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Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Engineering Multifunctional Hollow Titanium Dioxide Nanocarriers for Photodynamic
Therapy, and Targeted- Chemotherapy

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Materials Science and Engineering

by

Minerva Uribe-Robles

March 2022

Dissertation Committee:

Dr. Alfredo A. Martínez-Morales, Chairperson
Dr. Guillermo Aguilar
Dr. Kambiz Vafai

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Minerva Uribe-Robles
March 2022

The Dissertation of Minerva Uribe-Robles is approved:

Committee Chairperson

University of California, Riverside

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Chapter 2, in part, is rewritten from article: Uribe-Robles, M., Ortiz-Islas, E., Rodriguez Perez, E., Lim, T., & Martinez-Morales, A. A. (2019). TiO₂ hollow nanospheres functionalized with folic acid and ZnPc for targeted photodynamic therapy in glioblastoma cancer. *MRS Communications*, 9(4), 1242–1248.

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The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is a reprint from article: Uribe-Robles, M., Ortiz-Islas, E., Rodriguez Perez, E., Lim, T., & Martinez-Morales, A. A. (2019). TiO₂ hollow nanospheres functionalized with folic acid and ZnPc for targeted photodynamic therapy in glioblastoma cancer. *MRS Communications*, 9(4), 1242–1248.

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The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, is currently being prepared for submission for publication of the material. Uribe-Robles, M., Ortiz-Islas, E., Rodriguez Perez, E., Lim, T., Perez Severiano, F & Martinez-Morales, A. A. Targeted Delivery of Temozolomide by Folic Acid- Hollow TiO₂ -Nanospheres Nanocarriers for the treatment of Glioblastoma.

The dissertation author was the primary investigator and author of this paper.

DEDICATION

This dissertation is dedicated to my grandparents Antonio, Luis, Trine, and Paula for leaving me with their great example of perseverance and ethics, and immense respect towards all living things. To my mom Carmen for believing in the value of education, and to my dad Armando for imparting me the love for science. Also, to my brother Ulises for accompanying me in this trajectory, and to all my friends and family for all their encouragement, especially my aunt Elsa. Finally, I would like to thank my husband Juan Carlos for his unconditional love; for always believing in me, supporting me, cheering me up along with our pet chepis, and for all of his patience.

ABSTRACT OF THE DISSERTATION

Engineering Multifunctional Hollow Titanium Dioxide Nanocarriers for
Photodynamic Therapy, and Targeted- Chemotherapy

by

Minerva Uribe-Robles

Doctor of Philosophy in Materials Science and Engineering
University of California, Riverside, March 2022
Dr. Alfredo A. Martínez-Morales, Chairperson

In theranostics, nanocarriers are being engineered to overcome existing limitations in the diagnosis and treatment of a wide range of diseases and conditions such as cancer. Among the most significant deficiencies of contemporary treatment drugs are premature degradation, lack of cell selectivity, and poor control of pharmacokinetics. In this research, the development of a nanocarrier based on hollow TiO₂ nanospheres (HT) is investigated. The proposed system offers many advantages for theranostics in terms of stability, biocompatibility, low cost, low toxicity, high and versatile loading

capacity. Extensive research is being performed in the use of a variety of novel TiO_2 metal oxide morphologies for biomedical applications. Particularly, morphologies such as hollow nanospheres have been researched for the delivery of diverse agents, including chemotherapeutic, imaging agents, and therapeutic proteins.

However, overcoming key limitations of hollow nanospheres before they can be successfully utilized, as nanocarriers still exist, including a narrow absorption confined to the UV range, and non-selectivity towards affected cells. Methods to improve the properties of HT for biomedical applications include functionalization with photosensitizers to shift their absorption from UV to VIS, and functionalization with targeting agents to achieve cell selectivity.

This doctoral research investigates and demonstrates the development of HT nanocarriers. In particular, functionalization of this platform with zinc phthalocyanine (ZnPc) is shown to shift the light absorption spectrum for their use in photodynamic therapy (PDT) against Glioblastoma cancer (GBM), and functionalization with folic acid (FA), and temozolomide (TMZ) for targeted- chemotherapy. In summary, the work presented here will investigate and research the potential for the HT to be used as a nanocarrier for diverse agents in biomedical applications.

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CHAPTER ONE

Introduction

Engineered TiO₂ nanocarriers offer a promising solution to the transportation of compounds used for the diagnosis and treatment of diseases. Many diseases such as cancer, central nervous system (CNS), cardiovascular, and pulmonary are among the top causes of death and disability in the US [1]. Although, the development of new drug compounds and biologics have significantly improved their treatment, these free therapeutics suffer from poor payload, low water-solubility, premature degradation, short pharmacologic half-life, and a lack of selectivity, which hampers their transition to a clinical setting [2,3]. The formulation of compounds in nano material delivery systems can be used to increase their protection and solubility and hence improve their therapeutic and/or diagnostic capabilities.

Nanocarriers have the potential to overcome the current challenges free therapeutics face. Ranging in size from 1-1000 nm, their material composition acquires unique material characteristics with respect to their original bulk forms e.g., increased particle surface area. They include inorganic (gold, iron oxide, silica, carbon-based, quantum dots, etc.), organic (polymeric nanoparticles and micelles, liposomes, dendrimers, etc.), or hybrid compositions (Figure 1.1). Inorganic nanocarriers offer tunable size fabrication, biochemical inertness, mechanical stability, and unique optical, electrical, and magnetic properties. Organic nanocarriers have a higher biocompatibility and biodegradability. Hybrid nanocarriers composed of inorganic, and organic materials have the potential to combine and maintain the inherent advantages of each material [4-8]. The above-mentioned nano materials are promising delivering systems capable of transporting a variety of entities to specific tissues. These entities range from proteins, vaccines, and

imaging agents to phytochemicals, antibiotics, and chemotherapeutic drugs. The transportation method is determined by the physicochemical properties of the nanocarrier, single or multiple compounds can be transported by sorption, entrapment, or bonding [9-13].

Nano materials delivering systems can also be used for the transportation of photo sensible agents for light-based therapies such as photoimmunotherapy (PIT), photothermal (PTT), and photodynamic therapies (PDT). These therapies involve the utilization of molecules sensible to light e.g., photosensitizers. When exposed to light, these molecules, are activated to trigger changes in their immediate surroundings such as the generation of reactive oxygen species (ROS), and heat. These changes can then be used to treat diseases, primarily cancer [14-16].

The design of nanocarriers for the delivery of drugs or biologics can enhance their stability and solubility [17,18]. These formulations can also protect drugs from premature degradation, and hence prolong their circulation time [19]. Another advantage of this systems is their targeting capabilities, they can be engineered to deliver cargos to a specific site or can be fabricated with capabilities to cross or permeate biological barriers [9,20]. Currently there are dozens of nano systems approved by FDA, and many more ongoing clinical trials [21-23]. TiO_2 is one of the most promising nanocarrier materials.

TiO_2 is a metal oxide white powder, FDA approved for its use as food pigment [21CFR73.575, 21CFR73.2575]. Due to its stability, chemical inertness, surface properties, photocatalytic properties, and low fabrication cost [24-26]; TiO_2 has expanded from industrial applications in food, cosmetics, and plastics to biomedical applications

ranging from antibacterial treatment, medical implants, biosensing, and cancer treatment and diagnosis [27-30].

This dissertation focuses on exploiting the optical, structural, and chemical properties of TiO_2 material for its use as a nanocarrier in biomedical applications. Its functionality was studied for PDT, and targeted- chemotherapy against glioblastoma, as a cancer model. In chapter one, the design and engineering of Hollow TiO_2 nanospheres nanocarriers was investigated. Chapter two research the photo absorption of TiO_2 surface for Photodynamic Therapy, and chapter three, studies the capabilities of Hollow TiO_2 nanospheres as a nanocarrier for targeted chemotherapy.

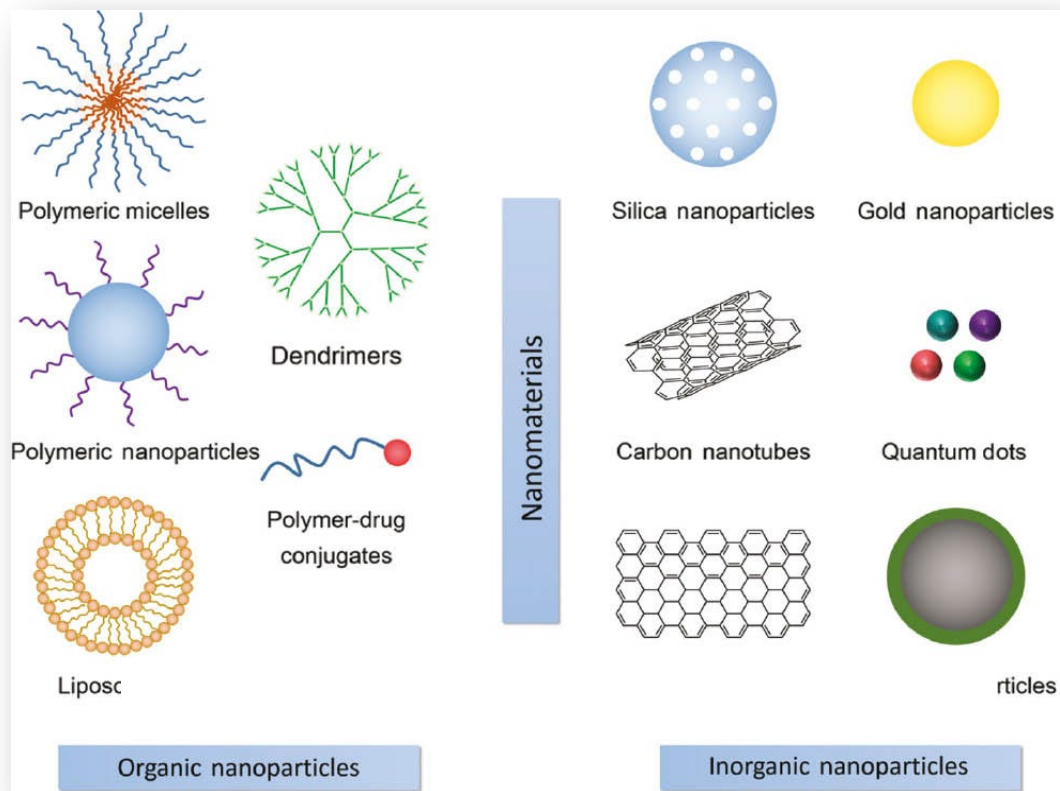


Figure 1.1 Nanocarriers classification

1.2 References

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CHAPTER TWO

Design and Engineering of Multifunctional Hollow TiO₂ Nanocarriers.

2.1. Introduction

Titanium dioxide (TiO_2) is a metal oxide inorganic material with properties that enable its use as a multifunctional nanocarrier. TiO_2 is currently approved by FDA as a color additive in food, drug, and cosmetic applications [21CFR73.575, 21CFR73.2575], and is being employed in many industrial applications ranging from food and plastic colorant, ink and paint additive, cosmetics, and pharmaceuticals [1,2]. It is found in nature in three crystal phase forms: brookite, rutile and anatase. Rutile and anatase are the most studied phases due to their thermodynamic stability [3,4]. Anatase is considered a better photocatalytic material due to its higher 3.2 eV energy bandgap, which is believed to raise the valence band to higher energies, thus facilitating the electron transfer. It is also considered a better photocatalytic material due to its higher conversion efficiency, higher crystallinity, and exposed reactive surface planes [5-8]. In the last decades, these inherent material properties have been a great interest of study, leading to the water splitting discovery in 1972 [9], and subsequent research in photovoltaic cells, gas sensing, and photodegradation of contaminants in water and air, [10-14]. More recently, TiO_2 nanomaterials ranging from 1-1000 nm have been synthesized in many morphologies: mesoporous nanoparticles, nanosheets, nanowires, nanotubes, nanorods, and hollow nanospheres [15-18]. Due to their, biocompatibility, UV-light triggered cytotoxicity, thermal and mechanical stability, high surface to volume ratio, and its low fabrication cost, TiO_2 nanomaterials have gained high attention for its promising potential in biomedical applications [3,4,19]. Utilizing TiO_2 alone, or in combination with organic compounds and biologics, biomedical applications range from implants in hip

replacements, as an antibacterial material for hospitals and orthodontics, as a platform for contrast agents in imaging technologies, as a detector of biological analytes in biosensing, as a photocatalytic material for cancer phototherapies, and as a nanocarrier for drugs and biologics [20-24]. Various methods have been employed for the synthesis of TiO_2 nanomaterials, among them are sol-gel, chemical and physical vapor deposition, anodization, template, and hydrothermal. Each fabrication method has their advantages and disadvantages. Sol-gel involves an acid mediated hydrolysis of a TiO_2 precursor followed by a condensation reaction. It produces highly homogenized nanostructures, however the use of acids in the process hinders its implementation. Chemical and physical vapor deposition consists of the condensation of substrates, under vacuum, from vapor to solid phase. These methods yield high quality coatings and films yet the control over nanoparticles uniformity is harder to obtain, also they involve a high energy process and require expensive equipment. The anodization or oxidation method is based on the oxidation of titanium foil to obtain TiO_2 , under an electric potential and oxygen. This method is popular to obtain ordered TiO_2 nanotubes however its application to other morphologies is more difficult to achieve [3,25-27]. The template method is a method in which a material is used as a mold, and the desired material is coated on the surface of the mold, the mold or template is then removed by etching or thermal treatment. The composition of templates ranges from silica to carbon materials. Although, many morphologies are obtained, it may require strong solvents e.g., hydrofluoric acid, if etching is employed to remove the solid core [28,29]. The hydrothermal synthesis method crystallizes material precursors in aqueous solution at specific temperature and pressure

(>100 C, > 1 atm) controlled by a furnace and autoclave. Hydrothermal method may be disadvantageous for other morphologies e.g., nanotubes nonetheless it has proven to be an easy, low-cost fabrication method to obtain hollow TiO₂ morphologies [30,31]. Compared to other techniques, hydrothermal method involves non-acidic aqueous solutions, has low energy requirement, low cost, and accessible equipment. Although, many TiO₂ morphologies have been achieved, most reported biomedical applications have been researched for mesoporous, and nanotubes morphologies [32-35]. In the present work, we design and engineered hollow multifunctional TiO₂ nanocarriers of spherical shape for biomedical applications. We propose a two-step cheap template - hydrothermal method to fabricate tunable size hollow TiO₂ nanospheres. The first step involves the synthesis of carbon spheres (CS) as sacrificial templates which are derived from low-cost and eco-friendly saccharide precursors and synthesized by a hydrothermal method at low temperature reaction conditions. In the second step, the coating of titanium precursor, and the removal of template by heat treatment take place. The effect of synthesis time, and solvent ratios on the CS size; and the influence of CS on final crystal size, and volume of HT is investigated. We also studied the effect of temperature on HT crystal phase and characterized its morphological and physicochemical properties. We hypothesized that the use of carbon spheres as sacrificial templates will yield a hollow TiO₂ core suitable for loading of pharmaceuticals and/or biologics. We also predict the surface of HT to be conformed by TiO₂ anatase nanocrystals and have a high surface area with the capability to be functionalized and/or coupled with biomedical entities.

2.2 Experimental Methods

2.2.1 Chemicals and reagents

Sucrose, gelatin, and glucose (dextrose) were obtained from Fisher Chemical™, β -Cyclodextrin (cyclo, $\geq 97\%$), and titanium (IV) isopropoxide (TTIP, 97%) were purchased from Sigma-Aldrich. All the materials were used as received without additional purification or treatment. Milli Q-Water from Synergy® water purification system and ethanol from Koptec were used.

2.2.2 Synthesis Methods

2.2.2.1 Carbon Sphere Template Preparation

The following commercial low-cost and eco-friendly saccharide precursors were used for the preparation of the carbon spheres: sucrose, gelatin, glucose, and β -Cyclodextrin. A sample of 1.0 g of each carbon precursor was dissolved at different ratios (DI water : ethanol) and stirred at 400rpm for 30 min to obtain a homogeneous solution [36-39]. The solution was then transferred to a sealed Teflon-lined stainless-steel autoclave (25 mL) and heated at a temperature of 200 °C and predetermined reaction times of 1-16 h for hydrothermal treatment. After cooling at room temperature, the CS were collected and washed three times with DI /ethanol solution at 1:1ratio by centrifugation at 3,000 rpm for 10 min. The carbon spheres were dried overnight on hot plate at 60 °C and collected for further use (Figure 2.1 (a)).

2.2.2.2 Hollow TiO₂ Nanospheres Preparation

After obtaining the desired size of CS templates, the HT was synthesized via a liquid phase deposition [40]. First, 0.03 g of the as prepared carbon templates and 2.2 mmol of

the TiO_2 precursor TTIP, were mixed in 15 mL of ethanol solution and ultrasonicated for 30 min. Then, to allow the titanium to be coated on the surface of the carbon spheres, the mixture was let to stand undisturbed for 6 h. The residue was then purified through centrifugation three times at 11,000 rpm for 10 min and dried on a hot plate overnight at 60 °C. The obtained CS- TiO_2 powder material was then collected and placed in muffler furnace for different thermal treatments at a temperature range between 450 °C - 650 °C for 4 h, were the burning of carbon templates, and crystallization of different HT phases takes place (Figure 2. 1(b)).

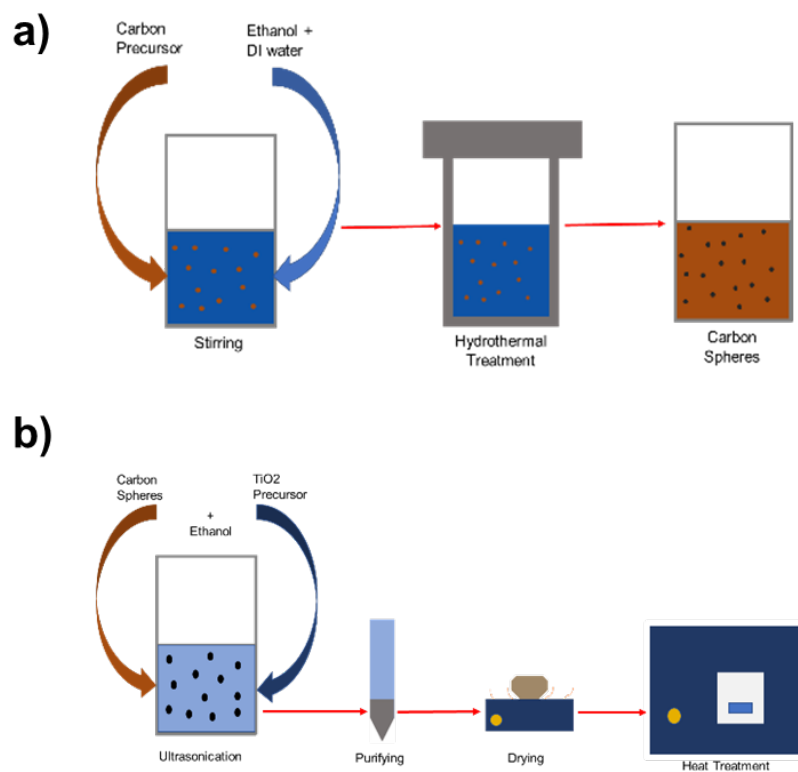


Figure 2.1 Two- step synthesis HT process. a) CS synthesis by hydrothermal method. b) HT synthesis by template mediated method.

2.2.3 Characterization Methods

To study the effect of synthesis time, and solvent ratios on CS size, distributions were obtained by dynamic light scattering (DLS, Nano-ZS90). CS surface morphology and size, and the formation of the hollow core and the outer layer, was further studied by transmission electron microscope (TEM, FEI Tecnai12). The evaluation of HT volume reduction from CS was also carried out by TEM, and scanning electron microscopy (SEM, TESCAN Vega3 SBH). To determine the burning temperature of CS and the phase formation of TiO₂, differential scanning calorimetry (DSC, Netzsch 214 Polyma), and Raman spectroscopy (ThermoFisher DXR SmartRaman) were performed. To investigate the effect of the calcination temperature in obtaining anatase crystal phase on HT, X-ray diffraction (XRD; PANalytical Empyrean Series 2) was used. To determine the surface characteristics of HT, N₂ adsorption-desorption isotherms (BelsorptII-BEL) were acquired.

2.3 Results & Discussions

2.3.1 Characterization of Carbon Spheres

2.3.1.1 Effect of Synthesis Reaction Time on CS Size

DLS measurements show size distributions of carbon spheres from distinct carbon precursors under different hydrothermal synthesis reaction times (Figures 2.2). For carbon precursors: sucrose, glucose, and gelatin, at 1h reaction time and constant temperature of 200°C, we detect a color shift from transparent to yellowish, and formation of aromatic compounds/and of particles in the size range of approximately 0

nm to 20 nm, with the majority in the 0 nm to 5 nm range (Figure 2.2.1 (a-c)). CS formation from gelatin carbon precursor at 16h reaction time and constant temperature of 200 °C is shown in (Figure 2.2.2 (a-d)). As the reaction time increases from 3h to 6h, 9h, and 12 h, the hydrodynamic diameter of gelatin CS increases from approx. 20 nm to 200 nm, 400 nm, and 800 nm respectively.

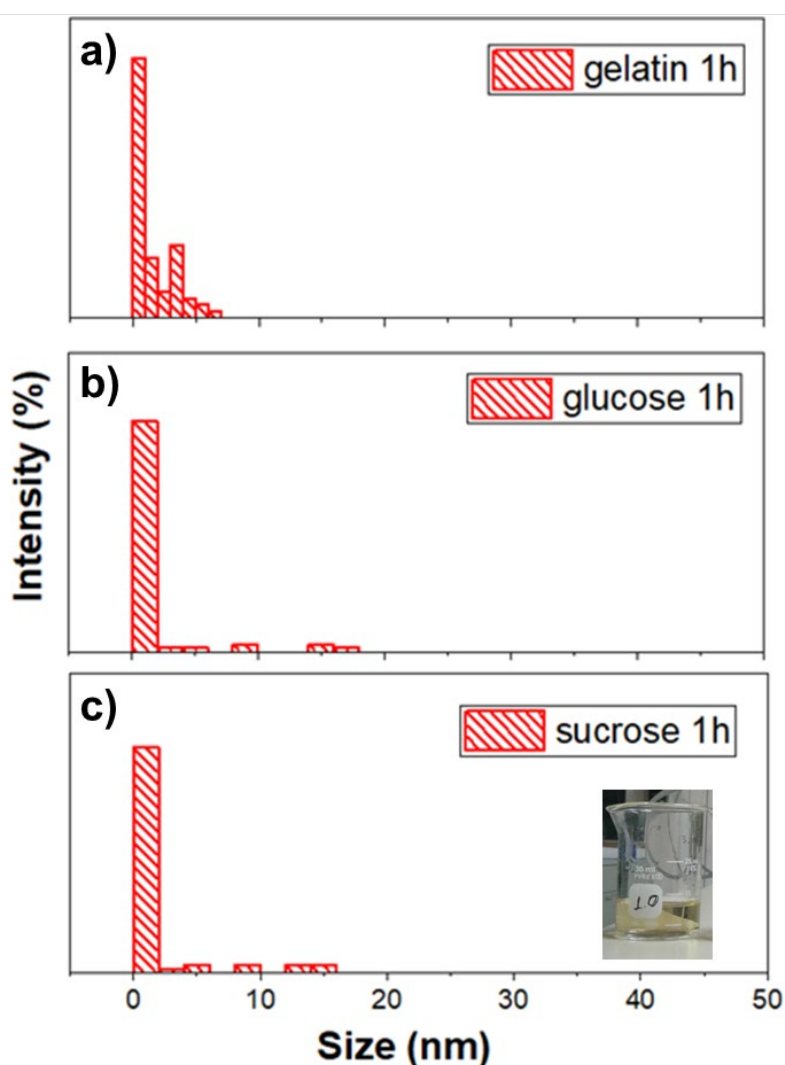


Figure 2.2.1 DLS size distributions of different carbon precursors under hydrothermal treatment at 1h reaction time and constant temperature of 200 ° C.

Gelatin CS

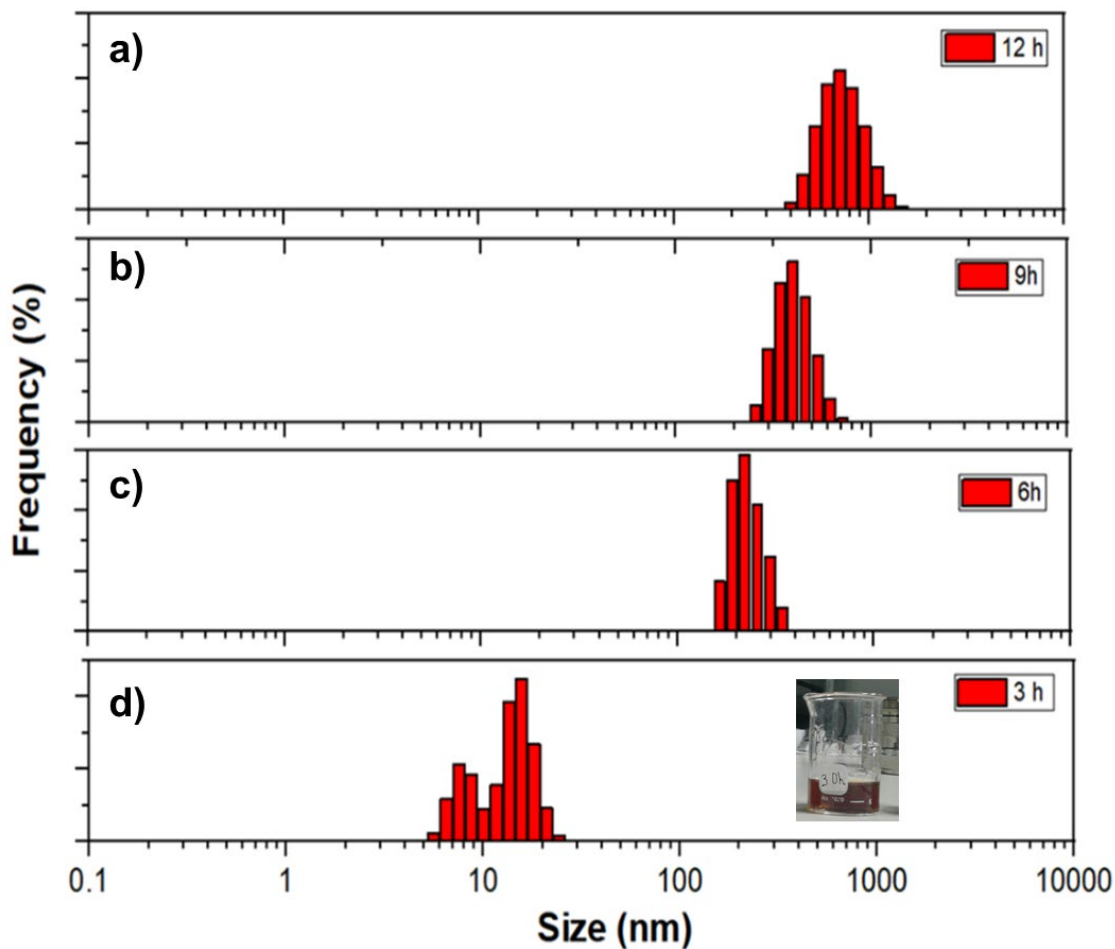


Figure 2.2.1 DLS size distributions of gelatin carbon spheres under hydrothermal treatment at 3,6,9, and 12 h reaction times and constant temperature of 200 ° C.

Carbon spheres were experimentally studied to investigate the effect of the synthesis reaction time on their final size. From our experimental results, and reports in the literature, carbon sphere formation occurs following the LaMer Model (Figure 2.3) [41]. First, the change of color from transparent to yellow, after one hour of reaction time, indicates the formation of oligosaccharides and/or particles through dehydration, this

process is referred to as polymerization. The model suggests a continued polymerization until supersaturation is reached, then a carbonization stage begins with a spontaneous burst of nucleation, although recent literature now proposes a spontaneous burst of growth [42]. This growth increases as dehydration continues, and the diffusion of the solutes toward the surface of the newly formed aromatic compounds, and/or particles continues. Resulting in the size increase of our CS from 20 nm to 800 nm, from 3 h to 12 h reaction times respectively. These results suggest the possible CS size tunability by controlling the hydrothermal reaction time.

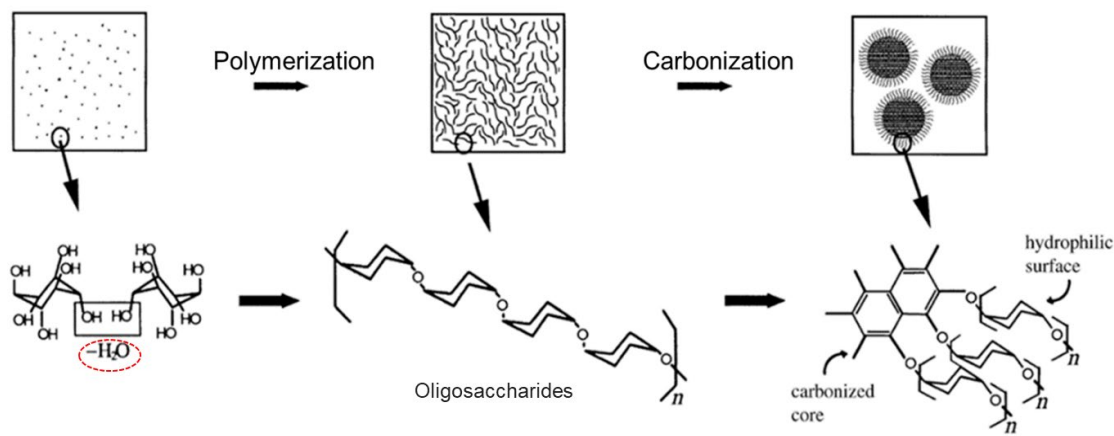


Figure 2.3 Carbon sphere formation following the LaMer Model.

2.3.1.2 Effect of Solvent Ratios on CS Size

TEM characterization reports carbon spheres obtained from β -cyclodextrin carbon precursor utilizing different solvent ratios of DI water and ethanol Figure 2.4 (a-c). Hydrothermal treatment experiments were performed using the following water/ethanol solvent ratios: (1:0), (2:1), and (0:1) at a constant reaction time of 16h and temperature of 200 °C. A dark brown solution was obtained after hydrothermal treatment with water

content, a transparent solution was observed for the hydrothermal treatment using only ethanol. Figure 2.4 (a). Ununiform size distribution of spherical CS were imaged with average sizes of approx. 760 nm for (1:0) Figure 2.4 (b), and more uniform size distribution of spherical CS with average sizes of approx. 1130 nm was observed for (2:1) Figure 2.4 (c). No CS were collected for ethanol solution (0:1) for (2:1).

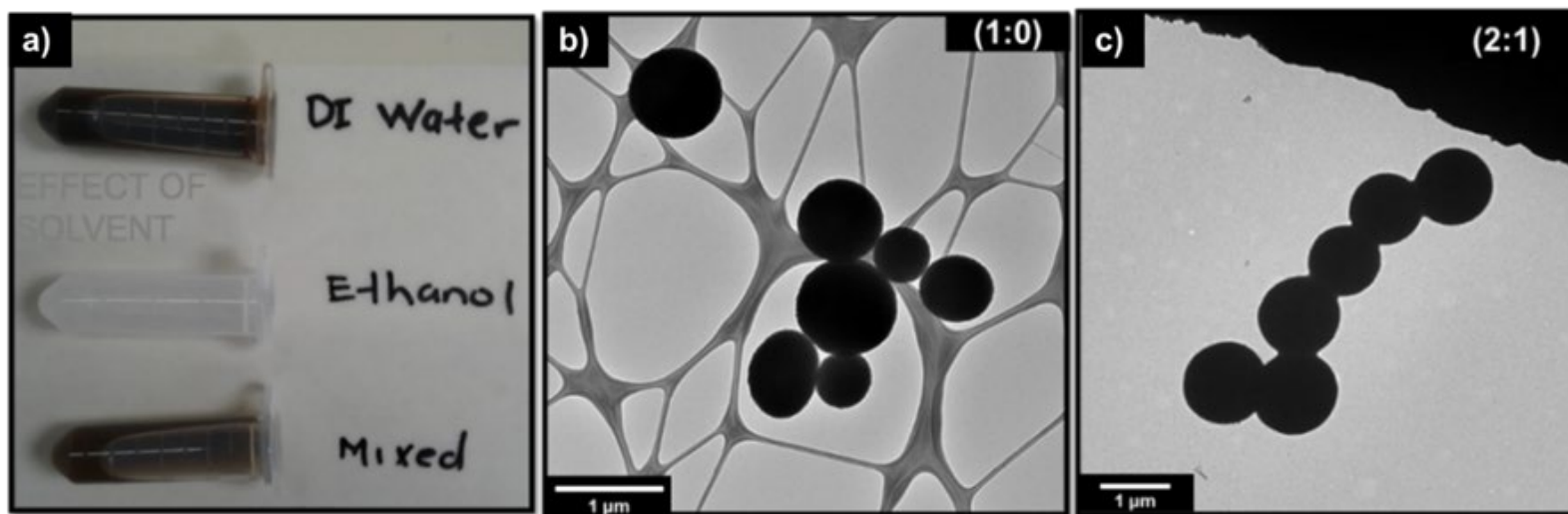
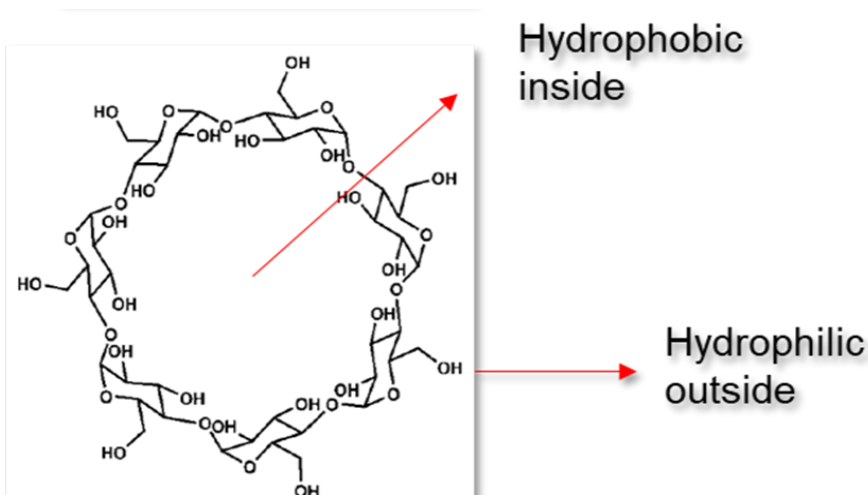


Figure 2.4 Characterization of CS from β -cyclodextrin synthesized with different water/ethanol solvent ratios at 16 h and 200 °C. (a) CS solutions for different solvent ratios. (b-c) TEM characterization of CS for (1:0), and (2:1) solvent ratios respectively.

The β -cyclodextrin carbon precursor is a cyclic oligosaccharide composed of seven glucose subunits, it has a cavity of 7.0 Å. It is amphiphilic with its outer surface being hydrophilic due to its outer OH groups Figure 2.5. The size uniformity of CS observed from hydrothermal reaction in water solution (1:0) may be due to the low solubility of β -cyclodextrin (1.8 %) affecting its dehydration and hence polymerization process [36]. The addition of ethanol may be contributing to its solubility and to other CS formation processes such as decarbonylation and aromatization, hence the more uniform size distribution



2.3.2 Characterization of Hollow TiO_2 Nanospheres

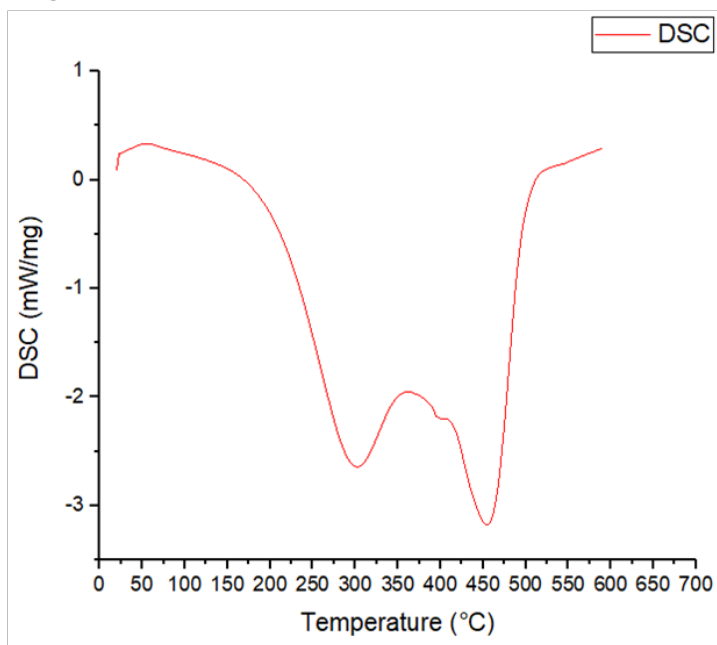
3.2.1 CS calcination and HT formation

The CS calcination and HT formation is investigated through DSC thermo analysis and Raman spectroscopy in Figure 2.6 (a-b). DSC thermogram of CS-HT material sample is shown in Figure 2.6 (a). The sample was heated, and the heat flow was measured with

respect to temperature in the 25 °C to 600 °C range. Two exothermic peaks are exhibited at approx. 300 °C, and 450 °C. Raman spectroscopy analysis shows the characteristic D- and G-bands for CS (inset of Figure 2.6 (b)), and the characteristic active modes of TiO₂ anatase phase: Eg at 145 cm⁻¹, and 639 cm⁻¹, and B1g, A1g at 397 cm⁻¹, 517 cm⁻¹ respectively.

The formation of HT takes place between the decomposition of CS upon the heat release at approx. 300 °C, which is in accordance with reported decomposition temperatures of carbon materials [43], and the crystallization of TiO₂ beginning at approx. 450 °C [44]. From Raman results we observe the disappearance of D and G-bands after the crystallization of TiO₂, which is indicative of no residual CS material left behind after the crystallization.

a)



b)

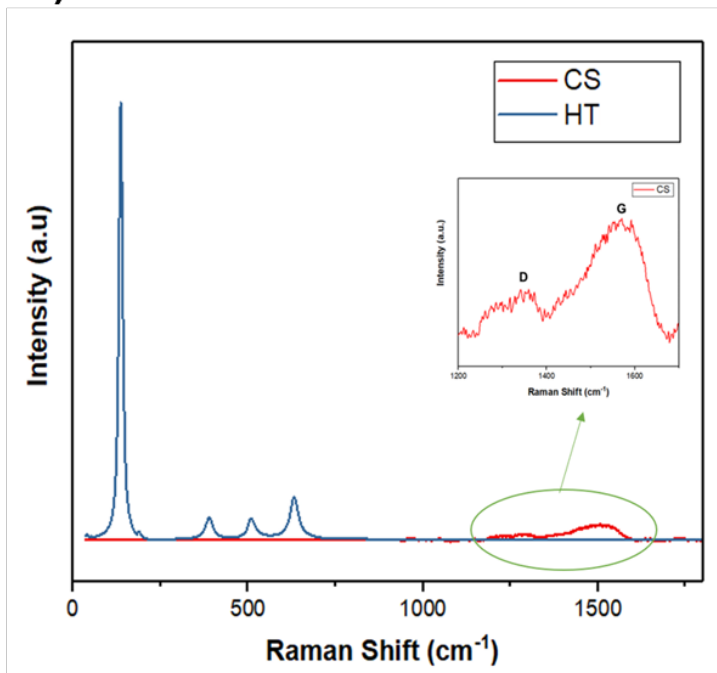


Figure 2.6 CS calcination and HT formation (a) DSC curve CS-HT sample. (b) Raman spectra of CS and anatase HT.

2.3.2.2 HT volume reduction from CS

SEM and TEM images show a HT volume reduction from CS volume utilizing glucose and β -cyclodextrin as carbon precursors Figure 2.7 (a-d). The resulting HT decrease in volume is of approx. 50% for glucose CS templates, reducing their diameter from approx. 970 to 500 nm, and of approx. 70 % for β -cyclodextrin reducing their diameter from approx. 1000 nm to 700 nm.

We observe a HT volume reduction of between 50% to 70% from CS using different carbon precursors. The difference in volume from one precursor to another may be due the different interactions occurring between CS surfaces and TiO_2 precursors in the coating material preparation phase. For example, glucose predominately has the following surface functional groups: O-H, C=O, and C=C, while β -cyclodextrin has: O-H, -C-H, C-C, C=C, and C-O functional groups on its surface [41,45]. Overall, HT of tunable sizes may be achieved by controlling the CS template size.

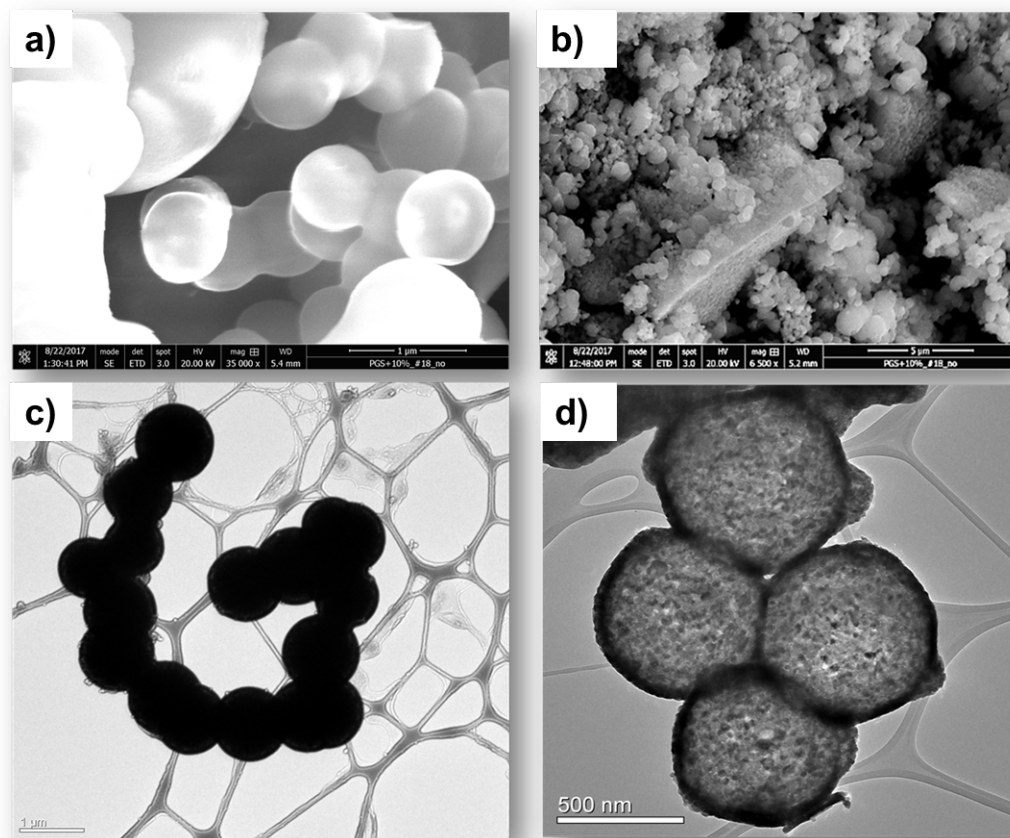


Figure 2. 7 SEM images of (a) CS synthesized at 200 °C from glucose carbon precursor at 12 h synthesis time, (b) HT calcinated at 550 °C for 4 h utilizing glucose templates. TEM images of (c) CS synthesized at 200 °C from β -cyclodextrin carbon precursor at 16 h synthesis time. (d) HT calcinated at 550 °C for 4 h using β -cyclodextrin templates.

2.3.2.3 Effect of Calcination Temperature on the Crystallinity of HT

XRD patterns and TEM images show the calcinated HT samples from 450 °C to 650 °C Figure 2.8 (a-c). Sample calcinated at 450 °C presents representative anatase phase peaks of Bragg reflections at $2\theta = 25.53^\circ$, 37.99° , 48.24° , and 62.88° attributed to (101), (004), (200), and (204) respectively, also a small peak indicative of rutile phase appears for (002), its corresponding TEM image shows an aggregated crystal distribution throughout the copper grid where some crystallites began to conform the outer spherical shell of an approx. avg. of 13 nm, the formed HT have a approx. avg. diameter of 245 nm. The XRD pattern from calcination at 550 °C shows all characteristic mentioned anatase crystal peaks. TEM characterization shows well conformed hollow sphere core with an outer shell of 20 nm crystallites, and final HT diameter of approx. avg. of 265 nm. Calcination at 650 °C presents all the characteristic peaks of anatase phase, however the peak of (002) suggest the beginning of rutile phase crystal formation, its TEM images show an outer shell with 35 nm crystallites and approx. avg. diameter of 475 nm.

The crystallinity and crystallite size of TiO_2 has been studied at different calcination temperatures. We observe the transition from amorphous material to anatase crystal phase at 450 °C, which is also supported by previous DSC results. Although representative peaks of anatase phase appear at 450 °C calcination temperature, from TEM images we observe the progressive formation of HT, as previously spread crystallites compact and conform the outer shell, and as their size increase from 13 nm to 20 nm when calcinated from 450 °C to 550 °C. The small (002) rutile peak appearing at 550 °C calcination temperature, and its evident increase at 650 °C suggest that for temperature $> 650^\circ\text{C}$

anatase phase begins transitioning to rutile. Overall, we see that at higher calcination temperatures, the crystallites increase in size which can be attributed to the thermally promoted crystallite growth [44,46]. We have successfully obtained well defined anatase TiO₂ spheres consisting of a hollow core and outer shell, as supported by TEM Figure 2.8.1.

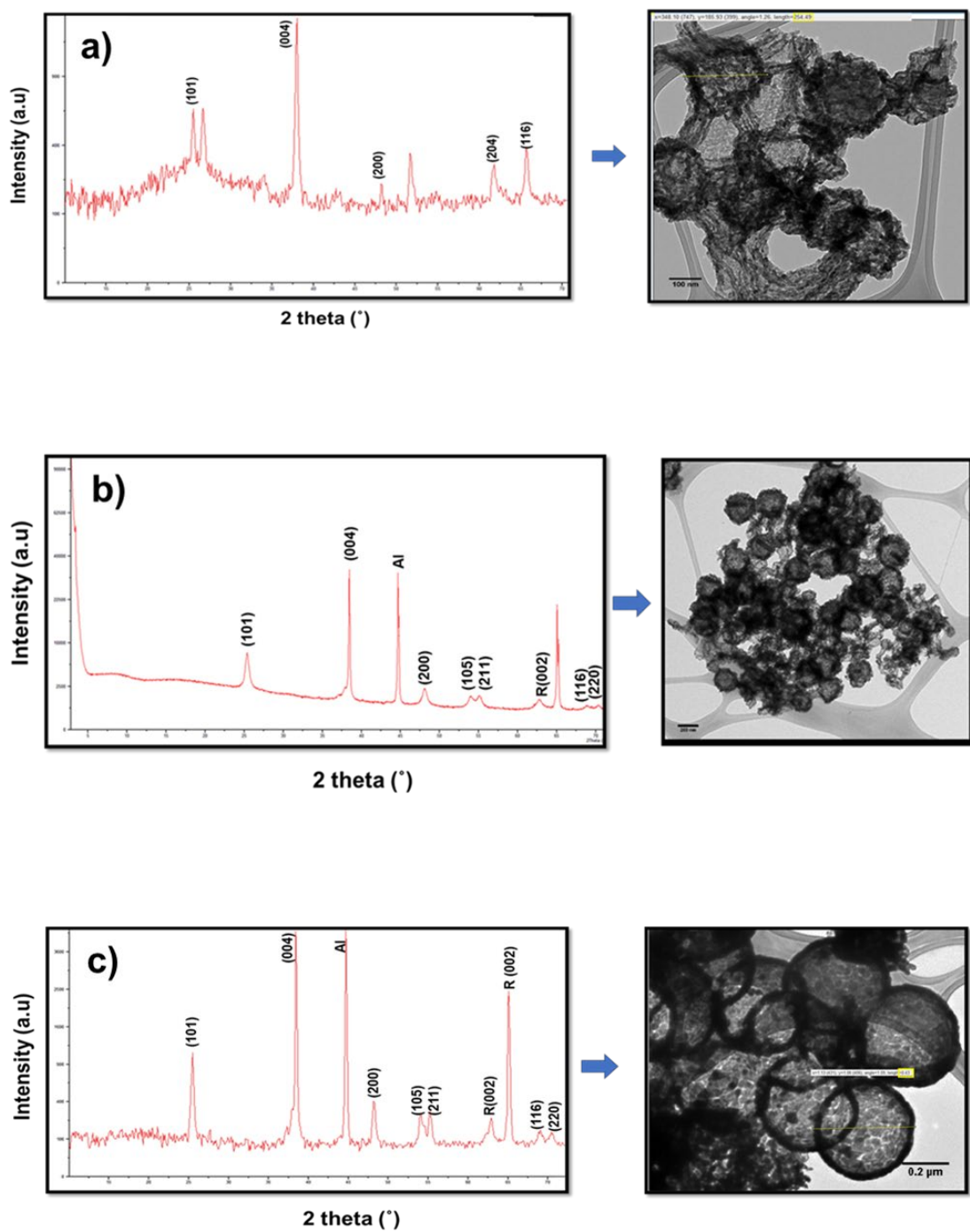


Figure 2.8 XRD Patterns and their corresponding TEM images of HT calcinated at: (a) 450 °C , (b) 550 °C, and (c) 650 °C.

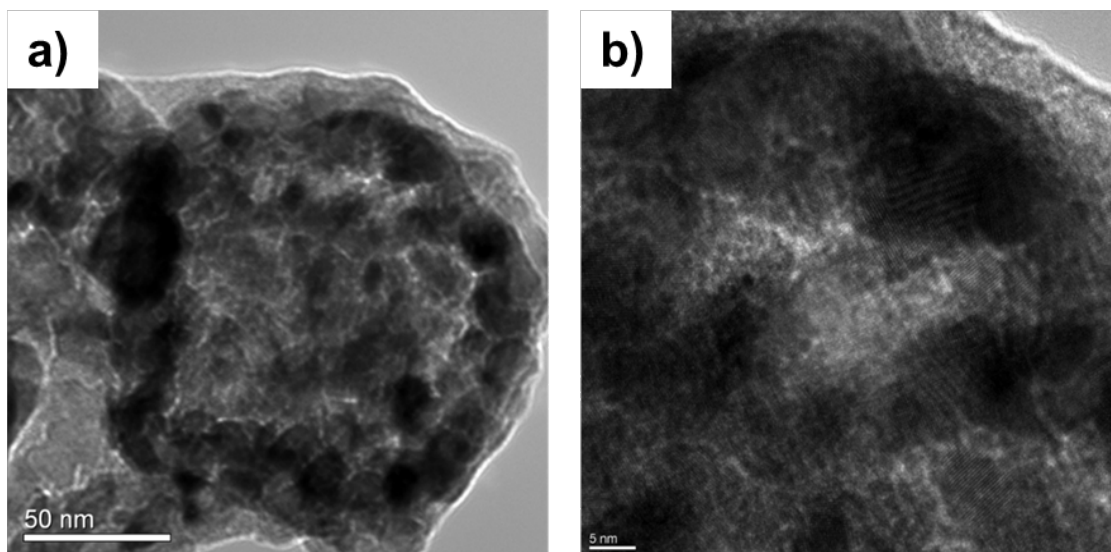


Figure 2.9.1 TEM images of (a) HT calcinated at 550 °C for 4 h, from cyclodextrin carbon precursor template, and (b) high-resolution TEM (HRTEM) of TiO₂ with an anatase crystal lattice.

2.3.3 HT Surface Area

The N₂ adsorption-desorption isotherms of HT are shown on Figure 2.9. These isotherm presents a narrow hysteresis loop on top of the P/P₀= 0.9 region indicating a mesoporous surface composition (IUPAC type IV classification) for HT. The textural values of surface area, average pore diameter, and pore volume of HT are of 14.515, 42.758, and 0.1552 accordingly .

We have obtained HT of high surface area and mesoporous nature. These properties may enable HT nanocarriers the capability to be couple different biomedical entities, either to enhance TiO₂ inherited properties e.g., photocatalytic, or to transport pharmaceuticals, and/or biologics.

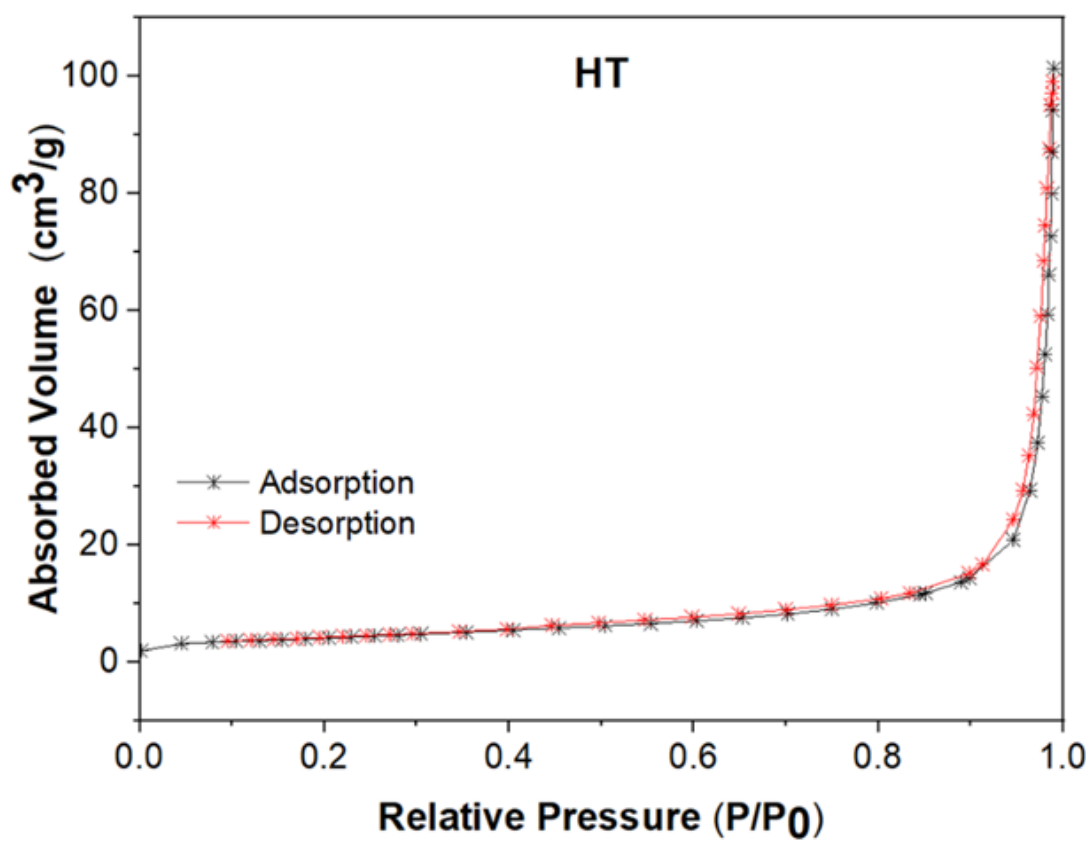


Figure 2.10 BET Nitrogen adsorption-desorption isotherms of HT.

2.4. Conclusions

CS were successfully synthesized by the hydrothermal method from the next carbon precursors: sucrose, glucose, gelatin, and cyclodextrin, following the LaMer Model. The increase in hydrothermal synthesis time increased the CS size. Utilizing a ratio of (2:1) DI water/ ethanol yielded uniform CS of approx. 1100 nm. The successful decomposition of CS was observed at 300 ° C, and a HT volume reduction of between 50% to 70% was observed from CS, possibly due to the nature of the CS surface functional groups and their interaction with titania precursor coating material. The successful calcination of anatase crystal phase HT was achieved at 550 ° C, with hollow core morphology and outer TiO₂ crystallite shell. A higher TiO₂ nanocrystal size was obtained by increasing calcination temperature. The HT surface is of mesoporous nature and possess a high surface area which enables its functionalization capability.

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CHAPTER THREE

TiO₂ hollow nanospheres

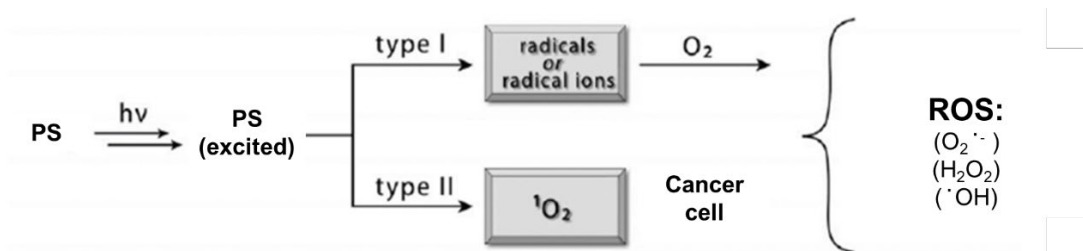
functionalized with folic acid and

ZnPc for targeted photodynamic

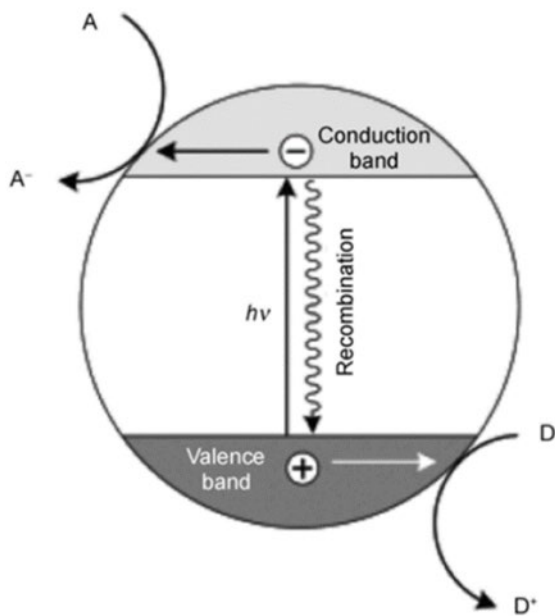
therapy in glioblastoma cancer.

3.1 Introduction

The functionalization of HT by FA and ZnPc make it an ideal nanocarrier for targeted Photodynamic Therapy (PDT), by adding targeting capabilities, enhancing the intrinsic photocatalytic properties of TiO_2 , and expanding its light absorption range. PDT has emerged as an alternative treatment for the removal of various types of cancers, including the brain, skin, lung, prostate, and others [1,2]. It consists of the following three major components to produce cytotoxic reactive oxygen species (ROS): light, a material sensible to light or photosensitizer (PS), and the presence of oxygen (O_2). First, PS is accumulated in cancer tissue, then upon light irradiation, the PS absorbs light at specific wavelength, depending on the material composition, and is excited to its triplet state (ground state) to follow type I or type II photochemical reaction mechanisms in the presence of body oxygen. In type I, PS interacts with biomolecules surrounding cancer cells to produce cations or ions that interact with oxygen to produce ROS such as ($\text{O}_2^{\cdot -}$), (H_2O_2), ($\cdot\text{OH}$), etc. In type II, PS interacts directly with the oxygen inside the cancer cell to produce ROS Figure 3.1 [3,4]. These ROS have been proven to cause photodamage of membrane and other cancer cell components, leading to their death by various mechanisms, including apoptosis and necrosis [5,6].



Although many organic PS have been used in PDT e.g., natural, synthetic dyes, and porphyrin derivatives, their efficacy has been limited due to their low water solubility, low photostability, low absorption, and poor cancer selectivity [7-10]. To mitigate these limitations, various nanocarriers have been employed for the transportation of PS. The incorporation of commonly used PS into hydrophilic nanocarriers enhances their solubility; also, the large surface area of nanomaterials enables their functionalization with targeting agents for cell selectivity [10-15]. TiO₂ nano materials have shown promising results in their application for PDT, their intrinsic photocatalytic properties, hydrophilicity, photostability, and nanomaterial properties enable its use as both PS and nanocarrier systems [16-19]. TiO₂ is an inorganic semiconductor material with an energy band gap of 3.2 eV, it absorbs light in the UV-A spectrum (315–400 nm). Upon light irradiation, electrons (e⁻) in its valence band are excited to the conduction band generating electron holes (h⁺) behind Figure 3.2. TiO₂ functionalization with organic photosensitizers increases the efficacy of the excitation process, expanding its wavelength absorption from UV to VIS or near IR spectrum range (400-1000 nm) thus extending into the therapeutic window to achieve deeper tissue penetration for PDT [20,21].



In this chapter we synthesized hollow TiO_2 nanospheres (HT) nanocarriers following the protocol established in chapter one. We investigate the capabilities of HT as a nanocarrier system for PS, using Zinc (II) tetranitrophthalocyanine (ZnPc) as the photosensitizer material. ZnPc is a second-generation PS used for PDT, which enhances the absorption wavelength to the near IR (600–800 nm) [22,23]. Also, as reported by Lopez et al., TiO_2 –ZnPc enables cellular uptake in cancer cells [24]. We also study the functionalization of HT with folic acid (FA) for its potential future use as a cancer targeting agent, FA is a synthetic form of vitamin B9 that naturally aids cellular proliferation, furthermore, FA is useful for targeted PDT applications because it exhibits a high affinity to folate receptors that are significantly overexpressed on the surface of cancer cells [25,26], further research will be presented in chapter 3. The in vitro production of ROS by our HT

nanocarriers for possible PDT under UV light was also investigated, and the cell viability of obtained HT configurations was tested by *In vitro* cytotoxicity against M059K cell line (Human GBM cancer cells) as cancer model.

We hypothesized that the incorporation of ZnPc to HT will expand HT light absorption capabilities from UV-A to VIS range and its functionalization with FA will enable HT with targeting capabilities. We also expect that upon activation with light, HT will generate ROS capable of killing GBM cancer cells. Overall, our approach has the potential to develop a viable HT-FA-ZnPc nanocarrier for the targeted treatment of cancerous cells by PDT.

3.2. Experimental Methods

3.2.1 Chemicals and reagents

β -Cyclodextrin ($\geq 97\%$), Titanium (IV) isopropoxide (TTIP, 97%), Zinc(II) tetranitrophthalocyanine (ZnPc, 97%), folic acid (FA, $\geq 97\%$), N,N'-Dicyclohexylcarbodiimide (DDC, $\geq 99\%$), 1,3-diphenylisobenzofuran (DPBF, 97%), Dimethyl sulfoxide (DMSO, 99.7%), 4'-Diamidine-2'-phenylindole dihydrochloride (DAPI), MTT and Isopropanol were all obtained from Sigma-Aldrich. Fluorescein isothiocyanate (FITC) was obtained from Invitrogen. For the cell culture and cell viability studies, M059K cell line was obtained from American Type Culture Collection (ATCC, CRL-2365). The Dulbecco's Modified Eagle's/F-12 Medium (DMEM/F-12), Phosphate Buffered Saline (PBS) buffer, antibiotic-antimycotic, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic) and sodium pyruvate were purchased from

Gibco. Fetal bovine serum was purchased from Biowest. All the water used was Milli Q-Water from Synergy® and the ethanol from Koptec.

3.2.2 Synthesis methods

3.2.2.1 Carbon sphere template-CS

Carbon spheres (CS), used as sacrificial templates for the HT, are synthesized via the dehydration and polymerization of a solution prepared from commercial saccharide precursor β -Cyclodextrin, following the synthesis protocol from chapter one. Briefly, the synthesis is performed in a closed system at low temperature [27,28]. 1g of carbon precursor is dissolved in a mixture of Milli Q-Water and ethanol (2:1). The precursor solution is stirred at 400rpm for 30 min to obtain a homogeneous solution. The solution is sealed in a 25 ml Teflon-lined stainless-steel autoclave and placed into a muffle furnace at 200 °C for 16 h. After the reaction, the autoclave is left to cool down under ambient temperature. To collect and purify the carbon spheres, the obtained solution is centrifugated at 8,000 rpm for 15 min and the CS are washed three times with ethanol and Milli Q-Water mixture (1:1). The CS are dried on a hot plate overnight at 60 °C.

3.2.2.2 Hollow TiO₂ nanospheres nanocarriers – HT

HT are synthesized by liquid phase deposition following the synthesis protocol from chapter one. 0.03g of as prepared CS templates are added to 15 mL of an ethanolic solution with 2.2 mmol of TTIP as the titanium precursor . The solution is sonicated for 30 min and is allowed to stand for 6 h undisturbed to allow the titanium to be coated onto the surface of the CS. The residue is purified three times through centrifugation at 8,000 rpm for 15 min and dried on a hot plate at 60 °C for 12 h. The dried residue is collected

and placed in a muffle furnace in air condition at 550 °C for 4 h for subsequent burning of the CS and formation of HT by calcination.

3.2.2.3. HT-FA nanocarriers

HT-FA structures are synthesized by activation of the carboxylic groups in FA. First, 20 mg of FA are mixed with 20 mg of DDC in 4 mL DMSO solution [29]. The solution is stirred for 2 h under a N₂ environment and dark conditions. Then, 40 mg of the previously prepared HT are added and stirred for an additional 2 h under same conditions. The solution is washed three times with DI water at 3,000 rpm for 10 min and left to dry under dark conditions on a hot plate overnight at 60 °C.

3.2.2.4 HT-FA-ZnPc nanocarriers

HT-FA-ZnPc structures were synthesized by a chemical impregnation method [29]. First, a solution with 20 mg of ZnPc and 20 mL of DMSO is prepared and stirred at 300 rpm for 30 min at room temperature and under dark conditions. Second, 300 mg of as prepared HT-FA are added and are dispersed by stirring for 6 h, under dark conditions. The obtained HT-FA-ZnPc product is washed three times with DI water by centrifugation at 3,000 rpm for 10 min and left to dry overnight at 60 °C.

3.3. *In vitro* Detection of ROS singlet oxygen

Detection of the generated ROS from HT, HT-FA, and HT-FA-ZnPc, under exposure to UV light was performed by detecting the singlet oxygen (¹O₂) generated, using a fluorescent probe method [30]. DPBF was utilized as the fluorescent probe, 5mg of DPBF was added to ethanol 2 mL and 1 mg of HT were added to 2 mL ethanol separately. Then, the prepared solutions were mixed and transferred to a 4 mL cuvette to

be irradiated by a 365 nm UV lamp for different times. The supernatant was collected for UV–VIS absorption measurement.

3.4 Physical-chemical characterization

The morphology, size, and hollowness of the nanocarriers was examined by transmission electron microscopy (TEM, FEI Tecnai12). The crystal phase of the prepared HT was analyzed by X-ray diffraction (XRD, PANalytical Empyrean Series 2). The chemical adsorption and the change in the surface groups of HT-FA-ZnPc was analyzed by infrared absorption spectroscopy (FTIR, ThermoFisher Scientific Nicolet iS10 spectrophotometer). To confirm the attachment of ZnPc onto the HT-FA surface, absorption spectra from ultraviolet–visible spectroscopy performed in DMSO solvent solution (UV-VIS, Agilent Cary 60 spectrophotometer) was employed. To evaluate the effects of nanocarriers on cellular morphology, optical microscope (Olympus, CKX41) was used. To assess the location of our synthesized nanocarriers in the cell, images were acquired with a fluorescent cell imager (ZOE™ Bio-Rad).

3.5. Biocompatibility Studies

3.5.1 Cell Culture

Human glioblastoma cell line M059K was grown in DMEM/F-12 medium with pH of 7.2, supplemented with 10% of fetal bovine serum, 1% of antimycotic antibiotic , 15mM of HEPES , 0.05 mM non-essential amino acids and 0.5 mM of sodium pyruvate. Cells are maintained in an incubator under humidified atmosphere at 37 °C and 5% CO₂. For the cytotoxicity and morphology experiments, M059K cells are seeded into 96-well plate at a density of 1.6×10^4 per well or at 1×10^5 cells per well on a 6 well plate, respectively,

and cultured overnight. The following day, the medium is removed, and fresh medium with nanocarriers is added into each well at different concentrations :5, 10, 50, 100, 500 µg/mL, and incubated for 24, 48 or 72 h.

3.5.2 Cytotoxicity

The in vitro cytotoxicity of nanocarriers is evaluated through 1) the cell metabolic activity by MTT assay [31]. After incubation times, the cells are washed twice with PBS buffer. MTT solution of 5 mg/mL H₂O is added to each well at 10 µl MTT/90 µl medium and incubated for 4 h under standard cell culture conditions at 37 °C and 5% CO₂. During incubation, the salt is reduced by viable cells to an insoluble formazan. After the incubation period is complete, the MTT solution is aspirated, and formazan crystals are dissolved by addition of HCl at 0.1N/ 100 µl (prepared in isopropanol) solution. The absorbance of each sample is measured at 570 nm using a microplate reader (Synergy HT, Biotek). These experiments are carried out on separated and in triplicate for each tested nanocarrier configuration. The cytotoxicity is expressed as the percentage of cell viability compared to control cells (average of each material concentration/average of cell control × 100%); or 2) Cell morphology analysis, is carried out by the cell's observation at 24, 48 and 72 h after nanomaterials treatment, and images are collected with a phase-contrast microscope (10 X).

3.5.3 Fluorescence Microscopy

To evaluate the nanocarriers uptake by the cell, 1mg of FITC solution and 10 mL of water was stirred for 24 h under dark conditions. The HT-FA-ZnPc were added and stirred for another 24 h under the same conditions. The excess of the dye was removed by

successive washes with DI water and centrifugation. The resulting powder was dried at 50° C for 12 h. M059K cells were treated for 24h with the previously stained HT-FA-ZnPc. The treated cells were fixed with 4% of paraformaldehyde for 30min, washed three times with PBS and incubated with 200nM of DAPI for nuclear staining for 5min at room temperature. Images were acquired by fluorescence microscopy.

3.6. Results & Discussions

3.6. 1 CS and HT Characterization

From the TEM characterization, it is observed that the morphology from the synthesized CS is a well-defined spherical shape with Avg. sizes of approx. 1050 nm, from cyclodextrin precursor, as shown in Figure 3.3 (a). The resulting HT decreased in volume by approximately 65 % from the CS templates Figure 3.3 (b). The obtained HT structure is composed of an outer shell (approx. 55 nm), and a hollow core as shown from the broken structure in Figure 3.3 (b).

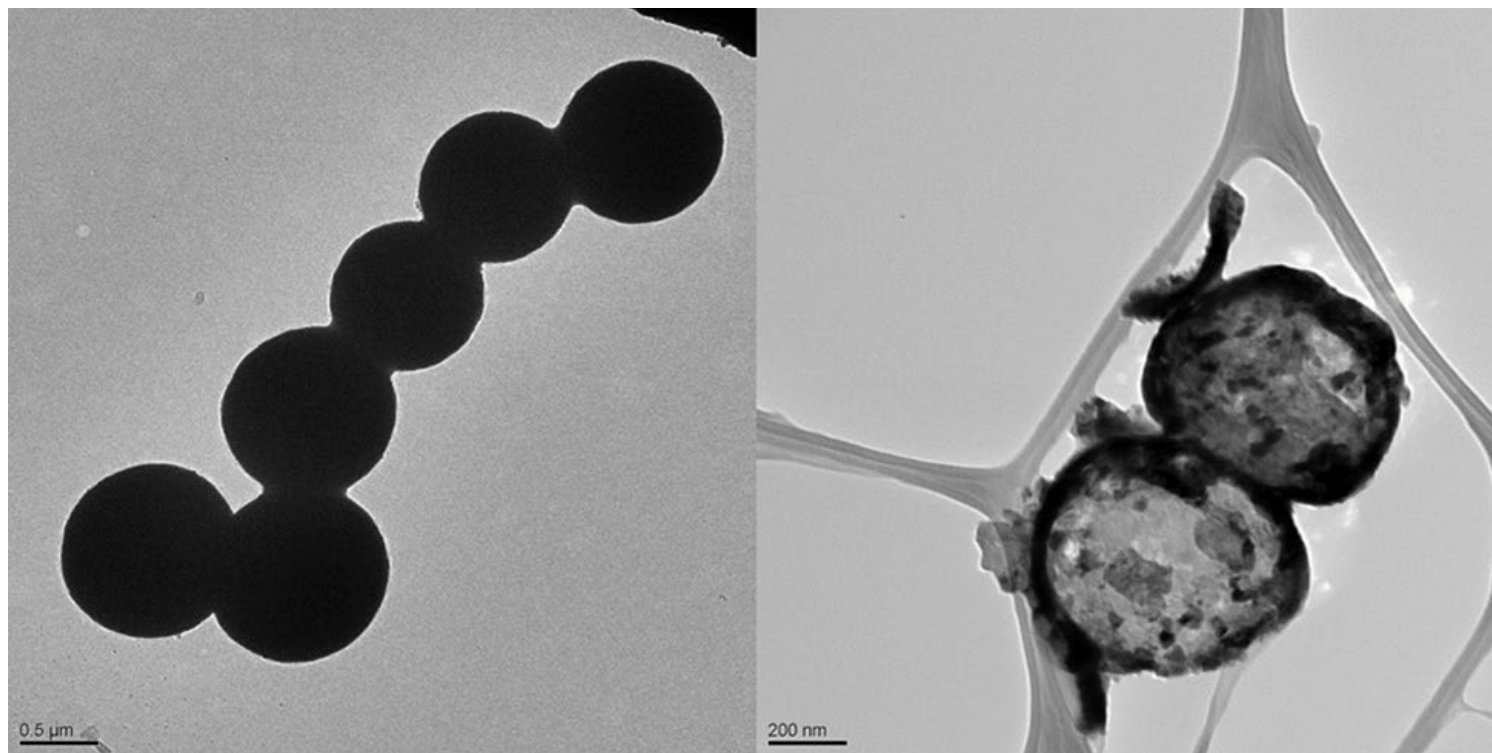


Figure 3.3 TEM images of (a) CS synthesized at 200 °C from cyclodextrin carbon precursor at 16 h synthesis time, (b) HT calcinated at 550 °C for 4 h, from cyclodextrin carbon precursor template.

3.6.2 Crystallinity

The obtained narrow peaks of Bragg reflections at $2\theta = 25.53^\circ$, 37.99° , 48.24° , 62.88° , and 76.64° are attributed to (101), (004), (200), (204), and (215). These peaks are indicative of anatase TiO_2 crystal phase, as shown in Figure 3.4. To further confirm the crystallinity of the TiO_2 , HRTEM was performed. Figure 3.4 (inset) shows the surface crystal lattice spacing of 0.35 nm corresponding to TiO_2 (101) facet.

Due to the presence of more functional groups on its surface than other crystal phases of TiO_2 , anatase phase has been shown to have a higher photocatalytic activity [32]. By synthesizing anatase HT and exposing it to UV light we expect to produce a higher quantity of ROS for PDT.

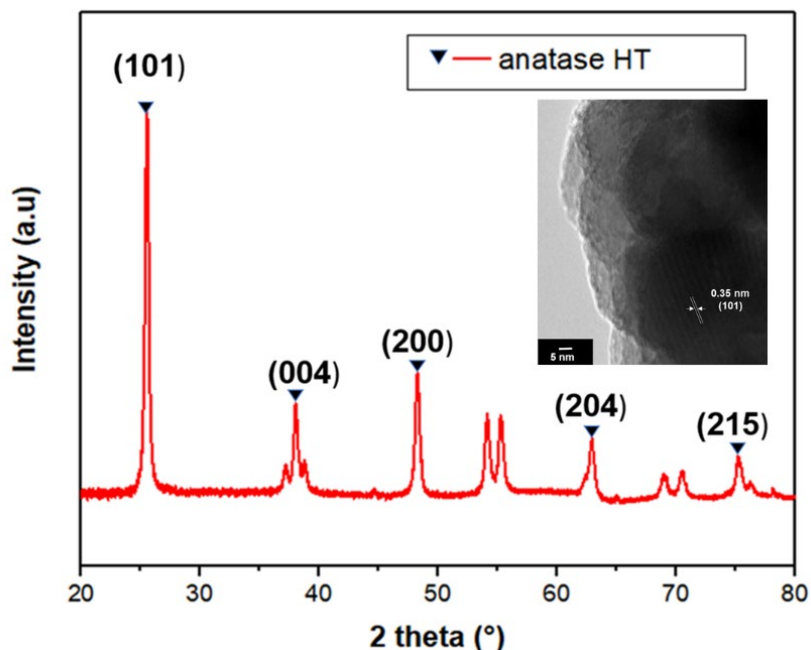


Figure 3.4 Powder XRD patterns of HT calcinated at 550°C for 4 h. Inset HRTEM of HT calcinated at 550°C for 4 h.

3.6.3 Functionalization of HT with FA and ZnPc

FTIR Study and analysis

The IR spectra of HT shows two peaks Figure 3.5. The peak at 2342 cm^{-1} is related to the $\text{O}=\text{C}=\text{O}$ bonds and is commonly observed from CO_2 from the environment. The broad peak below 1000 cm^{-1} is a peak due to the formation of Ti-O bonds from the network structure of TiO_2 . The HT-FA spectrum shows the characteristic absorption peaks of FA. The peak at 3329 cm^{-1} corresponds to the N-H stretching vibration from the pteridine ring. The peak at 2932 cm^{-1} is attributed to vibrational C-H of CH_2 groups from the pteridine ring and glutamic acid present in the FA. The peaks at 1625 and 1571 cm^{-1} belong to C=O and C=C vibrations in the folic acid, respectively. While the peak at 1533 cm^{-1} confirmed the formation of an N-Ti bond during the functionalization of HT with the DDC-activated folate, as shown in the schematic of Figure 3.5 [33].

Although the HT-FA and HT-FA-ZnPc spectra are very similar, experimentally during functionalization a change in color from white to yellow (characteristic of FA) is observed after attaching FA to the HT and a change from yellow to blue is observed after loading ZnPc to HT-FA. UV-VIS spectroscopy is used to further investigate the functionalization of HT-FA by ZnPc.

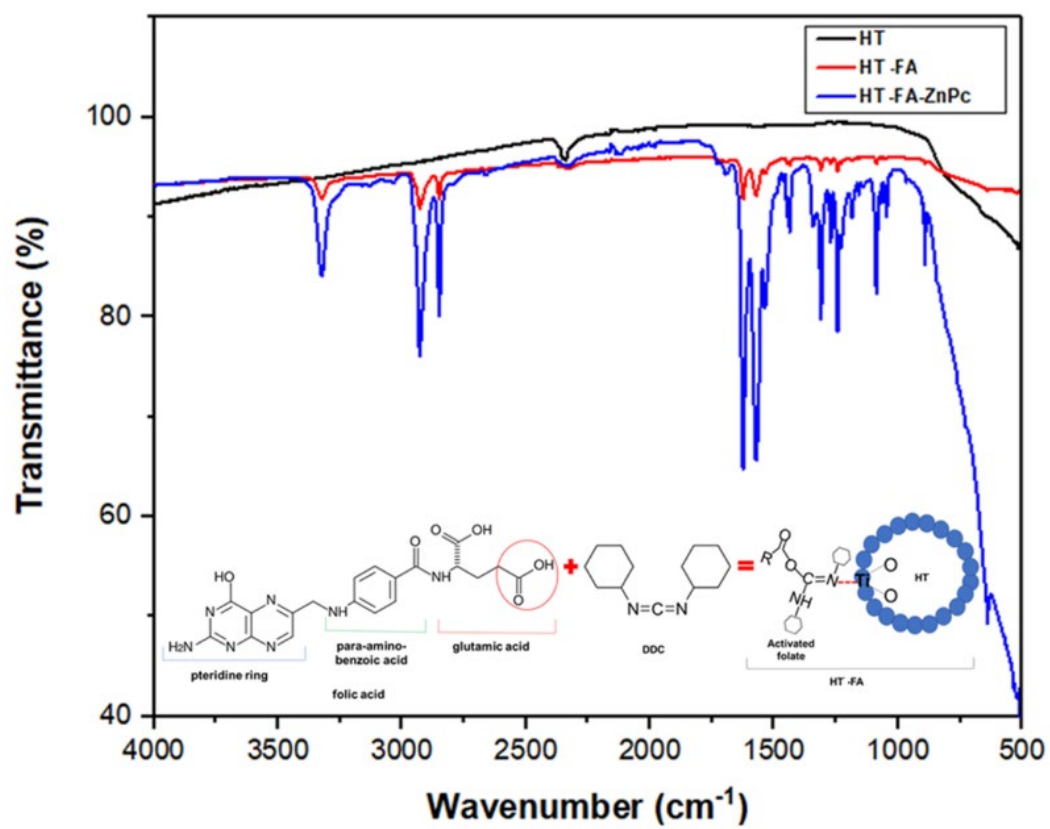


Figure 3.5 FTIR spectra of HT-FA-ZnPc and schematic of HT-FA functionalization.

UV-VIS Study

The UV-VIS absorption spectra of ZnPc and HT-FA-ZnPc are shown in Figure 3.6. In the ZnPc spectrum the typical energy bands for ZnPc: B-band (γ or Soret band), Q-band (α band in porphyrin) are observed [34,35]; with a maximum at 348 and 684 nm, respectively. Those bands result from the π - π electronic transitions of the phthalocyanine rings. In the HT-FA-ZnPc spectrum the B and Q bands appeared slightly displaced from the ZnPc spectra. Both peaks are broadened, and red shifted. These changes are indicative of the interaction between the ZnPc and the HT-FA.

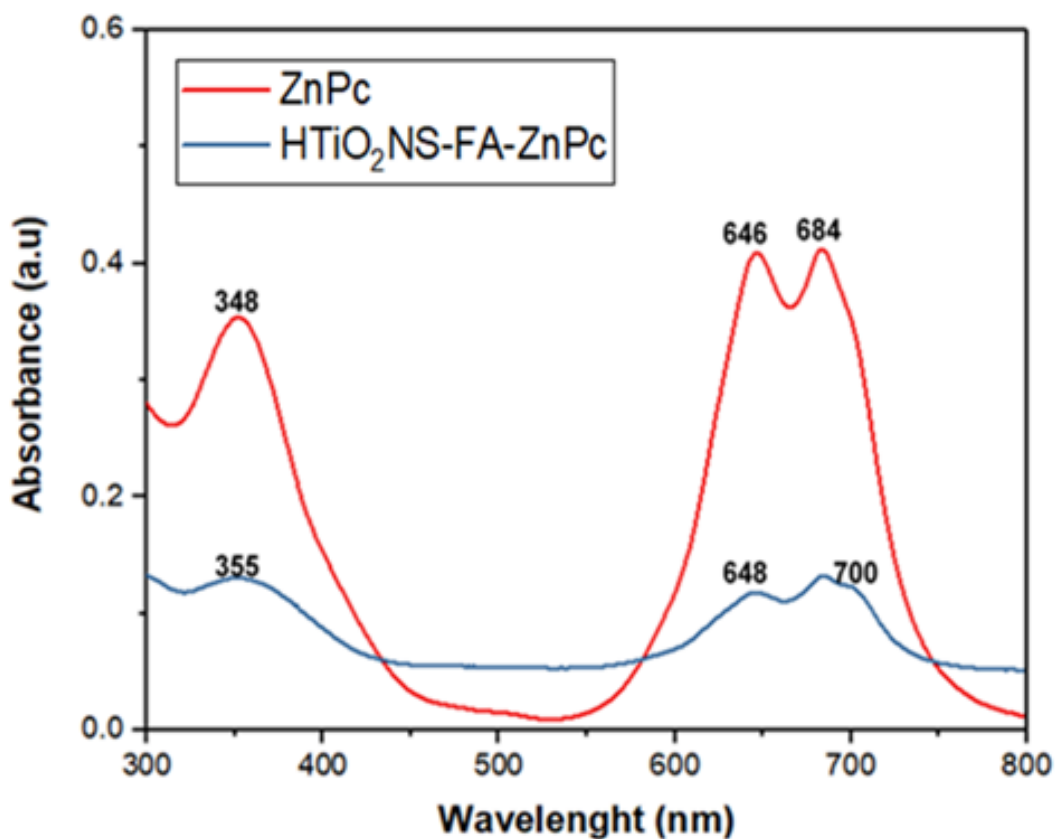
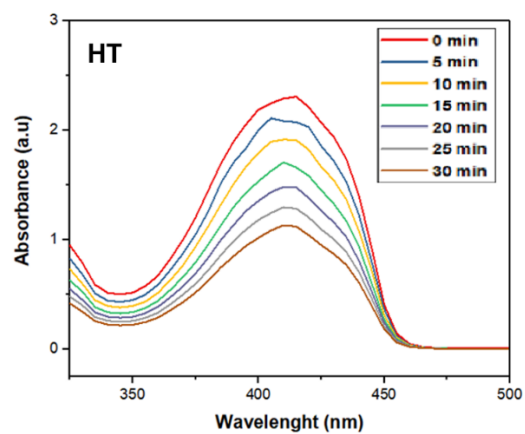


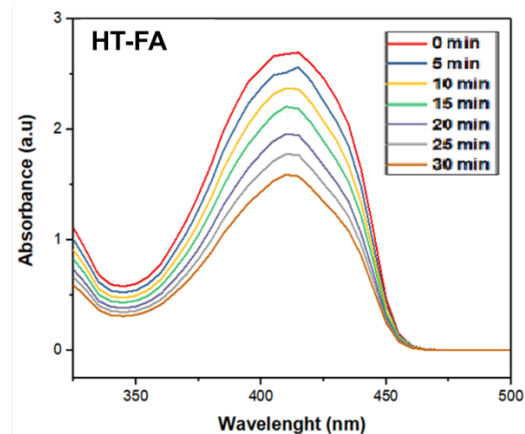
Figure 3.6 UV-VIS spectra of ZnPc and HT-FA-ZnPc solutions in DMSO.

3.6.4 Reactive Oxygen Species Detection

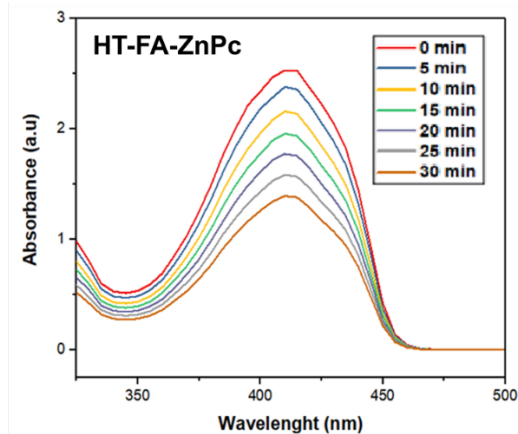
ROS detection was performed using DPBF as a chemical probe Figure 3.7 (a-c). To generate ROS ($^1\text{O}_2$), HT, HT-FA, and HT-FA-ZnPc were exposed to irradiation from a 365 nm UV lamp for different times in the presence of the chemical probe. DPBF is a reductant material that can react with $^1\text{O}_2$. It has a typical UV absorption at 415 nm when irradiated by the 365 nm UV light. An indication of ROS presence is the decrease of DPBF absorption, which reduces in concentration as it reacts with the $^1\text{O}_2$. The observable reduction of DPBF absorption as the irradiation time increases is seen on Figure 3.7 (a-c), demonstrating the successful generation of $^1\text{O}_2$ by our proposed structures.



(a)



(b)



(c)

Figure 3.7 (a–c) Detection of ROS generation via a DPBF fluorescent probe.

3.6.5 Cytotoxicity

GBM cell lines are commonly used as an *in vitro* brain tumor model to evaluate the anti-cancer effect of novel therapeutics. In vitro cytotoxicity was assessed using MTT as a cell viability indicator after treatment with ZnPc, HT, and HT-FA-ZnPc at different concentrations Figure 3.8 (a-c). Strikingly, nanocarriers treated with 1 to 100 $\mu\text{g/mL}$ concentrations had no significant effect on cell viability when compared to the control. However, in the case of HT-FA-ZnPc, significant viability decreases were found for 500 $\mu\text{g/mL}$ concentration. The data indicates that nanocarriers are biocompatible with MK cell line.

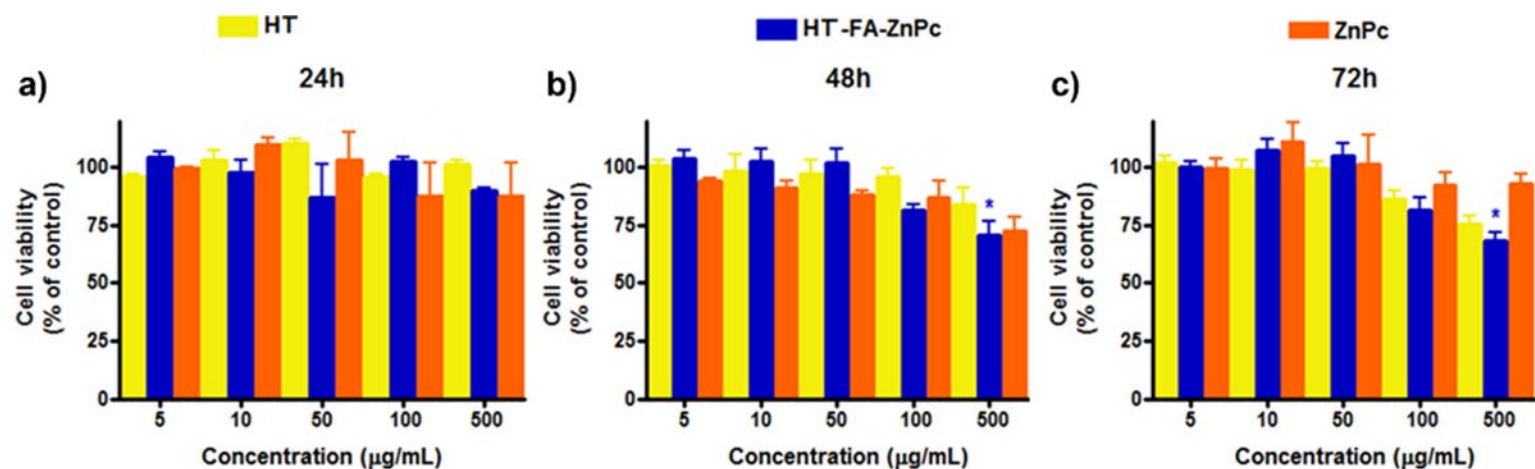


Figure 3.8 (a-c) Dose and time cytotoxicity effect of HT, HT-FA-ZnPc, and ZnPc over cell viability in M059K cell line. Cells were treated at 5, 10, 50, 100, and 500 µg/mL and assayed at 24, 48, and 72 h. Results are the mean $\pm$ SEM, $n = 3$. * $P < 0.01$ versus control.

3.6.6 Cell Morphology

To assess the effect of the nanomaterials over M059K cell line, we used untreated cells as control and Cis-Pt as a positive cytotoxicity control for comparison Figure 3.9. After incubation for 24 h with HT and HT-FA-ZnPc concentrations at: 1 µg/mL and 100 µg/mL, no growth inhibition or changes in morphology were observed. However, in the presence of CisPt, at a concentration of 100 µg /mL, the cells had shrunk, had irregular shapes and were detached from culture dishes. We also recognized the accumulation of nanoparticles aggregates mostly in the cell cytoplasm (green fluorescence) and differentiated from the cell nucleus (blue fluorescence) Figure 3.10. However, further experiments will be necessary to determine the exact organelle cell localization of the HT-FA-ZnPc nanocarriers.

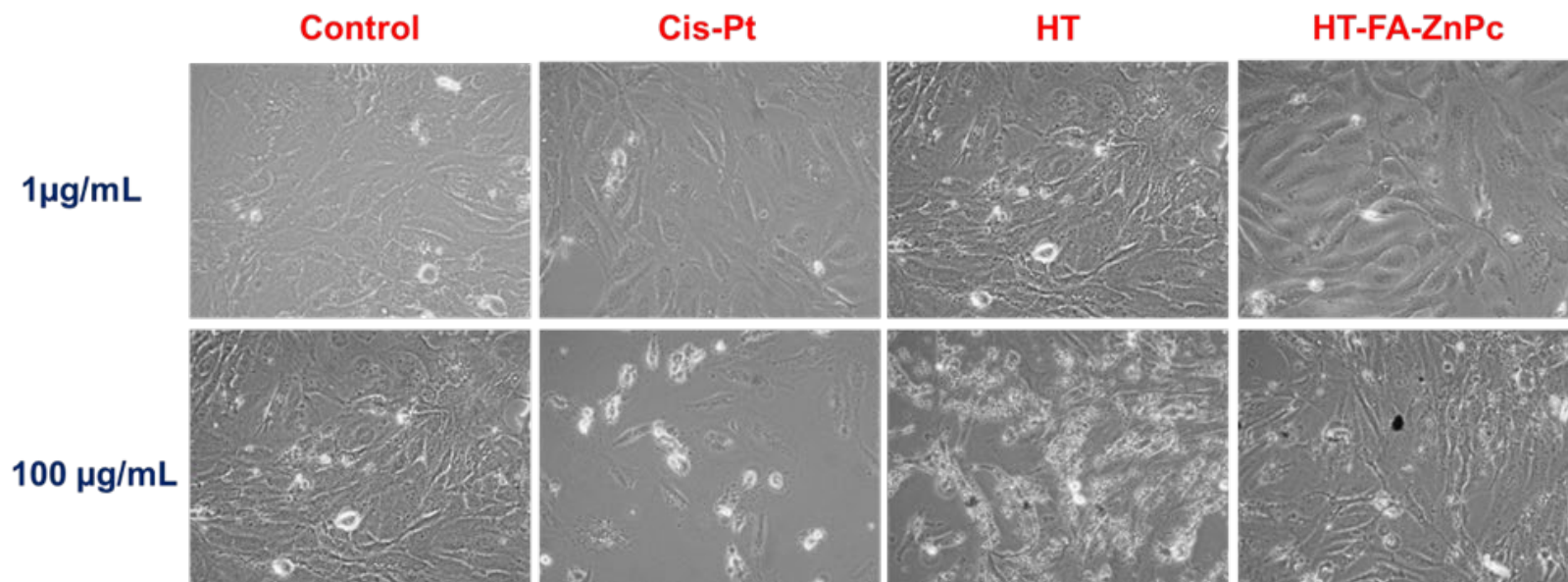


Figure 3.9 Representative images of the effects of nanocarriers on M059K cellular morphology after 24 h incubation time at 1 $\mu\text{g/mL}$ (top row) and 100 $\mu\text{g/mL}$ (bottom row).

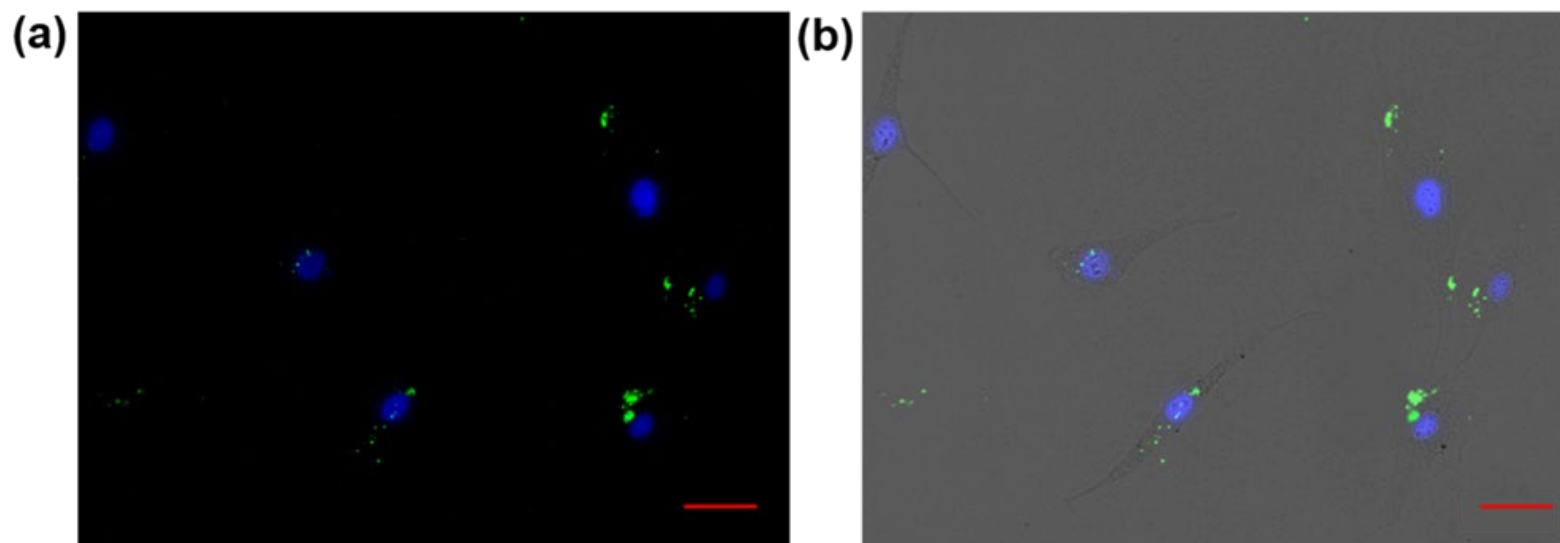


Figure 3.10 HT-FA-ZnPc nanoparticles uptake visualized in M059K cells. (a) image of overlapping fluorescent channels, and (b) merge of fluorescence and phase contrast images. In green HT-FA-ZnPc labeled with FITC and in blue nuclear stain with DAPI. Scale bar: 46um.

3.7. Conclusion

In summary, HT were synthesized using a facile and low-cost method. Functionalization of these hollow structures with FA and ZnPc for targeted PDT applications was achieved by modification of their surface. Viability of the HT-FA-ZnPc nanocarriers on MK cancer cells was demonstrated. Additionally, the *in vitro* activation of HT-FA-ZnPc by UV irradiation was successful. These results show promising use of HT as a nanocarrier for targeted treatment of GBM cancer by PDT.

3.8 References

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CHAPTER FOUR

Targeted Delivery of Temozolomide by Folic Acid- Hollow TiO₂ - Nanospheres Nanocarriers for the treatment of Glioblastoma.

4.1 Introduction

The targeted delivery of temozolomide by nanocarriers offers many advantages over its conventional delivery to glioblastoma cancer cells. Chemotherapeutic treatment to different cancer involves the delivery of free antineoplastic agents usually by oral or intravenous administration [1]. These free therapeutics are generally limited by a low chemical stability, low circulation time, and a lack of cell selectivity [2,3]. In particular, Glioblastoma multiforme (GBM) is a highly malignant brain tumor which derives from the star-shaped glial cells, astrocytes, that support the nervous system [4]. It is infiltrative, and hence extended throughout deep regions of the brain making the delineation of its margins impossible [5,6]. Its current treatment is a combination of surgery, radiation, and chemotherapy [7,8]. Despite the efforts to improve its treatment, the patient average survival is of only 12-15 months [9]. Temozolomide (TMZ) is an alkylating agent that offers many advantages for the chemotherapy of GBM. It has been the first line chemotherapeutic agent for GBM, since in 2005 the Food and Drug Administration (FDA) approved its use for GBM treatment, after a clinical study proved that in combination with radio therapy it increased the patient survival by a factor of 2.5 [10]. It is stable in acidic environments, thus can be administrated orally, making its administration non-invasive [11]. Although, TMZ has been proven to be highly cytotoxic to GBM cancer cells [12], several limitations hinder its administration: its non-polar properties make it insoluble in water, and consequently easily taken by the reticuloendothelial system [13]. Also, it has a low biological half-life of only 2 h due to its rapid degradation under physiological pH conditions [14]. Hence, it requires a high

dose administration to reach desired therapeutic concentrations [15]. Another major limitation for its successful administration is its inability to differentiate between healthy and cancer cells [16]. This along with its high dose administration requirements causes systemic toxicity throughout the liver, lungs, and blood responsible for a high bone marrow suppression, and undesired side effects such as vomiting, nausea, headaches, and severe fatigue [15]. In recent years, many nanocarrier configurations have been engineered to overcome the limitations of the conventional delivery of chemotherapeutics. Nanocarriers are transport systems (1-1000 nm) used to deliver different therapeutic cargos such as chemotherapeutic drugs. They have the capability to be functionalized by various agents to acquire multiple functionalities [17-19]. In particular, their surface can be functionalized with folic acid (FA), a synthetic form of folate (vitamin B). Due to its high affinity to folate receptors, which have been found to be 500 times overexpressed on the surface of cancer cells compared to healthy cells, FA has been used as a targeting agent in many nanocarriers configurations for the treatments of cancer [20-23].

For the last two decades, the use of nanocarriers and folic acid for the delivery of temozolomide has been under development to hinder the limitations of free TMZ administration. The encapsulation of TMZ by these systems offers protection from its rapid degradation, and its functionalization with folic acid increases its accumulation at specific target sites, and therefore decreases its overall toxicity [11,16,21]. However, some of the major disadvantages of these systems are their low loading capacity, instability, and low circulation time [24-29].

To address these problems, we report on the engineering of Hollow Titanium Dioxide Nanosphere (HT) as a nanocarrier for TMZ. Titanium Dioxide (TiO_2) is an inorganic hydrophilic material broadly used in biomedical implants [30], and FDA approved for its use in food and pigments [21CFR73.575, 21CFR73.2575] Due to its interesting properties, such as biocompatibility, chemical stability, and surface properties [31]. Its different morphologies have acquired great research interest for their use in the delivery of different agents for biomedical applications [32-35]. In particular HT is composed of a hollow core morphology with the capability to load chemotherapeutic drugs, it also possesses a high surface to volume ratio which allows for the functionalization of targeting agents [36]. To the best of our knowledge HT has not been used for the delivery of temozolomide for the treatment of GBM. In this work, HT have been synthesized by the hydrothermal method with the sacrificial carbon template. Their surface, and porous morphology was studied. Functionalization of its surface was performed through adsorption with FA as a targeting agent against GBM. The HT nanocarriers were loaded with TMZ through impregnation and its loading capacity, protection and retention time was investigated. Cell viability was performed with different HT formulations and concentrations to assess the anticancer activity or cytotoxicity of HT against LN18, U87, U373 and M059K GBM cell lines. Finally, the cell internalization of HT configurations was evaluated to study its targeting capabilities against GBM cancer.

We hypothesized that the hydrophilic properties of HT nanocarrier will prevent TMZ from rapid degradation and the high stability of HT will provide TMZ with a longer protection time. HT nanocarrier are expected to have a high loading capacity and thus

TMZ treatment will require less administration. We propose the nanocarrier functionalization with FA to target GMB cancer cells and lower the toxicity to healthy cells. Hence, HT nanocarriers were developed as a potential delivery system for the targeted chemotherapy of TMZ to GBM.

4.2 Experimental Methods

4.2.1 Chemicals and reagents

β -Cyclodextrin ($\geq 97\%$), titanium (IV) isopropoxide (TTIP, 97%), folic acid (FA, $\geq 97\%$), N,N' - N, N' -dicyclohexylcarbodiimide (DDC, $\geq 99\%$), dimethyl sulfoxide (DMSO, 99.7%), Temozolomide (TMZ, 98%), (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl (MTT), tetrazolium bromide isopropanol, glutaraldehyde, cacodylic acid, osmium tetroxide, and U-373 cell line were obtained from Sigma-Aldrich (St. Louis, MO, USA); Epon 812 and uranyl acetate were purchased from Merck; propylene oxide and Dodecenylsuccinic anhydride (DDSA) were purchased from Electron Microscopy Sciences (Hatfield, PA, USA); Nadic methyl anhydride (NMA), Tris(dimethylaminomethyl) phenol (DMP-30), and lead citrate were acquired from Polysciences (Warrington, PA, USA); LN-18 (CRL-2610), M059K (CRL-2365), and U-87 MG (HTB-14) cell lines were obtained from American Type Culture Collection (City of Manassas, VA, USA). The Dulbecco's Modified Eagle's (DMEM) and (DMEM/F-12) mediums, phosphate-buffered saline buffer (PBS), antibiotic-antimycotic, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES), trypsin-EDTA and sodium pyruvate were purchased from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Biowest (Riverside, MO, USA). Milli Q-Water

from Synergy®, Millipore Sigma (Burlington, MA, USA) and the ethanol was Koptec 200 was purchased from Deacon Labs (King of Prussia, MA, USA).

4.2.2 HT Synthesis and Functionalization

Preparation of Carbon Sphere templates (CS), hollow TiO₂ nanospheres (HT), and folic acid functionalization (HT-FA) was carried out using the synthesis protocol from chapter one. Briefly, CS were prepared from β -cyclodextrin carbon precursor through hydrothermal synthesis method. 1g of carbon precursor is dissolved in ethanol and DI water at a ratio of 1:3. The solution is stirred at 300 rpm for 30 minutes and transferred to a Teflon lined stainless steel autoclave (25mL) for hydrothermal treatment at 200°C for 16 hours. CS are then purified to remove any organic residues by washing three times through centrifugation at 8000 rpm for 15 minutes using ethanol and DI water mixture at 1:1 ratio and are placed on hotplate to dry at 60 °C overnight. Synthesis of HT was carried out by liquid-phase deposition. First, previously collected 0.03 g CS are added to 15 mL ethanol solution and ultrasonicated for 30 minutes. Then, 2.2 mmol TTIP, used as titanium precursor, is added in a dropwise manner. Solution is ultrasonicated for 30 min and is kept in ambient temperature for 6 hours for Ti coating to take place on CS surface. Afterwards, solution is purified and dried using the same purification and drying procedure as for CS. The collected powder residue is placed on muffle oven at 550 °C for 4 hours to burn the CS templates and the TiO₂ crystals to be calcinated, obtaining the final nanospheres (HT). The HT surface was functionalized by adsorption of FA. First, activation of the carboxyl groups of FA was carried out by adding 20 mg of FA and DCC mixture at 1:1 weight ratio in 4 mL DMSO, and the solution was stirred under N₂

atmosphere at ambient temperature and dark conditions for 2 hours. Then 40 mg of previously prepared HT is added to continue stirring under same conditions for 2 hours. The final solution is then collected, washed, and dried following same procedure used for CS.

4.2.3 Temozolomide Loading

TMZ was loaded to HT, and HT-FA nanocarriers by impregnation method. TMZ and nanocarriers at a 1:10 weight ratio were added to 5mL DI water and stirred for 24 hours under ambient and dark conditions. Following, the HT-TMZ, and HT-TMZ-FA precipitates of light purple and light yellow respectively were washed three times with DI water by centrifugation at 3000 rpm for 10min to remove any unloaded drug. Then, were dried on hot plate at 60 °C overnight.

4.2.4 Physicochemical Characterization

The size and surface morphology of CS and HT were analyzed by transmission electron microscopy (TEM, FEI Tecnai12; 120 kV) and imaging software (ImageJ). Samples were ultrasonicated in 2mL of ethanol for 10 min, mounted on copper grid with lacey carbon supporting film, and left to dry under tungsten lamp for 30 min before analysis. The surface properties were obtained from the N₂ adsorption-desorption isotherms (BelsorptII-BEL). The specific surface area was calculated from Brunauer–Emmett–Teller (BET) and the mean pore diameter (D_p) and the pore volume (V_p) were calculated from the desorption isotherm applying the Barrett-Joyner-Halenda (BJH) theory. The samples were vacuum-treated at 50°C for 12 h prior to the N₂ adsorption-desorption measurement at -196.15 °C.

The functionalization of our nanocarriers by FA was first characterized by X-ray diffraction (XRD, PANalytical Empyrean Series 2). All sample characterizations were attained under the same parameters (Cu Ka radiation at 40 mA and 40 kV). Further functionalization analysis was performed with X-ray photoelectron spectroscopy by analyzing the chemical state of nanocarriers before and after FA functionalization (XPS, Kratos AXIS ULTRA^{DLD} ; Al anode source, 165-mm mean radius electron energy hemispherical analyzer).

4.2.5 Loading of TMZ Characterization

The inspection of the loading and distribution of HT-TMZ nanocarrier was performed by TEM (FEI Tecnai12; 120 kV) with copper grid with lacey carbon supporting film. Also, TEM-energy dispersive spectroscopy (S/TEM-EDS, Titan Themis 300; 300 kV), coupled with Z-contrast imaging (Fischione HAADF detector) was employed with samples mounted on Silicon- Nitride Support Film grids. To investigate the thermal stability and loading capacity of HT-TMZ, and HT-TMZ-FA nanostructures, thermogravimetric analysis (TGA, Netzsch TG 209 F1 Libra) was employed. Samples were dried at 80°C for 20min, and measurements were carried out under N₂ atmosphere, at a temperature range of 25°C - 900 °C, and 20 °C/min heating rate. We calculated the difference between the mass loss of HT-TMZ, and HT-TMZ-FA compared to the unloaded nanostructures of HT, and HT-FA respectively. We related the loading capacities (%) of the nanostructures to be proportional to their associated TMZ weight loss (%) after thermal treatment.

4.2.6 Temozolomide Degradation Protection

4.2.6 .1 Stabilized System Formation

The stabilization of TMZ by our nanocarriers was investigated through TGA under the same measurements conditions as described in previous section; to better understand the stages of material loss over the heating range, first derivative thermal gravimetry, DTG, were carried out.

First, we prepared the baseline materials: HT, HT-FA, TMZ, and FA and measured their thermal stabilities and thermal decomposition temperatures. Following, we used a mortar to prepare a physical mixture with TMZ and the materials used for the unfunctionalized (HT+TMZ) and functionalized nanocarriers (HT+FA+TMZ) (1:1). We then measured their thermal stability and compared it with the degradation temperatures of baselines. We repeated the measurements with the nanocarrier systems (HT-TMZ, HT-TMZ-FA) . We expect the physical mixture to have the same degradation temperatures as the baselines, whereas in our proposed systems a change in their degradation behavior should occur.

4.2.6 .2 TMZ protection overtime

The protection of TMZ from degradation by our nanocarriers was tested by measuring the TMZ remaining after its release from HT-TMZ, and HT-TMZ-FA systems. We first suspended 5mg of loaded nanocarriers in 2mL of DI water, at ambient and dark conditions. We then allowed the drug release to take place for the following times: 0h, 24h, and 48h. After drug release, samples were centrifuged at 3,000 rpm for 10min and precipitates were collected and dried at 80 °C for 60 min. The drug remaining in the

nanocarriers was determined using TGA, under same parameters as in the loading section. The weight loss (%) is calculated for each sample time and is proportional to the unreleased TMZ from our systems.

4.2.7 Biological Studies

4.2.7.1 Cell culture

The LN18, U87, U373 and M059K human glioblastoma cell lines were cultured in DMEM or DMEM/F12 medium, respectively. The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were harvested using trypsin-EDTA at the logarithmic growth phase and maintained with complete medium until use at 37 °C and 5% CO₂ environment.

4.2.7.2 Morphology changes of cells

The morphology of LN18, U87, U373 and M059K cell lines was observed after treatment with HT, HT-TMZ, HT-FA, and HT-TMZ- FA nanocarriers. The cells were seeded onto 12-well plates at 0.1×10^6 cells per well and allowed growth for 24h at 37 °C and 5% CO₂. Afterwards, they were treated with nanocarrier suspensions at different concentrations between 10 to 500 µg/mL for 72 h at 37 °C and 5% CO₂. The morphology changes, of control and treated cells, were captured at 10x by inverted light microscope (CKX41 Olympus).

4.2.7.3 Cell Viability

The cytotoxicity of HT, HT-FA, HT-TMZ, and HT-TMZ- FA against LN18, U87, U373 and M059K cell lines, was assessed by tetrazolium reduction to formazan assay . Briefly, cells were seeded at 1×10^4 cells per well in a 96-well plate and incubated for 24h at 37

°C and 5% CO₂. After cell growth, the media was removed, and cells were treated by different nanocarriers concentrations, ranging from 0 to 200 µg/mL, contained in 100uL of fresh media. After 72h of nanocarriers exposure at 37 °C and 5% CO₂, the medium was removed and washed three times with PBS buffer. Afterwards, cells were incubated with medium containing MTT at 0.5 mg/mL for 3 h at 37 °C and 5% CO₂. Then, the supernatant was removed, and the formazan crystals were dissolved in isopropyl alcohol with 200 µL/ acidified 0.04 HCl. Finally, the absorbance was read at 570 nm using a multi-well plate reader (Synergy HT, Biotek).

4.2.7.4 In vitro Cellular uptake of nanocarriers

We assessed the cellular uptake of HT-TMZ-FA nanocarriers by LN18, U87, U373 and M059K cell lines through TEM ultrastructure cell analysis (JEM-1400 Plus, 100 kV).

4.2.7.4.1 Nanocarriers cell treatment

Cells were seeded onto T25-flasks at 0.8×10^6 cells and allowed to adhere overnight at 37 °C and 5% CO₂. Afterwards, cell lines were treated with HT-TMZ-FA at 50 µg/mL and incubated for 72h at 37 °C and 5% CO₂. The cells were then washed with PBS buffer, detached with trypsin, resuspended in growth medium, and gently centrifugated at 1200rpm for 5min. The supernatant was removed, and the pellet was washed two times with PBS buffer. The cells were collected and fixed with 2.5% glutaraldehyde in PBS buffer at room temperature for 1 h and then washed three times with cacodylate buffer. Finally, the samples were post-fixed in 0.5% osmium tetroxide in cacodylic acid buffer at room temperature for 2 h and washed again with cacodylate buffer.

4.2.7.4.2 TEM sample preparation

The preparation of the treated cells for TEM characterization, involved the dehydration of the cells by different grades of ethanol (50%, 60%, 70%, 80%, and 90%) (x1/10 min), and by 100% ethanol (x 2/10min), and the incubation in propylene oxide (x 2/10min) at 37 °C and CO₂ environment. Then, the cells were prepared by infiltration of epoxy resin solution at 2:1 for 1h and propylene oxide at 1:1 overnight. Afterwards, cells were embedded with epoxy resin to polymerize and were placed on conventional gravity oven at 60°C for 18h. After polymerization, blocks were trimmed and cut with an ultra-microtome in semi-thin sections of 70nm to 90nm, were placed on glass slides, and stained with 1% toluidine blue under light microscope. Thinner sections of between 50 nm to 60nm were cut using a diamond knife on an ultra-microtome and placed on copper grids. Afterwards, were stained with 3% uranyl acetate at room temperature for 15min, and 0.3% lead citrate at room temperature for 15min followed by three washes with ultrapure water. Finally, images were acquired at 5x, 10x and 25x magnification.

4.3 Results & Discussion

4.3.1 Physicochemical Characterization

TEM characterization shows the hydrothermally prepared CS to have a solid core, and spherical surface morphology with average size and standard deviation of 904.2805 ± 326.3061 Figure 4.1 (a). The HT synthesized by liquid-phase deposition present an average size and standard deviation of 489.6689 ± 203.4144 respectively Figure 4.1 (b). We recognize a hollow core composition from the clear color contrast between its core

and its outer shell. We observe the outer shell to have rough spherical-like surface, a thickness of approximately 20 nm, and to be conformed by TiO₂ nano crystals.

From TEM characterizations results we can observe an approximate 36% reduction from the volume of the carbon spheres, used as sacrificial templates, to the final Hollow Titanium Dioxide nanocarriers. These results suggest carbons spheres could be fabricated of tunable size, thus allowing for controllable size of HT nanospheres. As was mentioned in the introduction, a major problem for the use of TMZ against GBM is its solubility. We propose the utilization of the hollow cavity, observed on TEM, for the loading and protection of TMZ and for the possible enhancement of its solubility.

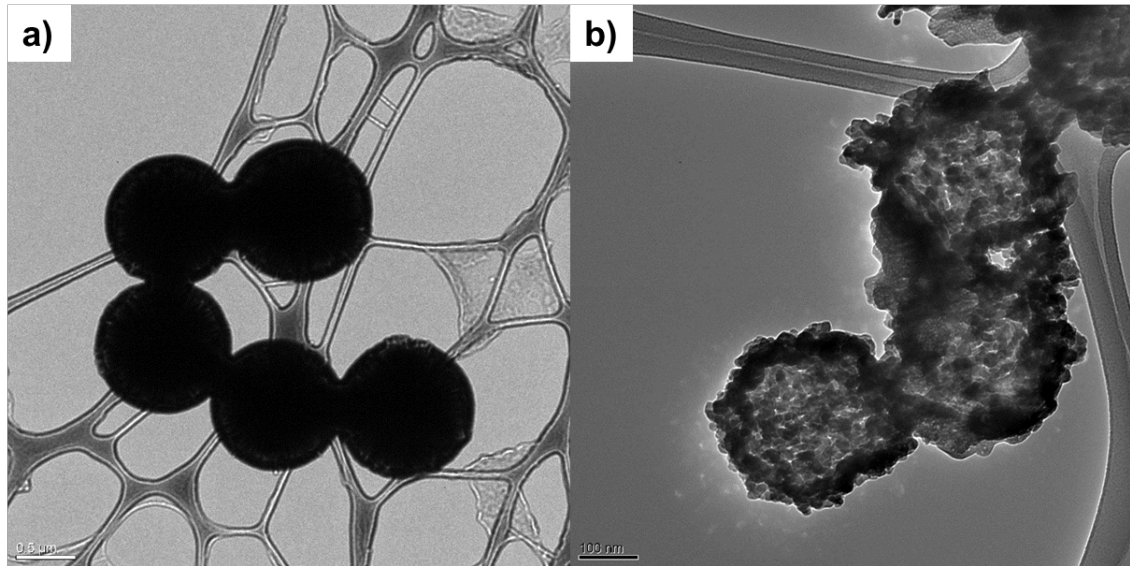


Figure 4. 1 TEM images of: (a) Carbon Spheres (CS). (b) Hollow Titanium Dioxide Nanospheres (HT).

4.3.2 HT Functionalization

4.3.2.1 BET Studies

The N₂ adsorption-desorption isotherms of HT, HT-FA, HT-TMZ, and HT-TMZ-FA samples were obtained Figure 4.2 (a). These isotherms present a narrow hysteresis loop on top of the P/P₀= 0.9 region indicating a mesoporous surface composition for all of our samples, with an IUPAC type IV classification. Figure 4.2 (b). shows the pore-size distribution (0-100 nm) plots obtained using the BJH equation from the desorption branch of the isotherms. Results show a broad peak in the range of 20-70 nm indicative of mesopores. Figure 4.2 (c). Presents a zoom image of pore distribution for 0-12 nm, which exhibits sample HT to have a considerable amount of micropore composition which decreases considerably after FA functionalization and TMZ loading.

The textural values of surface area, average pore diameter, and pore volume of our nanocarriers are reported on Table 1. Titania hollow nanospheres had a significant surface area of 14.515 m²/g. This area decreased, when the nanospheres were coupled to TMZ or FA, to 12.680 and 12.422 respectively. Similarly, the surface area decreased when the functionalized nanospheres (HT-FA) were loaded with TMZ from 12.422 m²/g, falling to 11.617 m²/g (approximately 6.48% surface area reduction). Interestingly, there is also an increase from 42.758 to 54.434 in the pore diameter of HT after couplings take place HT-TMZ- FA.

The porosity and surface properties of a material are of high importance when considering the delivery of chemotherapeutics. These can dictate their mechanisms of loading and protection. The results from N₂ adsorption-desorption isotherms, and textural

values show a presence of mesopores and micropores on our HT structures, as supported by TEM results roughness of surface. The increase in the mesopore diameters after the loading of the TMZ suggest the loading of TMZ is taking place primarily on the mesopores, which may correspond to the hollow cavity. However, the presence of micropores and the decrease of the HT surface area, after loading, suggest loading is also taking place on the surface of the hollow titania spheres. Further investigation is needed to confirm the distribution of TMZ in nanocarriers.

Table 4. 1 Textural properties data from N₂ adsorption-desorption isotherms of different HT samples.

Sample	S_{BET} (m ² /g)	D_P (nm)	V_P (cc/g)
HT	14.515	42.758	0.1552
HT-FA	12.422	52.078	0.1617
HT-TMZ	12.680	50.016	0.1585
HT-TMZ-FA	11.617	54.434	0.1581

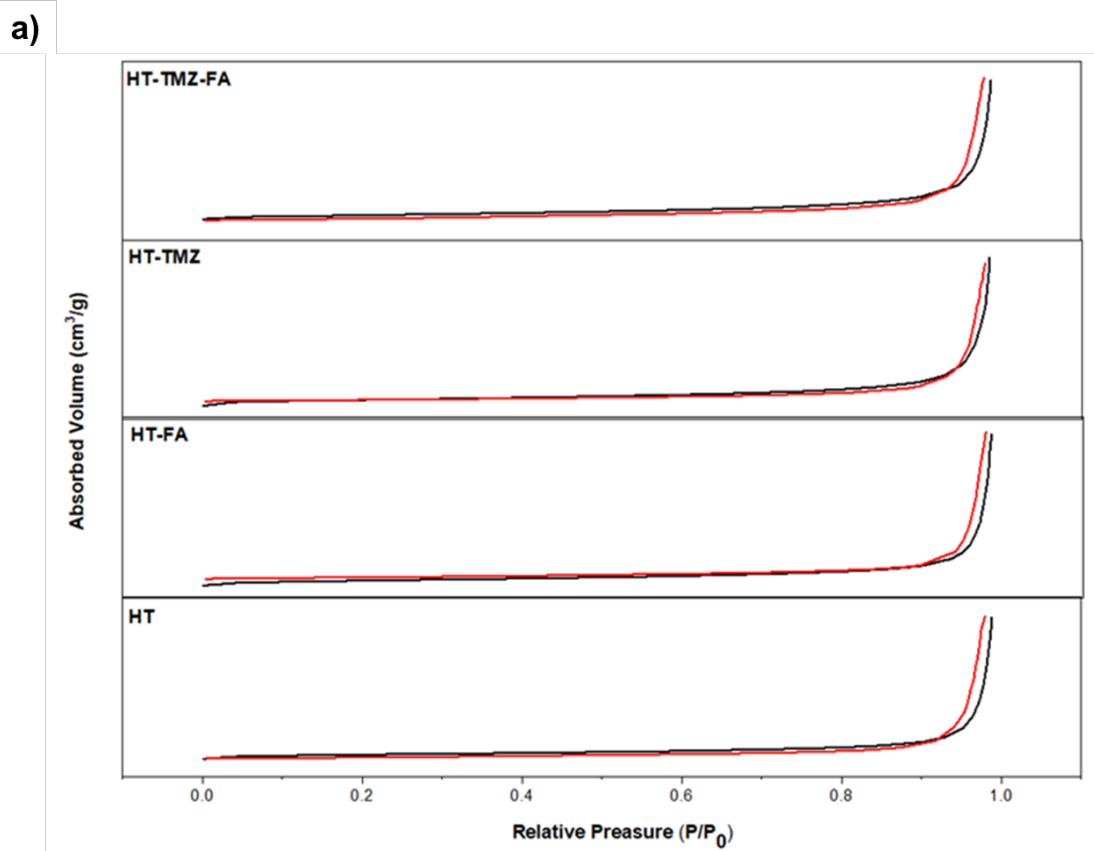


Figure 4.2 BET characterization of HT, HT-FA, HT-TMZ, and HT-TMZ-FA: (a) Nitrogen adsorption-desorption isotherms.

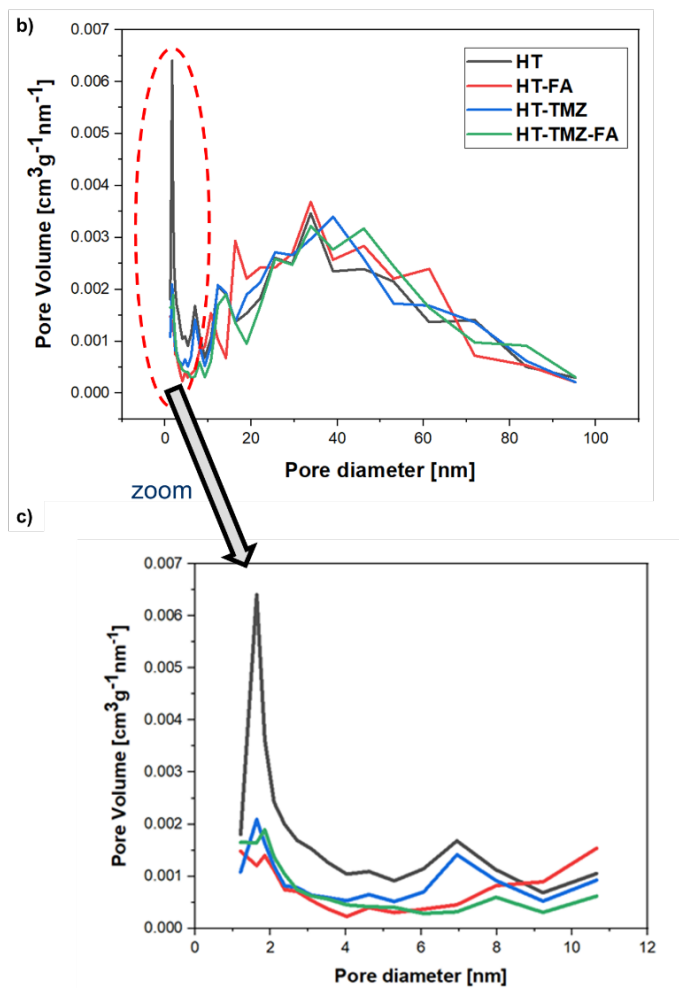


Figure 4.3 BET characterization of HT, HT-FA, HT-TMZ, and HT-TMZ-FA: (b) BJH pore size distribution. (c) Zoom image from 0-12 nm.

4.3.2.2 XRD Characterization

Folic acid functionalization was first characterized by XRD. The patterns of HT, FA, and HT-FA samples were analyzed Figure 4.3. The diffraction peaks of HT at $2\theta = 25.62$, 38.05 , 48.32 , 62.96 , and 75.20 correspond to the representative peaks of anatase TiO_2 crystal phase (JCPDS-21-1272). Diffraction peaks of pure FA at $2\theta = 5.63$, 11.08 , 16.53 , 21.96 , and 27.01 agree with data presented in the literature [37]. The peaks at $2\theta = 44.68$, 65.05 , and 78.16 correspond to the Alumina XRD holder (JCPDS-10-173).

FA diffraction spectra presents a crystal structure corresponding to the organic crystal structure of pure folic acid. After functionalization of HT by FA, the organic crystal loses its structure to become an amorphous material, as we can observe from the HT-FA profile. Whereas the anatase crystal phase of HT remains with no change. These results suggest the FA targeting agent to be homogeneously distributed and not aggregated in HT nanocarriers.

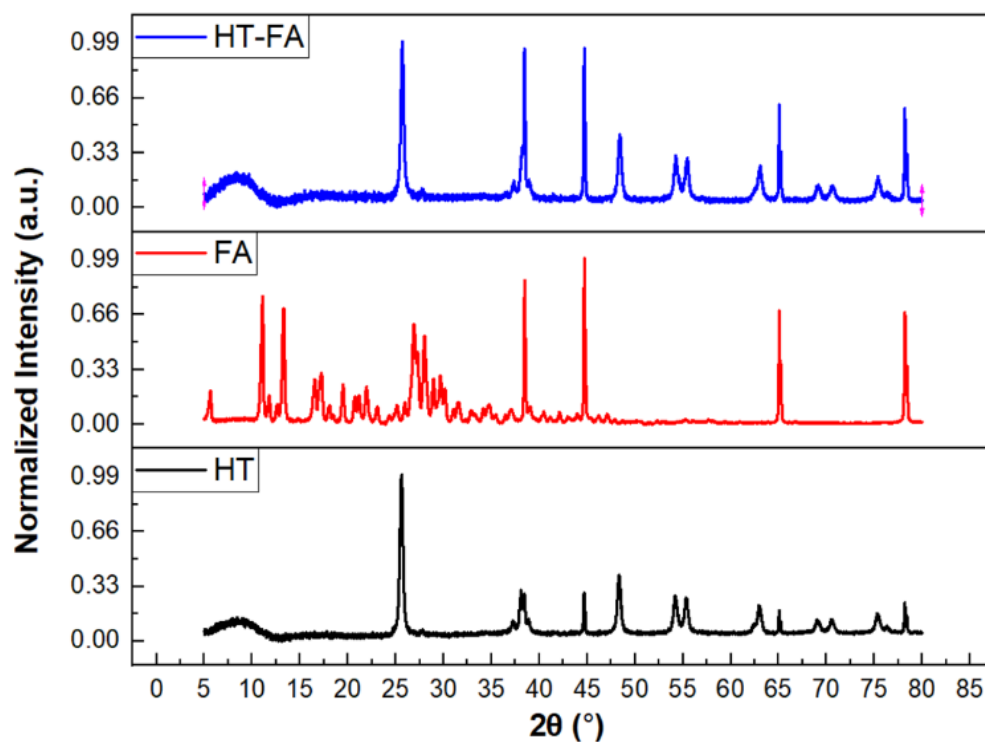


Figure 4.4 XRD patterns of HT, FA, HT-FA.

4.3.2.3 XPS Characterization

To further investigate the attachment between FA and TiO₂ nanospheres, the chemical states of our nanocarriers were studied through XPS characterization Figure.4.4. We compared the spectrums of unfunctionalized samples (HT) vs the functionalized (HT-FA). In the Ti 2p core energy level spectra, peaks 458.96 eV (2p 3/2), and 464.67 eV (2p 1/2) correspond to the Ti-O bonds, typical of TiO₂ material [38]. No change in peaks was observed after functionalization. In the O 1s energy level, a main peak presents at 530.05 eV. Its asymmetry indicates that several oxidation states may be present for oxygen. By deconvolution, we observe another peak at 531.60 eV. The first corresponds to the Ti-O bonding; and the second to the carbonylic group (C=O) . In both peaks, we observe a shift to a higher binding energy: (530.05-533.06) and (531.60- 534.80) respectively. The presence of carbon bonds may be indicative of carbon contaminants from XPS instrument, and residues from synthesis process. However, the shift to higher energies suggests an electrostatic interaction between the oxygen from TiO₂ and the carbonylic groups present in the carboxamide from the activated FA [39,40]. In the N 1s energy level, there is no presence of nitrogen on our HT sample. After functionalization however, an asymmetric peak appears at 400.05 eV. Two other peaks are observable after deconvolution : 400.6 eV and 398.28 eV. The first two, corresponding to the nitrogen atoms present in the C-N bonds from aromatic rings, and peak 398.28 eV to the amine groups (NH₂) . In the C1s energy level spectra, two asymmetric peaks appear at: 285.4 eV and 289.8 eV. The first corresponding to the C-C bond from possible carbon contamination and/or to the C-N bonds from the aromatic rings in FA. The second main

peak corresponds to the $\text{O}-\text{C}=\text{O}$, from possible attachment between HT and FA, as described in $\text{O}1\text{s}$ spectra. Deconvolution also shows the presence of peaks at: (286.0, 286.8 and 287.3) eV which correspond to $\text{C}=\text{O}$ bonds. The functionalized sample (HT-FA) shows an increase in the intensity for all peaks. Also, after functionalization, peak 287.3 eV shifted to the higher energy of 288.0 eV which corresponds to a change from $\text{C}=\text{O}$ to COOH . Peak 289.8 eV shifted to the lower energy of 288.7 eV indicative of a change from $\text{O}-\text{C}=\text{O}$ group to COOH bond.

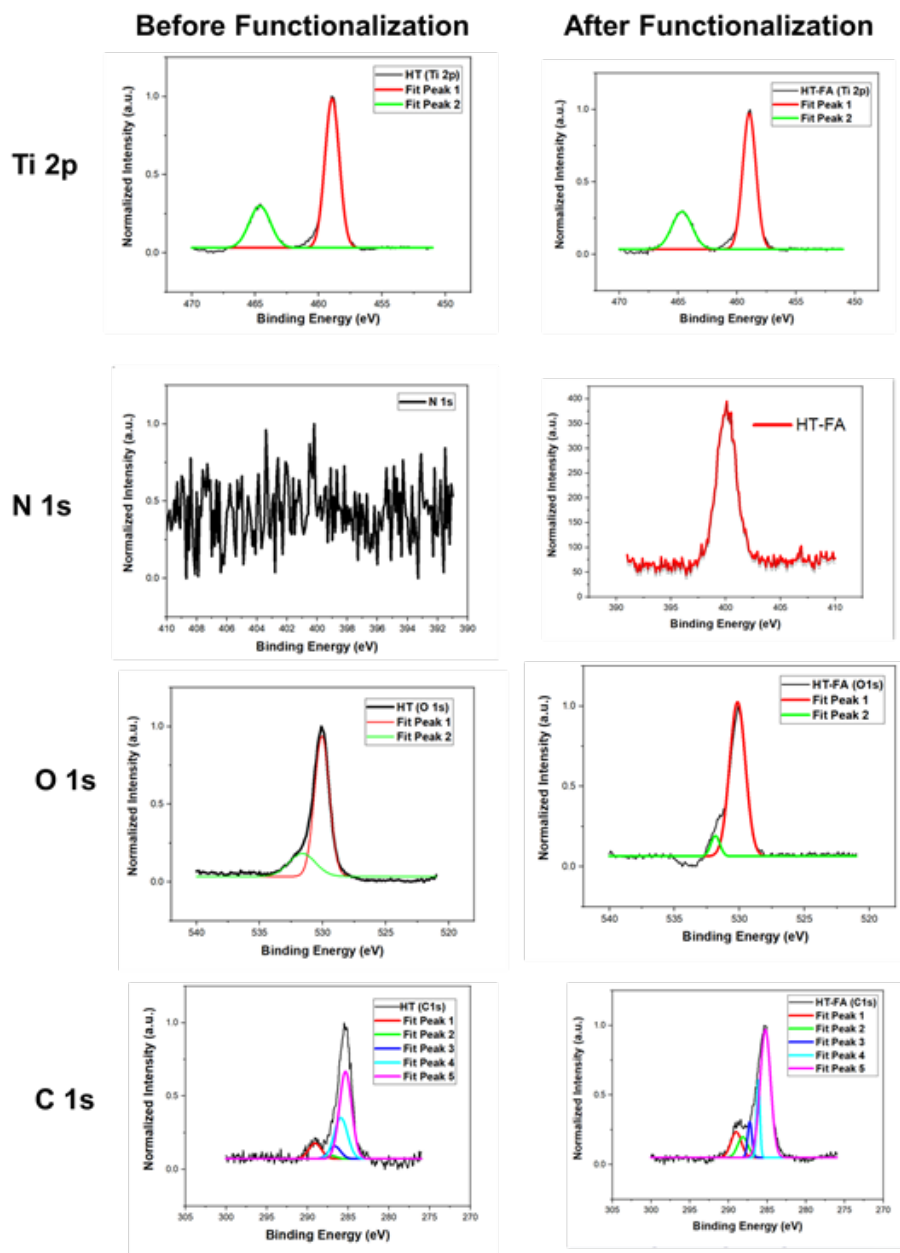


Figure 4.5 XPS spectrums of Ti (2p), O (1s), N (1s), and C (1s) before (HT) and after functionalization with FA (HT-FA).

The surface atomic compositions of HT, and HT-FA are presented in Table 2. The surface exposure of O, and Ti decrease after functionalization with FA, while the N and C increase considerably. These compositional changes are also visible in the increase of the atomic ratios from 0 to 0.58 for nitrogen, and from 0.83 to 2.79 for carbon both with respect to Ti.

Table 2. XPS surface composition of HT, and HT-FA.

	Atomic Concentration (%)				Atomic ratio		
	O	Ti	N	C	O/Ti	N/Ti	C/Ti
HT	56	24	0	20	2.3	0	0.83
HT-FA	37	14	10	39	2.6	0.58	2.79

The observation of no oxidation changes in Ti 2p spectra after FA functionalization, indicates that the functionalization does not cause disruptions in the anatase crystalline structure of TiO₂ nanocarriers, as supported by our XRD results. From N1s spectra, we observed the appearance of nitrogen on sample HT-FA vs HT which proves the presence of folic acid. This presence is further supported from the noticeable increase in the atomic ratio (N/Ti) in Table 2 of functionalized sample. The changes in the oxidation states in O1s, and the presence of carbonyl functional groups in both O1s and C1s spectra, suggest that the interaction between HT and FA is of electrostatic nature via these functional

groups. These results confirm the successful functionalization of FA onto HT surface by adsorption.

4.3.3 Loading of TMZ Characterization

To visualize the loading and distribution of TMZ onto the HT nanocarriers, we utilized TEM Figure 4.5 (a-b), and STEM elemental mapping Figure 4.5 (c-d). We observe the characteristic hollow core of HT Figure 4.5 (a) before the loading of TMZ. Whereas observation of a dense core appears after 24 h of drug loading, contrasting with the hollow core of the unloaded nanospheres Figure 4.5 (b). The uniform distribution of TMZ can be appreciated through the elemental mapping of the carbon contained in TMZ Figure 4.5 (c-d).

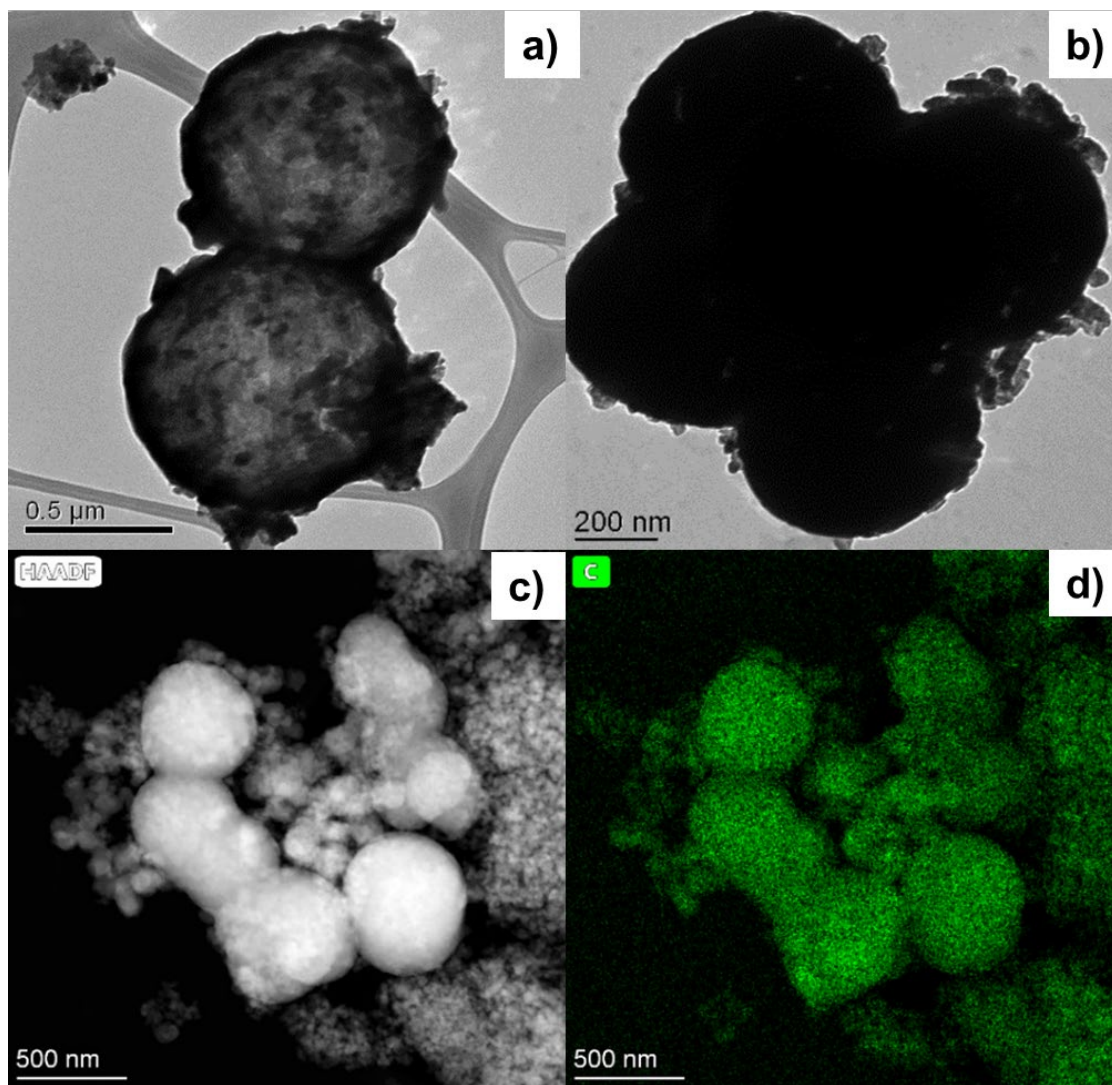


Figure 4.6 TEM images of TMZ loading characterization. (a) HT before loading (t=0 h). (b) HT-TMZ after loading (t=24h). TEM-EDS (c) Z-contrast imaging (d) Mapping of carbon in HT-TMZ sample.

To study the loading capacity of HT nanocarriers, TGA measurements were performed from 25 ° C to 900 ° C temperature range Figure 4.6. The weight loss of TMZ was ~ 6 % and was estimated from the difference between HT-TMZ and HT; and ~ 10 % difference between HT-TMZ-FA and HT-FA. Hence, considering the initial TMZ employed (10%), in the preparation our system, we infer a 60% TMZ loading capacity for HT-TMZ nanocarriers, and nearly 100% for HT-TMZ-FA.

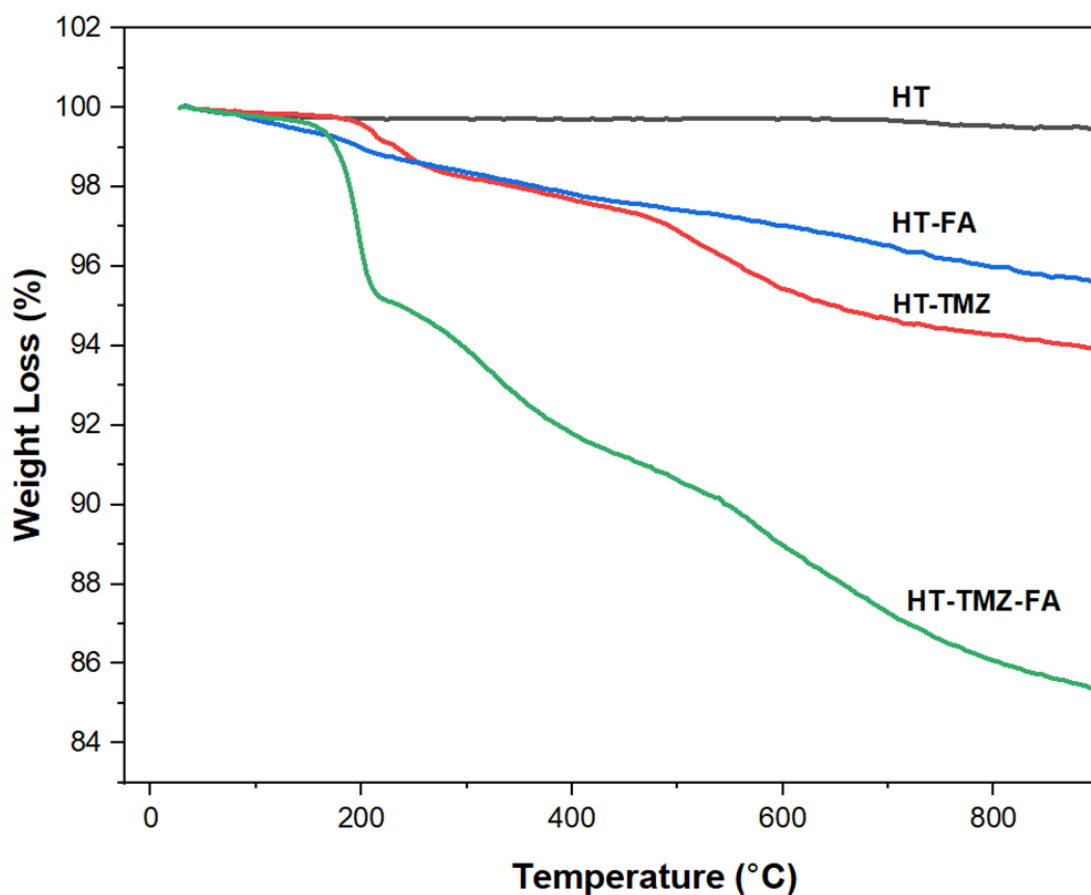


Figure 4.7 TGA curves for samples HT, HT-TMZ, HT-FA, and HT-TMZ-FA .

The aim to obtain nanocarriers with a high loading capacity has been one of the main research goals in recent years, as the encapsulation of temozolomide leads to a higher solubility and hence therapeutic efficiency [29]. From TEM results, we have visualized the loading of TMZ to take place in the hollow core of HT. However, from BET characterization, we find TMZ is also loaded in the surface of HT. These results were further confirmed by mapping the carbon contained in TMZ with TEM-EDS. Further analysis of TMZ loading can be drawn from TGA characterization, where the loading of TMZ was confirmed by the weight loss of loaded nanocarriers after thermal treatment.

4.3.4 Stabilized System Formation

The stabilization of TMZ by our nanocarriers was investigated through TGA and DTGA, as described in methods section. First, the degradation baseline temperature for TMZ was investigated. TMZ shows a mayor degradation temperature with DTG peak at 199.7 ° C Figure 4.7 (a), whereas the degradation of FA appears to happen in four mayor stages with derivative peaks at: 134.5 ° C, 269.5 ° C, 434.5 ° C , and 724.2 ° C Figure 4.7 (b), Results show a high thermal stability for HT thorough the whole thermal treatment Figure 4.7 (c-d). Physical mixtures present the degradation temperatures peaks of TMZ in HT+TMZ, and HT+TMZ+FA, and the degradation of FA is also confirmed at 124.5 ° C, 269.6 ° C, and 739.3.2 ° C for HT+TMZ+FA. We observe a shift in the degradation temperature of TMZ for our nanocarriers. The degradation temperature shifted from 199.7 ° C to 214.7 ° C in HT-TMZ system. The HT-TMZ-FA system presents a peak of 199.7, which can be attributed to TMZ or a shift in FA from 134.5 ° C. Other two peak

shifts were observed: from 269.5 ° C (FA) to 329.6 ° C , and from 409. 6 ° C (TMZ) and/or from 434.5 ° C (FA) to 574.2 ° C in Fig. 7(a-b).

The stabilization of TMZ by HT-TMZ, and HT-TMZ-FA nanocarriers was studied, comparison was made of their thermal behavior against a physical mixture configuration HT +TMZ, and HT+TMZ+FA respectively. As expected, the physical mixtures presented the similar degradation temperatures as the baselines, whereas our proposed nanocarriers showed a shift in the degradation behavior of TMZ and FA. This change is indicative of the formation of a new material arrangement, supporting our theory of the stabilization of TMZ by our nanocarriers.

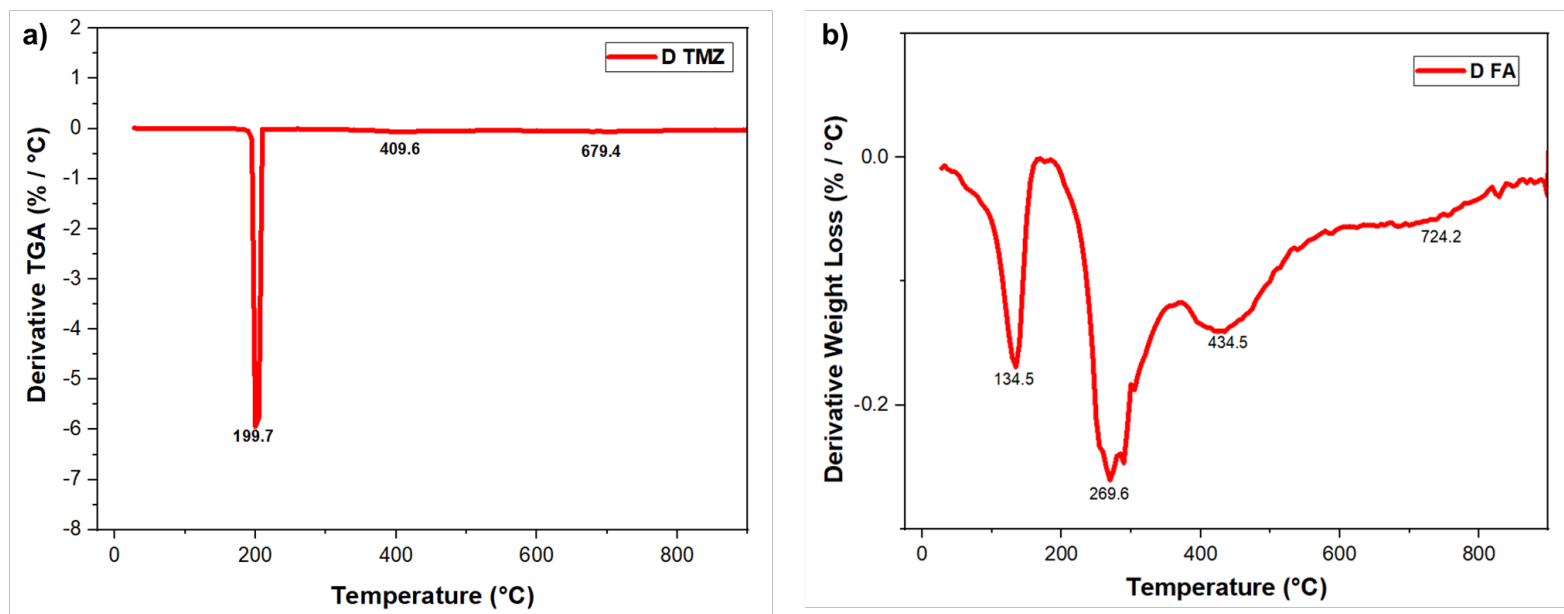


Figure 4.8 (a-d) Nanocarrier stabilization of TMZ by HT nanostructures. a) TMZ baseline derivative . b) FA baseline derivative.

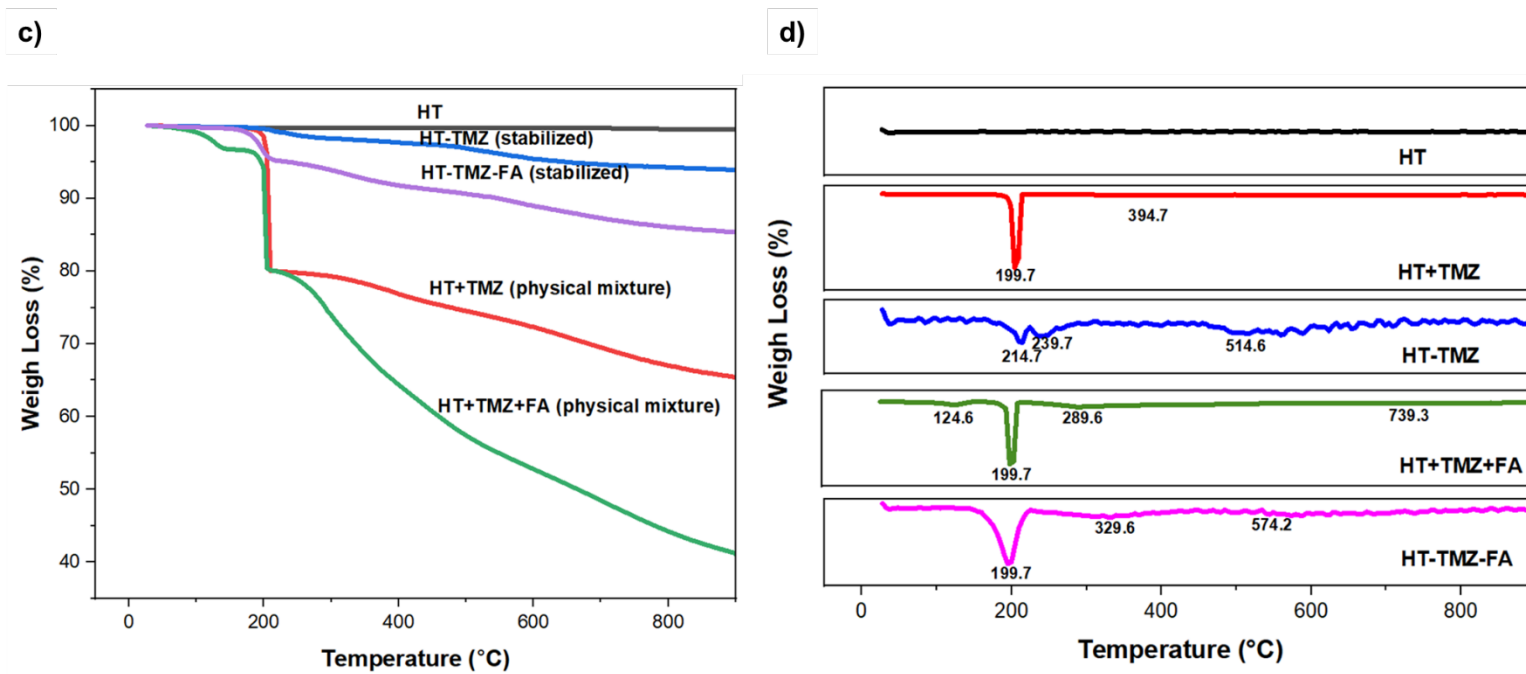


Figure 4. 9 (c-d) Physical Mixtures and Stabilized Systems. c) TGA d) DTGA.

4.3.5 TMZ protection overtime

The protection of TMZ from degradation, and its possible circulation time were investigated by TGA characterization Figure 4.8 , as described in methods section. After 24h, and 48h drug release from HT-TMZ nanocarriers, the remaining TMZ was estimated. Results HT-TMZ show a TMZ weight loss of $\sim 3\%$ for 24h and $\sim 2\%$ for 48h.

As previously mentioned, one of the disadvantages of TMZ administration are its high degradation and low circulation time. Utilizing the weight loss from loaded nanospheres, we have proven TMZ presence in nanocarriers after 48h release time. These results show a protection of TMZ from early degradation, and a promising circulation time.

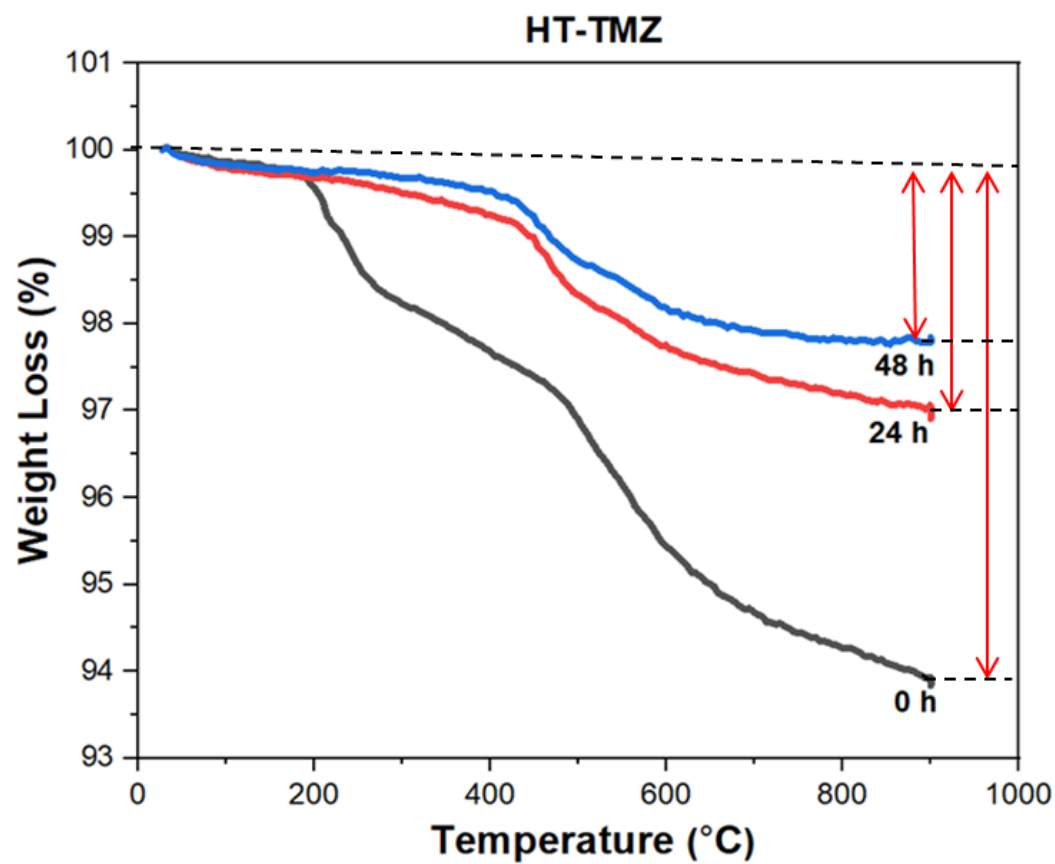


Figure 4.10 TMZ (%) remaining after 48 h of drug release in DI water.

4.3.6 Biocompatibility Studies

4.3.6 Morphology Changes of Cells and Cell Viability

Analysis of the morphological changes in glioblastoma cell lines by HT nanocarriers is shown in Figure 4.9. Change is observed in the morphology of cells exposed to the HT nanostructure in comparison with untreated cells, its more pronounced at 100 μ g/mL treatment, where after nanomaterial exposure for 72 h, the accumulation of nanocarriers on the surfaces of cells was noticeable. Cells begun to be detached from the surface and had lost their original morphology. By observation, the LN18 cells had the most damage. The light microscopy observations corresponded to the results of the MTT assay Figure 4.10 (a-d), where cellular cytotoxicity was determined to evaluate the effect of HT nanostructures at different concentrations. Firstly, we observed the cytotoxicity in the HT and HT-FA, not drug charged systems between 0.1 to 200 μ g/mL concentration range in GBM lines Figure 4.10 (a-b) and in human fibroblasts (BJ cell line) as no cancer cells control/example with no effect in cell viability (data no showed). The LN18 presented a higher decrease (between 20% and 40%) in their viability even at low concentrations of HT and HT-FA treatment. The results shows that M059K cell line was the less sensitive to TMZ delivery from HT-TMZ and HT-FA-TMZ contrary to the other cell lines analyzed here Figure 4.10 (c-d).

The HT nanocarriers are biocompatible nanomaterials that functionalized with FA are a good strategy to target brain tumors. Although, from the cytotoxicity studies in healthy fibroblasts, we observed that HT and HT-FA were very safe, surprisingly the LN18 cells were very sensitive unfunctionalized HT., this cytotoxicity was enhanced in presence of

HT-TMZ and HT -TMZ-FA. The LN18 cells are considered being a resistant glioblastoma cell line to TMZ , here we have demonstrated that HT formulations can sensitize LN cell line to the TMZ drug treatment. On the other hand, the M059K cell line had less effect, probably due to the higher activity of DNA-dependent protein kinase, therefore is less sensitive to TMZ effect. Interestingly, the IC₅₀ values observed for TMZ were approximately 10-fold times lower when was delivery from HT-TMZ and HT -TMZ-FA compared to reported in the literature. The glioblastoma cell line showed the following effect to TMZ: LN18>U373≥U87>M059K, that diverse response is due to the molecular heterogeneity nature of glioblastoma multiforme cancer.

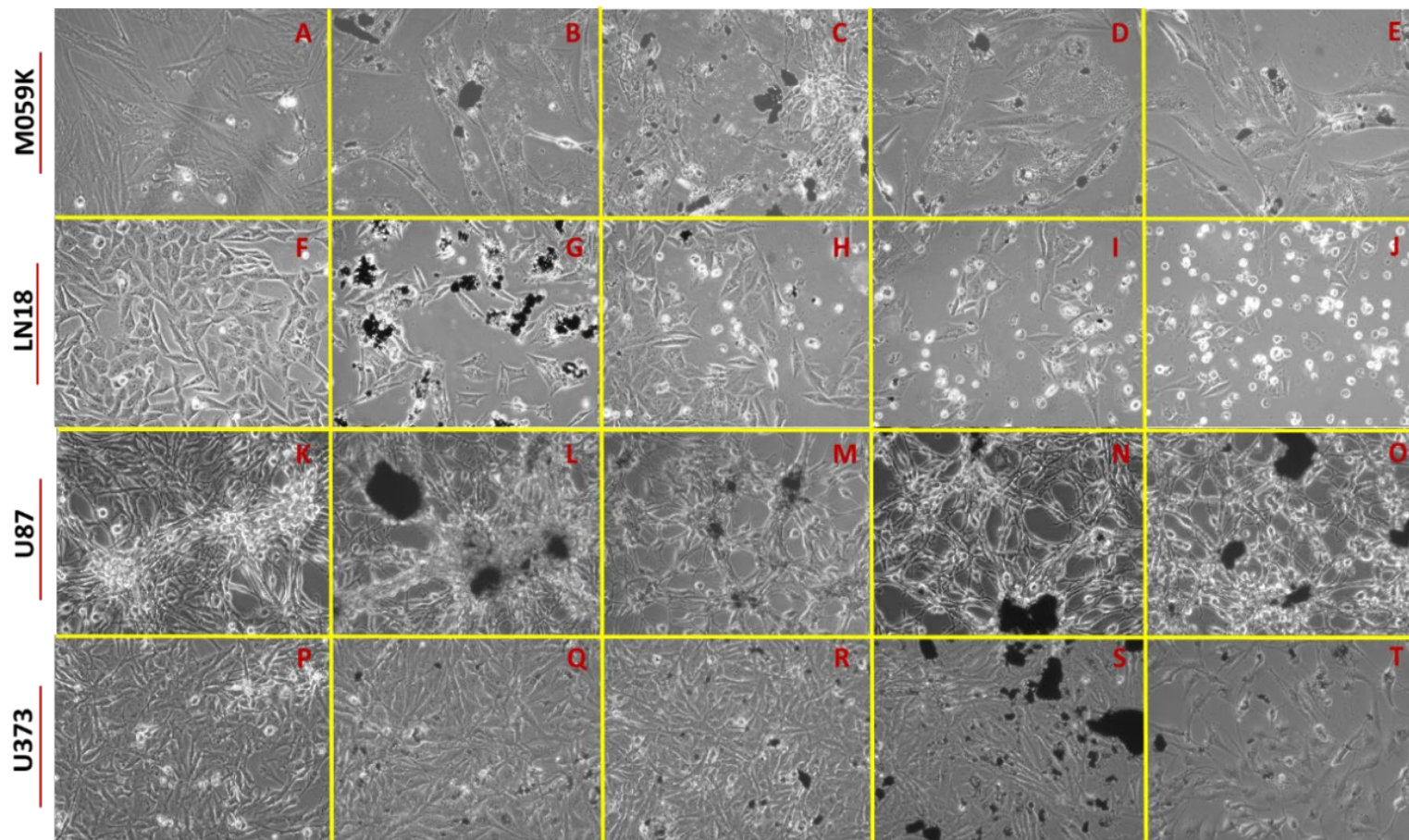


Figure 4.11 Representative light microscope images of glioblastoma cell lines. Control cells (A, F, K, P) and treated with 100 $\mu\text{g/mL}$ of HT (B, G, L, Q), HT-FA (C, H, M, R), HT-TMZ (D, I, N, S) and HT-TMZ-FA (E, J, O, T) for 72h.

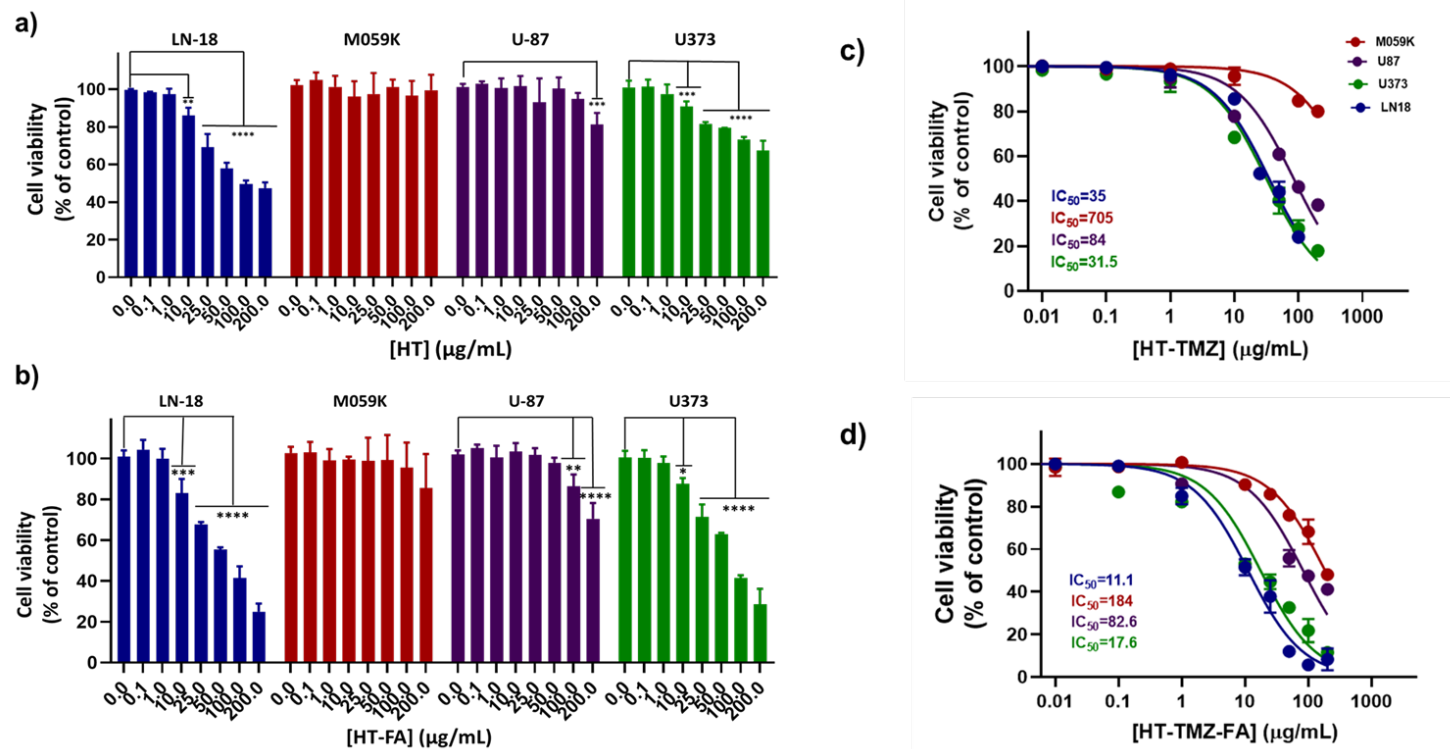


Figure 4.10. (a-b) Cytotoxicity of HT and HT-FA over cell viability in glioblastoma cell lines LN18 (blue), M059K (red), U87 (purple) and U373 (green). **(c-d)** Dose response curves of HT-TMZ and HT-FA-TMZ ; table inset of the IC₅₀ calculate value for HT-TMZ and HT-FA-TMZ with corresponding [TMZ] (μM) charged. The data are present as mean ± SD, n=3 or 5. The statistical significance is expressed as **** p<0.0001, *** p<0.001, ** p<0.001 and * p<0.05 compared to control (untreated) cells.

4.3.7 In vitro Cellular uptake of nanocarriers

The cellular localization of HT-TMZ-FA nanostructures was evidenced by ultrastructural TEM analysis Figure 4.11. After treatment for 72h, agglomeration of HT-FA-TMZ is observed in the cytoplasm involved inside of endosomes for all glioblastoma cell lines. Also, there is an apparent damage in mitochondrion and over population of autophagic vesicles.

The agglomeration of HT-FA-TMZ in the cell cytoplasm offers evidence of the ability of HT nanocarriers to deliver drugs into the cells. Also, the presence of autophagic vesicles suggest an autophagic and apoptotic cellular uptake mechanism.

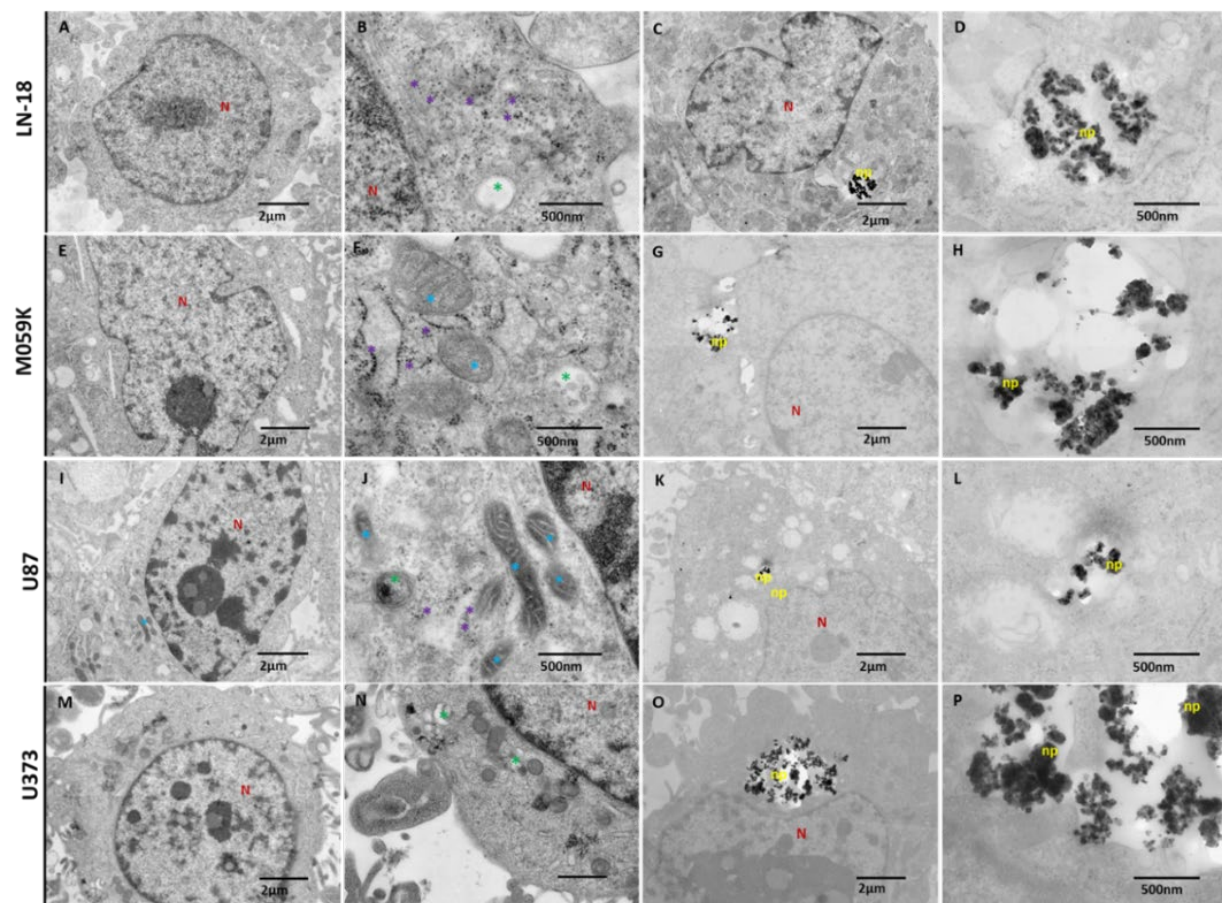


Figure 4. 12 Transmission electron microscopy representative images of HT nanocarriers uptake inside LN18, M059K, U87 and U373 cells. Control cells without nanostructures (A-B, E-F, I-J, M-N) and HT-FA-TMZ located inside the cytoplasm of LN18 (C-D), M059K (G-H), U87 (K-L) and U373 (O-P) cells. Scale bars in 5x and 25x magnification are 2μm and 500nm, respectively. Np, nanoparticles; Cellular organelles observed: N, nucleus; Blue asterisks, mitochondrion; Purple asterisks, ribosomes. Green asterisks, autophagic vacuoles.

4.4 Conclusions

In summary, hollow TiO₂ nanospheres nanocarriers of high surface area and mesoporous/microporous structure were synthesized. The targeting agent folic acid was successfully functionalized and uniformly distributed onto the surface of HT by absorption through electrostatic force attachment. The chemotherapeutic drug temozolomide was successfully loaded and uniformly distributed to HT nanostructures, with loading efficacies between 60%-100%. The synthesized HT nanostructures were thermally stable and offered TMZ protection from degradation for at least 48 h. The HT nanocarriers successfully deliver TMZ to glioblastoma cancer cells with cytotoxicity of up to less than 20% through autophagic and apoptotic cellular mechanisms. These results confirm the potential use of HT nanostructures as nanocarriers for chemotherapeutic drugs for the treatment of GBM cancer

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4.5 References

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CHAPTER FIVE

CONTRIBUTIONS

In summary, the research presented in this dissertation addresses the design and engineering of biocompatible Hollow TiO₂ nanospheres nanocarriers (HT) with a high anatase crystallinity and a high surface area, these properties allow for HT nanocarriers to be used as a multifunctional photocatalytic material and/ or delivery system. By modification of its surface with ZnPc photosensitizer, the absorption was successfully shifted from the UV (200 nm–400 nm) to VIS spectrum (400 nm to 700 nm). Also, the *In vitro* generation of singlet oxygen, by HT systems after UV irradiation was achieved. Therefore, the intrinsic photocatalytic properties of TiO₂ were enhanced and tested for its potential use in light biomedical therapies such as PDT. The loading capacities of HT nanocarriers were also investigated, exhibiting high loading capabilities of between 60%- 100%. HT nanocarriers were researched to test their capability to protect chemotherapeutic drugs from early degradation, results showed the protection of drugs for at least 48 h, prolonging the drugs circulation time. By functionalization with targeting agents, HT nanocarriers displayed the delivery of drugs to the cytoplasm of cancer cells by autophagic and apoptotic cellular mechanisms. Overall, HT systems are multifunctional biocompatible nanocarriers which show promising results for its potential use as a photocatalytic material for light biomedical therapies, and/or for the delivery of chemotherapeutic drugs to GBM cancer.