ANTI-CANCER MOLECULE QUERCETIN NANOPARTICLES FOR THERANOSTICS

M.Sc. Thesis

By

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DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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Submitted in partial fulfillment of the
requirements for the award of the degree
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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **ANTICANCER MOLECULE QUERCETIN BASED NANOPARTICLES FOR THERANOSTICS** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2017 to June 2019 under the supervision of Dr. Sharad Gupta, Asst. Prof.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Abhipsa Panda

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Dr. Sharad Gupta

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ABHIPSA PANDA

BSBE

DEDICATED TO

MY FRIENDS AND FAMILY

Abstract

Chemotherapy is a conventional treatment available for cancer. Mostly all chemotherapy drugs have some side effects due to non-targeted drug delivery. In this work, we studied for the possible therapeutic effects of a natural compound on cancer followed by the nano-encapsulation. Epidemiologic studies suggest that many of the plant-derived natural food products have potent protective activity against various diseases. In recent years many experiments have demonstrated the anti-cancer properties of flavonoids. Quercetin is a flavonoid, which is known for its anti-cancer activity and antioxidant properties. This molecule has shown its potential for treating cancer as a naturally occurring drug. However, one of the significant drawbacks of quercetin is its low bioavailability in the body due to its hydrophobic nature. In this work, we have encapsulated the quercetin in the polymeric nanoparticles to increase its bioavailability and application.

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NOMENCLATURE

rpm	revolution per minute
MW	Molecular weight
nm	Nano meter
mV	Milli Volt

ACRONYMS

PAH	Polyallylamine hydrochloride
MW	Molecular weight
DMSO	Dimethyl Sulfoxide
ddH_2O	Double distilled water
FESEM	Field emission scanning electron microscope
UV/VIS	Ultraviolet-visible
Qct-NPs	Quercetin nanoparticles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DMSO	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
PFA	Para formal dehyde
DLS	Dynamic light scattering

Chapter 1

Introduction

Cancer is one of the deadliest diseases that increase in an uncontrolled manner within the diseased body. Following heart disease, it is the second most common reason for mortality all over the world. Cancer has been a cause of death of humans since prehistoric time**.**[1]

1.1 History of cancer

History can never be overlooked because it has already shaped our present and is shaping our future. Describing the brief history, the word "CANCER" was acknowledged by Greek physician Hippocrates (460-370 BC), who is regarded as **"Father of Medicine."**[2] According to Hippocrates, the malignant tumor reminded him of a ***karkinos*** (Greek for crab) as the tumor cells resemble the hard shell and the swollen blood vessels as the legs of a crab. Another Greek physician, Gelen, used the term ***Oncos*** (swelling in Greek) to characterize a tumor.[3] Oncologists now use as a part of the name of cancer.[1] From prehistoric times, the presence of tumor masses in fossilized dinosaurs and human bones provides sustainable proof of the existence of disease in mammals. So, large scale studies were conducted over thousands of dinosaur species for the evidence and origin of tumor from ancient times. Out of the survey, duck-billed dinosaurs (***Cretaceous hadrosaurs***) whose existence was around 70 million years ago harbored benign tumor, and 0.2 % of the specimen displayed malignant tumor.[4] Earlier Egyptian manuscripts uncovered in the nineteenth century provide the earliest written record of human cancer. Predominantly surgical, pharmacological, and magical treatments are reported in Edwin Smith and George Eber papyri, which were written and published between 1500 and 1600 BC based on sources of thousands of years ago. Way back in 1500 BC, evidence of cancerous

growth in a human was found in Egyptian and Peruvian mummies. The oldest scientific documentation of cancer promulgation was the occurrence of a middle-aged Scythian king who used to reside in the steppes of southern Siberia dated back 2700 years earlier. Present-day microscopic and proteomic approaches, for instance: Digital radiography and multi-detector CT scans of his mummy confirmed that he was cancer affected. [5] Since age, many theories have been proposed regarding the origin and proliferation of cancer.[6]

1.2 Cancer Biology

Cancer is a disease which deals with the rapid growth of abnormal cells. It is a multistage process transforming the healthy cells into tumor cells.[7] These cells can start growing from anywhere in our body and spread throughout it.[8] In a healthy human being, normal cells grow, divide, and form new cells as a part of cell the cycle.[9] When a cell gets damaged, it receives instruction to die, and a new cell replaces it as per the natural phenomenon of apoptosis. But in the case of cancerous cells, they lack the instruction of natural cell death. New cells are formed, which were meant to replace them. Now, these new cells divide rapidly due to abnormal cell cycle and get transformed into tumor cells.

1.3 World cancer statistics[1]

According to the approximation given by World Health Organization (WHO) in 2015, cancer is the first or second principal factor of mortality, among people with age less than 70 years in 91 out of 172 countries. Among the other 22 countries it is placed as a third or fourth major cause of death. (Figure 1.1) However, the position of cancer to cause the premature death of people of many countries reflects their social and economic development.

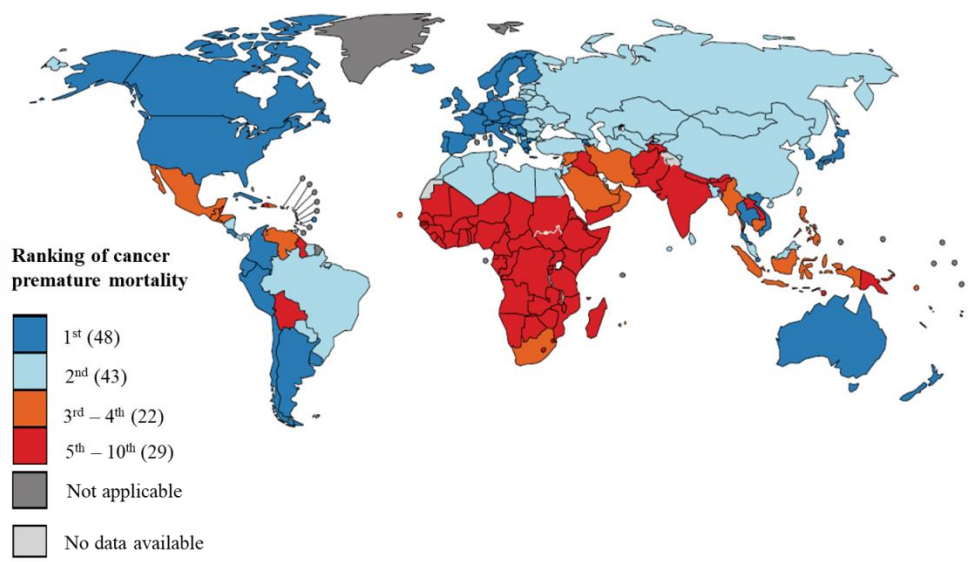


Figure 1.1: Global map presenting the national ranking of cancer as a cause of death at ages below 70 Years in 2015. The numbers of countries represented in each ranking group are included in the legend.[1]

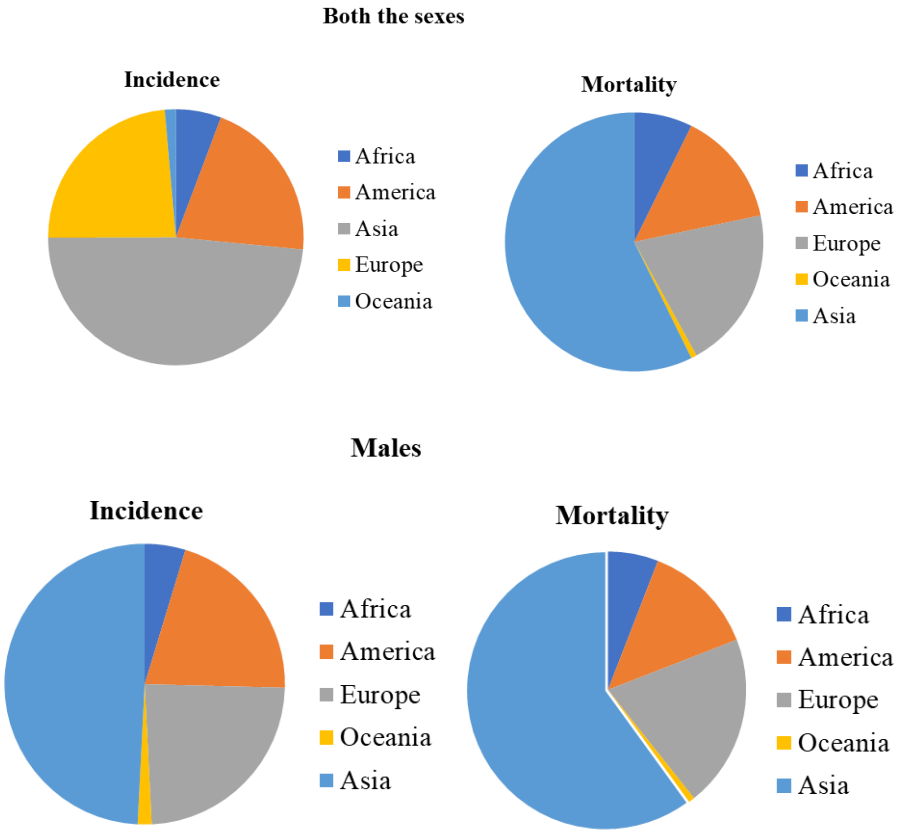
Day by day, the rate of occurrence and fatality of cancer is increasing. The justifications behind the exponential increase are however sophisticated which show the extensiveness and spreading of the leading risk factor as well as both aging and growth of population. Many of them are in combination with socioeconomic developments.[10] The cancer-related premature deaths show the socioeconomic development of a nation.[11] According to recent data of volume XI by IARC's (International Agency for Research On Cancer)[12] across five continents.[13][1] According to estimation, there were 18.1 million recent cases and 9.6 million deaths due to cancer worldwide in the year 2018. (Table 1.1) [12]

CANCER SITE	NO. OF NEW CASES(% OF ALL SITES)	NO. OF DEATHS (% OF ALL SITES)
Lung	2,093,876 (11.6)	1,761,007 (18.4)
Breast	2,088,849 (11.6)	626,679 (6.6)
Prostate	1,276,106 (7.1)	358,989 (3.8)
Colon	1,096,601 (6.1)	551,269 (5.8)
Non-melanoma of skin	1,042,056 (5.8)	65,155 (0.7)
Stomach	1,033,701 (5.7)	782,685 (8.2)
Liver	841,080 (4.7)	781,631 (8.2)
Rectum	704,376 (3.9)	310,394 (3.2)
Esophagus	572,034 (3.2)	508,585 (5.3)
Cervix uteri	569,847 (3.2)	311,365 (3.3)
Thyroid	567,233 (3.1)	41,071 (0.4)
Bladder	549,393 (3.0)	199,922 (2.1)
Non-Hodgkin lymphoma	509,590 (2.8)	248,724 (2.6)
Pancreas	458,918 (2.5)	432,242 (4.5)
Leukemia	437,033 (2.4)	309,006 (3.2)
Kidney	403,262 (2.2)	175,098 (1.8)
Corpus uteri	382,069 (2.1)	89,929 (0.9)
Lip, oral cavity	354,864 (2.0)	177,384 (1.9)
Brain, nervous system	296,851 (1.6)	241,037 (2.5)
Ovary	295,414 (1.6)	184,799 (1.9)
Melanoma of skin	287,723 (1.6)	60,712 (0.6)
Gallbladder	219,420 (1.2)	165,087 (1.7)
Larynx	177,422 (1.0)	94,771 (1.0)
Multiple myeloma	159,985 (0.9)	106,105 (1.1)

Nasopharynx	129,079 (0.7)	72,987 (0.8)
Oropharynx	92,887 (0.5)	51,005 (0.5)
Hypopharynx	80,608 (0.4)	34,984 (0.4)
Hodgkin lymphoma	79,990 (0.4)	26,167 (0.3)
Testis	71,105 (0.4)	9,507 (0.1)
Salivary glands	52,799 (0.3)	22,176 (0.2)
Anus	48,541 (0.3)	19,129 (0.2)
Vulva	44,235 (0.2)	15,222 (0.2)
Kaposi sarcoma	41,799 (0.2)	19,902 (0.2)
Penis	34,475 (0.2)	15,138 (0.2%)
Mesothelioma	30,443 (0.2)	25,576 (0.3)
Vagina	17,600 (0.1)	8,062 (0.1)
All sites excluding skin	**17,036,901**	**9,489,872**
All sites	**18,078,957**	**9,555,027**

According to the estimation of the year 2018, out of the total number of cases worldwide, 50% of deaths due to cancer occurred in Asia. This is because the world's 60 % population resides here. Figure 1.2 illustrates all of the cancer occurrence and death cases worldwide for both the sexes combined and separately. Figure 1.3 shows the top 10 types of cancer, leading to the highest number of cases and deaths worldwide for both the sexes, together and individually. For both male and female, combined lung cancer is the most prevalent disease. There was 11.6% of the total number of cases of lung cancer with 18.4% of total no deaths worldwide. The second leading cancer is female breast cancer (11.6%), followed by collateral cancer (10.2%), and prostate cancer (7.1%) for prevalence and collateral cancer (9.2%), stomach cancer (8.2%), liver cancer (8.2%) for

death rate worldwide. By sex, lung cancer is the most prevalent disease and a significant cause of mortality in men trailed by prostate cancer and collateral cancer in case of occurrence and followed by stomach and liver cancer in case of the death rate. In females, breast cancer is the most prevalent cancer. It is the leading cause of fatality trailed by collateral and lung cancer.



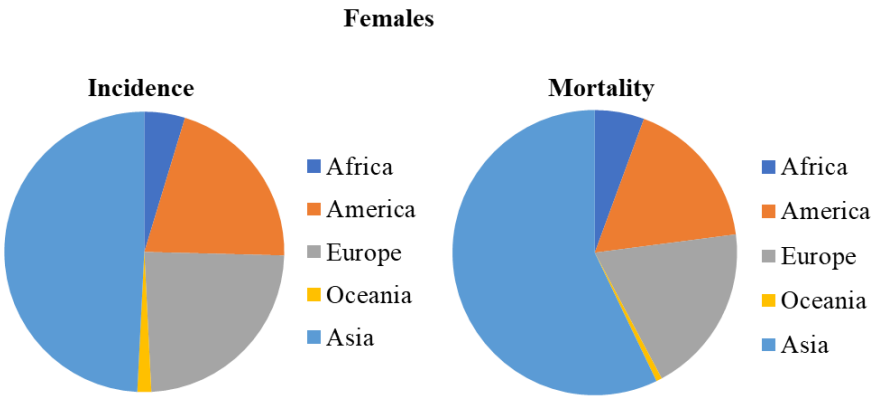
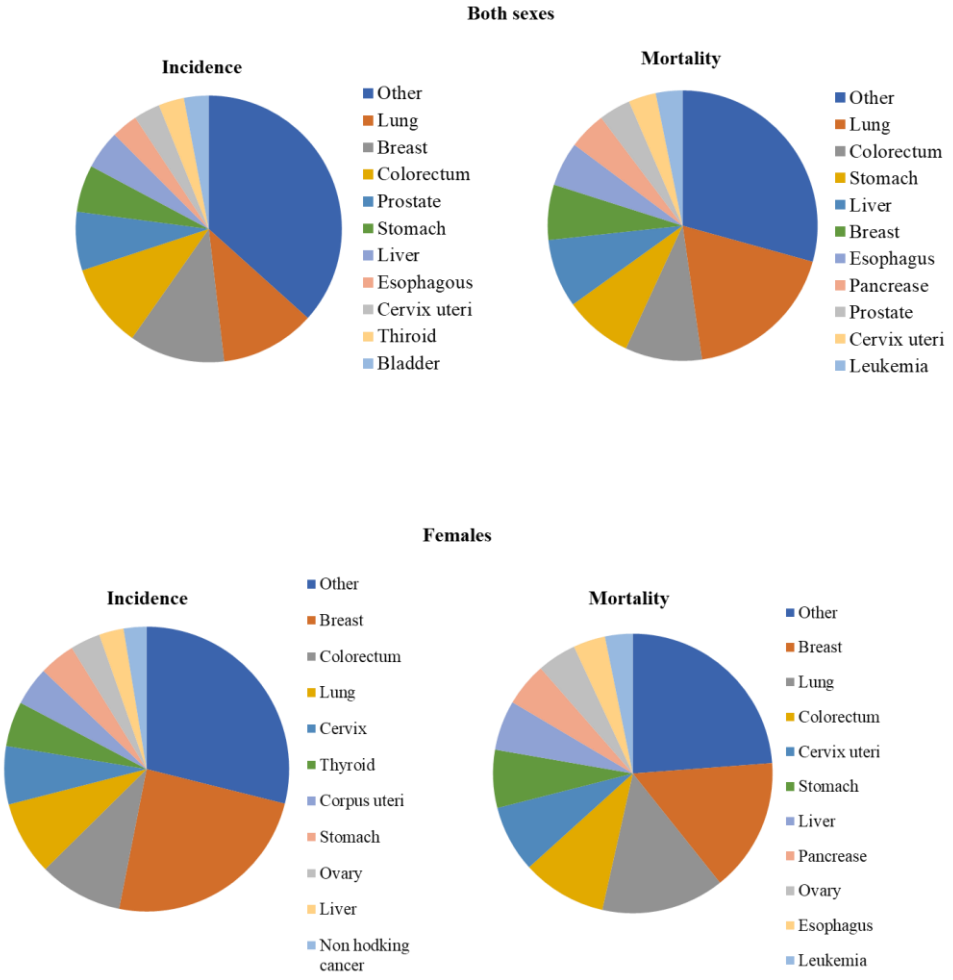


Figure 1.2: Number of cases and deaths according to world area in 2018 are shown through pie charts, (A) Both Sexes, (B) Males, and (C) Females.[12]



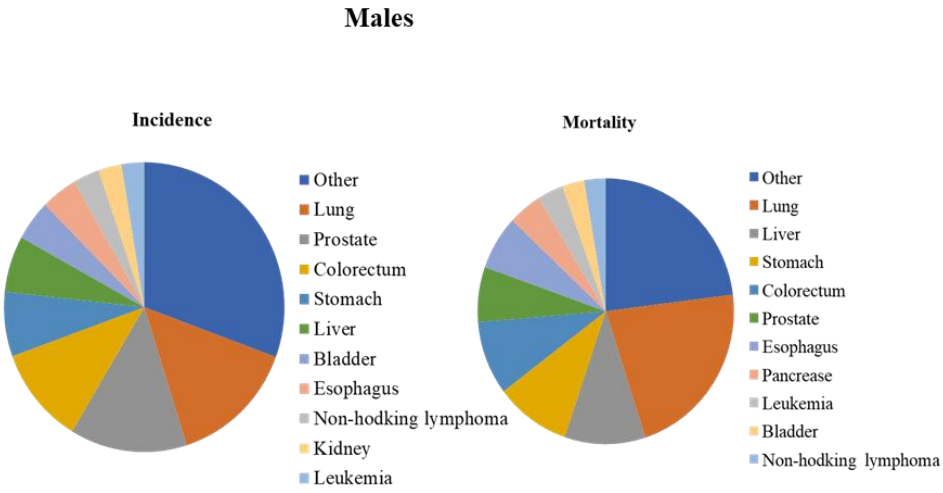


Figure 1.3: Number of cases and deaths according to world area for 10 Most Common Cancers in 2018 are shown through pie charts (A) Both Sexes, (B) Males, and (C) Females.[13]

According to an estimate prostate cancer is the commonly detected cancer in 105 countries. Lung cancer has been detected in 37 countries, followed by liver cancer in 13 countries. [1] Lung cancer is a significant cause of cancer death among men in 93 countries, followed by prostate cancer (46 countries) and liver cancer (20 countries). Among females, breast cancer is commonly diagnosed in 154 countries, and cervical cancer in 28 out of 31 countries. Worldwide the rate of occurrence of cancer is 20% higher in males than females. With regards to mortality, males have a 50% higher date rate than females.

1.4 Types of treatments for cancer

- **Surgery:**

  Surgery is concerned with the detection, treatment, and prevention of cancer. There are specific cancer operations for each cancer type.[14] Occasionally one surgery can take care of all of the above objectives, or more than one surgery is needed.[15] In the surgical diagnosis of

cancer, a biopsy procedure is used mostly. The method of biopsy is different for different types of cancer.[16]

- Surgery to detect the stage of cancer: Surgery for staging is critical because it not only can remove the tumour but also can tell about the spreading area of cancer. This information will help in deciding the treatment strategies (especially chemotherapy, radiation therapy) for cancer.[17]
- Curative Surgery: The primary surgery is preferable when cancer originates from a single part of the body. Surgery is the leading treatment if this is the case, and mostly all cancers are likely to be removed. It is used to gather with chemotherapy and radiotherapy.[18]
- Debulking Surgery: When removing the whole tumor part can damage the nearby organs or tissues, a debulking surgery is preferable. In this case, doctors try to remove cancer tissues as much as possible, and they treat the remaining part either by chemotherapy or radiotherapy.[19] For example, this technique is used in ovarian cancer and some type of lymphoma.[20]
- Palliative Surgery: This technique is used when advanced cancers create problems such as pain, discomfort, or disability. It helps in reducing the problem and make patients feel better. For example, in the case of abdominal cancer, cancer may grow and block the intestine. Then this technology is used to remove the blockage.[21]
- Supporting surgery: It is used as a support for many types of treatment methods.
- Restorative/reconstructive surgery: It upgrades the look of a person who has undergone many cancer surgeries and also redevelops the functions of organs or tissues after surgery. For example, remodeling of breast mastectomy.[22]
- Preventive (prophylactic) surgery: This kind of surgery is done basically to minimize the likelihood of cancer or to help in

arresting the threat of cancer by removing tissue or organs by surgery which may develop cancer later on. But the prevention is not guaranteed.

- **Radiation Therapy:**

It is also termed radiotherapy; radiation therapy primarily utilizes the waves or high-energy particles, such as gamma rays, electron beams, x-rays or protons.[23] Usually, the growths of cancerous cells are much faster as compared to healthy cells. So, the radiations affect the DNA of cancerous cells by creating small breaks and hinder their multiplication. It may affect the healthy cells nearby, but they recover to their previous state soon. Unlike chemotherapy, it provides localized treatments. In systematic radiation therapy, radioactive elements may be fed to the patient or injected through veins. [24]These elements travel throughout the body and get accumulated in the tumour area. With regards to pre-operative therapy or neoadjuvant therapy, radiation is given before surgery.[25] In the case of adjuvant therapy, radiations are given after therapy. Three ways of Radiotherapy are as follows.[26]

- External radiation/ external beam radiation: In this case beam is radiated outside of the body.
- Brachytherapy/ internal radiation: A radioactive source is put inside the body into or near the tumour.
- Systematic radiation: Radioactive elements are fed to the patient or injected through veins. These elements travel throughout the body get accumulated in the tumour area.[27]
- Targeted Therapy: There are some differences between cancer cells and healthy cells which help them to grow faster and spread. Targeting these differences with drugs is called targeted therapy. It is a specific kind of chemotherapy. These drugs side effects are also different from regular chemotherapy drugs. Targeted therapy may be used as the primary treatment,[28], but mostly it is used in

association with chemotherapy or surgery or radiation therapy.[29] Typically cancerous cells have many changes in their DNA, which make them different than healthy cells as they lose their original function and start proliferating abnormally.[29] The changes are also different for various cancer types. So different drugs have been developed to target them.[30]

- **Immunotherapy:**

Immunotherapy is a relatively newer therapy to treat cancer. Specific proportions of the immune system cells are used for the cancer treatment in immunotherapy. It can work in many ways, it can stimulate a person's immune system, or human-made immune proteins can be given.[31] Certain kinds of immunotherapy are called biology therapy or biotherapy. Four types of immunotherapies are being used nowadays, monoclonal antibodies, immune checkpoint inhibition, cancer vaccines, nonspecific immunotherapy.[32]

- **Chemotherapy:**

Chemotherapy is the most commonly used cancer therapy; it is used alone and/or in combination with other therapies. Chemotherapy is the use of drugs in the process of treatment of a disease. Unlike radiations or surgery, it works throughout the body. It includes varieties of drugs classified as antimetabolites and alkylating agents.[33] Targeted therapy is a specific form of chemotherapy in which different types of drugs are used for targeting a particular area.[34] In the treatment of cancer, chemotherapy has three considerable aims: cure, control, and palliation.

- Cure: Mostly, doctors use chemotherapy to destroy cancer. Still, the target is not assured. But it does not happen in that way. It generally takes many years to know if the person is restored to the healthiest state.[35]
- Control: If Cancer is not curable, the next measure is to control the disease. Some drugs are meant to shrink the size of tumours. In

numerous cases, it disappears completely. In some cases, it is disappeared for a while and comes back. In such cases, drugs can be repeated.

- Palliation/ Palliative Chemotherapy: It is used in case of advanced stage cancer when a cancer is not curable and does not remain under control. It improves the condition of one's life and makes him or her feel better.[36]

In this project, we have mainly focused on developing alternative treatment method (delivery of natural anti-cancer molecules with the help of nanotechnology), which has comparatively less side effect than chemotherapy. Here we have used natural plant product quercetin as an anticancer agent.

1.5 Flavonoids:

Plant-derived food products, particularly those, which are rich in fruits and vegetables, play a significant role in reducing the risk of several diseases in individuals.[37] Although they vary in compositions but carry many of the nutrients that are necessary for normal body metabolism for keeping an individual healthy and increasing average lifespan.[38] Flavonoids are one of the natural components present in various plant products like fruits, vegetables, flowers, stems, etc. More than 5000 flavonoids have been characterized from plants till now.[39]

Flavonoids are the secondary metabolic phenolic compounds of the plants. They are structurally composed of two benzene rings which are connected by an oxygen-containing pyrene ring.

Classification of flavonoid into different groups is done based on their molecular structure. If the carbon three position of the pyrene ring contains a hydroxyl group (OH), they are classified as 3-hydroxy

flavonoids. Flavonoids like flavanols, anthocyanidins, leucoanthocyanidins, and catechins belong to 3-hydroxyl flavonoids category. If they do not have a hydroxyl group at carbon three of the pyrene ring, they are called as 3-desoxyflavonoids (flavanones and flavones).[40] Another category is isoflavonoid in which one of the benzene rings is attached to the pyrene at carbon three position instead of carbon two position.[41] Anthocyanidin and catechins lack carbonyl group at the fourth carbon position.

In plants, flavonoids are mainly present as sugar linked residues by O-glycosidic linkage. Aglycons are the sugar lacking flavonoids and present in very trace amount. Sugar molecules are bound to the hydroxyl group at Carbon 3 or Carbon 7 positions of the flavonoids. The typical sugar moieties include L-glucose and D-rhamnose. At least eight different monosaccharides and their combinations can bind to the aglycons giving rise to different flavonoids.[42]

- Flavonols:

Quercetin is the most commonly consumed flavonol in a human body daily. Other kinds of flavonols found are kaempferol (broccoli), myricetin (berries), and isorhamnetin (onion). One of the essential and most studied flavonoids is quercetin (figure 1.4), which belongs to the flavonol group. It is the most commonly consumed flavonol daily.[43] It is found in many edible plant products but most commonly found in onion. In plants, they are present in many glycosidic forms such as quercetin-3-rhamnoglucoside (rutin), 4-glucoside and quercetin-3,4'-glucoside, quercetin galactosidase, quercetin arabinosides. **Quercetin is one of the highest studied flavonoids due to its antioxidative, anticarcinogenic, anti-inflammatory, anti-aggregatory, and vasodilating effects**.[44]

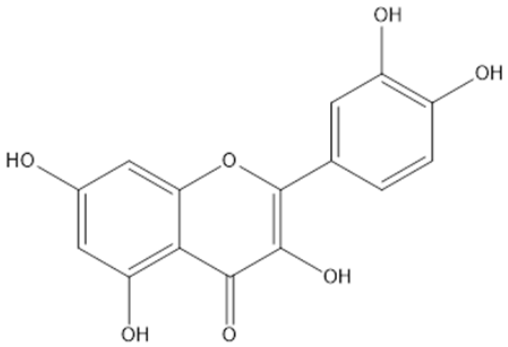


Figure 1.4: Structure of quercetin

- Flavones:

These include apigenin and luteolin. Their consumption is minimal as they are present in a tiny amount in daily consuming plants. Red pepper and celery are the significant sources of flavones.

- Catechins:

Mainly present in aglycon form or esterified with gallic acid. Primary sources are tea, red wine, apples, pears, grapes, peaches.

- Anthocyadinins/Anthocyanin glycosides:

These include pelargonidin, cyanidin, delphinidin, and malvidin. They provide blue, red, and violet colon to various foods.

- Isoflavonoids:

Most common sources are legumes, soybean.

Flavonoids are adsorbed to the gastrointestinal tract significantly due to their glycosidic form. But catechin is absorbed in aglycon form. So, there is controversy regarding the absorption of flavonoids.

1.6 Organization of thesis

Chapter 1: In this chapter history, current position, and statistics of cancer in causing death worldwide, different methods of treatment of cancer as well as classification of flavonoids.

Chapter 2: Project motivation and a review of past work.

Chapter 3: The materials, instrumentation, and procedure for the synthesis of quercetin-loaded PAH nanoparticles(Qct-NPs). This chapter also includes the experimental techniques employed to study the characterization of nanoparticles.

Chapter 4: This chapter comprises of the results obtained after the synthesis and application of quercetin nanoparticle.

Chapter 5: This chapter includes the conclusion of the work and discusses possible future scope and applications.

Chapter 2

Review of Past Work and Project Motivation

2.1 Side effects of chemotherapy: Chemotherapy is one of the most commonly used of treatment of cancer. It may cure patients, but it can also increase their emotional distress and many physical challenges. Resisting the crucial effect of chemotherapy is itself a challenge for the person as it as several severe side effects and mentioned below in tabular format. [45]

In **table 2.1,** the physical side effects are listed.[46]

- Being sick (vomiting)
- Itching at injection site
- Shaking all over
- Change in the way things taste
- Changes in how things smell
- Not having regular bowel action (constipation)
- Loss of liquid or frequent bowel action (diarrhea)
- Pins and needles in fingers or toes
- Numbness in fingers or toes
- Loss of weight
- Weight gain
- Increased hair growth on legs
- Constantly tired
- Giddiness on standing up
- Loss of appetite
- Sore mouth
- Sore throats
- Shortness of breath

- Pain passing water (painful urination)
- Colored urine
- Ringing in ears
- Deafness
- General aches and pains
- Tummy ache (abdominal pain)
- Swollen tummy (abdominal fullness)
- Periods stop
- Periods become irregular
- Changes in skin color
- Hot flushes
- Heart beating fast (palpitations)
- Headache/migraine
- Loss of hair
- Increased thirst
- Passing more water than usual
- (increased urination)
- Dry skin
- Acne (pimples)
- Increased appetite
- Trouble with swallowing
- Nose-bleeds
- Cannot taste things
- Fingernails go brown

Nonphysical effects of chemotherapy are listed in **Table 2.2.**[46]

- Loss of sexual feeling
- Loss of sexual ability (not getting aroused)
- Feeling low, miserable (depression)
- Thought of coming for treatment
- Length of time treatment takes at the clinic
- Feeling bad tempered (irritability)
- Having to have a needle
- Having to come to clinic rather than private doctor
- Affects my family or partner
- Feeling of not coping generally with treatment
- Feeling of having to have treatment which I don't think do any good
- Feeling of having to have treatment which I don't want
- Crying more often
- Feeling angry
- Cannot concentrate
- Affects my work/home duties affects my social activities
- Infertility (cannot have children)
- trouble finding somewhere to park near the clinic
- Trouble getting to the clinic
- Not having the chance to ask the doctor questions
- Forget things
- Not seeing the same doctor each time
- Cannot get clothes to fit
- Not understanding what is happening
- Feeling anxious or tense
- Having to wait for treatment with other patients
- Feeling that the treatment is damaging my body

Other than this, significant disadvantages are high dose and non-specificity of drugs. Along with killing cancer cells, these drugs also kill neighboring healthy cells since they cannot differentiate between normal and cancer cells.[47] Process of treatment is variable depending upon the type of cancer and the stage of cancer. It is not affordable by middle income and lower incoming family.[47]

Considering all the side effects of chemotherapy, it is required to look for an alternative therapeutic approach, which should be having the following properties:

- **Inbuilt anti-cancer property**
- **Specific to cancer cells**
- **Not cytotoxic to normal cells**
- **Cost-effective**

For this purpose, naturally occurring anti-cancer molecules might play a beneficial role against cancer.[38] Quercetin is a potent anticancer molecule, which comes under the flavonol group of flavonoids and might be used for cancer therapy.[44]

2.2 Why Quercetin?

Quercetin is a natural plant product, which is mainly found in dietary sources. Maybe in very less amount, but we consume it daily. In ancient times it has been used as medicine for various diseases such as cancer[44], cardiovascular and nervous disorders, obesity, and chronic inflammation. Some shreds of evidence demonstrate that it has antioxidant[44] as well as an anti-inflammatory effect.[48]

It can impede nuclear factor kappa (NF kappa B) stimulation, nitric oxide synthases (iNOS) regulation, expression of cyclooxygenase-2, C-reactive protein (CRP). It also has anti-bacterial, anticoagulative, anti-hypertensive properties. It can also regulate gene expression and can inhibit healthy cell

growth. Quercetin also participates in the antioxidant defense of blood plasma.[49] Exposure of the human body to methylene mercury can damage the blood cells. Quercetin shows a protective effect against methylene mercury.[50]

Ion-induced lipid peroxidation can be inhibited by quercetin. Melanin formation can be blocked by quercetin.[51] The miRNA 155i is a part of murine macrophage reacts to various types of inflammatory mediators such as lipopolysaccharides (LPS). Quercetin can regulate their expression.

The justification behind choosing quercetin is that it is cancer-specific and does not harm healthy cells. It has been used as an anti-cancer molecule since ancient times. Thus, quercetin can be used as a drug due to its various medicinal properties.

2.3 Limitations of quercetin

According to multiple studies, quercetin typically shows amphipathic behavior as the hydroxyl groups form the polar part where the phenyl groups form the non-polar part. Due to this reason quercetin is partially soluble in water.[52] At 20˚ C the solubility of quercetin is less than 10 mg in 1 liter of water.[53]

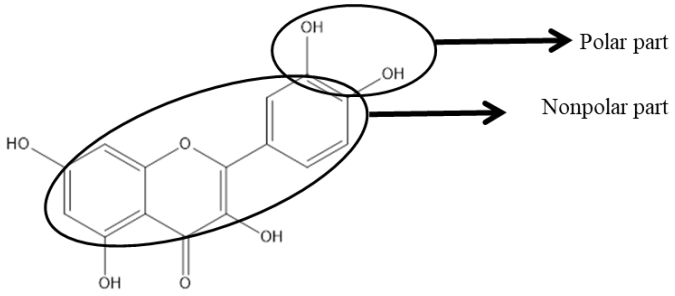


Figure 2.1: Polar and non-polar parts of quercetin

The human body consists of 70% of water. This reduces the bioavailability of quercetin. Gugler et al. fed 4 grams of quercetin to a person to examine

the absorption profile of quercetin into the body. However, surprisingly, he found all 4 grams of quercetin in the urine of the person, and nothing was detected in the blood plasma.[54]Due to the hydrophobicity, the bioavailability of quercetin is very low. So it readily gets discharged from the body after consumption.

To address this problem, we have planned to encapsulate quercetin into a nano-carrier (nanoparticle), which will improve the water solubility of quercetin and eventually improve the bioavailability within the body.

2.4 Reviews of previous work processes and our approach

Quercetin has been encapsulated in a variety of ways. Quercetin loaded polymer nanoparticles have been developed with the use of polyvinyl alcohol and Eudragit E by nanoprecipitation technology. With the use of anti-solvent precipitation method[55], Quercetin was also encapsulated into lipid using emulsification and low-temperature solidification procedure.[56] Chitosan has been used for preparing quercetin loaded chitosan nanoparticles in regards to gelation of chitosan with tripolyphosphate anions.[57] In case of use of metals for the preparation: gold, silver, copper, are also participated in the development of quercetin metal nanoparticles.[58]The literature review also suggests that quercetin also encapsulated along with quantum dots,[43] Graphene.[59]

2.4.1 Disadvantages

Majority of the quercetin loaded nanoparticles have several disadvantages. Metallic nanoparticles are thermodynamically unstable. They are highly reactive inside the body and mostly contain impurities. Furthermore, these kinds of nano-carriers are not biodegradable and can cause long term toxicity.[60] In the case of lipid nanoparticles, accumulation of lipids in the liver and spleen is a big problem.[61] Additionally, uncontrolled release of drug from the nano-carriers may bring toxic effects, no useful

human safety data available in case of lipid nanoparticles. Graphene nanoparticles, quantum dots are highly cytotoxic to the healthy cells.

2.4.2 Our approach

Considering all of the above disadvantages, we selected a polymer Polyallylamine Hydrochloride (PAH), which has been used in several biological applications along with drug delivery purpose.[62]

- All the constituents of nanoparticles are biodegradable.
- The synthesis was performed at room temperature in an aqueous environment.
- Fabrication time was less than one hour.

Chapter 3

Experimental Section

3.1 Reagents and Chemicals

For the synthesis of Quercetin nanoparticles (Qct-NPs) cationic polymer Polyallylamine hydrochloride (PAH, average MW 50,000 g/mole), salt sodium phosphate dibasic heptahydrate (Na_2HP0_4, MW 119.98 g/mole) and Quercetin (Qct) were purchased from Sigma-Aldrich. Deionized water (ddH_2O) was used as a solvent for the synthesis of Qct-NPs and obtained from Sartorius water purifier system.

3.2 Synthesis of Quercetin-loaded PAH nanoparticles:

Qct-NPs were synthesized by using a simple two step self-assembly method. Briefly, the stock solution of PAH (5.5 mg/mL), Quercetin (1 mg/mL) and Na_2HPO_4 (0.01 M) were prepared and stored at 4 ℃. For synthesis 20 µL of the cationic polymer was mixed with 60 µL of the anionic salt, which results in self-assembly of polycation. In the next step, Qct was added to the solution for nano-encapsulation of Qct in PAH NPs. Finally, the volume makeup was done up to 1 mL by adding ddH_2O. The fabricated Qct NPs were aged for 1 h at 4°C and differentially centrifuged for the collection of the formed Qct-NPs. Fresh NPs were prepared every time for further characterization.

3.3 Characterization of surface morphology by FESEM:

For morphological characterization the Qct-NPs were lyophilized using Alpha 1-2 LDT, CHRISTlyophilizer and stored for FESEM characterization. For field emission scanning electron microscopy (FESEM) the samples were mounted on glass stub using double-sided carbon tape and coated with 5 nm of gold using coating machine. For visualization, the glass slide was mounted on the aluminum stub by

double-sided carbon tape. FESEM images were captured using Supra55 Zeiss, Gemini FESEM.

3.4 DLS and Zeta potential measurements:

Hydrodynamic diameter and zeta potential of the Qct-NPs were measured by using NanoPlus zeta/nano particle analyzer, dynamic light scattering. The measurement of the zeta potential was also done by using NanoPlus zeta/nanoparticle analyzer, Particulate System. Both the measurements were performed at 25.0 °C and the scattering angle of the measurement was 15 °.

3.5 Absorption spectroscopic measurements of Quercetin-loaded PAH nanoparticles:

Spectroscopic measurements of the Qct-NPs and free quercetin were done using PerkinElmer UV/VIS/NIR spectrophotometer (Lambda 35). Both the free Qct and nano-encapsulated Qct were suspended in 1 ml of ddH_2O, absorbance measurement between300 to 700 nm.

3.6 Fluorescence spectroscopic measurements of Quercetin-loaded PAH nanoparticles:

For spectroscopic measurements, both free Qct and Qct NPs were suspended in 1 ml of ddH_2O. Fluorescence spectra of free Qct and Qct NPs were recorded with Horiba spectrophotometer and the excitation was done by xenon lamp. All the emission spectra were taken from 395 to 650 nm with an excitation wavelength of 375 nm.

3.7 Therapeutic efficacy assessment of Quercetin-loaded PAH in A549 and HeLa cell line:

Cytotoxicity studies of the Qct-NPs and free Qct were evaluated using MTT assay. Both HeLa and A549 cells were cultured (at 37 °C, 5% CO_2) using DMEM(1X) medium containing 10% FBS and 5% pen-strep for 24

hours in 24 well plates. Seeding density for this experiment was 80,000 cells per well. The cells were delicately washed with the 1X PBS for 3 to 4 times. For treatment, all the samples were dissolved in 500 µl DMEM medium. For cellular viability studies, cells were treated with the following conditions free Qct and Qct-NPs. After 30 minutes of incubation, all the media were aspirated from the wells and freshly prepared 500 µl MTT dissolved in DMEM medium was added. After 4 hours of incubation, MTT was also aspirated and 500 µl DMSO was added to each well. Before the optical measurement the plates were shaken properly, and the absorbance measurement was done at 570 nm using microplate reader.

3.8 Treatment process of A549 and HeLa cells for fluorescence imaging:

Both HeLa and A549 cells were cultured in 24 well plates having coverslips for imaging. The incubation was done at 37 °C supply with, 5% CO_2 using DMEM(1X) culture medium containing 10% FBS and 5% pen-strep for 24 hours. The seeding density for the imaging experiment was 80,000 cells per well. The cells were gently washed with 1X PBS for 3 to 4 times. All the samples used for the treatment were dissolved in DMEM medium. For imaging, cells were incubated with Qct and Qct-NPs. incubated with free Qct and Qct-NPs for 30 minutes. After incubation, the media were aspirated, and slides were prepared for the imaging. Slides were prepared after 4 to 5 times wash with 1X PBS and fixed with 4% PFA using 30% glycerol.

3.9 Fluorescence imaging of quercetin nanoparticles treated A549 and HeLa cell lines:

Fluorescence imaging of the treated cells was carried out using Nikon ECLIPSE Ti inverted fluorescence microscope with a 10x objective equipped. Bright field images were taken with a one-millisecond exposure

and analog gain 2x. Fluorescence images were taken after the exposure of 800 milliseconds and analog gain 2x. Both bright field and fluorescence images were taken at the same location for each condition. The excitation and emission specification were listed here such as long pass filter cube were used B-2A with filter diameter 25mm, excitation filter 420/40, dichroic mirror 505, barrier filter 580.

Chapter 4

Results and Discussion

4.1 Synthesis of Quercetin-loaded PAH nanoparticles (Qct-NPs):

The process of synthesis of Quercetin-loaded polymeric nanoparticles was done using a self-assembly method. The major constituents of these nanoparticles are PAH, sodium phosphate dibasic heptahydrate (Na_2HPO_4) and Quercetin. PAH has a positive charge, and Na_2HPO_4 has a negative charge. Therefore the addition of salt to the polymer results in electrostatic interaction between them, employing which they bind to each other and form PAH-Na_2HPO_4 cross-linked bare nanoparticles. This is followed by the addition of the Quercetin in the mixture of PAH and Na_2HPO_4, due to the hydrophobic nature Quercetin gets readily encapsulated within the nanoparticles. This step completes the formation of Quercetin-loaded PAH nanoparticles (Qct-NPs). Figure 4.1 shows the schematic representation of the synthesis of Quercetin-loaded PAH nanoparticles, synthesized by self-assembly method where following incubation, centrifugation at particular rpm results in the pellet of Quercetin-polymeric nanoparticles. (Yellow)

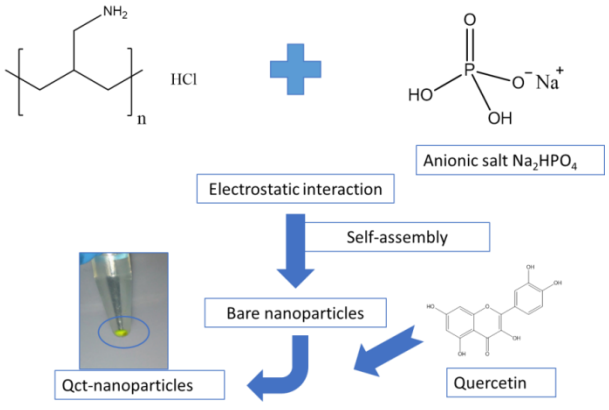


Figure 4.1: Schematic diagram of the process of synthesis of quercetin-loaded polymer nano-carriers

Encapsulation of Quercetin into nanoparticles results in the decrease of its hydrophobicity and making it solubilize in water. When free Quercetin is dissolved in water, it forms aggregation due to its hydrophobic nature as in Figure 4.2(a). However, nano-encapsulation of Quercetin results in a decrease of its hydrophobicity and making it uniformly dispersed in water without any aggregation. (Figure 4.2(b))

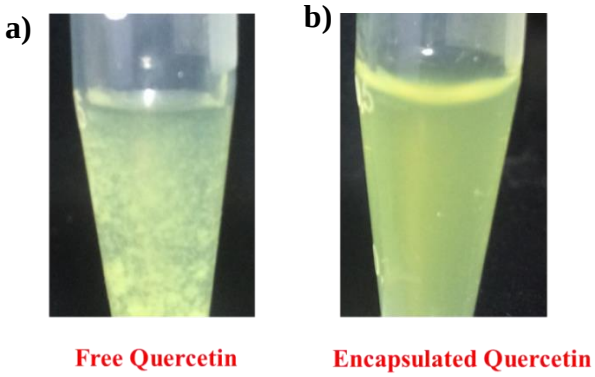


Figure 4.2: hydrophobicity of **a)** free Quercetin decreased after **b)** nano-encapsulation

Further, the PAH is biodegradable and biocompatible polymer. The process of nanoparticles synthesis was consistent, for each time, almost similar kinds of particles were produced. However, an attempt to load the higher concentrations of Quercetin within these nanoparticles led to the sticky aggregation of the nanoparticles, as shown in Figure 4.3.

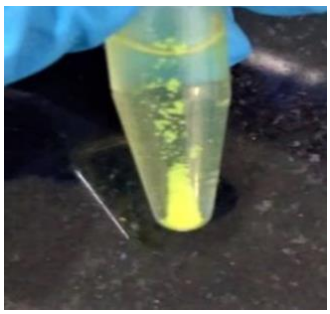


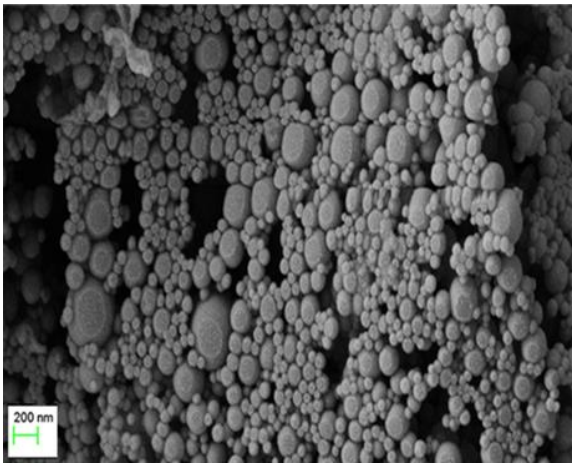
Figure 4.3: Sticky aggregation of nanoparticles when an attempt to load the higher concentrations of Quercetin within these nanoparticles

4.2 Morphological characterization of Quercetin-loaded PAH nanoparticles:

For the therapeutic applications of nanomedicine, particle size, and size distribution play a significant role. As it determines drug loading ability, stability, drug release, in vivo distribution, targeting ability, level of toxicity inside a body. Further, these nanoparticles were characterized in terms of morphology, size distribution, and size.

Figure 4.4a shows the SEM image of quercetin-loaded PAH nanoparticles, and figure 4.4b shows the SEM images of free quercetin in water. From these two images, we can differentiate the structure of Quercetin before and after encapsulation. In the case of figure 4.4b, Quercetin is forming an unusual aggregation when suspended in water. However, Figure 4.4a shows the Quercetin loaded nanoparticles are of spherical geometry. These nanoparticles get uniformly suspended in water, as shown above in Figure 4.2(b).

a)



b)

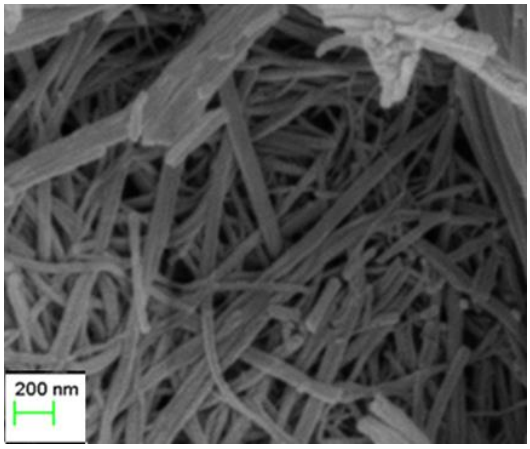
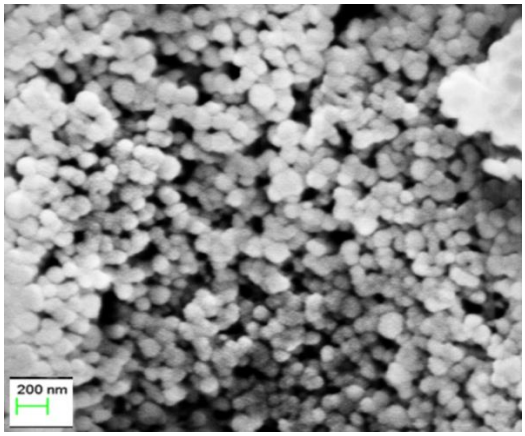


Figure 4.4: a) SEM image of Quercetin-loaded PAH nanoparticles **b)** The SEM images of free Quercetin in water, free Quercetin forms random aggregates in aqueous suspension.

As seen in Figure 4.4, the nanoparticle fabrication protocol provided the nanoparticles with huge polydispersivity where the size varies from 50 to 500 nm. Further, the monodispersivity of the sample was improved by optimizing the fabrication protocol, which successfully provided the nanoparticles of uniform size, as shown in Figure 4.5a and 4.5b. The diameter of optimized nanoparticles was ranging between **100 nm to 150 nm** in diameters.

a)



b)

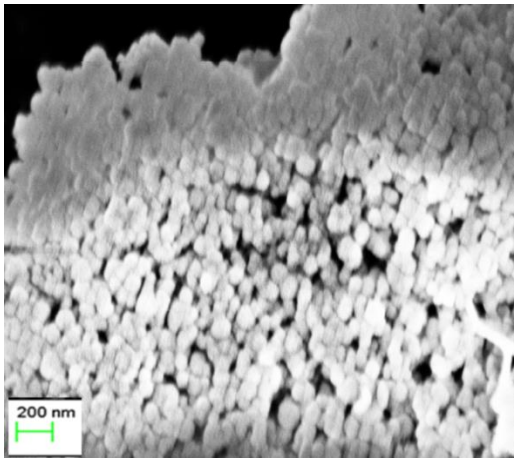


Figure 4.5: SEM images of quercetin nanoparticles with optimized protocol at different time points

4.3 Dynamic light scattering and zeta potential measurements of Quercetin-loaded PAH nanoparticles:

To study the size and surface charge of the Quercetin-loaded PAH nanoparticles in an aqueous environment, Dynamic Light Scattering measurements were performed. The hydrodynamic diameter of Quercetin-loaded nanoparticles was found to be 234 nm. Figure 4.6 shows the size distribution plot of quercetin nanoparticles in an aqueous environment.

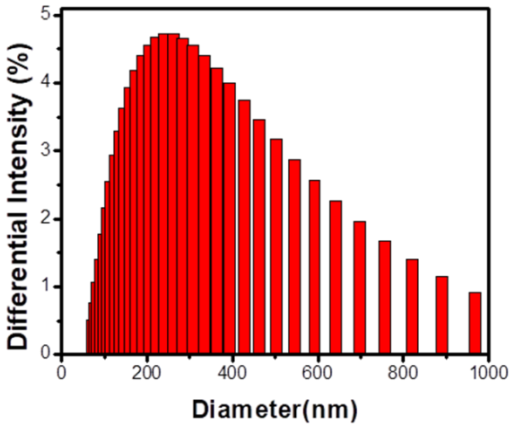


Figure 4.6: DLS measurement of Quercetin-loaded PAH nanoparticles

The zeta potential of the Quercetin-loaded PAH nanoparticles was found to be +14.4 mV as shown in Figure 4.7, which suggests that the particles are a stable and do not aggregate in an aqueous environment, which is good for their stability in an aqueous environment.

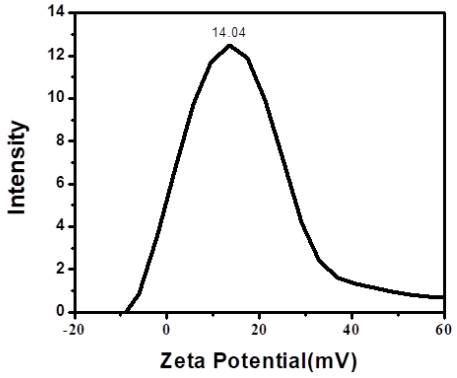
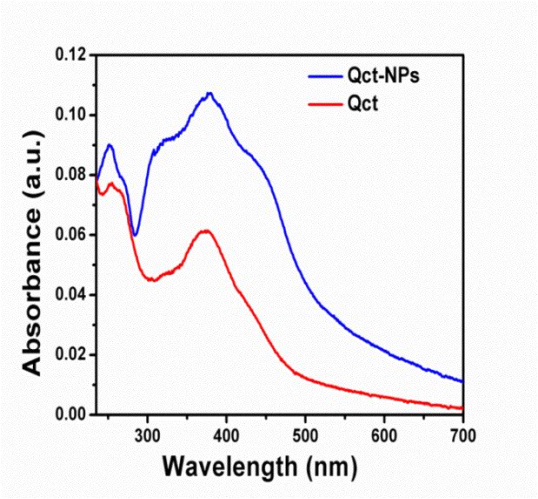


Figure 4.7: Zeta potential of quercetin loaded-PAH nanoparticles

4.4 Spectroscopic characterization of Quercetin-loaded PAH nanoparticles:

Absorption and emission spectra of both free and encapsulated Quercetin were taken. Generally, the absorption of the encapsulated molecules is the same (if loading is 100%) or less than its free form. However, in the case Qct-NPs, the absorbance of encapsulated quercetin has been increased, as shown in Figure 4.8a. This result suggests that Quercetin is successfully encapsulated within the nanoparticles. Followed by the absorption measurements, the fluorescence emission of free and nano-encapsulated Quercetin was also obtained. Similar to the absorption measurements, the fluorescence from nano-encapsulated Quercetin was found to be more than its free form, as seen in Figure 4.8b.

a)



b)

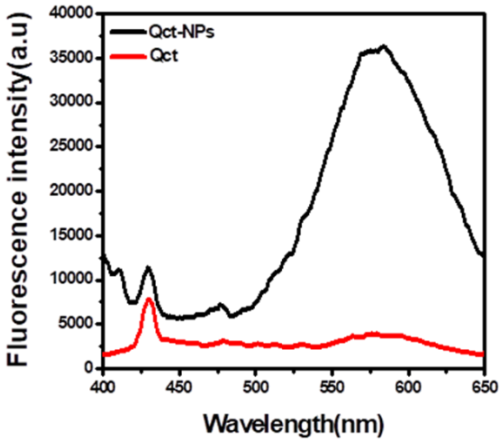


Figure 4.8: a) Absorbance spectra **b)** fluorescence spectra of Quercetin-loaded PAH nanoparticles

These results suggest that Quercetin is not only encapsulated within polymeric nanoparticles, but also it is improving the optical properties of Quercetin. This might be due to some interactions between Quercetin and polymer. Due to improved emission of Quercetin in the nano-encapsulated form, it could also be used for diagnostic applications.

4.5 Therapeutic efficacy assessment of Qct-NPs with A549 and HeLa cell line:

To assess the therapeutic efficiency of nano-encapsulated Quercetin, we incubated it with the different cell lines and cellular viability was estimated using MTT assay. The absolute purpose of MTT assay is to check cytotoxicity of different types of drugs or compounds in various cell lines. The viability of cells is positively associated with their mitochondrial activity as MTT get reduced to insoluble formazan by reaction with NAD(P)H-dependent cellular oxidoreductase enzyme of mitochondria, which is purple. Figure 4.9a shows the MTT assay of free and nano-encapsulated Quercetin along with the control in HeLa cell lines. In control, no treatment was given to the cells, and cells were found to be 100% viable. Cells treated with free quercetin showed a minor decrease in cell viability (approximately 90% of cells were viable). However, in the case of nano-encapsulated Quercetin cells, only 28% of cells were found to be viable.

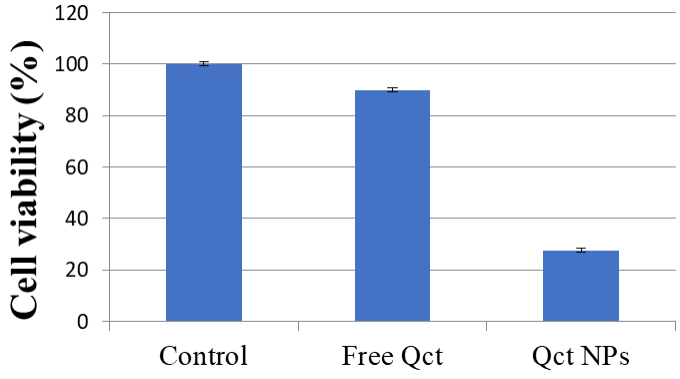


Figure 4.9: a) Cellular viability assessment of quercetin-loaded nanoparticles treated HeLa cells.

Figure 4.9b shows the comparison between the therapeutic efficacies of quercetin-loaded nanoparticles and free Quercetin. Here, control cells were not given any treatment and showed 100% cellular viability. Free quercetin-treated cells showed approximately 87% cellular viability in

comparison with ~35% cellular viability with Quercetin nanoparticle-treated cells.

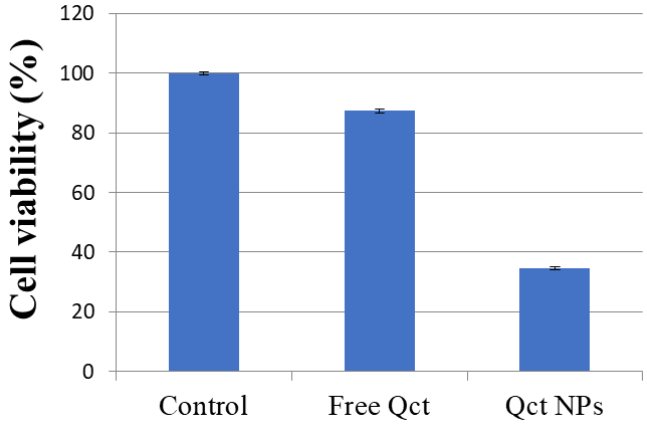


Figure 4.9b shows the comparison between the therapeutic efficacies of quercetin-loaded nanoparticles and free Quercetin.

From the results, it can be concluded that nano-encapsulated Quercetin is efficiently taken up by the cells and shows improved therapeutic efficacy in comparison with free Quercetin.

4.6 Fluorescence imaging of Quercetin-loaded polymeric nanoparticle treated HeLa and A549 cell lines:

Quercetin loaded polymer nanoparticles have improved the optical properties of Quercetin and could also be used for an imaging application. To study the imaging ability of nano-encapsulated Quercetin A549 and HeLa cell lines were used. Figure 4.10a shows the bright field and fluorescence images of A549 cells incubated with free and nano-encapsulated Quercetin along with control. It can be seen that Quercetin nanoparticle incubated cells show much higher contrast in comparison with free Quercetin incubated cells.

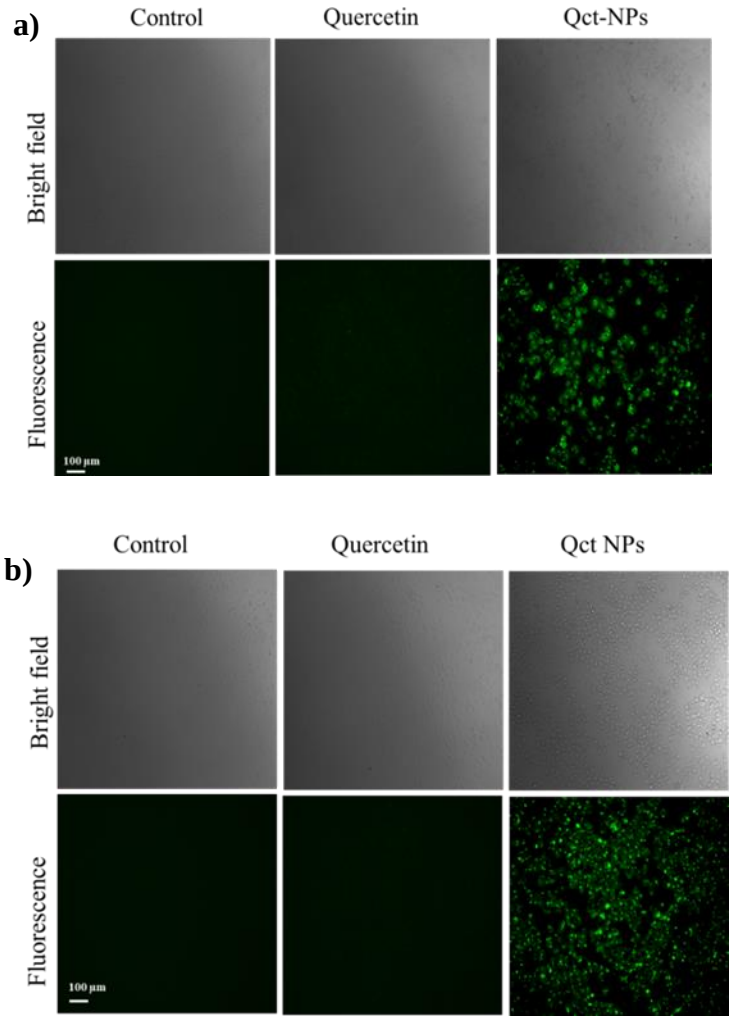


Figure 4.10: Fluorescence images of **a)** A549 cell line **b)** HeLa cell line after treatment with Quercetin-loaded PAH nanoparticles

Similarly, Figure 4.10b shows the bright field and fluorescence images of HeLa cells after incubation with free and nano-encapsulated Quercetin. The similar results were observed in this case also. The Quercetin nanoparticle incubated cells showed much higher fluorescence intensity in comparison with free Quercetin incubated cells. The control samples did not show any emission in both cases. These results suggest that nano-encapsulated Quercetin could also be used for imaging applications.

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