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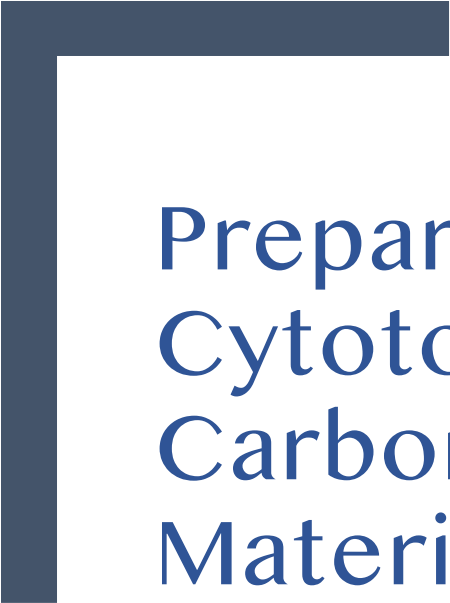
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Preparation and Cytotoxicity of Novel Carbon Nano-onion Materials

Cammie York
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Table of Contents

Abstract.....	4
Theranostics and Nanomedicine with Carbon Nanomaterials.....	4
Introduction to Project.....	9
Background.....	9
• What are Carbon Nano-Onions (CNOs)?	9
• Cisplatin in Chemotherapy	11
Methods.....	12
• Preparation of ox-CNOs	12
• Preparation of fluoro-CNOs	13
• Preparation of CNO-Pt	14
Characterization and Results	15
• Ultraviolet-Visible Spectroscopy	15
• Infrared Spectroscopy	18
• Fluorescence Microscopy	19
Cell Testing.....	21
• Alamar Blue Assay Procedure	21
• Alamar Blue Assay Results	22
Conclusions.....	23
Future Work.....	24
References.....	25

Table of Figures

Figure 1.....6

Figure 2.....8

Figure 3.....10

Figure 4.....11

Figure 5.....12

Figure 6.....13

Figure 7.....14

Figure 8.....15

Figure 9.....15

Figure 10.....16

Figure 11.....17

Figure 12.....17

Figure 13.....18

Figure 14.....19

Figure 15.....20

Figure 16.....20

Figure 17.....21

Figure 18.....22

Figure 19.....23

ABSTRACT

The applications of carbon nanomaterials (CNM), including graphene and its derivatives such as carbon nanotubes (CNTs) in nanomedicine is well established. These nanomaterials have been widely used as *theranostic* delivery systems with the potential to deliver bioactive agents and simultaneously detect selectively diseased tissues. A rather underexplored CNM for biomedical imaging and theranostics delivery are carbon nano-onions (CNOs). CNOs are carbon-based nanomaterials that can potentially be used in cancer therapy when they are functionalized. Recent studies on cellular fate of different CNMs, including CNOs, have demonstrated that the surface composition is critical for the *in vivo* application of these CNM. Current research discusses the preparation and characterization a novel CNO-Pt nanomaterial and the cell viability of U87 glioblastoma cells in the presence of this functionalized CNO. In order to form the desired CNO-Pt compound, the CNOs were first oxidized, followed by attachment of the *cis*-diammine platinum(II) dichloride. The novel CNO-Pt nanomaterial was characterized by IR and UV-Vis spectroscopies. Cytotoxicity of the material was tested on U87 glioblastoma cells. Following an Alamar Blue assay, the CNO-Pt material showed approximately 25% cell death at all concentrations after only 24 hours.

THERANOSTICS AND NANOMEDICINE WITH CARBON NANOMATERIALS

Theranostics and Nanomedicine

The field of medicine is constantly changing and evolving as new discoveries are made and technology becomes more advanced. Theranostics is an emerging field of medicine which combines therapeutics and diagnostics in an effort to both diagnose and

treat simultaneously (1). The use of nanomedicine is very important in the theranostic field as well. Nanomedicine can be defined as the medical application of nanotechnology. Nanotechnology can help medications to be delivered more directly to the target area and have less of an effect on the body as a whole, which is especially important in cancer therapy. Current chemotherapy has a major effect on the whole body, killing healthy cells as well as cancerous cells. Nanomedicine can help direct the drug directly to the tumor and decrease exposure throughout the body, eliminating some of the harsh side effects that are present with current treatments (2). This type of treatment allows for movement towards personalized medicine, which is the process of diagnosing, monitoring, and treating patients in a way that will best fit their exact personal needs. This would be much more effective for decreasing the tumor size, as well as give the patient a much better quality of life.

Utilizing nanomedicine can also reduce the frequency of taking medications. The body can typically remove medicines rapidly, but nanoparticles can be retained and continue to release a drug over a more extended period of time (3). Many nanoparticles have major advantages that make them great theranostic delivery agents. Several carbon nanomaterials have begun to show promise in the field of theranostics and they are the subjects of much ongoing research.

Carbon Nanomaterials

Carbon nanomaterials (CNMs) have recently become a very popular subject of chemistry research. They have many potential applications in bioimaging and theranostic delivery, but their poor solubility in some common solvents hinders some of their potential for use. Some CNMs can be functionalized to increase their solubility,

therefore increasing their potential for biological application. Many different types of carbon nanomaterials can be explored for this purpose.

Carbon nanotubes (CNTs) (Figure 1) are a type of CNM that resemble a hollow cylinder. CNTs can be formed by rolling up sheets of graphenes, or single layers of carbon atoms, into a tube-like shape. Nanotubes are now known to be one of the most stable carbon aggregates. CNTs can be single-walled (SWNT) or multi-walled (MWNT) depending on the number of graphene shells. SWNTs are longer tubes that are formed from the wrapping of a single sheet of graphene into a cylindrical shape. The sidewalls of the cylinders can be rolled in different ways to give specific nanotubes unique characteristics. MWNTs are made up of concentric SWNTs, giving them distinct properties compared to SWNTs.

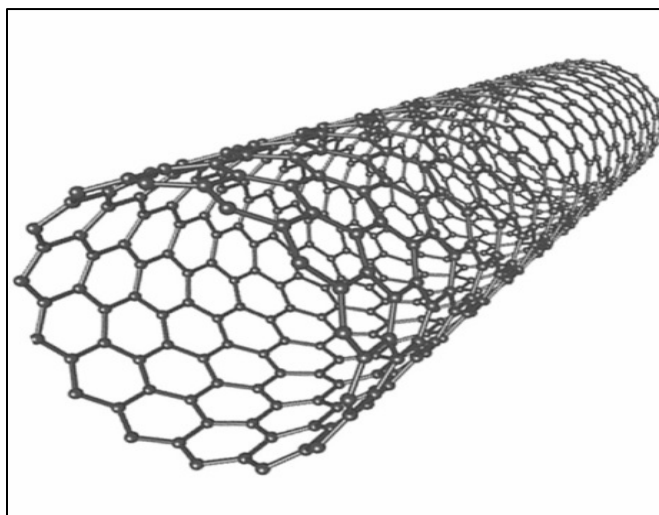


Figure 1. Structure of a carbon nanotube (4).

SWNTs have shown to be better for drug delivery than MWNTs because of their one-dimensional structure and very large surface area. Also, research has shown that anticancer drugs circulate much longer in the blood when attached to a SWNT than it does on its own. This allows for continuous uptake of the drug by a tumor for a

prolonged period of time, which is important in helping treat cancer more effectively. MWNTs tend to be more useful for thermal cancer treatment such as radiation. After being exposed to wavelengths of near infrared light, MWNTs release much vibrational energy into the tissue. This release of energy causes heating in the local area which can destroy cancer cells (5). Therefore, both types of nanotubes have much therapeutic potential.

Carbon nanotubes were discovered to have impressive chemical, electrical, thermal and mechanical properties that other carbon materials lack. They are also low in cytotoxicity, making them suitable for biological use. The chemical properties and great surface area of CNTs allow for modification of the surface of their outer shells, including the attachment of many functional groups. The surface can be functionalized to make them more water soluble, as well as to attach drug molecules. The structure and properties of the nanotubes could allow an attached drug to be released to a cell and enter via endocytosis or even passive diffusion (4). This makes CNTs seem very promising as delivery vehicles and gives them much potential for use in cancer therapy.

Drugs are not the only molecules that can be added to the surface of CNTs. Fluorescent materials can also be attached to nanotubes, allowing them to be used for imaging purposes as well (5). The therapeutic application along with their imaging abilities and low cytotoxicity make carbon nanotubes very desirable in the growing field of theranostics.

Carbon nanohorns (CNHs), sometimes called carbon nanocones (Figure 2), are a conically shaped type of CNM. They are very similar to nanotubes, but with closed ends. Nanohorns, like nanotubes, can be single-layered or multi-layered with each

having different properties. CNHs are very chemically stable, have high purity and low cytotoxicity along with great catalytic properties and good conductivity. These properties give CNHs a wide range of uses, including many biomedical applications. They have much potential as drug delivery vehicles because they are very porous, allowing small drug molecules like the anticancer drug cisplatin to be loaded into the interior spaces and slowly released. The surface can also be modified so that a drug can be attached, creating much potential for carbon nanohorns to act as double reservoirs. They can combine methods for treating both inflammation and tumors. Research has shown that nanohorns can release a bioactive drug over a longer period of time, making it very useful for treatment. CNHs tend to aggregate inside tumors, which would allow the drug to empty into the tumor directly over time (8). The surface of the nanohorns can be modified to allow the structure to become more water soluble so it can be dissolved in the blood and removed from the human body after use. Fluorescent molecules can also be attached to the nanohorns, allowing them to be used for imaging as well (6). This combination of properties makes carbon nanohorns very suitable candidates for use in the theranostic field.

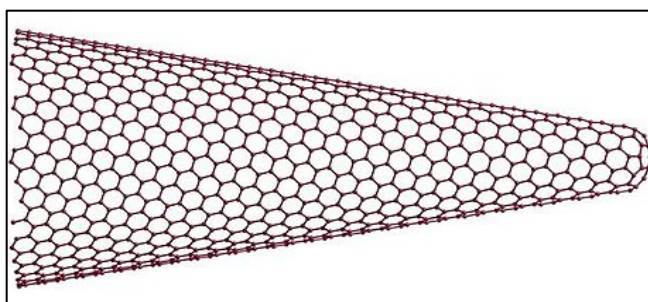


Figure 2. Structure of a carbon nanohorn (17).

In an experiment with mice, researchers injected modified CNHs into non-small cell lung cancer tumors and saw significantly retarded tumor growth. They also

observed the migration of these CNHs to the adjacent axillary lymph node—a major site of breast cancer metastasis. Due to these findings, researchers believe that these modified water-dispersed CNHs have the potential to be excellent drug carriers for chemotherapy, specifically against cancers that include lymph node metastasis (7).

Carbon nanomaterials are proven to have many advantages in the field of theranostics and nanomedicine. They continue to be the subjects of much research as they have their novel properties explored more in depth. CNMs seem to have much potential to help improve upon cancer therapy and diagnostics and may aid in making treatments more personalized as well. Hopefully carbon nanomaterials will fulfill their potential and become the major theranostic agents in the future.

INTRODUCTION TO PROJECT

As noted above, carbon nanomaterials have much potential in bioimaging and theranostic delivery for cancer treatment. The goal of this project was to prepare a novel carbon nano-onion platinum nanomaterial and test its cytotoxicity and potential for use in cancer therapy. This was achieved by attaching cisplatin to the outer shell of the CNOs and then testing it against U87 glioblastoma cells.

BACKGROUND

What are Carbon Nano-Onions?

Among many other types of carbon nanomaterials, carbon nano-onions (CNOs) (Figure 3) are particular CNMs of interest because they have shown to be less toxic than other CNMs and have the ability to be internalized by cells. Solubility of the CNOs

is greatly increased when their outer surface is functionalized, and functionalization increases their potential for theranostic application. Carbon nano-onions (CNOs) are characterized by a closed carbon shell and multiple layers which resemble an onion. The outer surface can be functionalized to make the material more soluble, which allows CNOs to be used for bioimaging and show potential for use in cancer therapy. CNOs have already proven effective as agents for cellular imaging. Fluorescent compounds can readily be added to the surface of the CNOs and visualized using fluorescence microscopy. The CNOs are readily taken up and can be localized to specific compartments of cells, which makes them well suited for use in bioimaging (9). CNOs are also useful as drug carriers and delivery vehicles. They are small enough to travel well through the circulatory system and are effectively taken up by cells. Due to their small size, they are able to access a wide range of biological targets. Some biomolecules can also be attached to the surface of the CNOs, which broadens their potential as intracellular carriers even more (9). In the future, the hope is to be able to attach a cytotoxic drug, such as cisplatin, which can be taken up by cancer cells and destroy them more effectively.

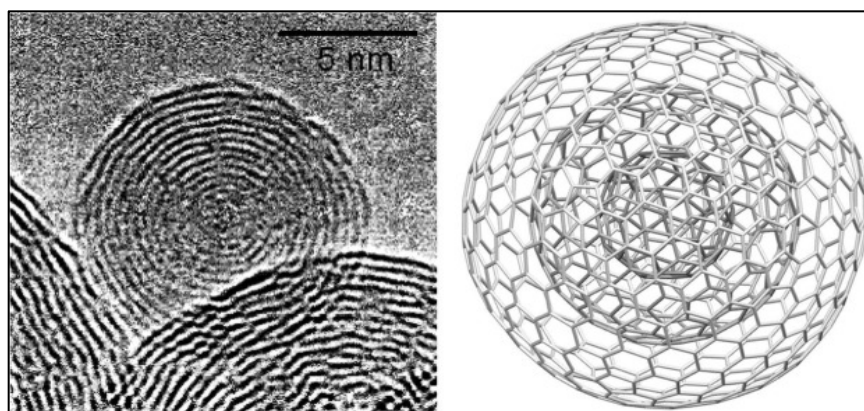


Figure 3. Structure and microscopic view of carbon nano-onions (16).

Cisplatin in Chemotherapy

Chemotherapy is a treatment that uses powerful chemicals to destroy rapidly dividing cells in the body. It is used in the treatment of cancer due to the fact that cancer cells grow and multiply much more quickly than most other cells in the body.

Unfortunately, current chemotherapy treatments affect the whole body, destroying many good cells along with the cancerous cells. Finding a way to utilize chemotherapy in a more localized area and decrease the side effects that accompany the destruction of healthy cells would be a major step forward in the treatment of cancer.

Cisplatin is a common drug used in chemotherapy. It was the first big chemotherapy drug to be used and is sometimes referred to as the “penicillin of cancer” (11). Cisplatin operates by forming a platinum complex inside a cell which binds DNA. When the DNA is crosslinked in this manner, the cell is signaled to undergo apoptosis and the cell dies. Unfortunately, cisplatin can affect the DNA of other cells in the body, which may lead to harsh side effects. Though it is one of the oldest chemotherapy drugs, is still widely used in treatment today either as the main drug or as part of a combination regimen (11). Carbon nanomaterials are now being explored as potential drug carriers for cisplatin. Many other new therapies using cisplatin continue to be explored with the goal of treating a more localized area to help reduce the harmful side effects that take such a toll on the body.

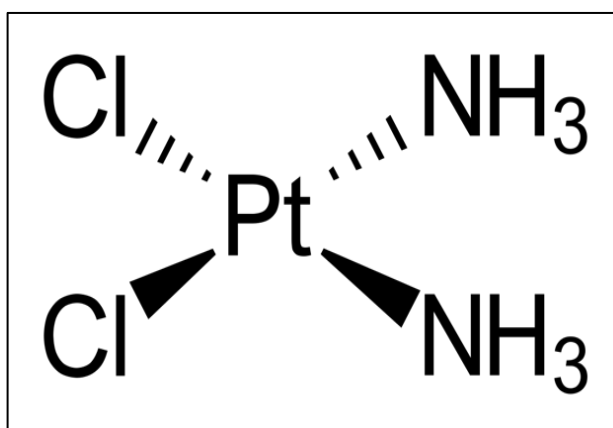


Figure 4. Chemical structure of cisplatin.

METHODS

Preparation of Oxidized-CNOs (ox-CNOs)

To prepare the oxidized carbon nano-onions, 0.04 g of CNOs were added to a 50 mL round bottom flask, along with 30 mL of 3M nitric acid. The reaction was then refluxed for 48 hours. After 48 hours, the reaction was removed from heat and allowed to cool. Next, the reaction was filtered using vacuum filtration to obtain the product. A Millipore 0.20 μm nylon membrane filter was weighed and then placed in the filter funnel. The reaction was diluted with deionized water and then poured through the filter. After filtering, the ox-CNOs were washed 3 times each with deionized water, methanol, and acetone. After washing, ox-CNOs were placed on a watch glass and allowed to air dry overnight.

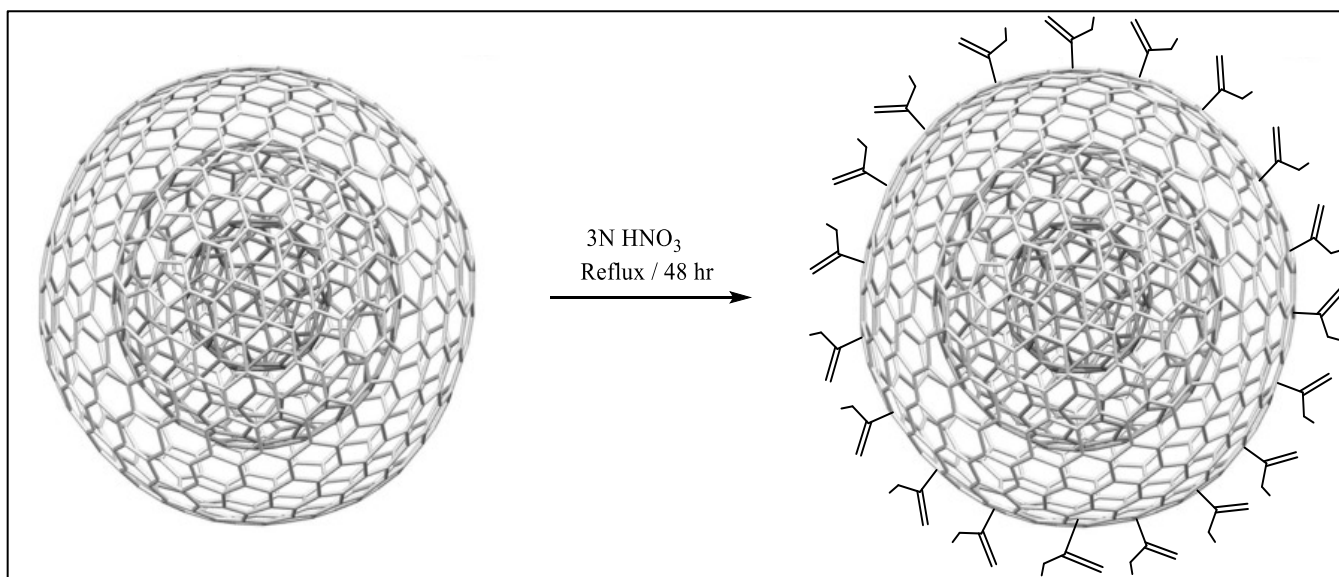


Figure 5. Reaction to prepare ox-CNOs from CNOs in 3M nitric acid.

Preparation of Fluorescent-CNOs (fluoro-CNOs)

To prepare the fluorescent-CNOs, 0.015 g ox-CNOs and dry tetrahydrofuran (THF)/dimethylformamide (DMF) (2:1 ratio) were added to a Teflon beaker and ultrasonicated for 20 minutes at 37 kHz. The mixture was then added to a 50 mL round bottom flask and deoxygenated under nitrogen. 4-dimethylaminopyridine (DMAP), 0.050 g, 0.050 g of N-hydroxysuccinimide (NHS), 0.080 g of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC-HCl), and 0.040 g of fluoresceinamine were added to the flask. The mixture was briefly sonicated and then refluxed under nitrogen for 4 days.

After 4 days, the reaction was stopped and allowed to cool. The product was obtained by using vacuum filtration. A 0.20 μm nylon membrane filter was weighed and then placed in the filter funnel on the vacuum. After filtering the original reaction mixture, the product was washed 3 times with both THF and acetone. Figure 6 shows the addition of fluoresceinamine to the ox-CNOs.

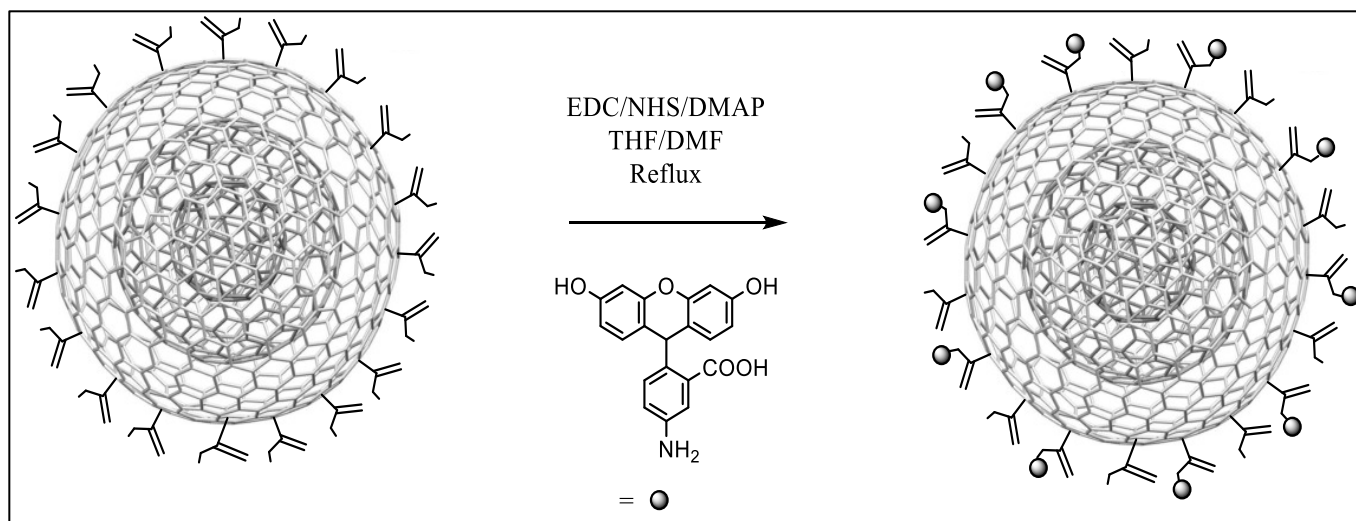


Figure 6. Reaction to prepare fluorescent-CNOs.

Preparation of cisplatin-CNOs (CNO-Pt)

To prepare the platinum CNOs, 0.015 g ox-CNOs and dry THF/DMF (2:1 ratio) were added to a Teflon beaker and ultrasonicated for 20 minutes at 37 kHz. The mixture was then added to a 50 mL round bottom flask and deoxygenated under nitrogen. DMAP, 0.050 g, 0.050 g of NHS, 0.080 g of EDC-HCl, and 0.036 g of cisplatin were added to the flask. The mixture was briefly sonicated and then refluxed under nitrogen for 4 days.

After 4 days, the reaction was stopped and allowed to cool. When cooled, vacuum filtration was performed to obtain the product. Again, a nylon filter was used to collect the product. After filtering the original reaction mixture, the product was washed 3 times with both THF and acetone. Figure 7 shows the addition of cisplatin to the ox-CNOs. The complete reaction to prepare fluoro-CNOs is shown using functional groups in Figure 8. The complete reaction for the preparation of CNO-Pt is also shown using functional groups in Figure 9.

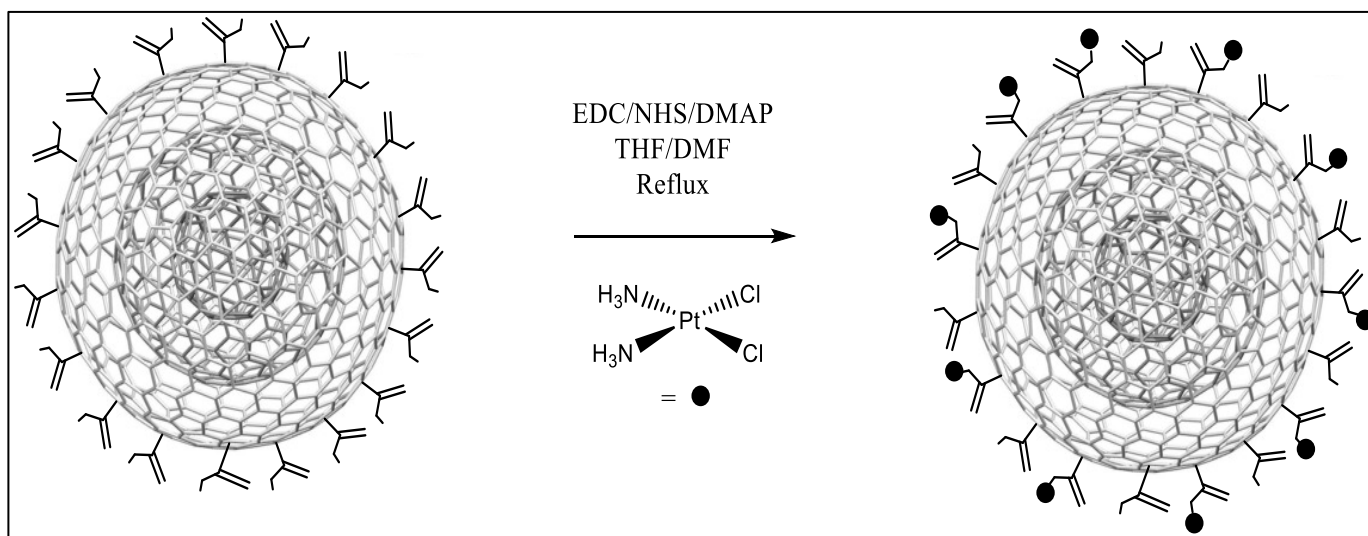


Figure 7. Reaction to prepare CNO-Pt.

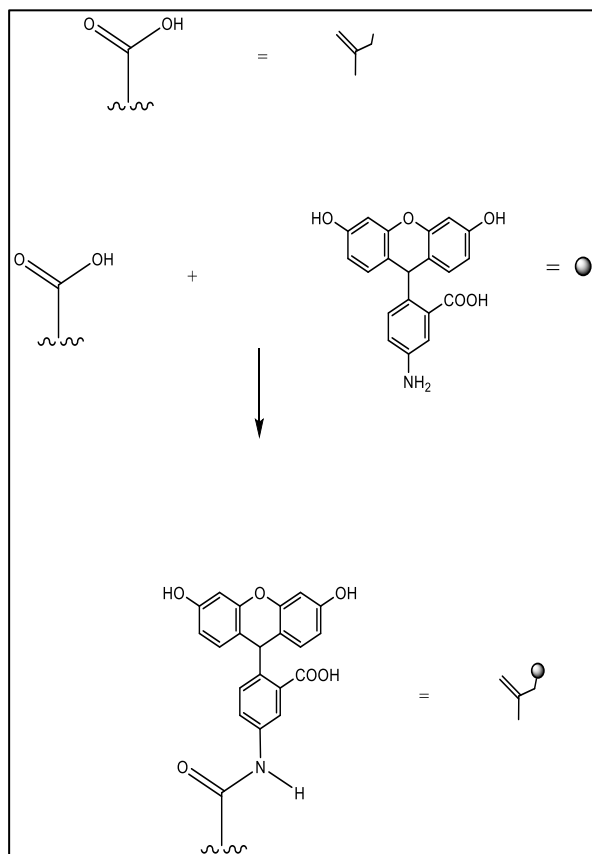


Figure 8. Complete reaction to prepare fluorescent-CNOs.

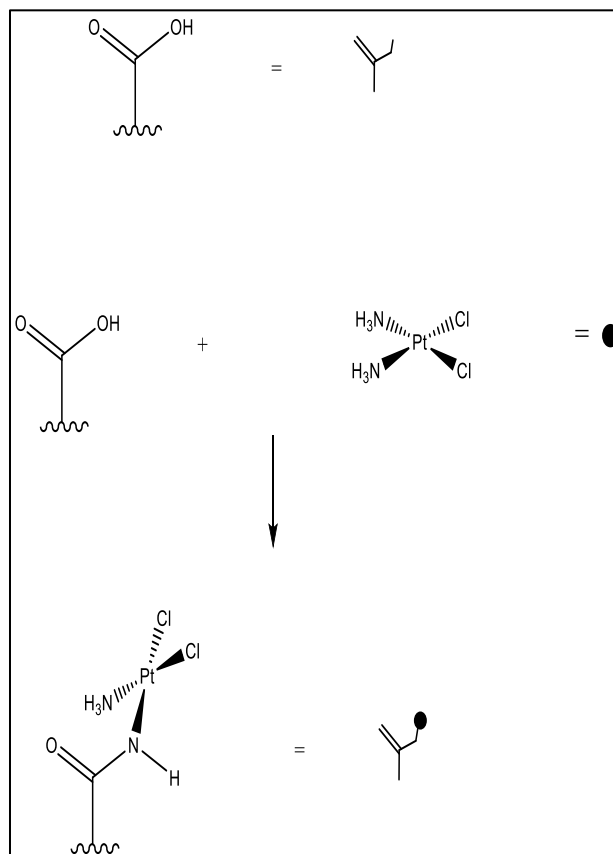


Figure 9. Complete reaction to prepare CNO-Pt.

CHARACTERIZATION AND RESULTS

Ultraviolet-Visible Spectroscopy

Ultraviolet-Visible (UV-Vis) spectroscopy is a useful tool to measure the absorbance of light by a sample. The perceived color of a molecule on either the ultraviolet or visible spectrum is indicated by a particular absorbance number. Energy from the different wavelengths of light is absorbed by molecules and can possibly excite electrons into higher energy orbitals. Easily excited electrons can absorb longer wavelengths of light. In UV-Vis, specific wavelengths of light are passed through a

solution and the absorbance of light is analyzed. The solution absorbs the light and is read and recorded by a detector (12).

Ox-CNOs (Figure 10) have characteristic peak near 280 nm. Fluoresceinamine has a characteristic peak near 485 nm, as seen in Figure 11. The UV-Vis spectrum of fluoro-CNOs is seen in Figure 12. The coupling of the ox-CNOs and fluoresceinamine is represented by the peak near 280 nm (characteristic of the ox-CNOs) and the new peak near 520 nm (characteristic of fluoresceinamine). Comparing the spectra, we can see that the fluoresceinamine was successfully coupled to the ox-CNOs to produce the fluoro-CNOs.

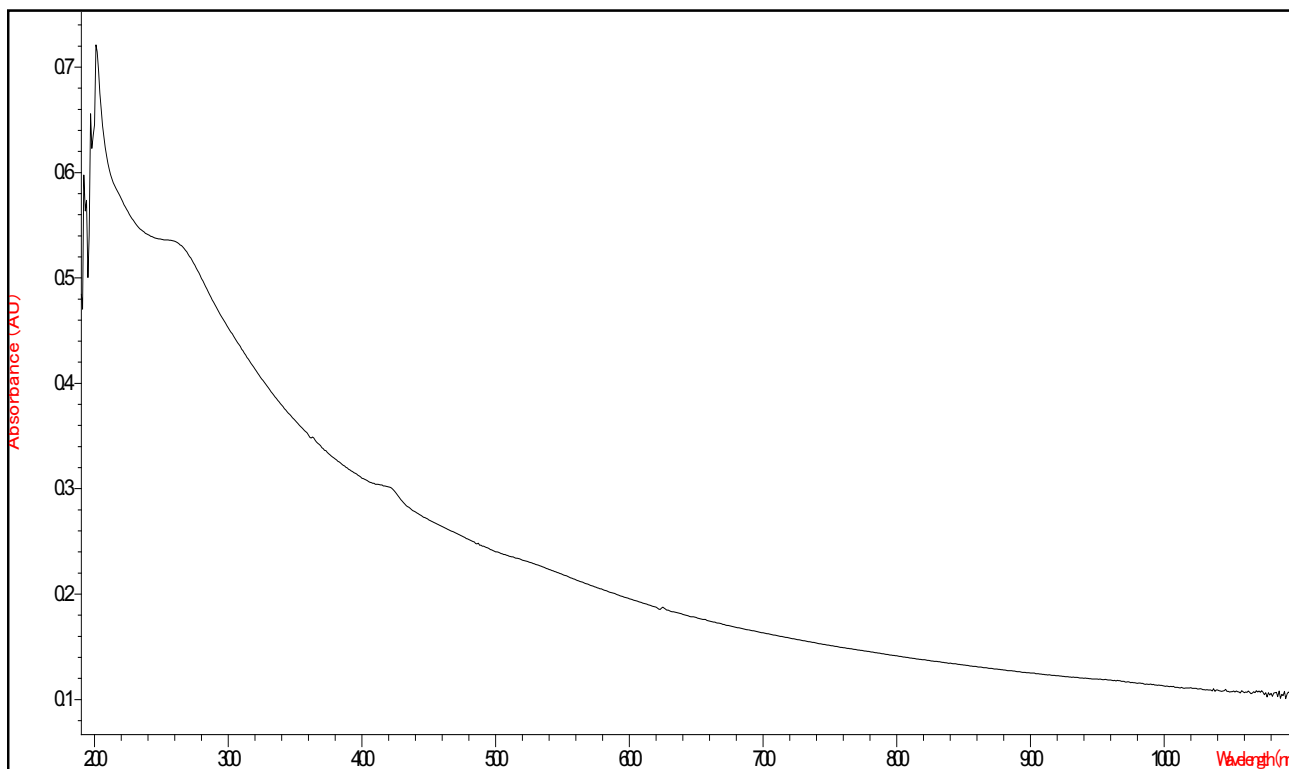


Figure 10. UV-Vis Spectrum of ox-CNOs in MeOH.

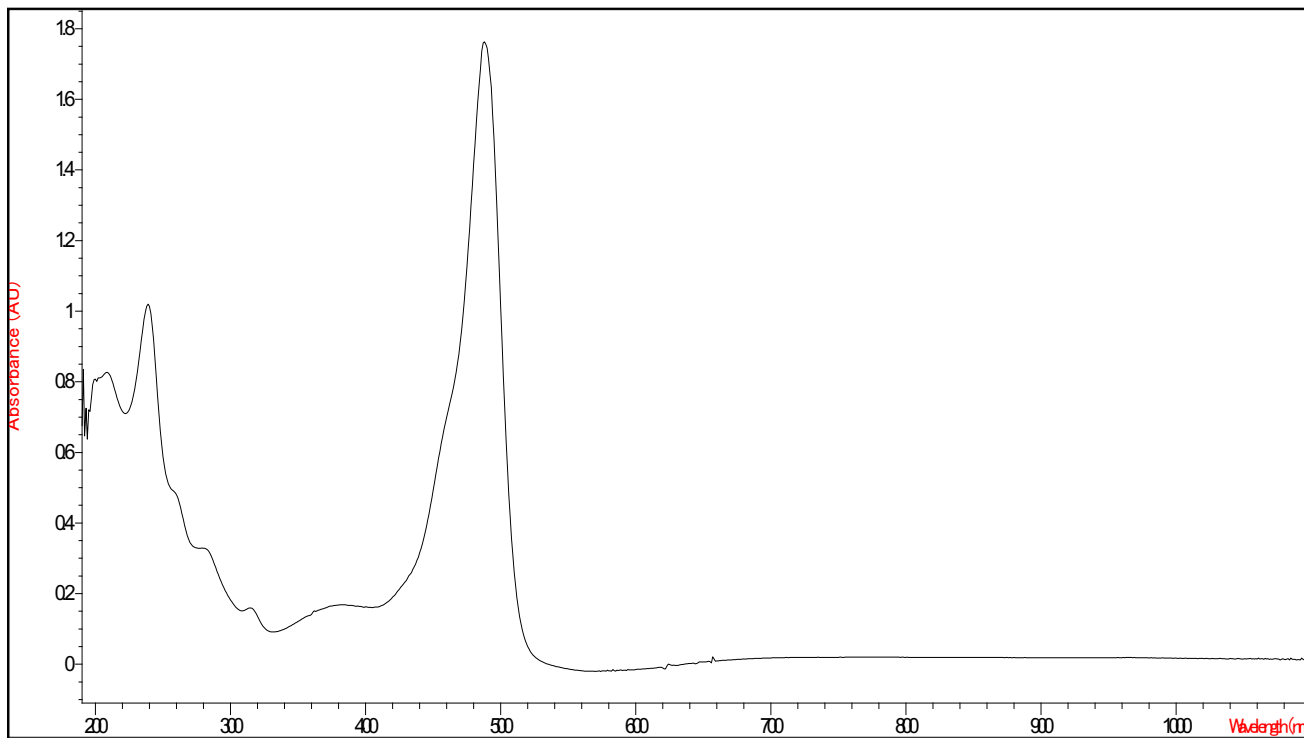


Figure 11. UV/Vis spectrum of fluoresceinamine in MeOH.

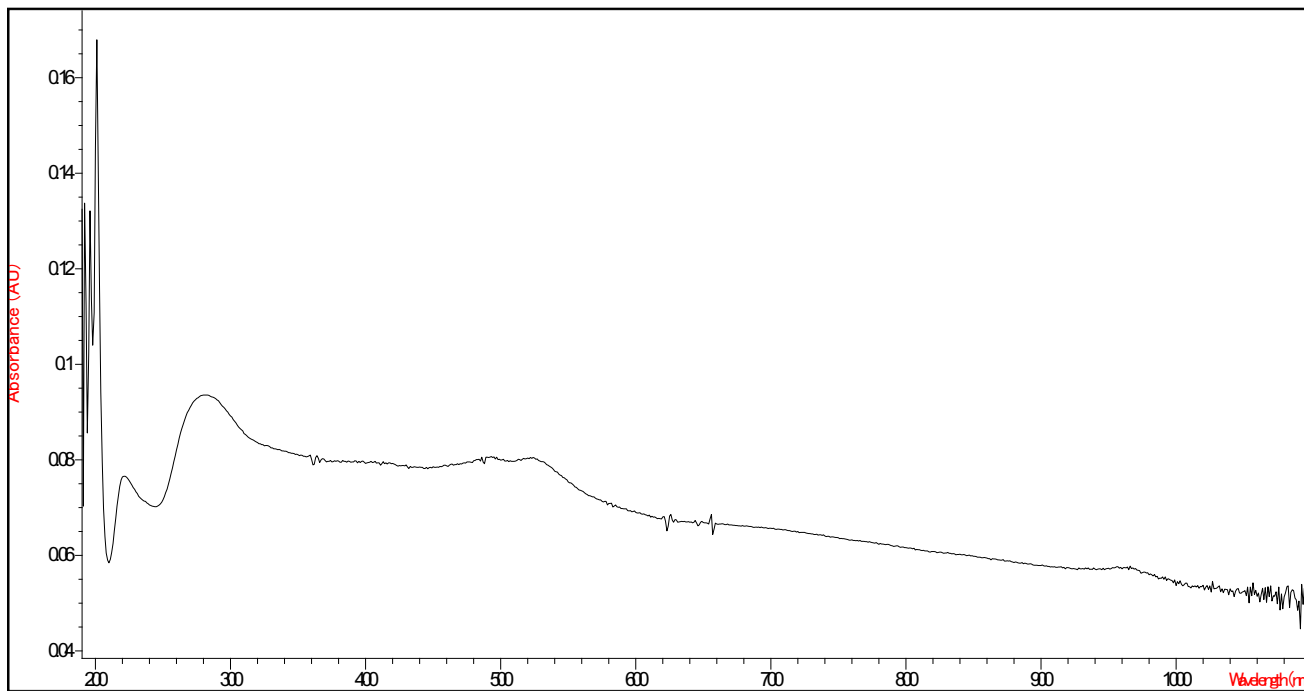


Figure 12. UV/Vis spectrum of fluoro-CNOs in MeOH.

Infrared Spectroscopy

Infrared (IR) spectroscopy is another useful tool in determining the structure of molecules. Different functional groups can absorb infrared radiation at different wavelengths based on their shape. As energy is absorbed by a functional group, it bends or stretches. The IR spectrometer creates a graph which records the wavenumber and transmittance of the stretch or bend (13).

The ox-CNOs have a peak near 1680 cm^{-1} that is characteristic of the carboxylic acids attached to the outer shell of the CNOs. Fluoro-CNOs displayed a new band near 1580 cm^{-1} which is characteristic of the attachment of fluoresceinamine as seen in Figure 13. This same set of peaks can be seen in the attachment of the Pt of cisplatin as observed in Figure 14. These peaks confirm the presence of the materials attached to the ox-CNO material.

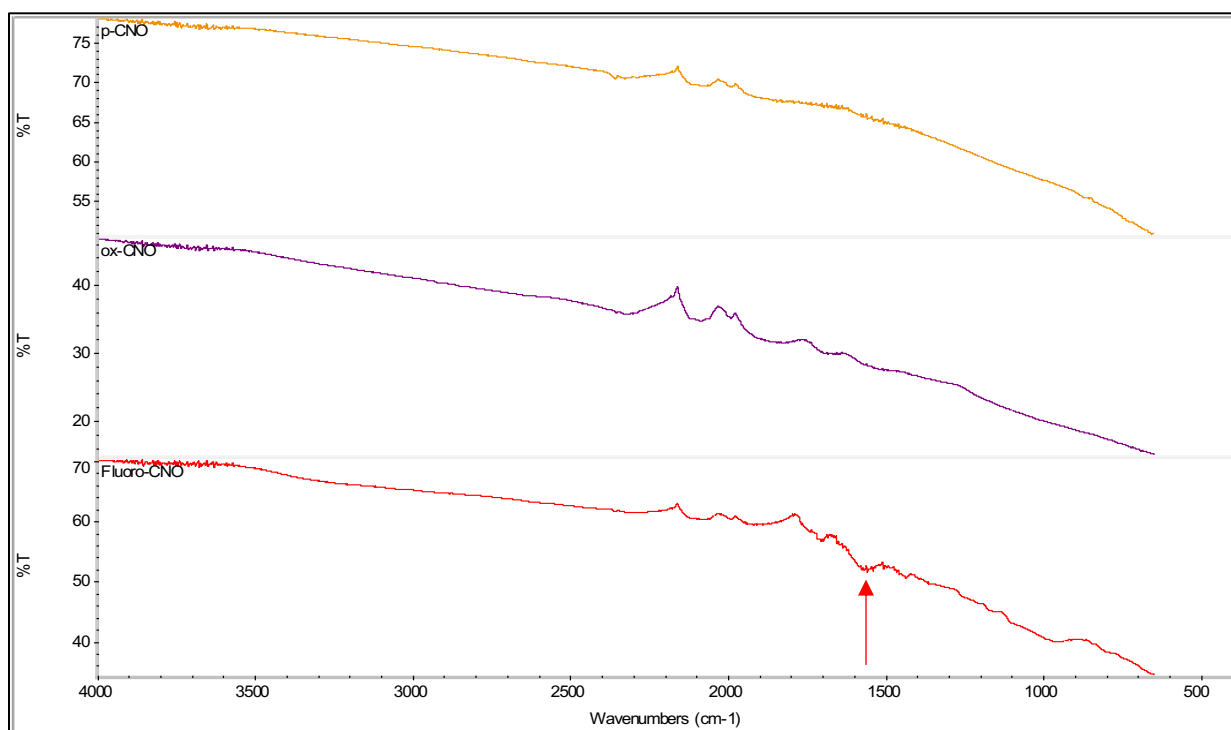


Figure 13. Overlay of the IR Spectra of p-CNO, ox-CNO, and fluoro-CNO

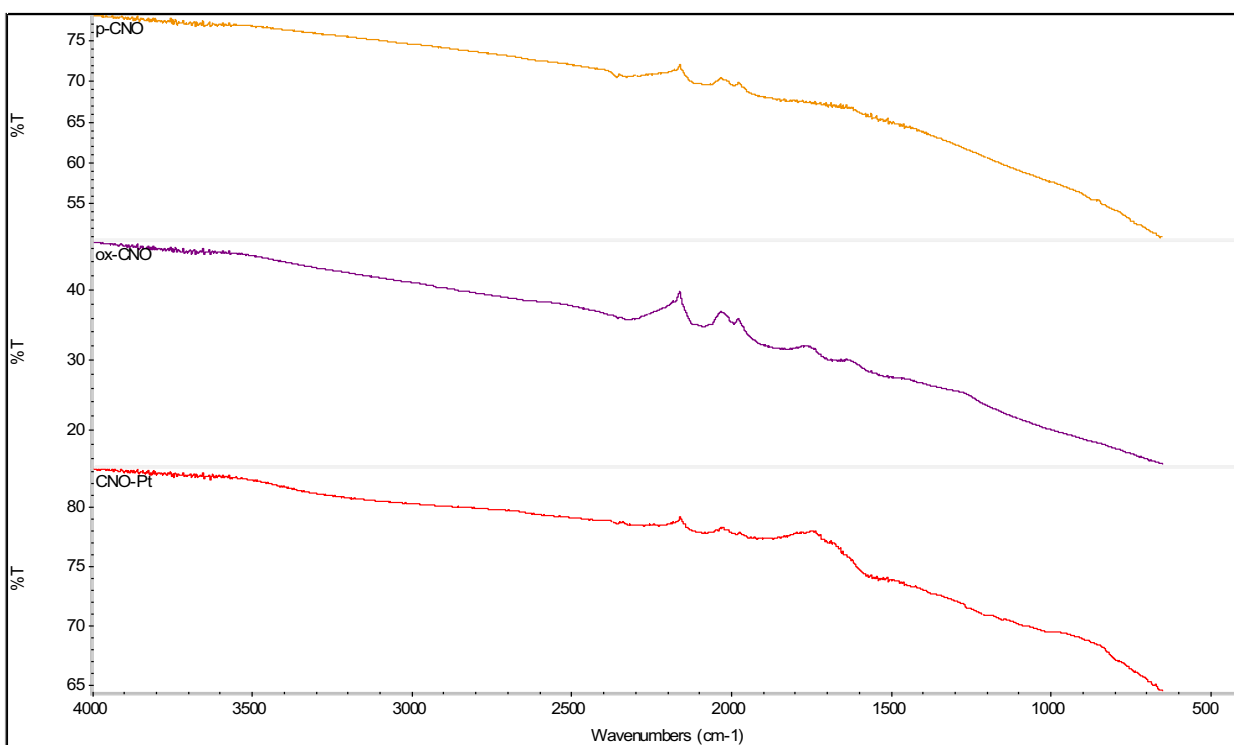


Figure 14. Overlay of the IR Spectra of p-CNO, ox-CNO, and CNO-Pt

Fluorescence Microscopy

Fluorescence microscopy uses fluorescence or phosphorescence to analyze the properties of a substance instead of reflection and absorption. Fluorescence can be observed in some substances that absorb light or electromagnetic radiation and in turn emit light. It is very beneficial because it makes it possible to visibly see specific parts of some substances (14).

Fluoro-CNOs were analyzed under regular light and at excitatory wavelengths to view fluorescence. Fluoresceinamine is excited and fluoresces at a λ_{max} of 496 nm. At nonexcitatory wavelengths, the fluoro-CNOs were seen as black spots only (Figure 15). Under the excitatory wavelengths of light, parts of the fluoro-CNOs were visibly fluorescing (Figure 16), confirming the attachment of fluoresceinamine to the outer CNO shell.

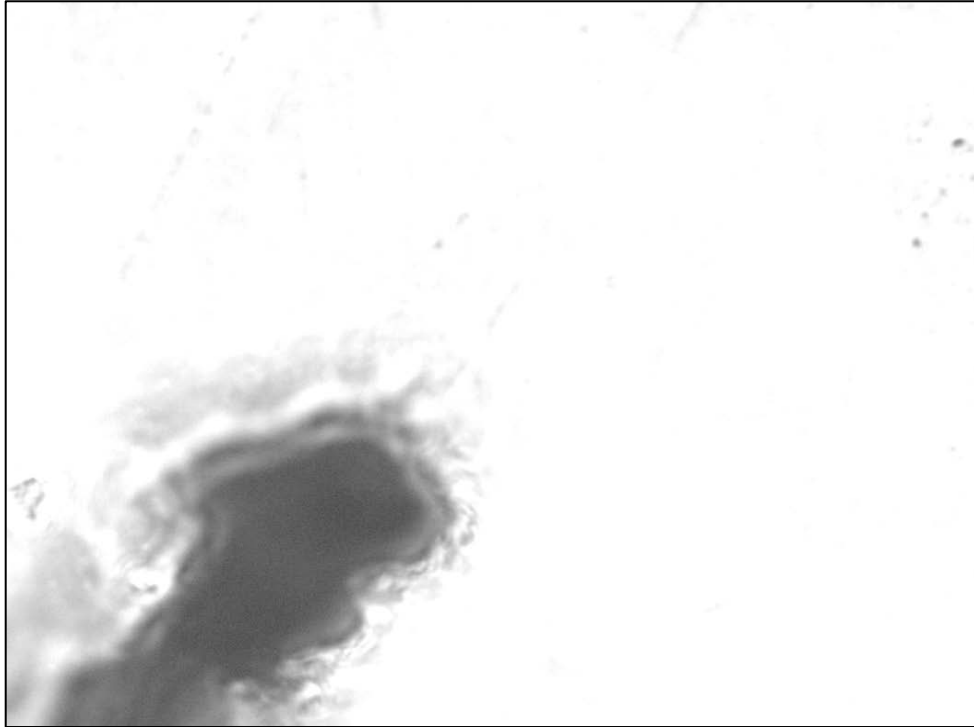


Figure 15. Fluoro-CNOs under white light.



Figure 16. Fluoro-CNOs under UV light.

CELL TESTING

Procedure

An *in-vitro* cytotoxicity assay was performed using Alamar Blue in order to measure cell viability. Alamar blue is a cell permeable reagent that contains blue fluorescent indicator dye called resazurin. This reagent is useful because it is non-toxic to cells, unlike the more commonly used MTT assay reagents. Resazurin changes colors in response to a metabolic reduction in cells and the reduced form fluoresces proportionally to the number of living cells present (15). Therefore, it can quantitatively measure cell viability in each well.

In this Alamar blue assay, U87 glioblastoma cells were plated onto a 96 well plate and allowed to grow for approximately 24 hours. Once cultured, the cells were treated with various concentrations of each of the ox-CNOs, fluoro-CNOs, and platinum-CNOs. The plate was incubated for 24 hours and then placed on a plate reader to determine cell viability.

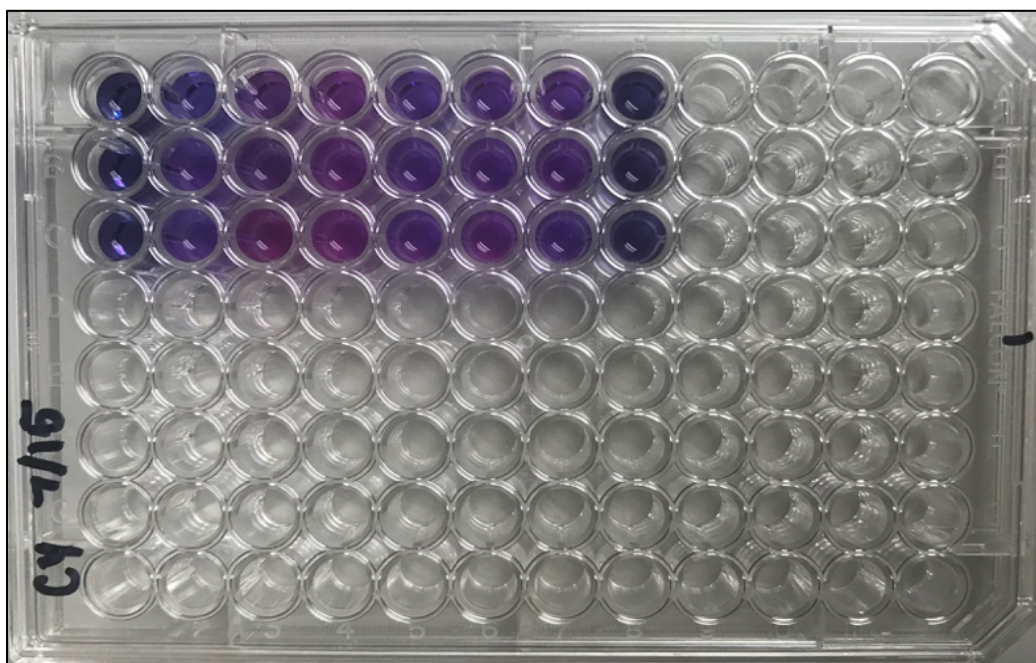


Figure 17. Alamar blue assay after 24 hours.

Results

As mentioned above, the Alamar Blue assay was used to test cell viability of U87 cells after treatment with the various CNOs. Ox-CNOs and fluoro-CNOs were tested against the cells each at a concentration of 10 $\mu\text{g}/\text{mL}$. Percent viability of the ox-CNOs and fluoro-CNOs was approximately 50% (Figure 18). The platinum-CNOs were tested against the cells at concentrations of 1, 5, and 10 $\mu\text{g}/\text{mL}$. Percent viability of the platinum-CNOs was near 75% (Figure 19).

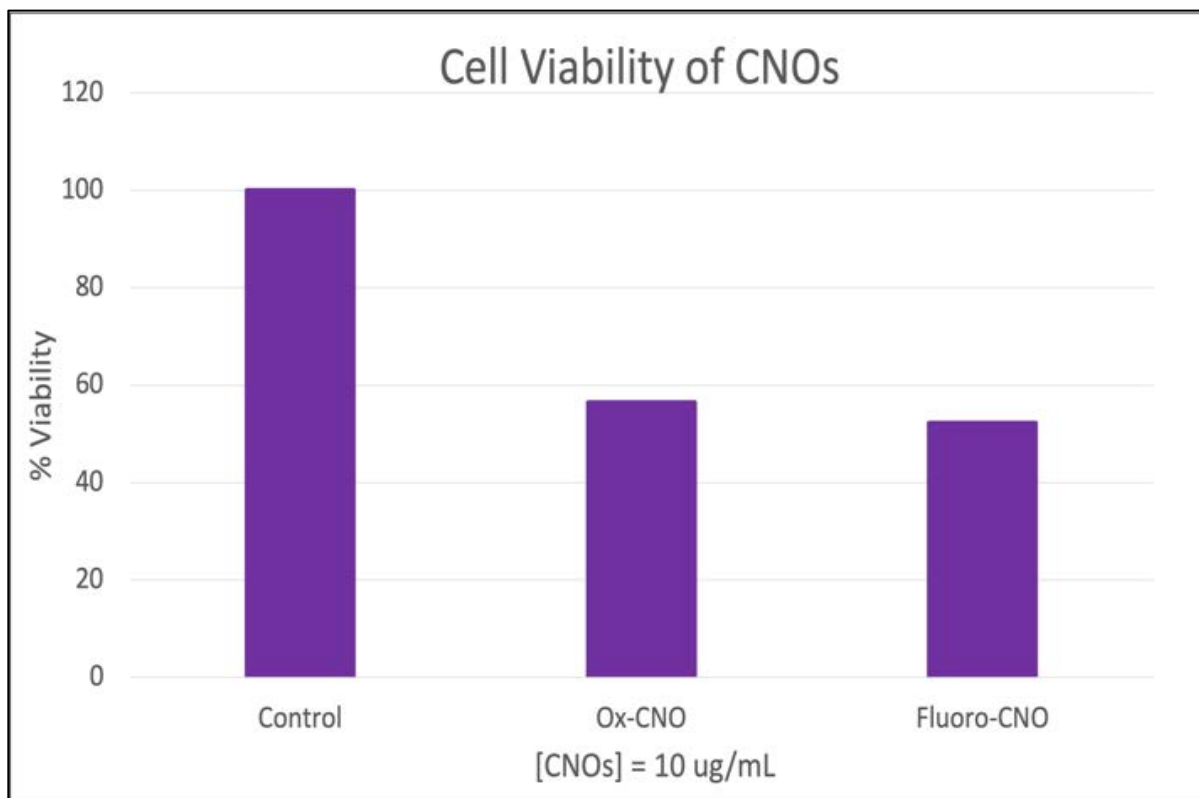


Figure 18. Results from testing cell viability of CNOs.

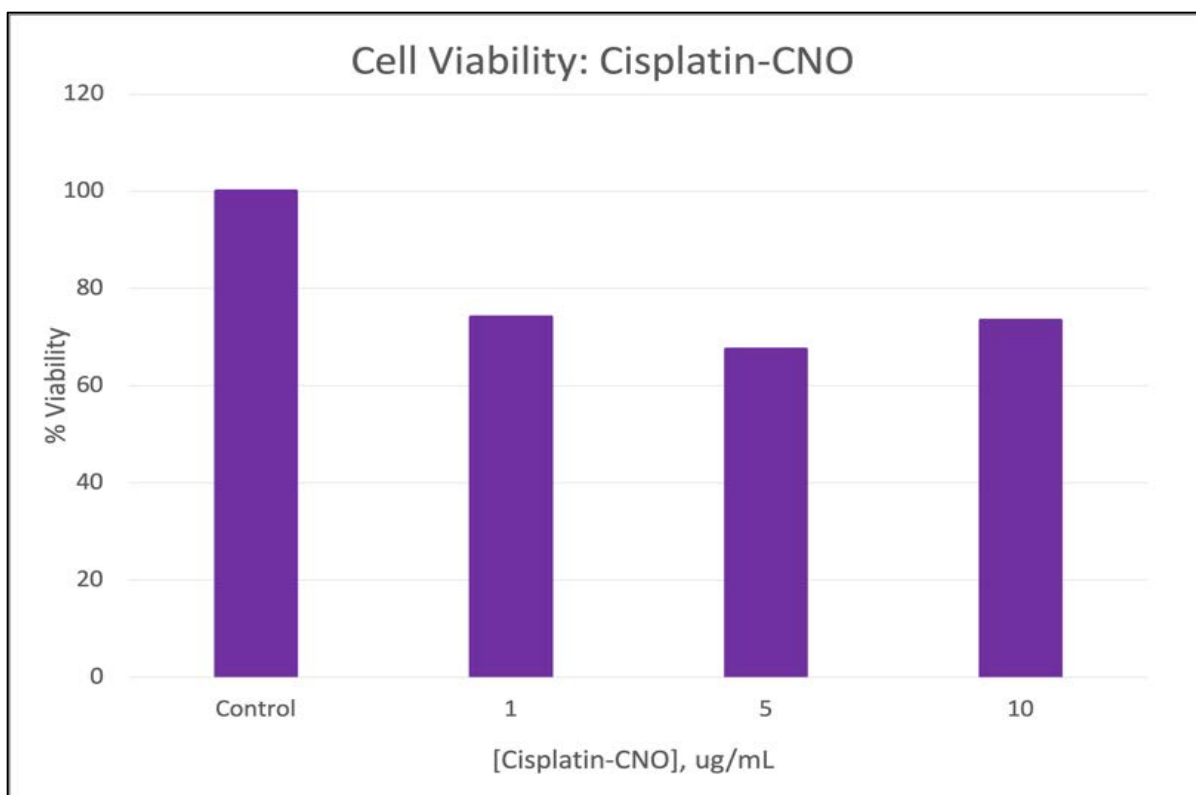


Figure 19. Results from testing cell viability of CNO-Pt.

CONCLUSIONS

In conclusion, CNOs were able to be successfully oxidized and functionalized with fluoresceinamine. This was confirmed by UV-Vis spectroscopy, IR spectroscopy, and fluorescence microscopy. UV-Vis confirmed the coupling of fluoresceinamine to the ox-CNOs to produce fluoro-CNOs, represented by the peak seen at 520 nm on the spectra. IR spectroscopy showed the characteristic peak of fluoresceinamine at 1580 cm^{-1} after coupling to the ox-CNOs. Fluorescence microscopy further confirmed the attachment of the fluoresceinamine as it could be seen visibly fluorescing under excitatory wavelengths. The novel CNO-Pt nanomaterial was also successfully prepared and characterized by IR spectroscopy. After running the IR on the CNO-Pt

nanomaterial, the spectrum showed an expected peak near 1580 cm^{-1} which confirmed the attachment of cisplatin to the ox-CNOs. Ox-CNOs and fluoro-CNOs tested against U87 glioblastoma cells showed approximately 50% cell viability after 24 hours. The platinum-CNOs showed near 75% viability at all concentrations.

The CNO-Pt material appeared to have killed near 25% of the U87 cancer cells at each concentration after only 24 hours. As previously mentioned, some carbon nanomaterials have been shown to stay in circulation and continue releasing their drug for an extended period of time. This data suggests that this novel CNO-Pt material could be a potential candidate for use as a theranostic delivery vehicle in cancer therapy. Further experimentation will help to provide more conclusive data on this CNO-Pt material and how useful it might be in nanomedicine.

FUTURE WORK

Carbon nano-onions, along with other types of carbon nanomaterials, appear to have much promise for use as theranostic agents. The CNO-Pt material shows potential, but more experimentation and data will be required to conclude their full capabilities as theranostic agents. In the future, more data will be collected through the use of Raman spectroscopy. A platinum analysis will be conducted using ICP (Inductively Coupled Plasma) spectroscopy to ensure the incorporation of the platinum in the CNO-Pt material. An MTT assay will be performed to assess cytotoxicity of the CNO materials in hopes of gaining more data to support the potential for use in the field of theranostics.

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