased from VWR chemicals, LLC. 2-(N-morpholino) ethanesulfonic acid (MES) and 3-morpholinopropane-1-sulfonic acid (MOPS) used in the activation of CNPs were purchased from Sigma-Aldrich.

2.2 Instrumentation and Equipment

Blue light transilluminator (Dark Reader Transilluminator, Clare Chemical Research, Inc.) was used to visualize dot blotting signals. M3800 Ultrasonic Bath (Branson Ultrasonics) was used to disperse aggregating CNP. Mupid-2plus System gel electrophoresis equipment (Takara Bio Inc.) was to determine if the concentration of DNA used in the preparation of CNP-DNA conjugates could be quantified on agarose gels. Spectra/Por 6 Dialysis Membrane (12-14

kD MWCO) (Repligen) and centrifuge (Zentrifugen, Germany), and centrifuge concentrator filters were used for purification of CNP conjugate. Thermocycler (Applied Biosystems) was used to anneal FITC-DNA to the biotinylated DNA.

Table 1. Details of oligonucleotides purchased from Integrated DNA Technologies, Inc. (IDT) for dot blotting tests.

Oligo DNA	Oligo DNA ID	DNA sequence	Molecular weight (g/mol)	T_m (° C)
FITC-DNA	Target 3-6-FAM_KY0001	5'ATG ACC TAT GAA TTG ACA GAC/3 6-FAM-3'	7007.7	49.7
Biotinylated-DNA I	ComplementToTarget-3-BiotinTEG-KY0002	5'-GTC TGT CAA TTC ATA GGT CAT/3 BIOTEG/-3	6980	49.7
Amine modified DNA	Target-3-AmMo-KY0003	5'-ATG ACC TAT GAA TTG ACA GAC/3 AmMo-3'	6649.4	49.7
Biotinylated-DNA II	Capture-3-Biotin-KY0004	5'-ATA GGT CAT/3 BIOTEG/-3'	3307.5	14.4

3. METHODS

3.1 Preliminary Test of Dot Blotting Procedure by Using FITC Labeled DNA

Preparation of stock reagents. All oligo DNAs were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). Sterile DI water (112.3 µl) was used to dissolve 112.3 pmol of FITC –DNA and 115.6 µl of sterile DI water were used to dissolve 115.6 pmol of biotinylated DNA I. 92.6 pmol of amine-modified DNA was dissolved in 92.6 µl of sterile DI water and 183.5 pmol biotinylated DNA II was diluted in 183.5 µl of sterile DI water. The oligonucleotide sequences were adapted from the work reported by Guodong and coworkers in the development of lateral flow biosensor.[42] To prepare a 2.5 mg/ml streptavidin solution, 1 mg of streptavidin was dissolved in 400 µl of PBS. The prepared 2.5 mg/mL streptavidin solution was then stored in 20 µl aliquots in -20 °C freezer until the time of use.

Immobilization of streptavidin on nitrocellulose membrane matrix. A small drop (0.5 µl) of streptavidin (2.5 mg/ml) was spotted on one nitrocellulose membrane (2 cm × 2 cm). The membranes were left to dry for ten minutes. A drop of HABA dye solution (0.25 mM) was then added to the nitrocellulose membrane.

Optimization of blocking conditions. Figure 9 shows the structure of the anticipated molecular complex[46] to be formed on the nitrocellulose membrane in our study that utilized nitrocellulose membrane, streptavidin, blocking with 0.5% BSA,[42] biotinylated DNA, and CNP-DNA conjugate. FITC-DNA was also used as a positive control.

Streptavidin solution (2.5 mg/ml, 0.5 µl) was spotted on three nitrocellulose membranes (1.5 cm by 1.5 cm) and left to dry for 15 minutes. The blocking was performed by incubating the membranes in 1.5 ml of microcentrifuge tube containing PBS containing 0.5% BSA for 1 hour, 2

hours, or 24 hours. The membranes were then transferred to a microcentrifuge tube containing 1.5 mL of PBS. To these tubes, 1 µl of pre-formed FITC-DNA/biotinylated DNA double-stranded DNA was added and incubated for 30 minutes at room temperature. After rinsing in 5 mL PBS for 2 minutes, the membranes were placed onto blue light transilluminator with an orange plastic filter board to observe the fluorescence.

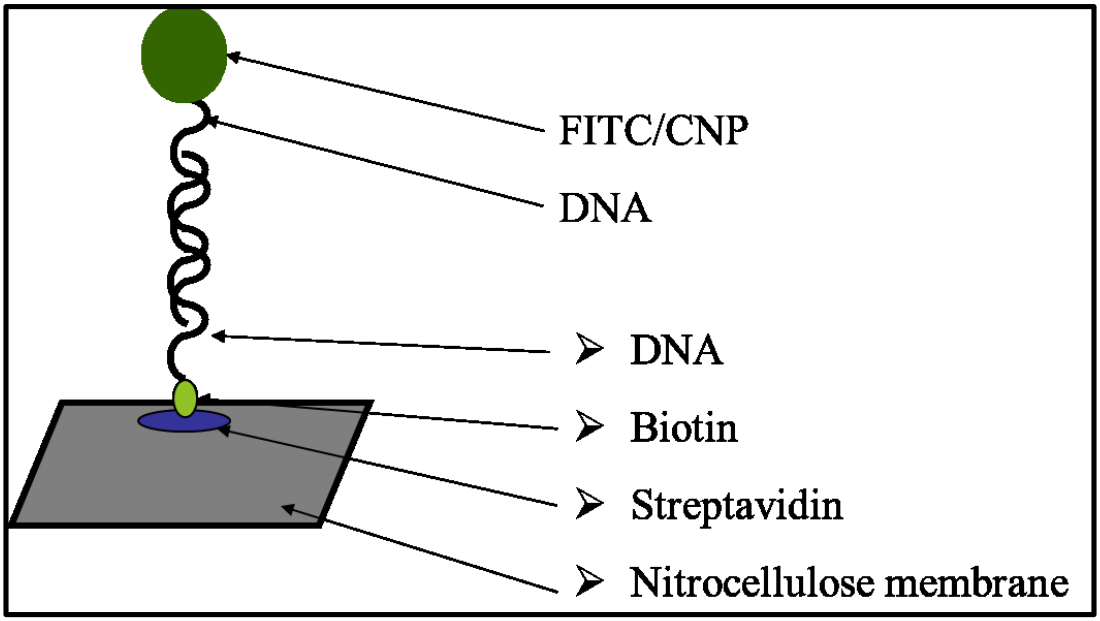


Figure 9: Description of the structure of the anticipated molecular complex to be formed on the nitrocellulose membrane in the preliminary test (proof of concept) on the left.

Effects of the presence of 0.5% BSA in other steps on binding test. Onto nitrocellulose membranes (1.5 cm by 1.5 cm), 1 µl of streptavidin was spotted at the center of the nitrocellulose membranes and left to dry for 15 minutes. The membranes were then immersed in a blocking solution (0.5% BSA in PBS) for 1 hour. The membranes were then washed in 10 mL PBS for 2 minutes before the first membrane was immersed in a solution containing biotinylated DNA I in 1 ml blocking buffer (0.25 mM) and then FITC-DNA in 1 ml blocking buffer (0.25 mM). The other membrane was immersed in solutions of biotinylated-DNA I in 1 ml PBS (0.25

mM) and then FITC-DNA in 1 ml PBS (0.25 mM). After rinsing in 10 ml PBS for 2 minutes, the membranes were placed onto blue light transilluminator with an orange plastic filter board to observe the fluorescence.

3.2 Synthesis of Carbon Nanoparticles

Synthesis of unoxidized carbon nanoparticle. Unoxidized carbon nanoparticles were synthesized by the group of Dr. Wanekaya at Missouri State University. In brief, unoxidized carbon nanoparticles (CNP) were prepared by autoclaving a solution containing citric acid, ethylenediamine, and formamide at 180 °C for four hours and cooled down to room temperature.[47] The reaction solution was then filtered through a 0.22 μm filtration membrane. CNP in the filtrate was then precipitated in acetone and collected by centrifugation at 10000 rpm for 10 minutes. The carbon nanoparticles were washed with 1:1 acetone and ethanol mix and centrifuged 3 times. The hydrodynamic diameter of oxidized carbon nanoparticles was 126.42 nm with a zeta potential of -22.11 mV. UV-Vis. Fluorescence spectra were recorded using an F-4600 spectrofluorometer. FT-IR spectra of these CNP were also recorded using a spectrometer. The optical data is shown in Appendix B1-B3 respectively.

Synthesis of oxidized carbon nanoparticles. To oxidize the CNP, 0.1 g of the prepared CNP was added to 15 ml of 3 M HNO_3 and refluxed for 12 hours then neutralized using Na_2CO_3.[47] Acquired product was then dialyzed using Snake Skin 3.5 kD MWCO dialysis tubing with 35 mm inner diameter. Dialysis was done for 3 days, replacing the water every 24 hours. The product was then frozen and lyophilization used to remove the water from the product. The final product was a brown solid. UV-Vis, fluorescence, and FTIR spectra of these CNP were shown in Appendix B4-B6, respectively.

3.3 Attempts to Activate Carbon Nanoparticles

Activation of unoxidized CNP. EDC (9.6 mg), NHS (5.43 mg) and 1 mL of CNP in water (3.25 mg/ml) were mixed with 1 ml of 0.1 M MES buffer (pH 4.7). The mixture was incubated at room temperature for 30 minutes.[43] 1 ml of 0.1 M MOPS buffer (pH 7.0) was added to the mixture to adjust it to pH 7.0. 2-mercaptoethanol (50 µl) was added to the acquired product to inactivate the excess EDC. One (1) µl of amine-modified DNA stock solution was then added to the activated DNA and incubated overnight. Centrifuge concentrator filter (MWCO: 10 K) was used to wash the CNP-DNA conjugate for ten minutes at 5000 RPM. After each centrifugation, the unit was refilled with 0.1 M MOPS buffer (pH 7.0). These steps were repeated for four times.

One step activation of CNP using EDC and conjugation to DNA. EDC (10 mg) was dissolved in 1 ml of water and 100 µl of this EDC solution added to 400 µl of MES buffer (pH 6.0). 200 µl of the CNP (0.325 mg/ml) was added to the solution and the 296 µl of PBS buffer then added to raise pH to 7.0. Amine-modified DNA stock solution (4 µl) was added to the solution and the mixture was incubated at room temperature for 3 hours.[43, 48]

Two-step activation of CNP using EDC, NHS, and conjugation to DNA. EDC (0.4 mg) and NHS (0.6 mg) was dissolved in 500 µl of CNP dispersion (0.325 mg/mL) and 500 µl 0.1 M MES (pH 6.0). The mixture incubated for 30 minutes. Amine-modified DNA stock solution (4 µl) was added to the solution and the mixture was incubated at room temperature for 3 hours.[49]

3.4 Attempts to Conjugate Activated Carbon Nanoparticles with DNA and Purification of the CNP-DNA

Conjugation of unoxidized CNP with amine-modified DNA and purification of the CNP-DNA conjugate. After the activation of CNPs by using the procedure described in section 3.3, 3 µl of amine-modified DNA stock solution (1 µM) was added and incubated at room temperature for 3 hours. The unreacted DNAs were removed by using a centrifugal filter unit (MWCO: 10 kD). The unit were subjected to centrifugation at 5000 rpm at room temperature for ten minutes. After each centrifugation, the unit was refilled with PBS. These steps were repeated for four times.[50]

Conjugation of activated oxidized CNP with amine-modified DNA and purification of CNP-DNA conjugate. Four (4) µl of amine-modified DNA stock solution (1 µM) was added to the mixture of activated, oxidized CNPs. The mixture was incubated at room temperature for 2 hours. The obtained mixture was dialyzed using a dialysis membrane with MWCO 12 – 14 kD against 0.1 M phosphate buffer (pH 7.0).

DNA conjugation at different CNP concentration. EDC (9.6 mg) and NHS (5.4 mg) was added to 500 µl of CNP (0.325 mg/ml) and 500 µl MES buffer (pH 5.5) and incubated for 30 minutes. A portion of this stock solution was used to prepare diluted CNP solutions, 0.35, 0.035, 0.0035, and 0.00035 mg/mL respectively. The pH of the solution was then raised to 7.0 by the addition of an equivalent volume of 0.1 M MOPS buffer pH 7.0. Ten (10) µl of 2 – mercaptoethanol was added to quench excess EDC. Three (3 µl) of amine-modified DNA (1 µM) was then added and the solution and then incubated for 3 hours. The obtained mixture was dialyzed using a dialysis membrane with MWCO 12-14 kD against 0.1 M phosphate buffer (pH 7.0) for three days. The buffer was replaced once per day.

Conjugation of DNA by using One-step activation at pH 5.5, 6.0, 6.5. EDC (10 mg) was dissolved in 1 ml of water. One hundred (100) µl of this EDC solution was added to 200 µl CNP dispersion (0.325 mg/mL). To this mixture, 0.1 M MES buffer (196 µl) at pH 5.5, 6.0, 6.5 and 4 µl of DNA (1 µM) was added. The reaction mixture was incubated for 2 hours. The obtained mixture was dialyzed by using a dialysis membrane with MWCO 12-14 kD against 0.1 M phosphate buffer (pH 7.0).

3.5 Dot Blotting Test Using DNA Conjugated Carbon Nanoparticles and FITC-DNA

Test on CNP-DNA conjugate binding on nitrocellulose membrane with and without a wash. The different concentrations (0.35, 0.035, 0.0035, and 0.00035 mg/ml) of CNP conjugates were directly spotted on nitrocellulose membrane. The fluorescence was observed under blue light transilluminator before and after rinsing with 10 ml deionized water three times.

Control experiments using FITC-DNA. Onto three nitrocellulose membrane (1.5 cm by 1.5 cm), 2 µl of streptavidin solution (2.5 mg/ml) spotted at the center. These membranes were left to dry for 15 minutes before being immersed in a blocking solution (0.5% BSA in 20 mM phosphate buffer) for one hour. The first nitrocellulose membrane was transferred to a solution containing following components double-stranded DNA formed by hybridization of FITC-DNA (112.3 pmol) and biotinylated-DNA I (183.5 pmol) in 1 ml PBS (20 mM); 2) FITC-DNA (112.3 pmol) in 1 ml PBS (20 mM); and 3) biotinylated-DNA I (183.5 pmol) in 1 ml PBS (20 mM). The membrane that was incubated with biotinylated DNA I was subsequently incubated in a solution containing FITC-DNA (92.6 pmol) in 1 ml PBS for another 30 minutes. The fluorescence of all membranes were then observed on a blue light transilluminator.

Binding test using CNP-DNA conjugates. Onto two nitrocellulose membranes (1.5 cm by 1.5 cm), 0.5 µl streptavidin solution (2.5 mg/mL) was spotted at the center of the membrane and allowed to dry for 15 minutes. The membranes were then immersed in a blocking solution (0.5% BSA in PBS) for 1 hour. After washed with PBS, the membrane was incubated in PBS containing biotinylated DNA I (1 µM) and then in PBS containing CNP-DNA conjugates (0.325 mg/mL) for 30 minutes. After another wash in 10 ml PBS for 2 minutes, the membrane was incubated in PBS containing CNP-DNA conjugates (0.325 mg/ml) for another 30 minutes. The membranes were then both immersed for an extra 30 minutes in 1 µl of FITC-DNA in 0.25 mM PBS and observed under blue light transilluminator again.

Binding test of CNP-DNA conjugate on nitrocellulose membrane at different CNP-DNA conjugate concentrations. Onto ten pieces of nitrocellulose membrane (2 cm by 2 cm), 2 ml of streptavidin solution (2.5 mg/ml) was spotted at the center of the membranes and allowed to dry for 15 minutes. Five of them were used as a negative control that omits the step of the incubation with the biotinylated-DNA solution. One was used as a positive control by incubating with solution containing biotinylated-DNA (1 µM) and then FITC-DNA (1 µM). The other four membranes were used as the test samples. Blocking of these membranes was carried out by the incubation in PBS solution containing 0.5% BSA at room temperature overnight. The membranes were then washed in PBS and immersed sequentially in PBS containing biotinylated DNA (1 µM) and CNP-DNA (0.325 mg/ml) conjugate in PBS. Fluorescence on the membrane was observed on the blue light transilluminator.

Attempt to detect amine-modified DNA on an agarose gel. One (1) µl of amine-modified DNA stock solution was mixed with 9 µl of sterile DI water to prepare a 10-fold diluted DNA sample. One (1) µl of the 10-fold diluted DNA sample was then mixed with 9 µl of

DI water to prepare 100-fold dilution. Table 2 shows the volumes of the mixture that were loaded on the wells. The amount of amine-modified DNA loaded to each well was 1.24 ng, 0.0124 ng, and 0.44 ng, respectively. The gel was run for 25 minutes. It was then stained with SYBR Safe dye and observed on the blue light transilluminator.

Table 2. Volumes used for the gel electrophoresis

	1/10 sample	1/100 sample	Ladder
Loading dye (µl)	2	2	2
DI water (µl)	7	7	9
Sample (µl)	3	3	1
Total (µl)	12	12	12

3.6 Spotting Non-oxidized and Oxidized CNP on Cellulose Paper to Investigate Potential Usage as Ink

Adsorption of CNP on cellulose membrane. Three (3) µl oxidized and non-oxidized CNP (approximately 3.25 mg/ml) was spotted on cellulose-based filter paper and the fluorescence was observed on a blue light transilluminator. The cellulose-based filter papers were rinsed with 30 ml of deionized water and observation repeated.

Adsorption of CNP on filter paper at different pH values. Two (2) µl of 0.325 mg/ml of CNP was spotted on five pieces of paper and allowed to dry for 15 minutes. The filter papers were rinsed three times with buffers with different pH (0.1 M MES buffer 4.7, 5.5, 7.0, 0.1 M phosphate buffer 8.0 and 0.1 M carbonate buffer 9.6). The fluorescence was then observed on the blue light transilluminator.

4. RESULTS AND DISCUSSION

4.1 Preliminary Test of Dot blotting Procedure by Using FITC Labeled DNA

Confirmation of streptavidin immobilization on nitrocellulose membrane. The purpose of the first experiment was to confirm the feasibility of immobilizing streptavidin on a matrix by the dot blotting technique. Elpidio Cesar has reported a streptavidin-biotin- enhanced nitrocellulose membrane enzyme immunoassay used to detect rhabdovirus of penaeid shrimps from infected animals.[51] Their work confirms that there is a strong noncovalent interaction between biotin and streptavidin as it can be used to enhance the signal. Additionally, it indicates that streptavidin could be stably immobilized on the nitrocellulose membrane. Hence, we chose to immobilize streptavidin on the nitrocellulose membrane (matrix) *via* the physical adsorption. This was for the subsequent immobilization of biotinylated DNA by the strong non-covalent interaction between streptavidin and biotin. In order to confirm immobilization of streptavidin solution was spotted on a nitrocellulose membrane and the membrane was incubated in a solution of 0.25 mM HABA dye. The result showed that the location where streptavidin was spotted turned into pink, confirming the immobilization of streptavidin on the nitrocellulose membrane.

Incubation of streptavidin-immobilized membrane with pre-formed biotinylated DNA/FITC-DNA double-stranded DNA solution. Dot blotting technique, that is popularly used in the analysis of proteins[52] was used to investigate the efficiency of biotinylated DNA *via* streptavidin-biotin interaction. Streptavidin-immobilized membranes were immersed in a solution of pre-formed biotinylated-DNA/FITC-DNA double-stranded DNA, a modified procedure that was used by Sunitha *et al.* in the development of a lateral flow biosensor.[42] The

illustration described on the left of Figure 10 shows the outline of the dot blotting assay that was used for this research. In our initial study, biotinylated DNA I that is complementary to FITC-DNA was used. Hence, if the hybridization of biotinylated DNA I and FITC-DNA occurs, the fluorescence is detected under blue light transilluminator. It is important to note that this combination of DNAs was used only to confirm the feasibility of the methods. In order to use this method in practical applications, the biotinylated DNA and fluorescently labeled DNA would be designed to be partially complementary to the target DNA (right side on Figure 10).[42]

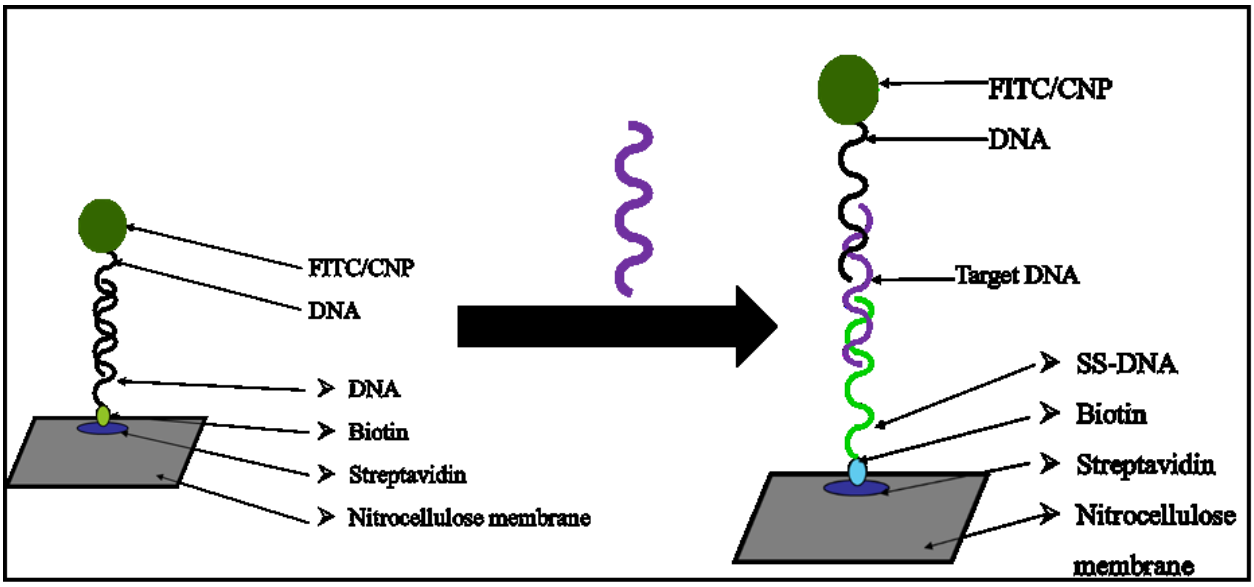


Figure 10. Description of the structure of the anticipated molecular complex to be formed on the nitrocellulose membrane in the preliminary test. On the right, description of sandwich anticipated hybridization of target DNA by capture and detection DNA (Ref 46).

The preformed double-stranded DNA was prepared by annealing of biotinylated DNA and FITC-DNA on a thermal cycler. Figure 11 shows the image of the membrane placed on a blue light transilluminator. While faint fluorescence could be observed on the streptavidin spot the contrast between the streptavidin spotted area and surrounding area was not very high. This appears to indicate that the streptavidin/biotinylated DNA/FITC-DNA complex might be formed

as described in Figure 10. We attributed the observed low contrast between the streptavidin spot and the surrounding area to a high background caused by non-specific adsorption of biotinylated-DNA/FITC-DNA double-stranded DNA on nitrocellulose membrane. We, therefore, investigated the blocking conditions to reduce the non-specific binding of DNAs.

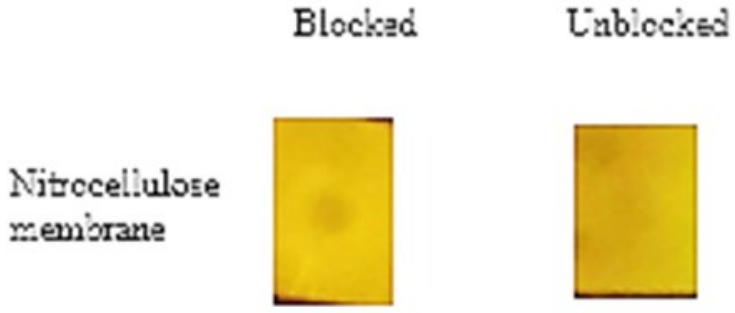


Figure 11. Binding test on immobilized streptavidin on nitrocellulose membrane using preformed FITC-DNA/biotinylated DNA.

Optimization of blocking conditions. We investigated the effectiveness of PBS containing and 0.5 % BSA as the blocking solution.[42] In this study, the streptavidin-immobilized membranes were incubated with the blocking solution for different durations, 1 hour, 2 hours, or 24 hours. It was determined that 1 hour of blocking sufficiently suppressed the background and improved the contrast (Figure 12). The contrast of the fluorescence intensity at the streptavidin area over surrounding could be enhanced, indicating the significant reduction of non-specific binding. Since blocking for 2 hours and 24 hours did not show further improvement, we used 1 hour of blocking with PBS containing and 0.5 % BSA in the subsequent experiments.

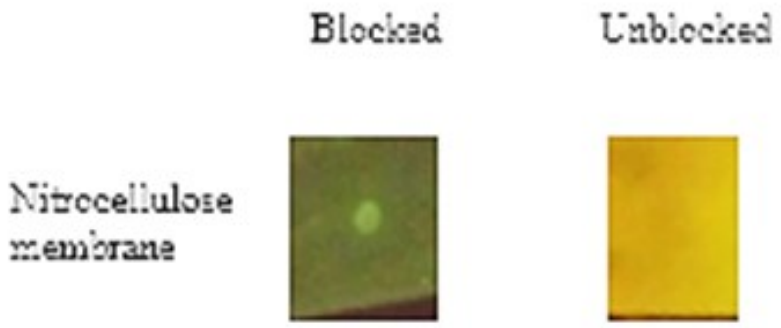


Figure 12. The effect of blocking with 0.5% BSA in PBS for 1 h at room temperature.

Effect of the presence of BSA during incubation with Biotinylated-DNA/FITC-DNA double-stranded DNA. Since the blocking of nitrocellulose membrane with 0.5% BSA was effectively reduced background, we studied if the addition of 0.5% BSA during the incubation with DNAs would allow for further reduction of background. It was observed that the presence of 0.5% BSA during the incubation with Biotinylated-DNA/FITC-DNA double-stranded DNA resulted in a reduction of the fluorescence. We attributed the decreased fluorescence intensity to the degradation of FITC-DNA/biotinylated DNA double-stranded DNA. We hypothesized that the degradation might have been due to prolonged and continual exposure of the oligonucleotides to the buffer/blocking reagent mixture. Therefore, for subsequent assays, we only blocked the matrix.

Capturing of FITC-DNA by immobilized biotinylated DNA. The previous sets of experiments confirmed that the optimized blocking conditions allows to reduce non-specific binding and biotinylated DNAs can be captured by streptavidin. Therefore, we next investigated if our procedure allowed for capturing of complementary DNA by the immobilized biotinylated DNA. In this experiment, streptavidin was spotted onto a nitrocellulose membrane using a spatula to imitate the straight line on lateral flow assays. The membrane was incubated with

single-stranded biotinylated DNA and subsequently incubated with FITC-DNA with a sequence that is complementary to biotinylated DNA. As shown in Figure 13, the membrane that was sequentially incubated with biotinylated DNA and FITC-DNA showed stronger fluorescence at the streptavidin area, indicating that the blocking enhanced signal over the background and immobilized biotinylated DNA efficiently captured FITC-DNA. These results agree with reports published on biosensors and bioassays.[53, 54]

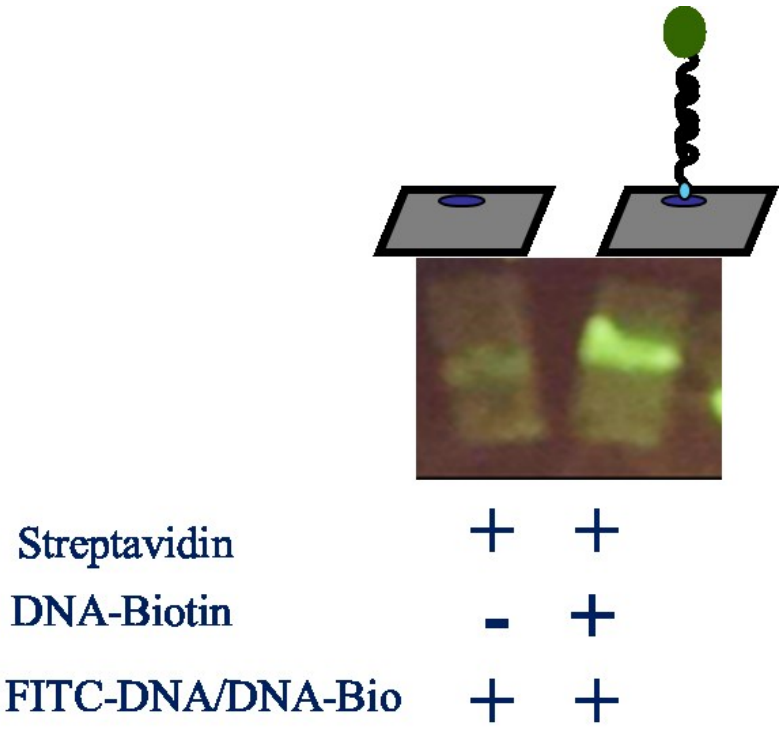


Figure 13. Effective capturing of FITC-DNA by immobilized biotinylated DNA I.

4.2 Attempts to Activate and Conjugate Carbon Nanoparticles with DNA

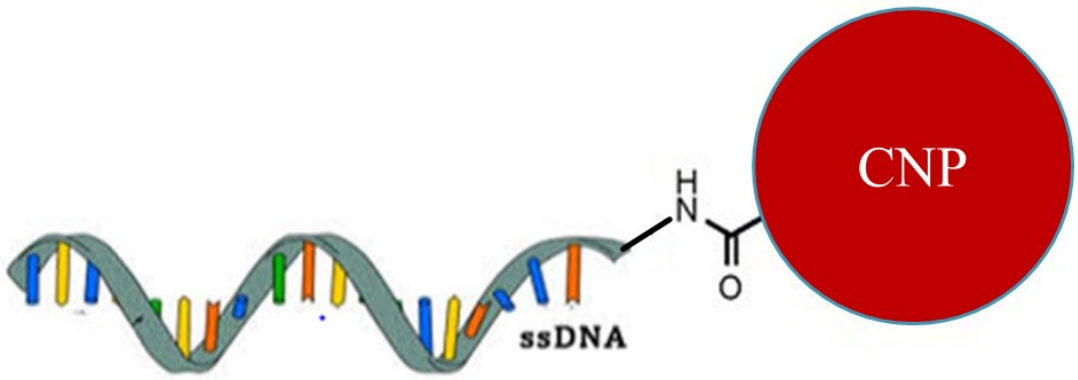


Figure 14. Illustration of carbon nanoparticle that is conjugated with single-stranded DNA *via* an amide bond. Reprinted from Ref 55 with permission from John Wiley & Sons, Inc.

Conjugation of DNA to unoxidized carbon nanoparticles. Figure 14 shows the illustration of a carbon nanoparticle that is conjugated *via* amide bond with amine-modified DNA.[55] The first batch of the carbon nanoparticle-DNA conjugate was prepared with the method described in section 3, MES buffer (0.1 M, pH 4.7) was used for the activation of the carbon nanoparticles with EDC and subsequent reaction with NHS and then MOPS buffer (0.1 M, pH 7.0) was used to raise the pH of the mixture prior incubation. The obtained crude CNP was then incubated with amine-modified DNA. Excess EDC was inactivated using 2-mercaptoethanol. In this experiment, no purification was performed before the binding test. The result of the binding test was shown in Figure 15. No signal was obtained for the binding test with DNA-conjugated

carbon nanoparticle as had been reported by Guodong and coworkers.[42] We, therefore, included the purification step to remove the excess unreacted amine-modified DNA from the mixture.

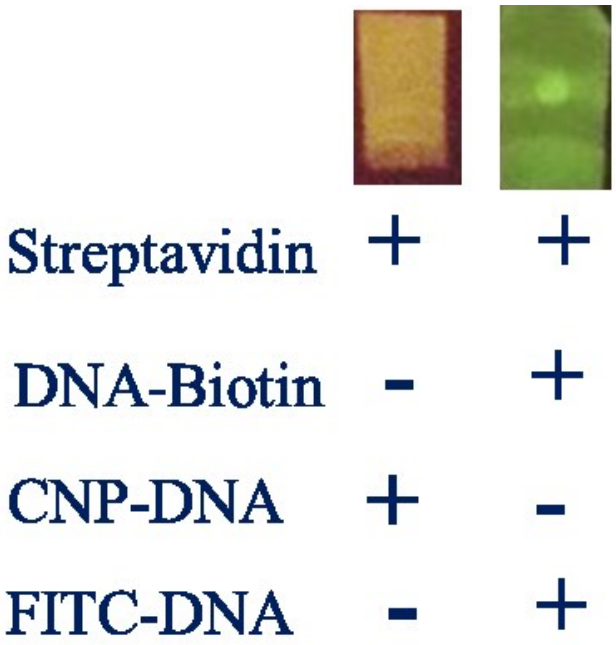


Figure 15. Showing binding test assay with unoxidized CNP and FITC-DNA

Purification of CNP-DNA conjugate using dialysis. We speculated that unreacted DNA might be competing with CNP-DNA conjugates on our previous experiment, resulting in the low binding of CNP-DNA conjugate. Therefore, dialysis was included in the subsequent sets of experiments. Guodong and coworkers reported to have used centrifugal filter units (3 KD) for purification of their fluorescent carbon nanoparticle conjugate, however, this technique was not ideal for us because our DNA was larger.[42] Since the molecular weight of oligonucleotides used for the assay ranged from 4000-6000 g/mol, the CNP-DNA conjugates were purified to remove excess DNA by dialysis using dialysis membranes with 6-8 KD and 12-14 KD molecular weight cut off (MWCO). Figure 16 shows the dialyzed CNP-DNA conjugates and dialysate. As the

result showed that majority of CNP-DNA conjugated retained in the tube, 12-14 KD MWCO dialysis membrane was determined to be ideal for the purification process. We, therefore, used it in subsequent experiments for purification of the CNP-DNA conjugates. Having determined the 12-14 KD dialysis membrane as appropriate, we proceeded to optimize various other parameters; concentration of carbon nanoparticles, pH at conjugation, and different methods of DNA conjugation conditions.

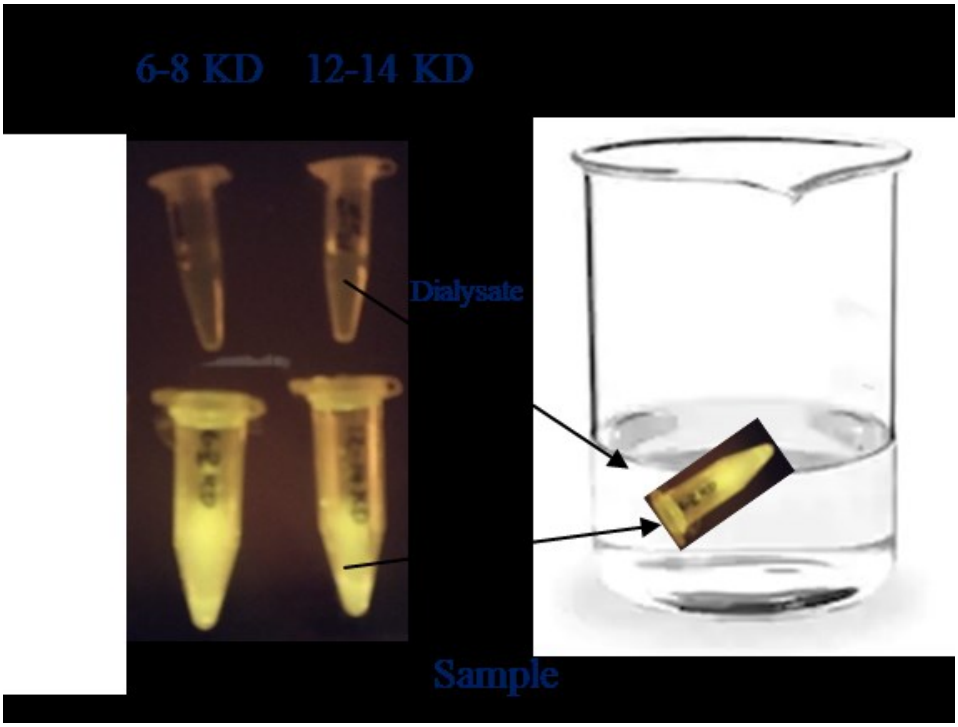


Figure 16. Fluorescence of unoxidized CNP-DNA conjugate samples after dialysis (with MWCO 12-14 KD membrane).

Binding test on nitrocellulose membrane using dialyzed CNP-DNA conjugates.

Figure 17 shows the result of the binding test with the dialyzed CNP-DNA conjugate. Streptavidin was spotted at the center of two nitrocellulose membranes. The nitrocellulose membrane to the left was directly incubated in PBS solution containing FITC-DNA in PBS. The nitrocellulose membrane was incubated in PBS solution containing biotinylated DNA I before

being transferred to PBS containing FITC-DNA. Both nitrocellulose membranes were then observed under blue light transilluminator. The absence of fluorescence at the streptavidin area on the nitrocellulose membrane to the right indicated that the CNP-DNA did not bind to the biotinylated DNA immobilized on the nitrocellulose membrane. On the other hand, the nitrocellulose membrane to the left no fluorescence was observed as was expected.[42]

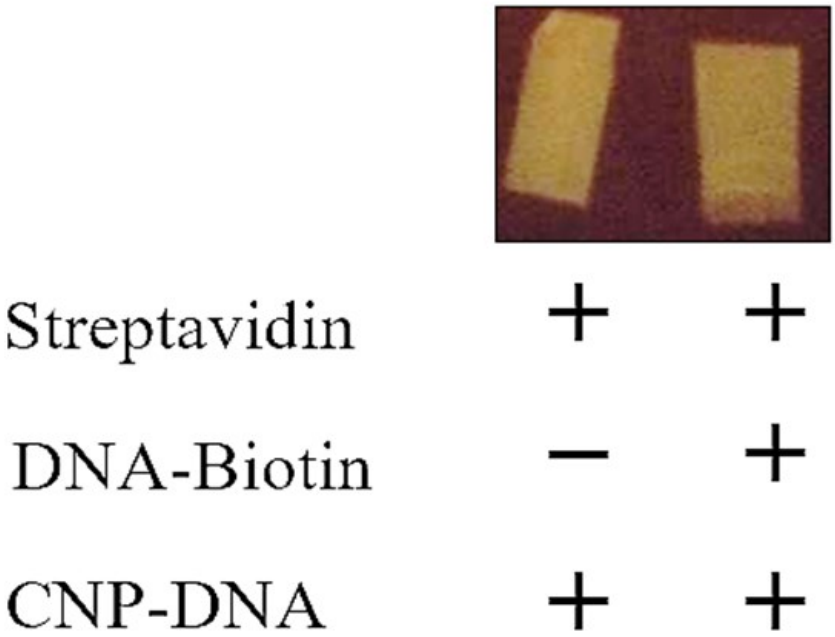


Figure 17. Binding test results using CNP-DNA conjugates that were purified by dialysis

To confirm the presence of biotinylated DNA on nitrocellulose membrane, the membranes were subsequently incubated with FITC-DNA in PBS buffer. As shown in Figure 18, fluorescence was observed in the streptavidin area of the nitrocellulose membrane to the right, confirming successful immobilization of biotinylated DNA with streptavidin on the membrane. Therefore, it was inferred that the conjugation of activated CNP with amine-modified DNA was not successful. There was a need to optimize conjugation conditions for successful conjugation to be achieved. One of the hypothesized possibility was to oxidize CNP. This would increase carboxyl groups on the surface of the CNP which were essential in conjugation reaction.[47]

Next, we attempted to couple amine-modified DNA to oxidized CNPs that exhibit a larger number of the carboxyl groups on the surface. We hoped that oxidation of CNP would increase the number of conjugated amine-modified DNA.

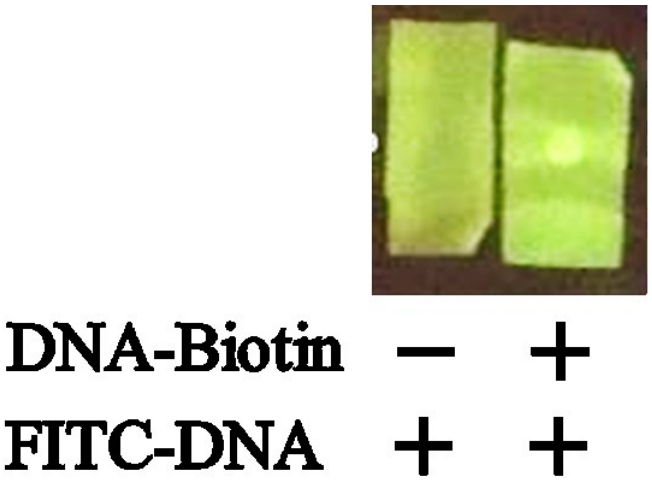
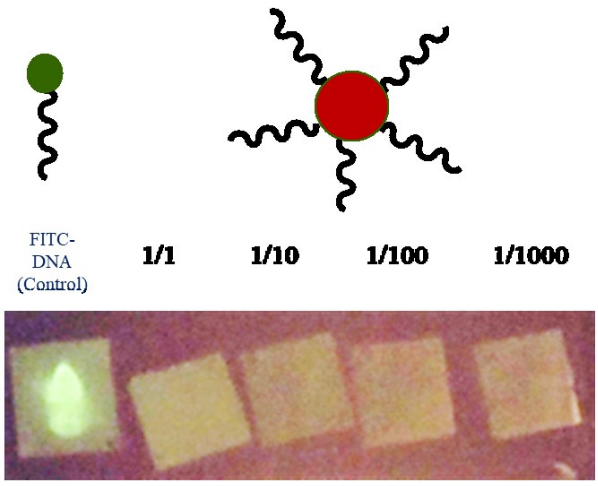


Figure 18. Binding test with CNP-DNA after the addition of FITC-DNA.

Binding test on nitrocellulose membrane at different concentrations of oxidized CNP-DNA conjugate. Oxidized CNPs were synthesized by the group of Dr. Wanekaya at Missouri State University. UV/VIS, fluorescence, and FTIR spectra are shown in appendix B. Appendix B6 depict the presence of key functional groups: hydroxyl (3200 cm^{-1}) and carbonyl carbon (1700 cm^{-1}), that were essential for the conjugation reaction. The CNP-DNA conjugate was prepared as described in section 3.5 and purified by dialysis. On the other hand, use of the excess amount of CNP-DNA conjugate might have contributed to the non-specific adsorption that was observed in the previous assay. Therefore, membrane with immobilized biotinylated DNA I was incubated with different concentrations of CNP-DNA conjugates. As shown in

Figure 19, fluorescence could only be detected on the positive control, the membrane that was incubated with FITC-DNA.

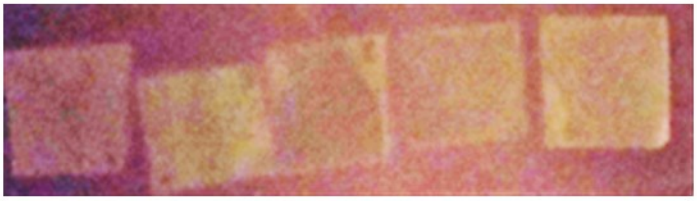


Streptavidin	+	+	+	+	+
Blocking with 0.5% BSA	+	+	+	+	+
Biotinylated-DNA	+	+	+	+	+
FITC-DNA	+	-	-	-	-
CNP-DNA	-	+	+	+	+

Figure 19. Incubation of DNA-immobilized nitrocellulose membrane after incubation with different concentrations of CNP-DNA conjugates and with FITC as a positive control.

In another control experiment, biotinylated DNA was omitted. As anticipated, no fluorescence was observed for any of the tested membranes when incubation with biotinylated DNA was omitted. This confirmed that the signal observed with the positive control with FITC-DNA is as a result of biotinylated DNA-FITC DNA hybridization. On the other hand, no signal was observed for the membranes that were incubated with any concentration of CNP-DNA conjugates, indicating that CNP-DNA conjugate could not be captured by streptavidin. This is a clear indicator that the binding of the detection DNA (FITC-DNA and CNP-DNA) is not non-

specific in both cases, as it depends on the capture DNA (biotinylated DNA) to bind through hybridization.



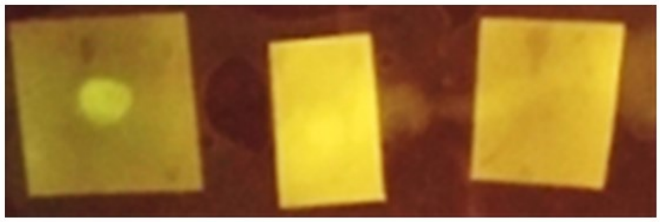
Streptavidin	+	+	+	+	+
Blocking with 0.5% BSA	+	+	+	+	+
Biotinylated-DNA	-	-	-	-	-
FITC-DNA	+	-	-	-	-
CNP-DNA	-	+	+	+	+

Figure 20. Showing binding results for negative controls of different concentrations of CNP conjugates and FITC.

One step coupling (EDC) versus two-step coupling (EDC/NHS). The results described above indicated that the immobilization of streptavidin, blocking with BSA, immobilization of biotinylated DNA, and hybridization of FITC-DNA could be achieved with the developed procedure. However, the selective binding of CNP-DNA conjugate to the biotinylated DNA on the membrane could not be observed. We attributed this to the failure in conjugating CNP with amine modified DNA.

As the two-step conjugation method with EDC and NHS apparently could not yield an efficient conjugation of amine-modified DNA with CNPs. We, therefore, examined if a one-step conjugation method with EDC alone would give a more efficient coupling of the CNP to DNA. As seen in Figure 21, incubation of the DNA-immobilized membrane with CNP-DNA conjugate

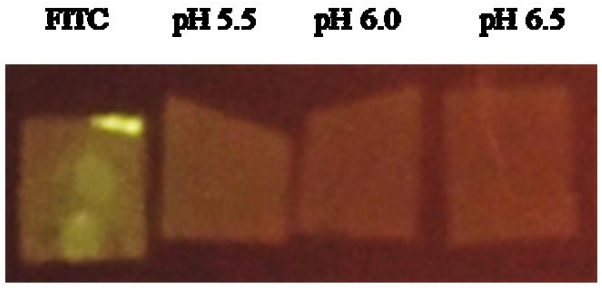
that was prepared by a one-step conjugation method with EDC showed a very faint fluorescence signal in one experiment. However, the results were not reproducible in the subsequent tests. According to the literature, the use of reaction buffers with lower pH (pH 4.5 to 6.0) is typically recommended while the two-step conjugation method with EDC/NHS was recommended to increase pH to near neutral before the addition of amine-containing compounds. Therefore, we performed DNA conjugation reactions at different pH values. Fluorescence was only observed



	FITC	EDC	EDC/NHS
Streptavidin	+	+	+
Blocking with 0.5% BSA	+	+	+
Biotinylated-DNA	+	+	+
FITC-DNA	+	-	-
CNP-DNA	-	+	+

Figure 21. Showing binding test for one step (EDC only) and two-step (EDC-NHS) CNP-DNA conjugates in 0.1 M MES (pH 6.0).

One-step activation of CNP using EDC and conjugation to DNA (pH 5.5, 6.0, 6.5). for the positive control that was incubated with FITC-DNA (Figure 22). Conjugation of amine-modified DNA with CNP at these tested conditions did not yield effective binding of CNP-DNA conjugate onto the DNA-immobilized membrane.



Streptavidin	+	+	+	+
Blocking with 0.5% BSA	+	+	+	+
Biotinylated-DNA	+	+	+	+
FITC-DNA	+	-	-	-
CNP-DNA	-	+	+	+

Figure 22. Results showing binding test for CNP conjugates at different pH.

Detection of amine-modified DNA on an agarose gel. The dot blotting assay did not show the evidence of CNP-DNA conjugation. This could have been due to the lack of the presence of enough DNA on the CNP surface or difficulty of the larger CNPs to remain on the membrane during the rinsing steps. We, therefore, considered an alternative method to study the efficiency of the CNP-DNA conjugation reaction. As one of the possible methods, we investigated if the concentration of unreacted DNA used during the coupling step could be quantified using agarose gel electrophoresis. In Figure 23, 1 and 2 represents the ladder and the amine-modified DNA respectively. No bands were detected for the amine-modified DNA (Figure 23), indicating that the concentration of amine-modified DNA used in the conjugation

reaction is too low to be detected by agarose gel electrophoresis.

Figure 23. Showing the gel results. The 3rd lane from left: 1 kb ladder (NEB), the 4th form left: 1/10 (Amine-modified DNA), 5th from left 1/100 (Amine-modified DNA)

CNP on amine-modified paper. As an alternative approach to identifying the optimal DNA conjugation condition, we prepared an amine-modified filter paper and studied it to screen the optimal conditions for the conjugation of CNP to amine-modified DNA. Interestingly, we discovered that unoxidized CNP strongly bound to the filter paper even after several times of rinsing with water. Figure 24 shows persistent adsorption of fluorescence of CNP on cellulose-based filter paper after several washes. Good Building upon this finding, we decided to explore the possibility to evaluate the applicability of unoxidized CNP as a fluorescent ink.

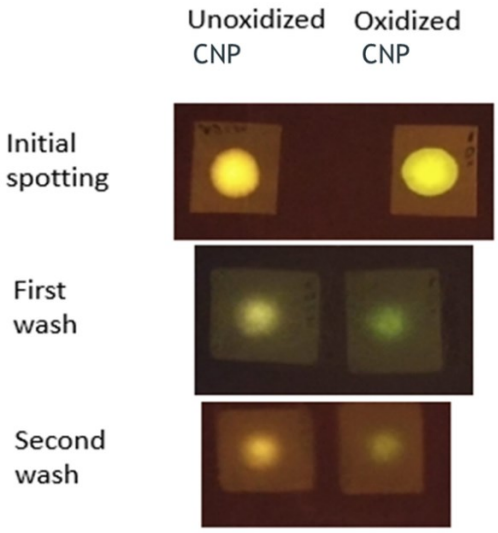


Figure 24. Persistent adsorption of unoxidized and oxidized CNP after several washes with water visualized on a transilluminator.

4.3 Carbon Nanoparticles as Fluorescent Ink

Carbon nanoparticles on cellulose membrane at different pH. To further investigate the potential utility of the unoxidized CNP as an ink, 0.035 mg/mL of the CNP sample was spotted onto a small piece of filter paper. The filter paper was subsequently rinsed by immersing into different buffer solutions; MES buffer (pH 4.7, 5.5, or 7.0); phosphate buffer (pH 8.0) and carbonate buffer (pH 9.6). As it can be seen in Figure 25, unoxidized CNP resisted to three times of rinsing with all the tested buffers. This showed that CNP is persistently adsorbed onto the filter paper and resistant to rinsing with buffer solution with a range of different pHs, suggesting their potential utility as a new type of fluorescent ink.[45]

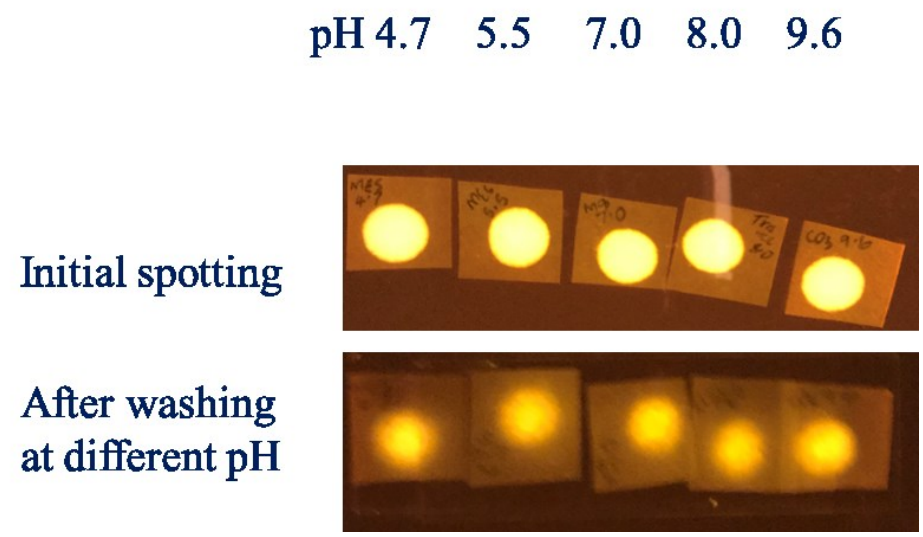


Figure 25. Adsorption of unoxidized CNP stickiness after repeated rinsing with buffers with different pH

5. CONCLUSION

In this work, we performed a series of experiments toward the development of a DNA detection assay method that utilizes visible light excitable CNP. Control experiments showed the hybridization of FITC-modified DNA with biotinylated DNA on the nitrocellulose membrane. The blocking condition was optimized by using FITC-modified DNA. Another result showed that 0.035 μg of CNPs could be detected on the nitrocellulose membrane with blue excitation light. Dialysis using dialysis membranes with MWCO 12-14 KD could be used for the purification of CNPs. However, the capturing of the detectable amount of CNP-DNA by the biotinylated DNA on nitrocellulose could not be accomplished up to date, presumably due to the low efficiency of the DNA coupling reaction. On the other hand, our efforts to develop a method for screening the conditions for the coupling reaction led to discover the potential utility of CNPs as a fluorescent ink. It was observed was that the fluorescence emission from CNPs could be observed even after several rinsing with various aqueous buffers with several different pHs. This result suggested a great promise to use of CNPs as a water-resistant fluorescent ink.

6. ABBREVIATIONS

CNP: carbon nanoparticles,
DNA: deoxyribonucleic acids
NHS: *N*-hydroxysuccinimide
PBS: Phosphate Buffered Saline
HABA: 4'-hydroxyazobenzene-2-carboxylic acid
MOPS: 3-(*N*-morpholino) propanesulfonic acid
FITC: fluorescein isothiocyanate
BSA: Bovine Serum Albumin
MES: 2-(*N*-morpholino) ethanesulfonic acid

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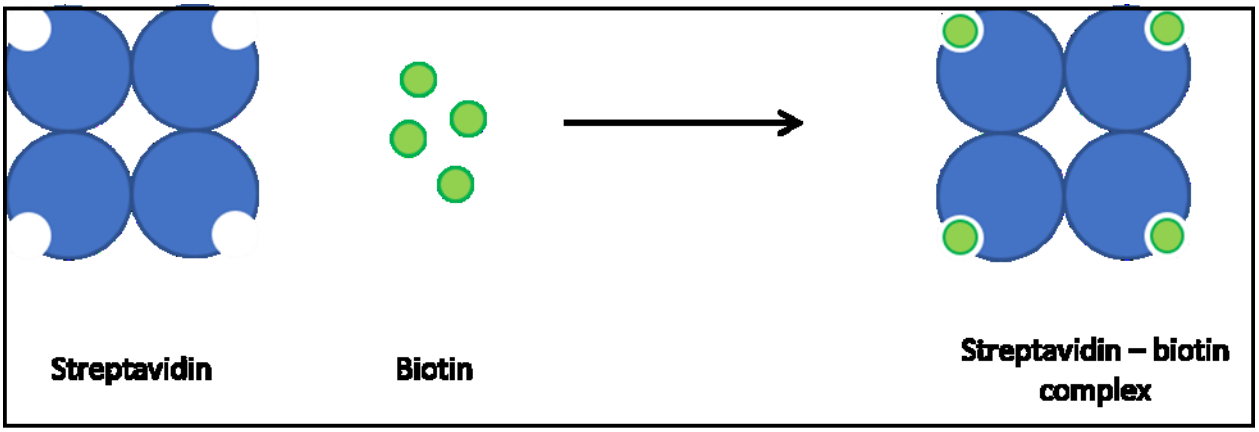
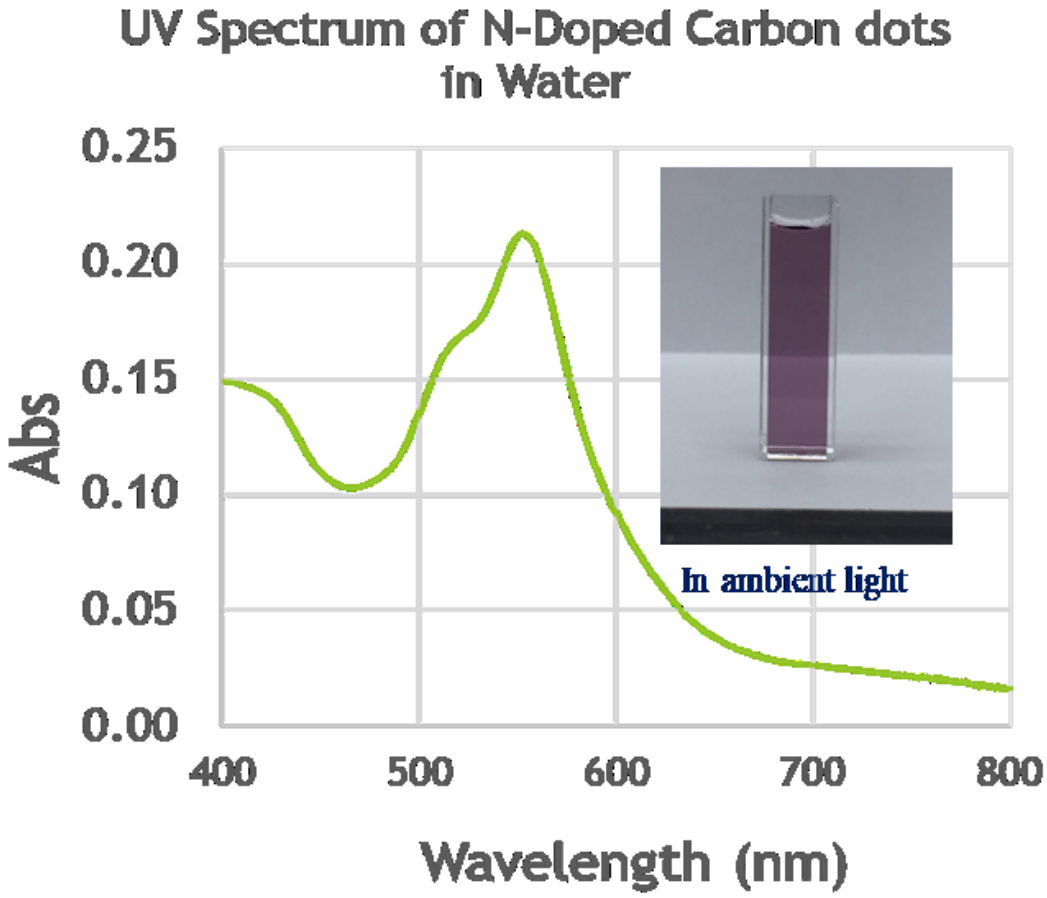
8. APPENDICES

Appendix A. Streptavidin-biotin Interaction

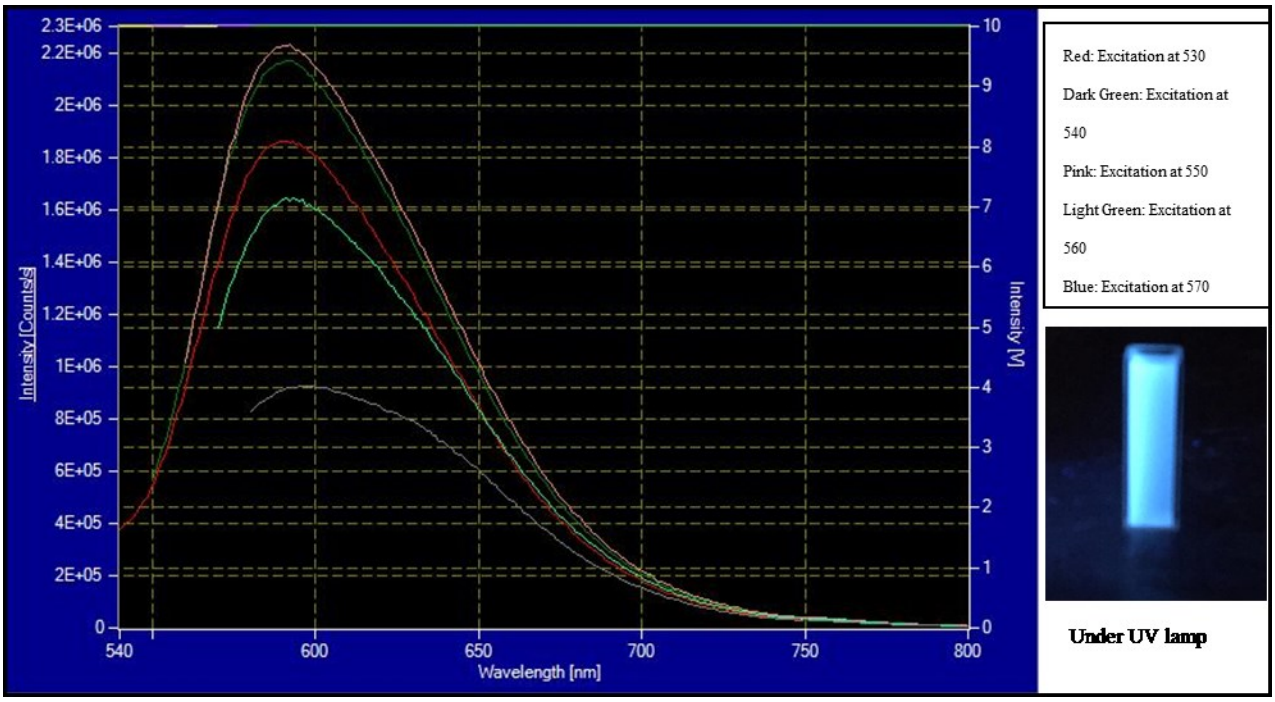


Appendix B. Characterization of Carbon Nanoparticles

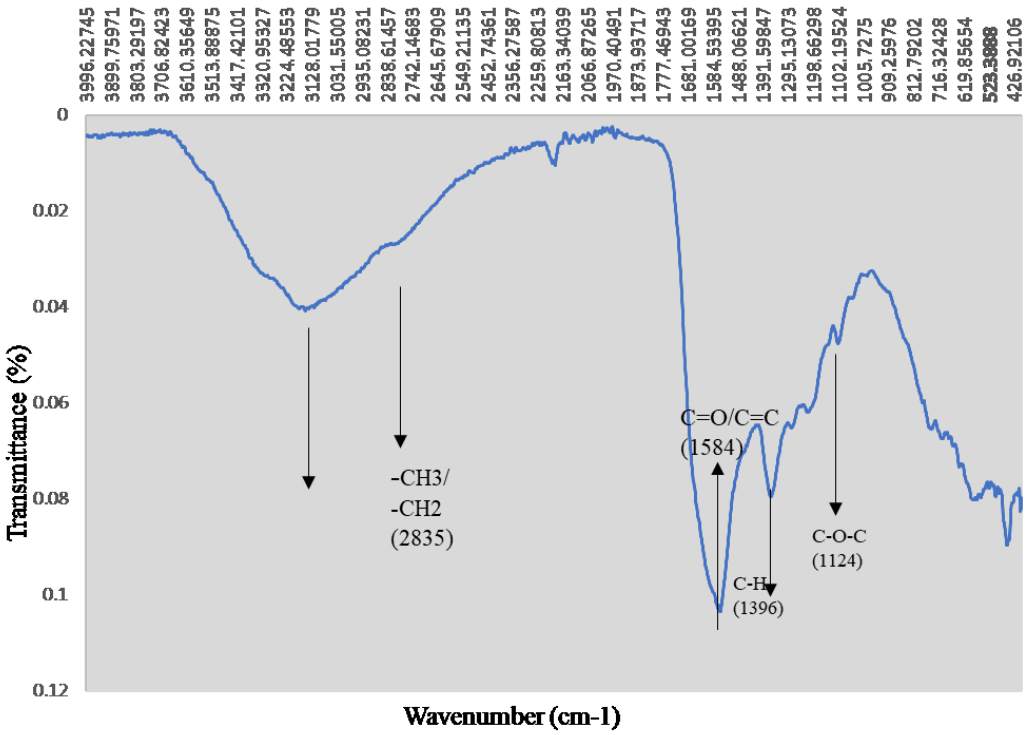
Appendix B-1. UV spectrum of nitrogen-doped unoxidized carbon nanoparticles in water.



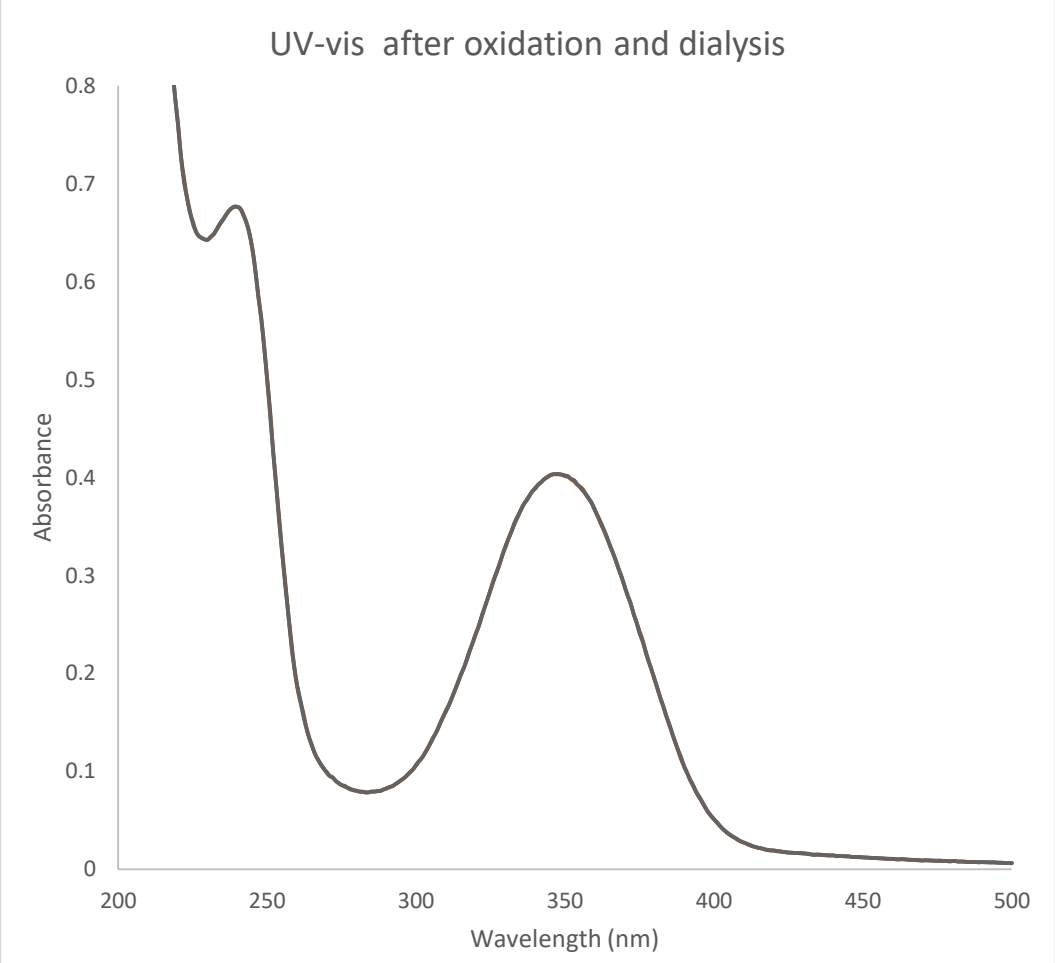
Appendix B-2. Fluorescence emission spectra of unoxidized carbon nanoparticle in water.



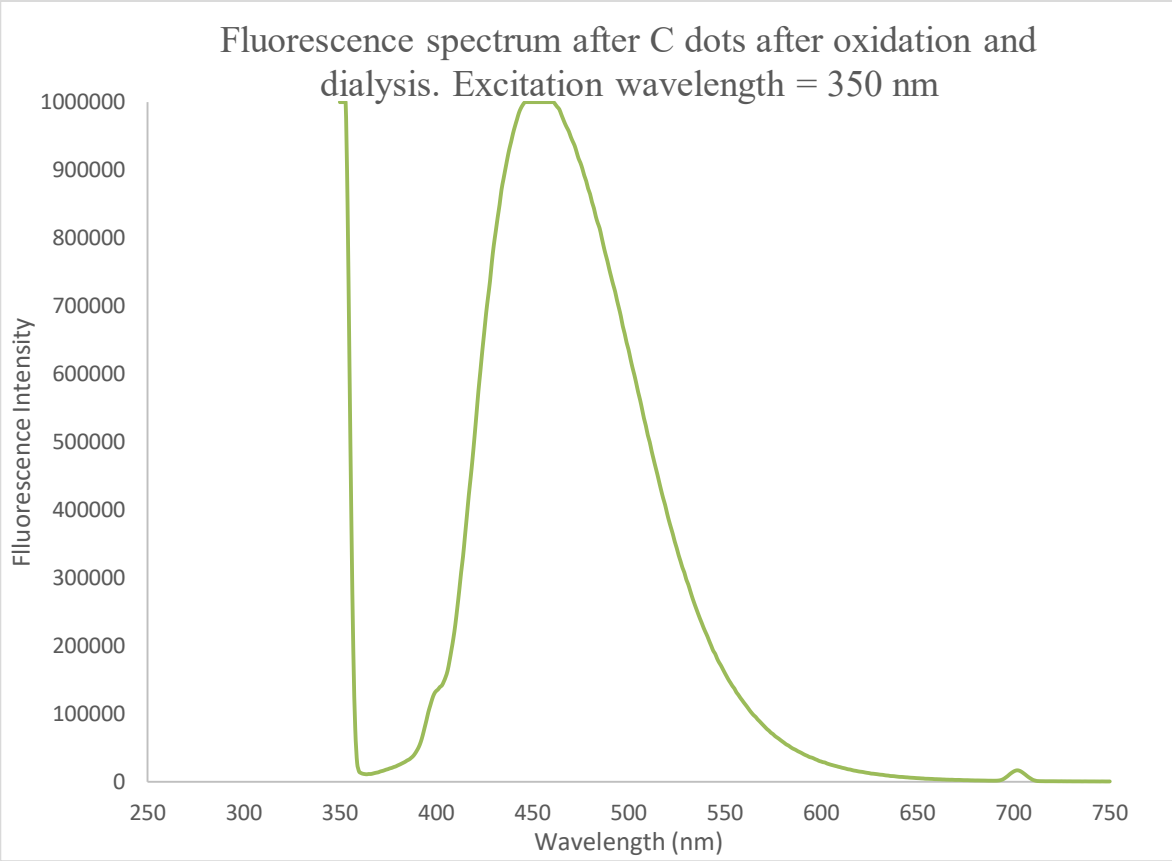
Appendix B-3. FTIR spectrum of unoxidized carbon nanoparticles.



Appendix B-4. UV spectrum of oxidized carbon nanoparticles in water



Appendix B-5. The fluorescence emission spectrum of oxidized carbon nanoparticles in water.



Appendix B-6. FTIR spectrum of oxidized carbon nanoparticles.