2009

Connect the Dots: Coupling Quantum Dots with Water-Soluble Porphyrins

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Connect the Dots: Coupling Quantum Dots with Water-Soluble Porphyrins"

written by

Kenley C. Singleton

and submitted in partial fulfillment of the requirements for completion of the Carl Goodson Honors Program meets the criteria for acceptance and has been approved by the undersigned readers.

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April 15, 2009
Connect the Dots: Coupling Quantum Dots with Water-Soluble Porphyrins

By: Kenley C. Singleton

August 2007 – April 2009
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ABSTRACT:

Currently, advances in the area of photodynamic therapy (PDT) using porphyrin molecules are being made and not only in this arena, but in science and society overall, nanoparticles are of high interest. For perspective on the size of nanoscale products (such as porphyrins or quantum dots) present in society today, consider that 2 g of 100 nm-diameter nanoparticles contains enough material to provide every human worldwide with 300,000 particles each (Hardman 2006). Porphyrins are better suited for PDT than their organic dye predecessors due to their fluorescence intensity and longevity. Currently, Photofrin® and Visudyne®, both porphyrin-type derivatives are used in PDT. However, the fluorescence and longevity advantages could possibly be further improved by the successful coupling of the porphyrin molecule to nanoparticles known as quantum dots (QDs). QDs, types of phosphors, are miniscule crystals of semiconductor material such as zinc sulfide, cadmium sulfide, cadmium telluride, or cadmium selenide, with their overall structure being composed of a core surrounded by an outer shell. The nature of QDs may be altered upon modification of or additions to this outer shell – including the attachment of porphyrin molecules (Zenkevich 2006). The resulting QD-porphyrin complex is expected to have increased photo-efficiency and more selective biological cell imaging. Porphyrins are known to be tumor specific and therefore the QD-porphyrin complex should be as well.

Continuing research may include the addition of zinc to the porphyrin complex followed by attempted coupling with QDs. A second area of further research will include the addition of hexadiamine through the aide of a BOC protecting group followed by
attempted coupling with QDs, as well as investigations concerning the cellular uptake of many of these QD-porphyrin compounds.
INTRODUCTION:

Protocols and methods for compounds to be used in cellular imaging and photodynamic therapy are areas of medicine in which advances are continually being made. Not simply in these procedures, but in any facet of medical use, compounds that are developed in a laboratory setting must be correctly characterized along with determining that they are soluble in water. If a compound is water-soluble, then the newly developed compound can be further analyzed for use in vivo - for example, the human body.

In most cases concerning abnormal cell growth, the most effective route for obtaining a correct diagnosis as well as issuing an accurate prognosis, is for physicians to consult images of the abnormal cell growth occurring within a patient’s body. Currently, successful cellular images are being obtained through the use of organic dyes. The drawback to utilizing organic dyes is that they only provide an adequate amount of fluorescence for a very limited amount of time while in the body. One option that is being researched and developed to improve the quality output of cellular imaging and to improve longevity of fluorescence is the possibility to make use of quantum dots (QDs). Quantum dots are nanoparticles (2-10 nm) whose structure is comprised of a core of semi-conductor material such as Zn or CdSe, surrounded by an outer shell (Shiohara 2004, Medintz 2005). At a size of 10 nm, 3 million QDs would be able to fit within the width of the average human thumb! QDs are capable of a highly effective level of fluorescence which can be modified and tuned by making alterations to their outer shells (Sui 2005). The goal of this project was to perform a successful alteration to the outer shells of QDs by the addition of large molecules known as porphyrins.
The structure of a porphyrin is based on the heterocyclic combination of four pyrrole rings. This base compound can then be modified through variations of substituents on its outer regions and/or the addition of a (typically) metal ion at its center. Familiar porphyrin structures include chlorophyll a in plants which contains a magnesium atom at its center and the heme molecule within the body that holds iron in its interior (Figure 1). Porphyrins are ideal for medical use since their outer substituents may be modified to ensure solubility in water, as is the case for porphyrin-based products currently being used in photodynamic therapy and cellular imaging. Many forms of porphyrins are also tumor specific, which makes them ideal for tumor marking and cellular imaging in cancer research and treatment (Ferrari 2005).

Figure 1: Familiar Porphyrins

The goal of this project is to be able to successfully couple porphyrin structures onto the outer shells of QDs. This surface modification will serve two purposes. First, the
highly insoluble QDs will be covered with a water-soluble porphyrin ‘envelope’ that can provide a way for the QDs to be transported safely into a biological environment, as well as resulting in a compound that exhibits excellent fluorescence and tumor specificity which would be unmatched by the QD and porphyrin components on their own (Hoshino 2004).
BACKGROUND INFORMATION:

Humans have always been intrigued by the complex world contained within our own bodies – this curiosity is continually being fueled and answered through modern medical techniques that provide cellular imaging of the inner regions of the human body. These advances not only provide a clearer picture of how the body works as well as supporting educational endeavors, but it also provides doctors and scientists with the means to visualize internal inconsistencies that may occur within a patient’s body. To obtain internal cellular images, a variety of compounds and methods such as organic dyes, ultrasounds, and MRIs, have been previously used and are currently being improved upon in order to make the process of procuring such images easier, faster, and safer.

One such method that is currently being used in medicine is photodynamic therapy (PDT) which makes use of porphyrin-based compounds in labeling tumors so that they can be more accurately located and destroyed (Salata 2004). The porphyrin-based photosensitizer is injected into the patient and allowed to concentrate in abnormal cells over time. Those labeled cells can then be exposed to a certain wavelength of light to activate the photosensitizer and in turn, destroy the tumor (Figure 2).

Figure 2: Photodynamic Therapy
Two photosensitizers that are currently on the market are Photofrin® and Visudyne®. Both of these products exhibit levels of fluorescence characteristic of porphyrins and aid physicians in targeting abnormal cells associated with lung and esophageal cancers and in treating macular degeneration, respectively. Photofrin® is injected into the patient and remains inactive until exposed to a laser beam of particular wavelength. Before becoming activated by the laser, the compound is allowed time to concentrate in the tissues – it is particularly fond of high-grade dysplasia and cancer cells which are destroyed when Photofrin® is activated (Axcan Pharma Inc.). Visudyne® can also be injected into a patient via the bloodstream which transports the compound to the blood vessels (involved in sight) affected by wet macular degeneration that are located on the backside of the eye that affect the person’s ability to see. Then a laser is shone into the affected area, Visudyne® is activated, and the vessels are sealed shut (Novartis). Both of these PDT treatments are relatively harmless and are sufficiently capable of relieving the patient of symptoms associated with their respective disease. Although porphyrins, as well as traditional organic dyes, provide a way of targeting and destroying abnormal cells within the body, their efficiency and longevity are only moderately impressive. As noticed with these two products, PDT is effective in areas of the body that can be easily exposed to light activation directly or by way of a scope. More defined cellular imaging and tumor labeling may be achieved through further modification of the porphyrin-based complexes, or by the use of a different compound entirely.

One such compound that is of high interest to scientists and physicians alike is the quantum dot (QD). A quantum dot is a nano-sized particle consisting of a core made of semiconductor material such as Cd/Se and an outer shell. QDs exhibit excellent
fluorescence capabilities, making them potential candidates for intracellular imaging and tumor marking – however, these dot structures are not water soluble, making them less than ideal for treatments and procedures that occur within the body (Palaniappan 2005).

By comparing the two, QDs prove to be much more efficient in maintaining fluorescence than their organic dye predecessors (Sungjee 2003).

Figure 3: Longevity of Fluorescence Comparison
Top Row – Organic Dye
Bottom Row – Quantum Dots

As seen in this comparison of illuminated frog embryos, the QDs (row b) display a highly effective level of fluorescence over time as compared to the organic dye (row a) which gradually dissipates (Figure 3).

Fluorescence - Fluorescence occurs when exposure to a particular wavelength of light excites a compound’s electrons. As an excited electron jumps to a higher energy level known as the excited state, the compound is said to exhibit absorbance of the light. The electron then emits visible color known as fluorescence as it falls back to its original ground state (Figure 4). This cycle of absorption and fluorescence happens only while the compound is exposed to the particular excitation wavelength and stimulation is induced.
Phosphorescence, on the other hand, is when the excitation of a compound's electrons is essentially stored over time and its illumination occurs whether or not the original stimulation is continued. The principle of phosphorescence is seen in glow-in-the-dark objects which show continual illumination that is more particularly visible to the naked eye in the absence of light. Fluorescence is a phenomenon which is seen in both porphyrin structures and QDs, both of which encompass the possibility of enduring modifications that may allow for the fine-tuning of the fluorescence of either compound.

The fluorescence of many compounds can be detected, measured and verified using an instrument known as a fluorescence spectrophotometer (Figure 5). A cuvette containing a solution of the compound of interest is placed in the instrument, which can be specifically programmed to expose the solution to a specified excitation wavelength. The instrument can then read the level of fluorescence of the solution and provides a printout with a corresponding peak displayed at the level of fluorescence.
Quantum dots may exhibit varying colors of fluorescence depending on their size and composition (Figure 6). As the size of a QD is increased, its exhibited fluorescence changes from blue to red in color. The QDs used in this particular project were classified as 620 nm – the wavelength at which the dots would be expected to display optimal fluorescence for their size. As seen in Table 1, 620 nm is located at the division between orange and red fluorescence and upon excitation the vial of QDs did in fact exhibit an almost neon-orange glow. Conversely, smaller dots (and therefore those at higher energy) show colors in the blue-green family.

<table>
<thead>
<tr>
<th>Color</th>
<th>Wavelength (nm)</th>
</tr>
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<tbody>
<tr>
<td>violet</td>
<td>380–450</td>
</tr>
<tr>
<td>blue</td>
<td>450–495</td>
</tr>
<tr>
<td>green</td>
<td>495–570</td>
</tr>
<tr>
<td>yellow</td>
<td>570–590</td>
</tr>
<tr>
<td>orange</td>
<td>590–620</td>
</tr>
<tr>
<td>red</td>
<td>620–750</td>
</tr>
</tbody>
</table>
As previously mentioned, despite the excellent efficiency of QDs for cellular imaging (Figure 7), the QD nanoparticles are not water soluble and are therefore less than ideal for use within a biological system such as the human body. Current use of QDs within the body is closely regulated with use of the nanoparticles being limited to areas of the body where they can be removed or expelled quickly, avoiding potential harm to the patient.

Figure 7: Intestinal Images Using QDs

Recent research concerning QDs has typically been focused on their structure – most specifically, modifications and alterations to the outer shell including the addition of biomolecules as seen in Figure 8.

Figure 8: QD Modification

Through such alterations of the outer shell, the solubility and reactivity of QDs may be changed and more specifically tuned for medicinal use (Sui 2005). Change in the composition of the QD's outer shell is the main focus of the majority of research projects,
papers, and articles within modern scientific magazines and meetings, as well as this thesis.
METHODS:

Synthesis of the Porphyrin, H₂TPPC - To begin, a porphyrin structure was synthesized and thoroughly purified. Pyrrole, 1.5 mL, was combined with 3.0 g of 4-carboxybenzaldehyde (also known as 4-formylbenzoic acid) in approximately 250 mL of propionic acid in a round bottom flask, and refluxed for 3 hours (Reaction 1). The flask was then removed, allowed to cool to room temperature, and placed in a freezer overnight. Observed color changes happened as the mixture refluxed with changes ranging from a white, cloudy appearance to a dark red, finishing with a seemingly black color. Upon removal from the freezer, the solid product was filtered and washed with dichloromethane on a course frit and allowed to stand and thoroughly dry. Next, the dried product was weighed and dissolved into solution with methanol. Purification of this solution continued through the use of a rotovap machine to evaporate the methanol under reduced pressure, followed by column chromatography. The purified product from the rotovap was dissolved back into solution using methanol and run through a pressurized column containing Sephadex LH-20. Each varying fraction that was expelled from the column was collected in 50 mL flasks with the light colored fractions from the very beginning and very end of the purification being discarded as well as a few dark green solutions from the beginning of the column. The fractions that appeared pink in color were retained from the column and were obtained as the final porphyrin product following one final rotovap purification.
Synthesis of H$_2$TPPEA began by combining H$_2$TPPC, 0.25 g, 0.313 g of 1-Hydroxy-benzotriazole hydrate (HOBT), 0.205 g of 1,3-dicyclohexylcarbodiimide (DCC), and 0.160 g diethanolamine in approximately 50 mL of tetrahydrofuran (THF) and stirred for 1 hour in a round bottom flask. Then the reaction was modified by the addition of 2 mL of water, THF was decanted off, and then the flask was allowed to sit overnight to facilitate complete evaporation of the THF that remained. The resulting product was then dissolved in 50/50 methanol/H$_2$O and purified by column chromatography using Sephadex LH-20. The red portion of the solution that eluted from the column was then collected and rotovapped. This purification was repeated using Sephadex LH-20 utilizing H$_2$O as the eluent. The pink porphyrin fraction was collected and evaporated under reduced pressure to yield the purified H$_2$TPPEA (Reaction 2). Sephadex LH-20 is a chromatography material that separates a solution based on its lipophilicity, while Sephadex G-50 focuses on separation as a direct result of size. By performing preliminary reactions, the need for the inclusion of water to the synthesis of H$_2$TPPEA was found to be unnecessary. In the end, coupling with QDs occurred using not H$_2$TPPEA, but H$_2$TPPC.
Synthesis of Quantum Dots – Although no QDs were directly synthesized for this project, the synthesis of most variations of QDs happens by way of a two-step process. Prolysis of organometallic precursors (such as dimethylcadmium and trioctylphosphine selenide) was conducted in trioctylphosphine oxide (TOPO). The precursors were injected into a flask at temperatures within the range of 340 to 360 °C followed by the addition of TOP, which generated small dots grown between 290 and 300 °C that were later collected as powders using size-selective precipitation with methanol and completed by redispersion in hexane. The resulting product constitutes the Cd/Se cores of many QDs. The second step served to generate a shell around the initial core. For example, a Zn/S shell was synthesized by using the precursors diethylzinc and hexamethyldisilathiane. Equimolar amounts of the precursors were dissolved in TOP inside an inert atmosphere glovebox, which were then transferred to a funnel onto the top of the reaction flask that already contained the Cd/Se cores under a heated atmosphere of N₂. The precursors were carefully injected at specific temperatures according to the desired final dot size. This two-fold process of core and shell creation is the general process for the synthesis of most QDs (Medintz 2005).
Synthesis of QD-Porphyrin Complex — To attempt the conjugation of porphyrins with QDs, a primary protocol from Invitrogen (2005) was studied and modified. Trials without quantum dots, containing only diethanolamine, DCC, HOBT, and porphyrin, were performed to determine the amounts of each reactant that would be sufficient for the detection and viewing of a final product on UV-vis and the fluorescence spectrophotometer, and to assess which technique for the reaction set-up/environment was best.

The best experimental set-up for coupling the QDs with the porphyrin structures was determined strictly through trial and error — although the previous reactions using diethanolamine were conducted in fairly small amounts, the reactions with QDs would have to be on a nano scale. As a result, the same weights of DCC, HOBT, and H$_2$TPPC were used, with those amounts being dissolved and diluted in volumetric flasks using methanol, from which microliter amounts of each were used. The initial reaction equipment set-up included a microscale flask and small stir bar. This, however, proved to be too large for the microliter amounts of reactants being used and a 1 mL centrifuge tube held to a vortex mixer by a ring stand (to ensure constant shaking) was decided upon.

The first round of dilutions and reactions proceeded as follows:

H$_2$TPPC, 0.25 g, was dissolved in a 100 mL volumetric flask, 0.313 g DCC in a 10 mL volumetric flask, 0.205 g HOBT in a 5 mL volumetric flask, and 0.160 g of diethanolamine in a 5 mL volumetric flask. Then, 1 mL of the H$_2$TPPC was diluted from the first flask to a second 100 mL volumetric flask. These dilutions were then followed by an initial trial reaction (with no QDs), which consisted of 250 μL of diethanolamine and 10 μL of DCC, HOBT, and H$_2$TPPC each. This mixture was stirred in a microscale
flask for 2 hours and then purification was accomplished by running the product through a column made of a 1 mL syringe containing Sephadex LH-20 and utilizing 50/50 methanol/H$_2$O as the eluent. The initial drops to elute from the column were assumed to be unreacted DCC and HOBT and were discarded. The following collected fraction was then diluted using methanol and analyzed using UV-vis spectroscopy. This first trial reaction was successful in that a UV-vis absorbance maximum was observed at 415 nm, which is indicative of the presence of porphyrin in the solution (the porphyrin soret band is located at 415 nm). From this information, it was determined that dilutions of the original weights of reactants and the microliter amounts used were indeed appropriate for the reaction to be detected by UV-vis spectroscopy and fluorescence spectroscopy. A few more practice reactions were completed with less than adequate results, but a final determination of reactant amounts and apparatus set-up was eventually reached.

Due to the limited volume of QDs available for use, UV-vis techniques were used to once again determine approximate concentrations that would be appropriate for achieving a successfully coupled product that could be characterized using the fluorescence spectrophotometer. The previously mentioned solution that displayed the characteristic porphyrin peak at 415 nm was used as a reference in concentration and color to approximate the necessary amounts of not only QDs, but the more dilute version of the porphyrin to be used in the coupling reactions (Table 2).
Table 2: Trials to Compare Color of Reference

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Porphyrin (µL)</th>
<th>Methanol (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>990</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>995</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>992.5</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>988</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>980</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>985</td>
</tr>
</tbody>
</table>

After running 6 trials, trial number 1 was selected as being the closest in color and concentration to the earlier solution and it was easily detected by UV-vis spectroscopy. This process helped to determine that 10 µL would be an appropriate amount to use in the coupling reactions for both the porphyrin compound and QDs.

Following trial reactions void of QDs, UV-vis techniques were used to determine the approximate concentrations of QDs and porphyrin that were used. UV-vis analysis also led to the determination that the more concentrated porphyrin from the original 5 mL volumetric dilution (versus a dilute version in a 100 mL volumetric flask) worked best in trial reactions using QDs. Successful coupling was immediately achieved using the following components:

Ten µL each of QDs and concentrated porphyrin as well as 50 µL of diluted DCC and HOBT were added to a centrifuge bullet, wrapped in aluminum foil, clamped on a ring stand, and shaken on a vortex mixer for 2 hours. When using the QDs, care was taken to handle them with minimal light exposure while wearing protective gloves at all
times. After being shaken for 2 hours, the solution was purified through microscale column chromatography using Sephadex G-50 and methanol as the eluting solvent. As before, the first drops to elute from the column were assumed to be unreacted DCC and HOBT and were discarded, and the final product was collected for characterization.

Characterization of the QD-porphyrin Complex – To accurately determine whether or not successful coupling of the porphyrin structure(s) with QDs occurred the collected reaction product was analyzed using a Hitachi F-2000 fluorescence spectrophotometer. The reaction mixture was transferred from the small glass HPLC vial in which it was collected from column chromatography, to a plastic cuvette followed by the addition of methanol to dilute the solution to the appropriate level to be analyzed by the spectrofluorimeter (approximately 2/3 of the 1 mL cuvette). The QDs being used were labeled as 620 nm Cd/Se quantum dots, so Ocean NanoTech LLC was consulted to determine which excitation wavelength would exhibit the desired fluorescence at 620 nm. The company verified that in order to obtain the desired optimal fluorescence of those particular dots at 620 nm, the excitation wavelength would need to be set at 500 nm.

Various solutions were run using the fluorescence spectrophotometer, including a methanol blank, 10 µL of QDs in methanol, 10 µL of porphyrin in methanol, and finally the reaction product. The first three solutions were analyzed in order to obtain reference/standard peaks at 500 nm, 620 nm, and 650 nm respectively. Data for four of the solutions’ emission spectra were obtained and viewed, with appropriate rescaling of the y-axis incorporated in the analysis if necessary.
RESULTS:

Before attempting a coupling reaction, UV-vis techniques were used to verify that the porphyrin complex had in fact been synthesized and to determine appropriate amounts of reactants to be used to ensure detection by the fluorescence spectrophotometer. The porphyrin did display the characteristic soret band at 415 nm, validating the assumption that H\textsubscript{2}TPPC had been successfully synthesized and ensuring that the methods for generating both H\textsubscript{2}TPPC and H\textsubscript{2}TPPEA were correct (Figure 9).

Figure 9: UV-vis Spectrum of H\textsubscript{2}TPPC

Reactions were then used to attempt coupling of porphyrin structures with the QDs, with the reaction product being analyzed by a fluorescence spectrophotometer. First, the separate reaction components were analyzed using the instrument, including methanol, 10 μL of H\textsubscript{2}TPPEA in methanol, and 10 μL QDs in methanol. The following emission spectra were obtained.
Figure 10: Emission Spectra

10 A: Methanol only - peak at 500 nm

10 B: Quantum Dots in Methanol - peak at 620 nm

10 C: Porphyrin in Methanol - peak at 650 nm
As expected, the coupled complex exhibited a strong peak at 650 nm and a shoulder at 620 nm, indicative of the presence of a conjoined product of porphyrin and QDs respectively.
CONCLUSIONS:

Basic conclusions as a result of this project include the following:

1. H$_2$TPPC was successfully synthesized and characterized using UV-vis spectroscopy.
2. H$_2$TPPEA was successfully synthesized and characterized using UV-vis spectroscopy.
3. A protocol was developed for coupling QDs with porphyrins.
4. H$_2$TPPC was successfully coupled to QDs as determined by fluorescence spectroscopy.

Future research may consist of synthesis, purification and characterization of a metallated porphyrin complex, which would then be coupled with QDs – more specifically, the addition of Zn to the porphyrin complex followed by attempted coupling with QDs and the addition of hexadiamine through the aide of a BOC group followed by attempted coupling with QDs. Cells could then be treated with the coupled complex, exposed to light and resulting changes in their growth and potential fluorescence could be monitored and analyzed. The outlook for this area of research appears hopeful as more specific coupling protocols are developed, continued variations in the structure and components of both porphyrins and QDs are synthesized and used, and methods for further tuning of fluorescence and tumor specificity are achieved. Overall, as porphyrin-based compounds can become more water soluble, then they are in turn more appropriate for use in medical treatments and procedures. The possibility that these successfully safe compounds can someday be used in conjunction with highly efficient quantum dots will continue to serve as a catalyst in generating interest in the coupling of the two components for use in medical treatments that seek to improve the well being of patients.
REFERENCES:


ACKNOWLEDGEMENTS:

- Carl Goodson Honors Program, Ouachita Baptist University
- Dr. Joseph Bradshaw
- Dr. J.D. Patterson
- Dr. Marty Perry
- Dr. Tim Knight
- Ocean NanoTech, LLC. Springdale, Arkansas
- NASA

This project would not have been possible without the guidance and direction provided by the groups and individuals listed above, not to mention the support given to me by my friends and family over the course of the past year and a half. From my directed study, begun in the fall of 2007, to presenting this research at the SW Regional and National American Chemical Society meetings, as well as Scholar’s Day 2009 at Ouachita Baptist University, it has been an honor and privilege to participate in cutting-edge research as an undergraduate student and present my findings in multiple academic arenas.