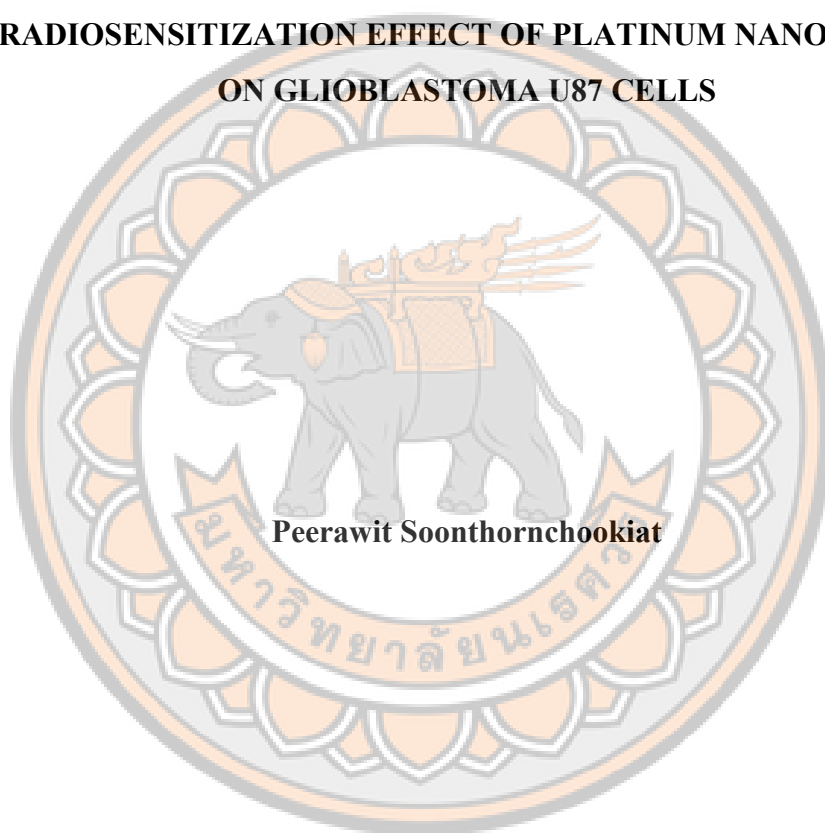




**FABRICATION, CHARACTERIZATION,
AND RADIOSENSITIZATION EFFECT OF PLATINUM NANOPARTICLES
ON GLIOBLASTOMA U87 CELLS**



**A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master's Degree in Medical Physics
2025**

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Peerawit Soonthornchookiat

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by Peerawit Soonthornchookiat

has been approved by the Graduate School as partial fulfillment of the requirements
for the Master's Degree in Medical Physics of Naresuan University

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ABSTRACT

Glioblastoma (GBM) is an aggressive and radioresistant brain tumor with limited response to conventional radiation therapy (RT). High atomic number (high-Z) nanoparticles have emerged as promising radiosensitizers due to their ability to enhance radiation-induced reactive oxygen species (ROS) generation and DNA damage. In this study, platinum nanoparticles (Pt-NPs) were synthesized and evaluated for their radiosensitizing effects in U87 glioblastoma cells. Physicochemical characterization revealed that Pt-NPs exhibited a spherical morphology with a spiky surface, a hydrodynamic diameter of 121 ± 13 nm, and a highly negative zeta potential (-42 ± 1.4 mV), indicating good colloidal stability. Cytotoxicity was assessed using the MTT assay, which demonstrated a concentration-dependent decrease in cell viability, and an IC_{10} of approximately $55 \mu\text{g/mL}$ was selected for subsequent experiments to minimize intrinsic nanoparticle toxicity. Clonogenic survival assays showed that Pt-NPs combined with 2 Gy irradiation significantly reduced the surviving fraction compared with Pt-NPs or irradiation alone, yielding a sensitizer enhancement ratio (SER) of 2.66. Consistently, increased γ -H2AX expression and intracellular ROS levels were observed following combined treatment, indicating enhanced DNA double-strand break formation. Furthermore, Pt-NPs in combination with irradiation induced cell cycle perturbation and increased apoptotic cell death. These findings demonstrate that Pt-NPs effectively improve the radiosensitivity of U87 glioblastoma cells through ROS-mediated DNA damage and apoptosis, highlighting their potential as nanomaterial-based radiosensitizers for glioblastoma radiation therapy.

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Peerawit Soonthornchookiat

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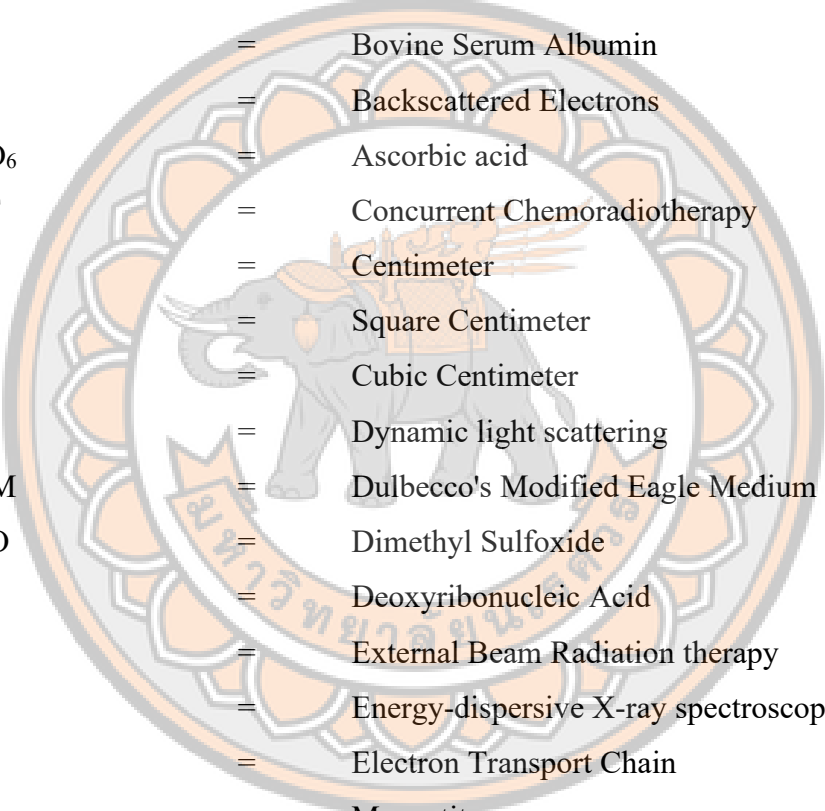
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Abbreviation



3D-CRT	=	Three-Dimensional Conformal Radiation therapy
Ag	=	Argentum (Silver)
AgNPs	=	Silver Nanoparticles
Au	=	Aurum (Gold)
AuNPs	=	Gold Nanoparticles
BBB	=	Blood-Brain Barrier
BSA	=	Bovine Serum Albumin
BSE	=	Backscattered Electrons
C ₆ H ₈ O ₆	=	Ascorbic acid
CCRT	=	Concurrent Chemoradiotherapy
cm.	=	Centimeter
cm ²	=	Square Centimeter
cm ³	=	Cubic Centimeter
DLS	=	Dynamic light scattering
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethyl Sulfoxide
DNA	=	Deoxyribonucleic Acid
EBRT	=	External Beam Radiation therapy
EDX	=	Energy-dispersive X-ray spectroscopy
ETC	=	Electron Transport Chain
Fe ₃ O ₄	=	Magnetite
G0/G1	=	Gap 0 and Gap 1
G2/M	=	Gap 2 and mitotic phase
GBM	=	Glioblastoma
Gy	=	Gray
h	=	hours
H ₂ DCFH-DA	=	2',7'-dichlorodihydrofluorescein diacetate
H ₂ O ₂	=	Hydrogen Peroxide
H ₂ PtCl ₆	=	Chloroplatinic acid

Abbreviation (continue)



Ic ₁₀	=	Inhibitory Concentration at 10%
IMRT	=	Intensity-Modulated Radiation therapy
IR	=	Irradiation
KBr	=	Potassium Bromide
LD ₂₀	=	Lethal dose at 20%
LINAC	=	Linear Accelerator
MeV	=	Mega electron volt
MLC	=	Multileaf Collimator
mL	=	Milliliter
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MU	=	Monitor Unit
MV	=	Mega Voltage
NADPH	=	Nicotinamide Adenine Dinucleotide Phosphate
NPs	=	Nanoparticles
NSCLC	=	Non-Small Cell Lung Cancer
•OH	=	Hydroxyl radical
O ₂ ^{•-}	=	Superoxide
p53	=	Protein 53
PBS	=	Phosphate Buffered Saline
PE	=	Plating Efficiency
PEG	=	Polyethylene glycol
Pt	=	Platinum
Pt-NPs	=	Platinum nanoparticles
RFI	=	Relative fluorescence intensity
ROS	=	Reactive oxygen species
rpm	=	Revolutions Per Minute
RT	=	Radiation therapy
S	=	Synthesis phase

Abbreviation (continue)

SAD	=	Source to Axis Distance
SE	=	Secondary Electron
SEM	=	Scanning electron microscopy
SER	=	Sensitizer enhancement ratio
SiO ₂	=	Silicon Dioxide
SF	=	Survival Fraction
SRS	=	Stereotactic Radiosurgery
Ti	=	Titanium
TMZ	=	Temozolomide
μL	=	Micro liter
μM	=	Micromolar
VMAT	=	Volumetric Modulated Arc Therapy
WHO	=	World Health Organization (WHO)
ZP	=	Zeta potential



CHAPTER I

INTRODUCTION

Background and rationale

Glioblastoma (GBM) is aggressive and the most common primary malignant brain tumor in adults, which develops from glial cells (1). Additionally, the survival rate of patients with glioblastoma is relatively low, approximately 15 months (2). Typically, patients receive a combination of treatments, such as chemotherapy, surgery, or radiation therapy (RT), to improve the survival rate (3). Surgery plays a crucial role in the treatment of GBM by focusing on obtaining the most secure removal of the tumor. However, the GBM diffusely infiltrates normal brain tissue, making it difficult to undergo surgery (4). Thus, radiation therapy remains a commonly used and effective treatment method for unresectable GBM. However, some GBMs exhibit a degree of radioresistance, leading to RT failure and subsequent recurrence. Therefore, the minimal response of GBM to radiation, despite combined novel approaches and RT, has led to improvements in GBM treatment.

Nowadays, radiosensitizers have become the most reliable approach, as they are inexpensive and widely available and can still be further explored and developed to increase the sensitivity of cancer cells to radiation, enhancing the effectiveness of RT without increasing the radiation dosage (5). According to Gong et al. (6), radiosensitizers can be classified into 3 different groups: (I) Small Molecules (Oxygen, Oxygen mimics, etc.), (II) Macromolecules (Proteins and Peptides, miRNAs, siRNAs, etc.), (III) Nanomaterials (Noble Metal nanomaterials, Heavy Metal Nanomaterials, etc.). Among these, nanotechnology-based radiosensitizers have emerged as up-and-coming tools for enhancing the efficacy of radiation therapy. Nanoparticles made from high-Z materials, such as gold, bismuth, and gadolinium, can be used as radiosensitizers when combined with external beam radiation therapy, as they can absorb significant radiation energy. The interaction of ionizing radiation with nanoparticles results in various outcomes through photoelectric interactions and Compton scattering. The

electrons generated by these interactions transfer energy to the surrounding water, causing radiolysis and the formation of reactive oxygen species (ROS), which can damage DNA and other cellular components (7, 8).

Among high-Z nanomaterials, porous platinum nanoparticles, as high-Z, show their potential as radiosensitizers. The radiosensitizing effects of platinum nanoparticles (Pt-NPs) are mediated by three mechanisms: physical, chemical, and biological. Physically, at kilovoltage (kV) energies, the photoelectric effect predominates, generating secondary and Auger electrons that lead to localized high-LET damage (9). At clinical megavoltage (MV) photon energies, where Compton scattering dominates, Pt-NPs can still improve dose delivery through low-energy electron production, photon energy downshifting that locally triggers photoelectric-like events, and γ -ray-induced clustered DNA damage (10, 11). Chemically, Pt-NP surfaces catalyze ROS production through water radiolysis and redox reactions, especially effective near nuclear DNA (12). Biologically, Pt-NPs increase oxidative stress, inhibit DNA repair, and disrupt the cell cycle (13). In addition to these biological effects, their radiosensitizing capacity is evaluated using the sensitizer enhancement ratio (SER), defined as the ratio of the radiation dose required without a sensitizer to that required with a sensitizer to achieve the same biological effect. This metric displays a size-dependent trend (14, 15), emphasizing nanoparticle size as a pivotal factor in radiosensitization. Collectively, these mechanisms support the hypothesis that Pt-NPs can exert significant radiosensitizing effects even at MV photon energies, primarily through the combined contributions of Compton-generated secondary electrons, photon energy downshifting, and enhanced ROS production from their catalytic surfaces.

Therefore, the objectives of this study were (I) to fabricate and characterize Pt-NPs, and (II) to investigate their radiosensitizing effects in glioblastoma U87 cells. Specifically, this study aimed to evaluate the effects of Pt-NPs on cell viability, clonogenic survival, ROS generation, DNA double-strand break formation, cell cycle distribution, and apoptosis following irradiation. Through this comprehensive biological assessment, the present work aims to provide mechanistic insight into the potential of Pt-NPs as effective radiosensitizers to improve radiation therapy outcomes in glioblastoma.

Research scope

This thesis investigates the radiosensitizing effect of platinum nanoparticles (Pt-NPs) on glioblastoma U87 cells. Initially, Pt-NPs are synthesized by chemical reduction and subsequently characterized for their physicochemical properties. The morphology of the synthesized nanoparticles is examined using scanning electron microscopy (SEM), while their hydrodynamic size distribution and surface charge are determined by dynamic light scattering (DLS). The cytotoxic effects of Pt-NPs at various concentrations are evaluated using the MTT assay, and the 10% inhibitory concentration (IC_{10}) is determined for subsequent experiments. To assess the radiosensitizing potential of Pt-NPs, cells are treated with IC_{10} concentrations of Pt-NPs in combination with an LD_{20} of X-ray irradiation delivered by a 6 MV linear accelerator (LINAC). Clonogenic survival assays are performed to determine the surviving fraction and to assess radiation response enhancement. In addition, further mechanistic studies are conducted to elucidate the biological effects underlying radiosensitization, including apoptosis analysis, cell cycle distribution analysis, immunofluorescence staining for DNA damage assessment, and intracellular reactive oxygen species (ROS) detection. All experimental data are statistically analyzed using GraphPad Prism software, and each experiment is performed in triplicate across three independent biological replicates to ensure reproducibility and reliability of the results.

Research Hypothesis

Pt-NPs act as effective radiosensitizers that enhance the biological effects of ionizing radiation in glioblastoma U87 cells.

CHAPTER II

LITERATURE REVIEW

2.1 Glioblastoma

Glioblastoma multiforme, or Glioblastoma (GBM), is a brain tumor that is classified as the most common type of malignant brain tumor in adults. Its origin is from the astrocyte of glial cells, shaped like a five-pointed star, and acts as a support unit of neurons, both for feeding and repairing (16).

In 2007, the World Health Organization (WHO) classified the severity of brain tumors into four levels

1. Grade 1 Pilocytic Astrocytoma
2. Grade 2 Oligodendroglioma, Astrocytoma
3. Grade 3 Anaplastic oligodendroglioma/ astrocytoma/ oligoastrocytoma
4. Grade 4 Glioblastoma

Grade 1 brain tumors have low proliferative potential and can be cured by surgical resection, but grade 2 to 4 brain tumors are highly malignant and invasive. GBM is the most aggressive, invasive, and undifferentiated type of tumor and has been designated Grade IV by WHO. The patient with GBM has a median survival rate from diagnosis of only 9.2 months. The symptoms depend on the size, location, and related anatomical structure of the brain. Patients often come with an increase in intracranial pressure, including headache, nausea, vomiting, seizures, paralysis, or muscle weakness (17). GBM is one of the most radio-resistant tumors, so there is a low response to radiation therapy. GBM can be classified into two types

1. Primary glioblastoma is a tumor that occurs without changing from a grade 1 or 2 glioblastoma and is the most common in older patients.
2. Secondary glioblastoma is a tumor that has changed from a Grade 2 brain tumor and tends to occur in younger patients. Glioblastoma (U87) is a cell line derived from human astrocytes.

2.2 Treatment for Glioblastoma

The standard treatment for glioblastoma is surgical resection to remove as much of the tumor as possible; it's suitable for the cases where the cancer does not spread to other nearby organs or metastasis. Patients who undergo surgery may experience side effects such as headaches, nausea, stupor, and unconsciousness because of intracranial hypertension. After surgery, treatment is usually followed by concurrent chemoradiotherapy (CCRT), a treatment that gives chemotherapy and radiation therapy simultaneously.

2.2.1. Surgical resection

The purpose of surgical resection in glioblastoma is to remove as much tumor as possible to alleviate mass effect and to obtain brain tissue for pathological analysis (18). Advanced imaging techniques, such as intraoperative MRI and intraoperative ultrasound, play an essential role in glioblastoma surgical resection. They provide real-time visualization and guidance during surgery to separate normal brain tissue from cancer tissue, improve the accuracy of tumor resection, and reduce the risk of residual disease (4).

2.2.2. Chemotherapy

Chemotherapy is a treatment that uses a variety of chemicals and drugs, either separately or in combination, to destroy tumor cells. It is a systemic treatment that affects the entire body rather than a specific area, unlike localized treatments such as radiation therapy or surgery. Chemotherapeutic drugs frequently target all rapidly dividing cells because they cannot distinguish between tumor and normal cells. As a result, they can cause side effects such as gastrointestinal disturbances, bone marrow suppression, and hair loss, so optimizing the dosage administered is essential to achieving the best possible outcome (19). Chemotherapeutic drugs can be given in various ways, including intravenously or orally; the method of administration depends on the specific drug and the type of cancer being treated.

The administration of chemotherapy in brain cancer has limitations due to the blood-brain barrier (BBB), a specialized barrier; this makes it difficult to deliver therapeutic agents to the site of the tumor and effectively treat cancer (20). Temozolomide (TMZ) is the most common chemotherapeutic drug for glioblastoma, as a small lipophilic molecule allows TMZ to pass through the blood-brain barrier

(BBB); TMZ is one of the alkylating agents, which can lead to DNA double strands break by transferring a methyl group to purine base of DNA (21).

2.2.3. Radiation therapy (RT)

A medical treatment that uses high-energy ionizing radiation in several forms, such as X-rays or gamma, to kill cancer cells and reduce the size of the cancer cells. This treatment aims to maximize damage to cancer cells' DNA, preventing them from dividing and leading to cell death, while surrounding normal tissues receive minimal dose and low toxicity. Unlike chemotherapy, which is systemic, radiation therapy is typically a localized treatment, focusing on specific areas of the body. It can be administered to help provide local control for microscopic diseases that can't be treated by surgical resection (22).

2.2.3.1 X-ray beam treatment

X-rays are produced by bombarding a target with energetic electrons. As shown in Figure 1, these X-rays are made up of bremsstrahlung photons, created from interactions between electrons and atomic nuclei, and characteristic photons, which arise from electrons moving between different nuclear energy levels. A variety of X-ray energies (or wavelengths) can be generated. X-ray tubes emit X-rays at kilovolt energies, categorized as superficial X-rays (50-150 kV) or orthovoltage X-rays (150-300 kV); in contrast, linear accelerators (LINACs) generate X-rays at megavolt energies (23).

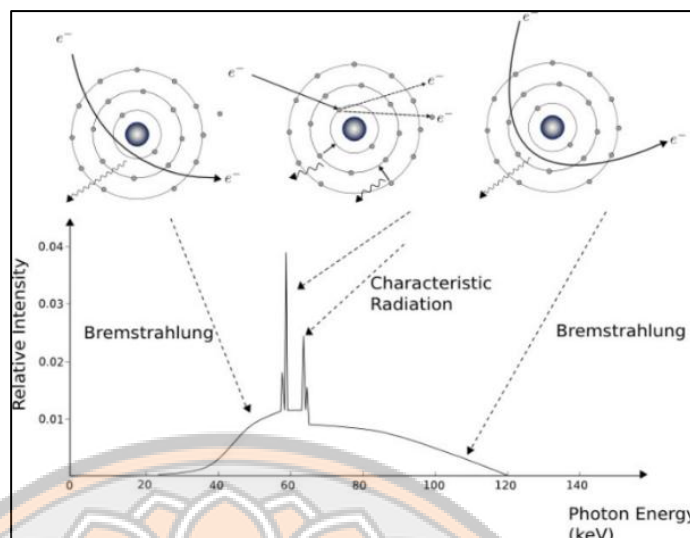


Figure 1 The mechanisms of incident electrons interact with the target atoms resulting in X-ray production and X-ray spectrum
(Source: Maier A et al., Medical Imaging Systems: An Introductory Guide, 2018)

Kilovoltage X-ray beams have limited penetration into the body, delivering most of the X-ray dose near the patient's surface; because of beam attenuation and scattering, the dose decreases rapidly with increasing depth. Currently, kilovoltage X-rays are primarily used to treat skin cancers. Megavoltage X-rays, being more penetrating, have replaced mainly kilovoltage treatments. Given its widespread application and varied uses, megavoltage X-rays represent the beam energy selected for this thesis.

2.2.3.2 Megavoltage X-ray Physical Properties

In a typical treatment, electrons are accelerated to 6 MV and impinge on a tungsten target, producing X-ray photons with an energy spectrum from 0 to 6 MV. An absorbed dose is a quantity that measures the energy deposited in matter by ionizing radiation per unit mass. It varies in depth through the patient. At the skin entry point, the dose is D_s , as shown in Figure 2. At the surface, an initial dose buildup occurs due to the contribution of secondary photoelectrons, reaching a maximum value, D_{max} , at a maximum depth. and then decreases almost exponentially until it reaches a value of D_{ex} at the patient's exit point (24).

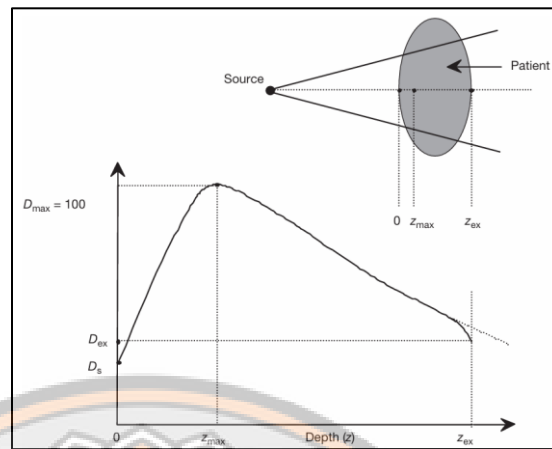


Figure 2 Dose deposition from megavoltage photon beams in a patient

(Source: Podgorsak et al., IAEA, 2015)

2.2.3.3 Type of radiation therapy

Radiation therapy can be divided into two types depending on the use of radiation beams.

2.2.3.3.1 External beam Radiation therapy (EBRT) is the most widely used form of radiation therapy, where high-energy radiation is delivered from outside the body to a targeted area, such as a tumor. Treatment planning techniques that can be used in the treatment of glioblastoma include,

- Three-Dimensional Conformal Radiation therapy (3DCRT) patients receive 3D conformal dose distribution, RT, which uses x-ray photons directed at the tumor target from several different angles.

- Intensity-modulated radiation therapy (IMRT). This technique generates varying radiation intensity on each beam using a multi-leaf collimator (MLC). Each beam is split into hundreds of beamlets, each with its own intensity level, to create a highly conformal dose distribution. Normal tissues may be further spared, and a curved target can be precisely shaped (25).

- Volumetric modulated arc therapy (VMAT) is a new radiation technology that made it possible to change three parameters during therapy delivery simultaneously: dose rate, treatment aperture shape by movement of MLC leaves, and

gantry rotation speed, that can attain exceptionally conformal dose distributions, providing better coverage of the target volume while protecting surrounding healthy tissues in comparison to traditional radiation therapy methods (26).

All those techniques can deliver higher doses to patients using standard fractionation, 1.8-2 Gy daily up to 60 Gy, whose survival rate increases with the radiation dose (27).

- Stereotaxic radiosurgery (SRS) is a technique that gives up high radiation dose in a single fraction, which depends on the tumor size and ranges from 15 to 24 Gy (28).

2.2.3.3.2 Brachytherapy is a type of internal radiation treatment in which radioactive materials are positioned directly within or close to a tumor. This method enables the administration of a high radiation dose to cancer while reducing exposure to nearby healthy tissues. For glioblastoma, the source will be placed into the tumor site during surgical intervention. Which Iodine-125 and Iridium-192 are the most frequently used isotopes for brachytherapy in cases of brain tumors (22).

2.3 Radiosensitizers

Radiosensitizers are drugs or chemical compounds administered along with radiation therapy to enhance the effectiveness of radiation treatment; they lead to a greater reduction in tumors or cancer cells than radiation or chemotherapy alone. Typically, radiosensitizers affect abnormal tissue more than normal tissue. The main mechanisms of action for radiosensitizers are now assumed to be (29).

1. Preventing the repair of DNA damage caused by radiation, thereby enhancing the extent of DNA damage.
2. Disrupting the cell cycle and organelle functionality to enhance cytotoxic effects
3. Suppressing the expression of genes related to radiation resistance or enhancing the expression of genes sensitive to radiation.

Radiosensitizers can be divided into three groups according to their structures (6, 30).

1. Small molecules such as oxygen, a potent radiosensitizer, enhance the generation of free radicals due to their distinctive electronic structure. Oxygen is the most electrophilic molecule in cells, which can react readily with electrons produced by the incoming radiation. Following the irradiation of oxygenated tumors, energy transfer leads to the radiolysis of water, initially creating an ion radical that subsequently reacts with another water molecule to produce the highly reactive hydroxyl radical.

2. Macromolecules such as proteins and peptides, like antibodies and short peptides, possess a strong binding capability with antigens and receptors that are overexpressed on the surface of tumor cells. SYM004, an antibody targeting the epidermal growth factor receptor, inhibits DNA double-strand break repair and triggers apoptosis by downregulating MAPK signalling, thereby enhancing radiosensitivity in cancer cells.

3. Nanomaterials, including noble metal nanomaterials like gold (Au, $Z=79$), silver (Ag, $Z=47$), and platinum (Pt, $Z=78$), can effectively absorb X-ray energy and interact with radiation in tumor cells, subsequently generating photoelectrons, Auger electrons, Compton electrons, and various other secondary electrons. These secondary electrons not only directly interact with DNA but also with water, leading to increased ROS production and further enhancing tumor cells' radiation sensitivity.

2.4 Nanoparticles-based radiation therapy

Nanoparticle -based radiation therapy is an innovative approach to cancer treatment that enhances the effectiveness of conventional radiation therapy by leveraging nanotechnology. This approach uses nanoparticles as radiosensitizers to amplify the damage to cancerous cells while protecting adjacent healthy tissues.

Metal and metal oxide NPs such as AuNPs, AgNPs, ZnO NPs, and Pt-NPs can increase the damage effectiveness on tumor cells by improving radiation absorption, and the mechanisms underlying radio-enhancement of nanoparticles can be categorized into various stages (8, 31, 32), as shown in Figure 3, including physical, chemical, and biological enhancement.

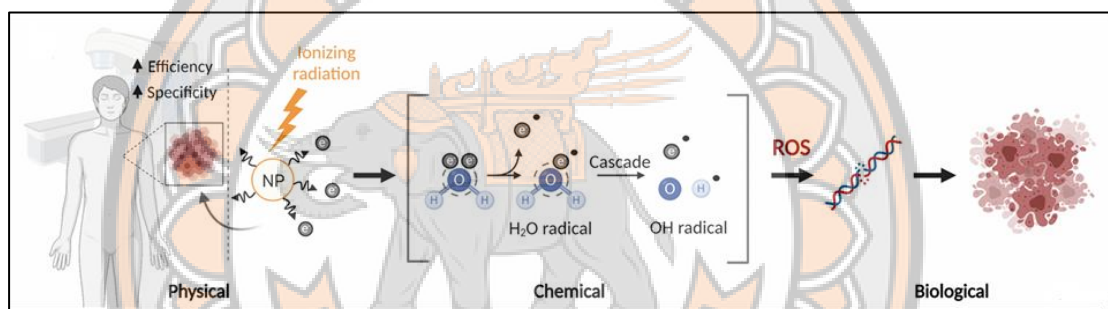


Figure 3 Mechanisms of nanoparticle radio enhancement
(Source: Lukas R. H. Gerken et al., Materials Horizons, 2023)

Physical enhancement is the interaction between NPs and ionizing radiation, generating secondary electrons that induce localized ionization and DNA damage. Low-energy photons (<500 keV) interact with NPs via photoelectric absorption, resulting in the ejection of photoelectrons and Auger electrons, and the emission of characteristic x-rays. The probability of the photoelectric effect is approximately proportional to Z^4/E^3 , making high-Z materials appealing for radio enhancement with low-energy photon beams. However, practical clinical applications generally involve higher energy levels, and interactions between mega electron volt (MeV) photons and nanoparticles (NPs) mainly lead to Compton scattering or pair production, where the interaction probabilities are primarily linearly dependent on the atomic number Z . The

physical dose enhancement for MV photons was modeled to be minimal in cellular environments, even when high-Z NPs are considered.

Chemical enhancement, the generation of reactive oxygen species (ROS) in the surrounding medium through secondary electron emission, is the primary mechanism of radiation-induced cellular damage. ROS that form can interact with cellular biomolecules, leading to apoptosis or cell death while also inhibiting tumor development. As a result, adequate generation of reactive oxygen species (ROS) is essential for inducing DNA damage and hindering DNA repair mechanisms. Moreover, nanoparticles are not only able to increase ROS generation, inhibiting tumor development and causing cellular damage when interacting with ionizing radiation, but also involve increasing the local oxygen levels through the use of external oxygen supply or the catalytic breakdown of hydrogen peroxide (H_2O_2) to alleviate hypoxic conditions, or converting H_2O_2 into more harmful hydroxyl radicals ($\bullet\text{OH}$) via Fenton or Haber–Weiss reactions. From Li et al. (13), experiments show that porous platinum nanoparticles can increase tumor oxygenation in the NCI-H460 human NSCLC cell line by converting endogenous H_2O_2 to O_2 , thus significantly enhancing the efficacy of conventional radiation therapy.

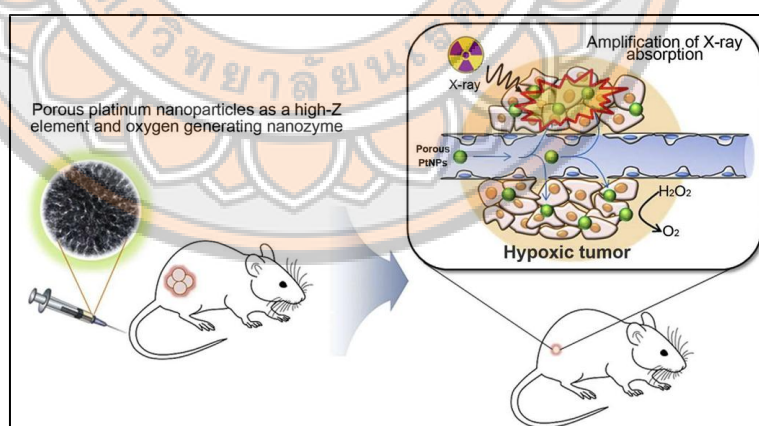


Figure 4 Schematic illustration of enhanced radiation therapy using porous PtNPs
(Source: Li et al, Biomaterials, 2019)

Biological sensitization mechanisms in radiation therapy: factors such as total dose, dose per fraction, number of fractions, intervals between fractions, and overall treatment duration influence both tumor control and the toxicity of normal tissues. While increasing the dose per fraction, reducing the time intervals between them, and shortening the overall treatment duration can enhance tumor control, these changes can also lessen the protective effects of fractionation on healthy tissues. The differing responses of tumors and normal tissues may be related to the 5 Rs of radiation therapy.

(I) Repair nanoparticles (NPs) have demonstrated the ability to disrupt a cell's DNA repair mechanisms, thereby reducing cancer cells' ability to respond to DNA damage induced by ionizing radiation, ultimately enhancing the effectiveness of treatment. In Mirjolet et al. (33), experiments demonstrated that Ti nanotubes reduced the effectiveness of DNA repair in human glioblastoma cells. (II) Redistribution refers to the alteration of the cell population's distribution across different cell cycle phases after radiation exposure. Since cell sensitivity to radiation varies with cell cycle phase. In AshaRani et al. (34), experiments demonstrated that using nanoparticles (AgNPs) can also arrest the cell cycle to the G2/M phase in U251 human glioblastoma cells, causing a change in the distribution of the cellular cell cycle, moving it into a phase that more sensitive to radiation, thus enhancing the response to irradiation. (III) Repopulation refers to the ability of both normal and tumor cells to divide and repopulate during or after radiation therapy. Since tumor cells can rapidly proliferate after sublethal irradiation, NPs can slow cell growth and inhibit repopulation. (IV) Reoxygenation, the process by which hypoxic tumor cells become reoxygenated during or after radiation therapy, in which hypoxic cells tend to be more radioresistant than normoxic cells, and can be explained by the oxygenation fixation theory. Li et al.'s experiments demonstrated that nanoparticles in NCI-H460 (Human NSCLC cell line) can overcome hypoxic cells by increasing tumor oxygenation by converting endogenous H_2O_2 to O_2 (13). In normoxic conditions with high oxygen levels, oxygen acts as a "fixative" for radiation-induced DNA damage by chemically stabilizing the free radicals formed during irradiation, rendering the damage permanent and irreparable, thereby increasing the rate of cell death. (V) Intrinsic radiosensitivity genetic background, tumor cells, and normal tissue influence the treatment response to irradiation; acquired mutations in some genes can render cells prone to programmed cell death, a prominent example

being p53. Therefore, nanoparticles that can modify the cell's intrinsic radiosensitivity would be interesting for therapy; Au NPs have been implicated in inducing apoptosis, for instance, by activation of caspases and, as a result, triggering the rupture of the mitochondrial membrane and the release of cytochrome C thereby aiding in counteracting intrinsic radiation resistance effects (35).

Moreover, nanoparticles can be modified by surface coating with different molecules to enhance biocompatibility. PEG is one of the most used for in-vitro and in-vivo applications. Kostiv et al. (36), demonstrated the biocompatibility of PEG by modification of $\text{Fe}_3\text{O}_4/\text{SiO}_2$ nanoparticles with PEG, showing that the highest concentration of $\text{Fe}_3\text{O}_4/\text{SiO}_2$ -PEG nanoparticles did not significantly affect either the survival or viability of mNSCs, but $\text{Fe}_3\text{O}_4/\text{SiO}_2$ nanoparticles without PEG coating at lowest possible concentration decreased cell viability by more than 50% compared to the control. Furthermore, PEGylation of NPs can also reduce aggregation, increase NPs stability in biological fluids, enhance circulation time, and reduce phagocytosis. Moreover, modification of NPs with PEG also results in a more positive shift in the surface charge of NPs, and with increasing NP size, this is related to the efficiency of PEG coverage (37).

2.5 Radiation-induced ROS generation mediated by nanoparticles

Reactive oxygen species (ROS) are highly reactive forms of molecular oxygen that are important in cell signaling and homeostasis, including singlet oxygen ($^1\text{O}_2$), superoxide radical anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot\text{OH}$), etc. (7, 38). In radiation therapy, the use of ionizing radiation generates ROS through water radiolysis, which the excessive ROS leading to oxidative stress, potential cellular damage and activation of cell death pathways, such as apoptosis and autophagy (39). When combined with high-Z metal nanoparticles, radiation can exert a greater tumor-inactivating effect than radiation alone (40).

The mechanism of radiosensitization of high-Z metal nanoparticles is mainly achieved by increasing the ROS generation, which high-Z metal nanoparticles can absorb more photons during irradiation due to their large photoelectric cross-section, resulting in greater energy deposition and enhanced the emission of 2nd electron including photoelectrons, Compton electrons and Auger electrons, which these 2nd

electron will be further interact with oxygen-containing molecules, such as water or oxygen in the surrounding medium, producing additional ROS, thereby significantly improving the overall effectiveness of radiation therapy (7, 8). These ROS can directly attack the DNA backbone and nucleobases, inducing oxidative base lesions and single-strand breaks (SSBs). When two SSBs occur in proximity on opposite DNA strands these lesions can be converted into DSBs. Furthermore, ROS can also promote DSB formation indirectly by interfering with DNA replication and repair processes (41). Collectively, these effects lead to the accumulation of unrepaired or mis-repaired DSBs, activation of the DNA damage response, and subsequent cell cycle arrest or apoptosis. The various mechanisms described above can be summarized as shown in Figure 5.

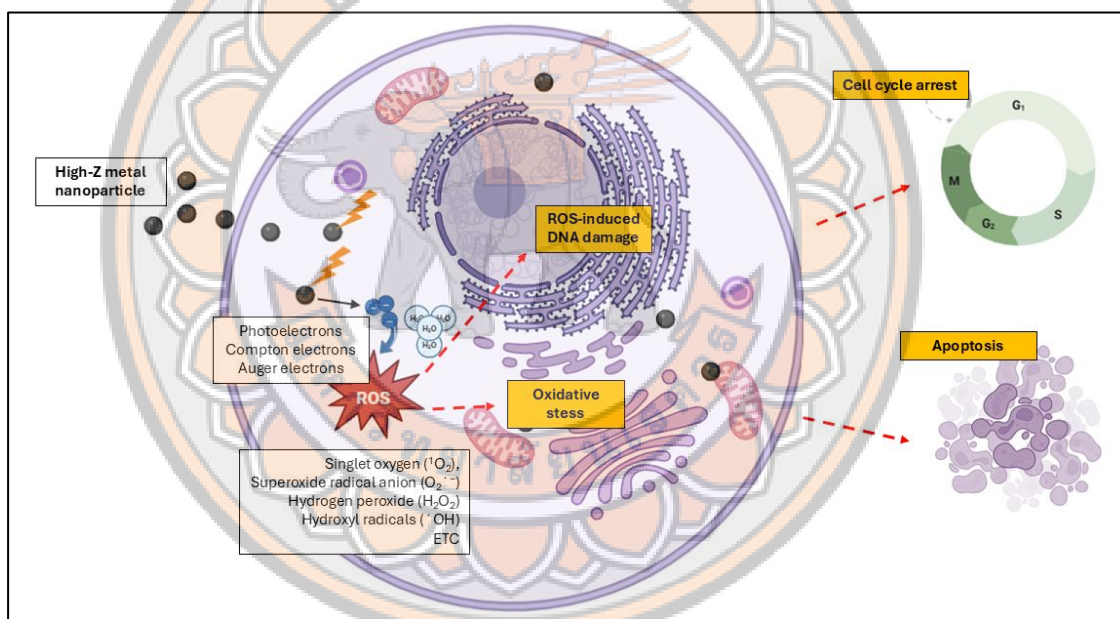


Figure 5 Radiation-induced ROS generation mediated by nanoparticles and cellular effect

Furthermore, radiation not only induces the immediate generation of reactive oxygen species (ROS) through water radiolysis, but also leads to a sustained elevation of intracellular ROS levels several hours after exposure, indicating the involvement of biological sources of ROS production (42). Although NADPH oxidase has been reported to contribute to radiation-induced ROS generation, accumulating evidence suggests that mitochondria also play a significant role in this process (43). In general,

electron leakage from the mitochondrial electron transport chain (ETC) to molecular oxygen results in the formation of superoxide ($O_2^{\bullet-}$), which can subsequently give rise to other reactive oxygen species, including hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$). These species, collectively referred to as mitochondrial ROS, are essential mediators of radiation-induced oxidative stress and cellular damage (44, 45).

2.6 Scanning Electron Microscope (SEM)

A scanning electron microscope (SEM) is a valuable tool for analyzing nanoparticles, a common and highly effective method for investigating their morphology, size, distribution, and surface features. SEM plays a crucial role in nanoscience due to its ability to image nanoscale structures with high resolution. The SEM scans the surface of the sample by focusing the high-energy electron beams direct toward the sample, once electron hits the sample, x-rays and three types of electrons are emitted, backscattered electrons (BSE), secondary electrons (SE) , and Auger electrons, which backscattered electrons (BSE) and secondary electrons (SE) are useful for imaging in the SEM technique (46). The electron emitted from the scan sample can be detected by electron detector and amplified by electronic devices, which both SE and BSE will be collected if positive voltage is applied to the collector screen and only BSE will be collected if negative voltage applied on the collector screen. The signals are then displayed immediately on the screen, and the operator adjusts the brightness and intensity until a sufficiently clear image is achieved (47).

Preparing samples for scanning electron microscopy (SEM) is essential for acquiring clear, precise images, particularly for materials such as nanoparticles. If the nanoparticles are metallic, they do not require any preparation because they already conduct electricity when bombarded with electrons. However, the nonmetallic nanoparticles need to be coated with a thin conducting material layer such as gold. For all samples, if presented as a solution, it must be completely dry before imaging to prevent artifacts.

2.7 Dynamic light scattering (DLS)

Dynamic Light Scattering (DLS) is a widely used technique for characterizing nanoparticles by determining their size distribution in suspension. When laser light is applied to a sample containing nanoparticles, the incident light will scatter in all directions, and the intensity of the scattered light will be recorded and assessed using a detector. If a sample contains particles of varying sizes or is polydisperse, it may result in multiple scattering events, known as multiple light scattering, which can impact the accuracy of the particle size distributions derived from the measurements. To reduce multiple scattering, the most common approach is to dilute the sample.

The Z-average, hydrodynamic size, is the primary measurement utilized in dynamic light scattering (DLS) to assess the size distribution of a sample, often combined with the polydispersity index (PDI) to evaluate the sample's size heterogeneity. A high polydispersity index (PDI) indicates polydispersity resulting from size variations within the sample or from agglomeration or aggregation during analysis. For a monodisperse sample, the Z-average should equal to the mean of the single peak within the distribution. In a polydisperse sample, while the Z-average provides a single value, the intensity distribution will show multiple peaks (48).

2.8 Zeta potential

Zeta Potential, the potential at particle-fluid interface, is a crucial parameter for characterizing the stability and surface charge of nanoparticles in a suspension. It measures the electric potential at the slipping plane of a particle, which is the plane within the diffuse layer that marks the boundary between ions and the liquid during particle motion. Nanoparticles with surface charge in suspension, when an electric field is applied, a particle begins to move toward the electrode that has the opposite charge call "electrophoresis", due to the interaction between the electric field and the charged particle.(49) Which velocity of the nanoparticles is proportional to the electrical potential of the particle at the slipping plane (46). So, the velocity of the nanoparticles can be used to determine the zeta potential by using the following equation: (49)

$$\mu_e = \frac{V}{E} \quad (1)$$

where V represents the particle velocity ($\mu\text{m/s}$), and E denotes the electric field strength (Volt/cm), then the zeta potential (ZP) is subsequently determined using the obtained μ_e based on Henry's equation.

$$\mu_e = \frac{2\varepsilon_r\varepsilon_0\zeta f(Ka)}{3\eta} \quad (2)$$

where ε_r denotes the relative permittivity or dielectric constant, ε_0 represents the permittivity of free space, $\zeta = \text{ZP}$, $f(Ka)$ refers to Henry's function, and η indicates the viscosity at the temperature of the experiment.

The magnitude of the zeta potential can indicate the stability of the particles, which has a greater magnitude signifies enhanced stability because of stronger electrostatic repulsion.

- ZP values of $\pm 0\text{--}10\text{mV}$ highly unstable and tend to aggregate
- ZP values of $\pm 10\text{--}20\text{mV}$ relatively stable
- ZP values of $\pm 20\text{--}30\text{mV}$ moderately stable
- ZP values of $\pm 30\text{mV}$ highly stable

CHAPTER III

RESEARCH METHODOLOGY

This research was conducted at the Faculty of Allied Health Sciences and Department of Radiology, Faculty of Medicine, Naresuan University

3.1 Materials and equipment used in the research

Table 1 Cells used in the research

Number	Cell	Abbreviation	Manufacturer
1	Human Glioblastoma U87 Cells (ATCC No. HTB-14)	U87 MG	ATCC, US

Table 2 Chemicals used in research

Number	Chemicals	Abbreviation	Manufacturer
1	Potassium bromide	KBr	HIMEDIA
2	Poloxamer 407	Pluronic F-127	Sigma Aldrich
3	Chloroplatinic acid	H ₂ PtCl ₆	TCI
4	Ascorbic Acid	C ₆ H ₈ O ₆	Supelco
5	Polyethylene glycol	PEG	Sigma Aldrich
6	Dulbecco's Modified Eagle Medium	DMEM	Gibco, New York, USA
7	3-(4,5-dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide	MTT	Thermo Fisher Scientific, China
8	Phosphate Buffered Saline	PBS	Gibco, New York, USA

Table 2 Chemicals used in research (continue)

Number	Chemicals	Abbreviation	Manufacturer
9	Trypsin Polyclonal Antibody	Trypsin	Gibco, New York, USA
10	Dimethyl Sulfoxide	DMSO	Loba Chemie, India
11	Crystal Violet	-	Loba Chemie, India
12	70% Ethanol	-	RCI Labscan, Bangkok, Thailand
13	Muse® Cell Cycle Kit	-	Luminex, Texas, USA
14	Muse® Annexin V & Dead Cell Kit	-	Luminex, Texas, USA
15	Paraformaldehyde	-	Thermo Fisher Scientific, China
16	Triton X-100	-	-
17	Bovine serum albumin (BSA)	-	HIMEDIA
18	Alexa-Fluor-488 conjugated polyclonal goat anti-mouse IgG antibody	-	Life Technologies, Eugene, OR, USA
19	4',6-diamidino-2-phenylindole	DAPI	Life Technologies, Eugene, OR, USA
20	Reactive Oxygen Species (ROS) Detection reagents	H ₂ DCFDA	Molecular Probe TM , USA

Table 3 Equipment used in research

Number	Equipment	Manufacturer
1	Linear Accelerator, Clinac 2100 CD No. 5515	Varian
2	Bolus	Varian
3	Solid Phantom	Varian
4	Biosafety Cabinets	NuAire
5	CO ₂ Incubator	SL SHEL LAB
6	Muse Cell Analyzer	(Merck, Darmstadt, Germany)
7	Microplate Reader	Molecular Devices, USA
8	Pipette Controller	Falcon®
9	Microscope Olympus CKX31	Olympus, Tokyo, Japan
10	Vertex Mixer	Labnet, New York, USA
11	Centrifuge	Hettich
12	Hemocytometer	HBG® BLOOD COUNTING CHAMBER BRIGHTLINE
13	Carl Zeiss fluorescence microscope	Carl Zeiss AG, Germany

3.2 Synthesis of Platinum nanoparticles (Pt-NPs)

Pt-NPs were produced through a chemical reduction method following a procedure reported previously (50)

1. A total of 50 mg of KBr and 5 mg of Pluronic F127 were put in 0.5 ml of deionized water at 70 °C until it dissolved completely
2. Then, 0.2 ml of H_2PtCl_6 solution (20 mg/ml) and 0.5 ml of ascorbic acid solution (25 mg/ml) were added
3. The mixture solution was stirred overnight at 70 °C in a shaker at 1000 rpm.
4. After incubation, Pt-NPs were obtained by centrifuging the solution at 15,000g for 15 mins and washed 3 times: first with 1 ml of ethanol to remove residual Pluronic F127, and then with 1 ml of deionized water to remove non-reactive components.

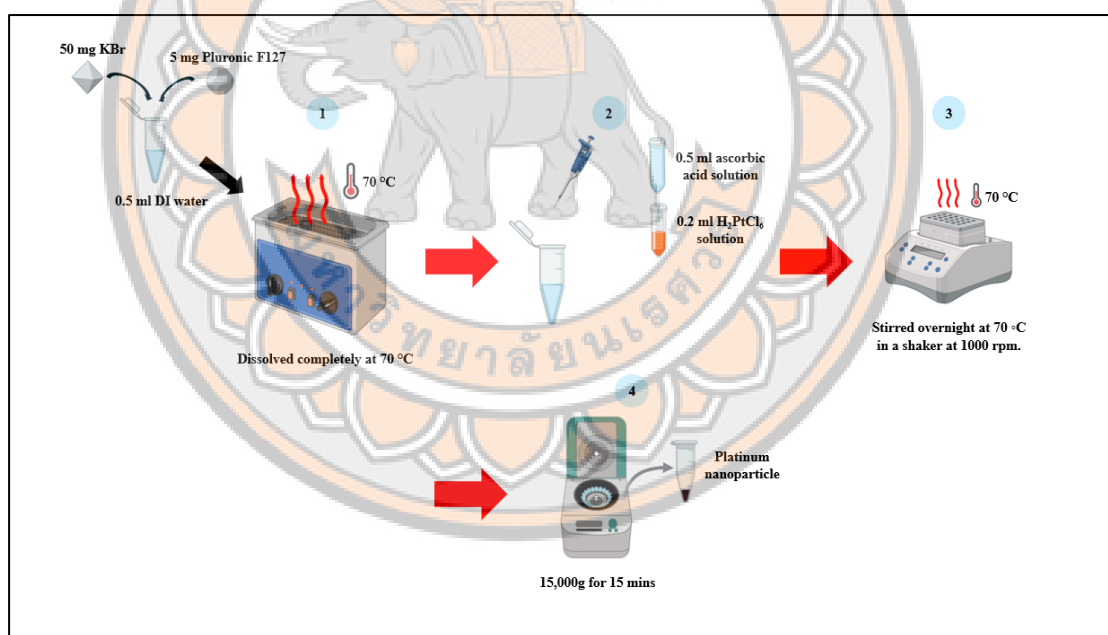


Figure 6 Schematic illustration of the synthesis of Pt-NPs

3.3 Characterization of Pt-NPs

3.3.1 Particle size and Zeta potential

Dynamic Light Scattering (DLS) is a widely used technique for characterizing nanoparticles by determining their size distribution in suspension, hydrodynamic size, and zeta Potential. (51) The hydrodynamic size and the surface charge of Pt-NPs will be determined through dynamic light scattering with Zeta-Sizer Ultra (Malvern Instruments Ltd., Malvern, UK), where the samples were prepared by diluting 2 μ l of Pt-NPs in 1 ml of deionized water, and the readings will be taken three times (n=3) to ensure accuracy.

3.3.2 Scanning Electron Microscopy (SEM)

The morphology of the Pt-NPs was analyzed using a field-emission scanning electron microscope (FE-SEM, Apreo S, Thermo Fisher Scientific) set to an accelerating voltage of 20 kV. A small amount of the Pt-NP suspension was placed directly onto a clean, conductive metal stub, which was then allowed to air-dry at room temperature. Additionally, elemental analysis was conducted using an energy-dispersive X-ray spectroscopy (EDX) detector integrated with the SEM system. EDX spectra were acquired from multiple sample regions to ensure uniformity and reproducibility. Characteristic X-ray emission peaks from platinum were detected, confirming its presence in the nanoparticles.

3.4 Culture of glioblastoma U87 cells

U87 MG cells (ATCC Number HTB-14) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin in a T75 flask in an incubator at 37°C, 5% carbon dioxide, and 95% relative humidity. The cell culture medium was changed every 3 days. At 70% confluence, cells were harvested by trypsinizing cultures with 0.25% trypsin-EDTA in Phosphate-Buffered Saline (PBS).

3.5 Cytotoxicity of Pt-NPs on glioblastoma U87 cells using MTT assay

An MTT assay was performed to assess the viability of glioblastoma U87 cells after 24, 48, and 72 hours of culture at varying concentrations of Pt-NPs. The percentage of cell viability was measured to identify the concentrations of Pt-NPs that caused a 10% decrease in the growth of glioblastoma U87 cells (Inhibitory concentration; IC₁₀), which will be used in further experiments and can be done using the following procedures (52):

1. Glioblastoma U87 cells were seeded into 96-well plates at a density of 1×10^4 cells per well in 100 μ L of complete culture medium and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell attachment.
2. After incubation, the culture medium was removed, and the cells were gently washed with phosphate-buffered saline (PBS). Pt-NPs were then added to the cells at final concentrations of 5, 10, 20, 40, 80, 160, and 320 μ g/mL, followed by incubation for an additional 24 h under the same culture conditions.
3. Subsequently, the medium was removed, and the cells were washed with PBS. A volume of 100 μ L of MTT solution (0.5 mg/ml) prepared in serum-free DMEM was added to each well, and the plates were incubated for 3 h in the dark at 37°C.
4. After incubation, the MTT-containing medium was carefully removed and replaced with 100 μ L of dimethyl sulfoxide (DMSO) to dissolve the formed formazan crystals, and the absorbance was measured at 570 nm using a microplate reader.
5. Cell viability was expressed as a percentage relative to untreated control and calculated using the following equation.

$$\% \text{Cell viability} = \frac{\text{absorbance of samples} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \times 100 \quad (3)$$

6. The relationship between Pt-NP concentration and cell viability was plotted, and the 10% inhibitory concentration (IC_{10}) was determined using GraphPad Prism software.
7. All experiments were performed in triplicate ($n = 3$).

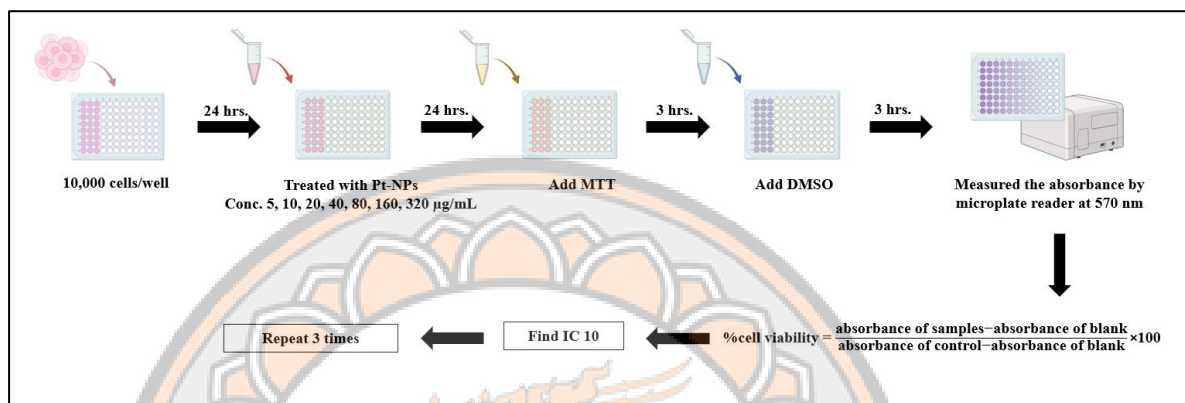


Figure 7 Schematic illustration of cytotoxicity of Pt-NPs on glioblastoma U87 cell (MTT Assay)

3.6 Radiation effect on glioblastoma U87 cells

3.6.1 Clonogenic assay

A clonogenic assay was performed, and the survival fraction was then calculated to determine the radiation dose that reduced approximately 20% of the survival fraction of glioblastoma U87 cells (LD₂₀), which will be further used in combinations with nanoparticles to investigate the radiosensitization effects of Pt-NPs and to evaluate the effects of ionizing radiation or radiosensitizer on the growth and proliferative characteristics of cells. The clonogenic assay can be done using the following protocol (53):

3.6.1.1 Determination of LD₂₀ of glioblastoma U87 cells

1. Glioblastoma U87 cells were seeded into 6-cm culture dishes at a density of 2×10^5 cells per dish in 2 mL of complete culture medium for five experimental groups as shown here

- Control group
- Exposed to 1 Gy irradiation (IR-1Gy)
- Exposed to 2 Gy irradiation (IR-2Gy)
- Exposed to 4 Gy irradiation (IR-4Gy)
- Exposed to 6 Gy irradiation (IR-6Gy)
- Exposed to 8 Gy irradiation (IR-8Gy)

The radiation doses of 1, 2, 4, 6, and 8 Gy were selected to cover a broad range of cellular responses, enabling accurate construction of the survival curve and fitting to the linear-quadratic model.

2. The cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell attachment.
3. Irradiation was performed by placing the 6-cm culture dishes in a 1.5 cm-thick bolus, which was positioned on a 30x30x15 cm³ solid phantom and covered with another 1.5 cm-thick bolus. Cells were irradiated using a 6 MV linear accelerator (Clinac 2100 CD, serial number 5515; Varian Medical Systems) at the Department of Radiology, Naresuan University Hospital.

4. The irradiation parameters included a source-to-axis distance (SAD) of 100 cm, a depth of 3 cm, a dose rate of 400 MU/min, and a field size of $20 \times 20 \text{ cm}^2$. The gantry angle was set to 0° , and the isocenter was positioned at the midpoint of the culture dish bottom.

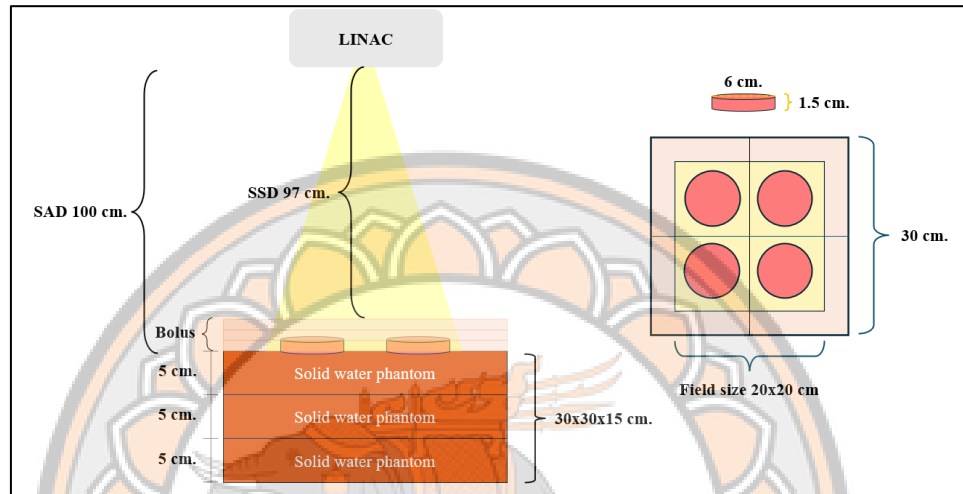


Figure 8 Schematic illustration of irradiation conditions

5. Following irradiation, the cells were returned to the incubator and cultured for an additional 24 h.
6. Subsequently, the culture medium was removed, the cells were washed with phosphate-buffered saline (PBS), and detached using 750 μL of 0.25% trypsin–EDTA for 3–5 min.
7. Trypsin activity was neutralized by adding a double volume of complete culture medium. The cell suspension was collected into 15-mL Falcon tubes, centrifuged at 1500 rpm for 5 min, and resuspended in 1 mL of medium.
8. Cell numbers were determined using a hemocytometer with trypan blue staining.
9. For colony formation, cells were reseeded into 6-well plates at 200 cells per well for the control group and 400 cells per well for the irradiated groups. The cells were incubated at 37°C with 5% CO_2 .

and 95% relative humidity without changing the culture medium to allow colony formation.

10. After the incubation period, the medium was removed, and the cells were washed with PBS, fixed with 2 mL of 100% methanol for 10 min, and stained with 0.05% crystal violet for 30–60 min.
11. Excess stains were removed by washing with distilled water, and the plates were air-dried at room temperature.
12. Colonies containing 50 or more cells were counted under a light microscope at 10×–40× magnification.
13. Plating efficiency (PE) and surviving fraction (SF) were calculated using the following equations (53), and the radiation dose corresponding to LD₂₀ was determined.

$$\%PE = \frac{\text{No.of colonies formed}}{\text{No.of Cells Seeded}} \times 100\% \quad (4)$$

$$SF = \frac{\text{No.of colonies formed after treatment}}{\text{No.of Cells Seeded X PE}} \times 100 \quad (5)$$

14. All experiments were performed in triplicate (n = 3).

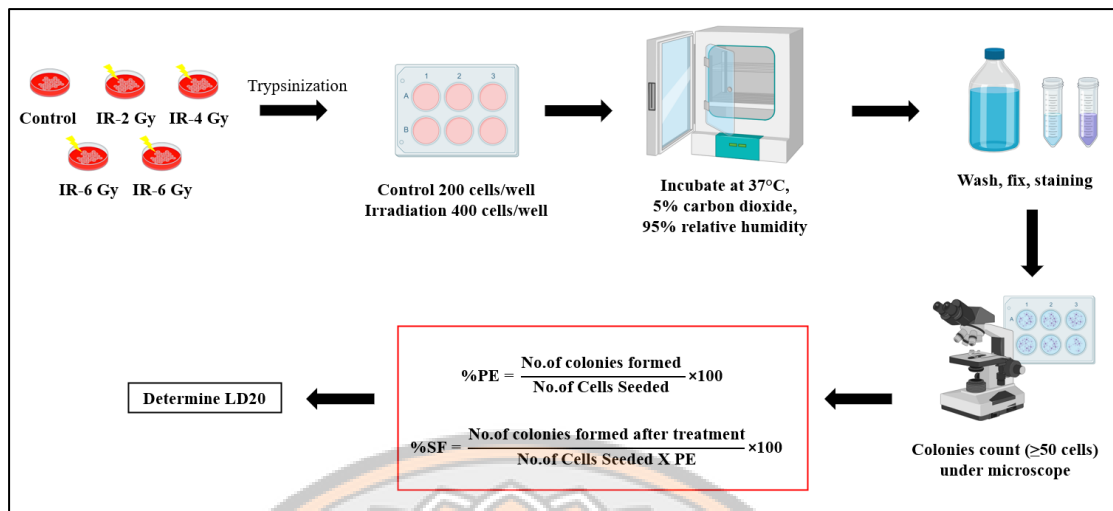


Figure 9 Schematic illustration of the clonogenic assay for determination of LD₂₀ glioblastoma U87 cells

3.6.1.2 Determination of the radiosensitization of Pt-NPs

1. Glioblastoma U87 cells were seeded into 24-well plates at a density of 2×10^5 cells per well in 2 mL of complete culture medium for four experimental groups:

- Control group
- IC₁₀ of Pt-NPs
- Irradiation at LD₂₀
- IC₁₀ of Pt-NPs and irradiation at LD₂₀

2. Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell attachment.

3. Irradiation was performed by placing a 24-well plate in a 2 cm-thick bolus, which was placed on a 30x30x15 cm³ solid phantom and covered with another 1.5 cm-thick bolus. Radiation was delivered using a 6 MV linear accelerator (Clinac 2100 CD, serial number 5515; Varian Medical Systems) at the Department of Radiology, Naresuan University Hospital.

4. The irradiation parameters included a source-to-axis distance (SAD) of 100 cm, a depth of 3.5 cm, a dose rate of 400 MU/min, and a field size of $20 \times 15 \text{ cm}^2$. The gantry angle was set to 0° , and the isocenter was positioned at the midpoint of the bottom of the 24-well plate.

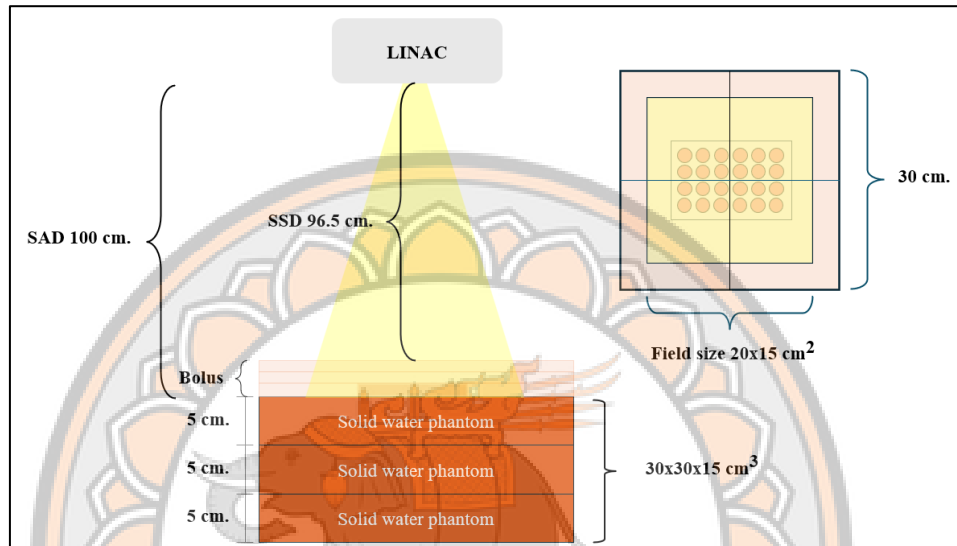


Figure 10 Schematic illustration of irradiation conditions

5. Following irradiation, cells were returned to the incubator and cultured for an additional 24 h.
6. Subsequently, the culture medium was removed, the cells were washed with phosphate-buffered saline (PBS), and detached using 300 μL of 0.25% trypsin–EDTA for 3–5 min.
7. Trypsin activity was neutralized by adding a double volume of complete culture medium. The cell suspension was collected into 15-mL Falcon tubes, centrifuged at 1500 rpm for 5 min, and resuspended in 1 mL of medium.
8. Cell numbers were determined using a hemocytometer with trypan blue staining.

9. For colony formation, cells were reseeded into 6-well plates at a density of 200 cells per well for the control group, 400 cells per well for the irradiated groups, and 600 cells per well for the Pt-NPs and a combination of Pt-NPs with 2 Gy irradiated groups.
10. The cells were incubated at 37°C with 5% CO₂ and 95% relative humidity without changing the culture medium to allow colony formation.
11. After the incubation period, the medium was removed, and the cells were washed with PBS, fixed with 2 mL of 100% methanol for 10 min, and stained with 0.01% crystal violet for 30–60 min.
12. Excess stains were removed by washing with distilled water, and the plates were air-dried at room temperature.
13. Colonies containing 50 or more cells were counted under a light microscope at 10× – 40× magnification.
14. Plating efficiency (PE) and surviving fraction (SF) were calculated using the following equations (53), and the radiosensitization effect of Pt-NPs was evaluated based on sensitizer enhancement ratio (SER) by determining the ratio of radiation doses required to achieve the same survival fraction in the absence and presence of Pt-NPs (54).

$$\%PE = \frac{\text{No.of colonies formed}}{\text{No.of Cells Seeded}} \times 100\% \quad (4)$$

$$SF = \frac{\text{No.of colonies formed after treatment}}{\text{No.of Cells Seeded X PE}} \times 100 \quad (5)$$

$$SER = \frac{\text{Dose of radiation alone}}{\text{Dose of radiation + sensitizer}} \quad (6)$$

15. All experiments were performed in triplicate (n = 3).

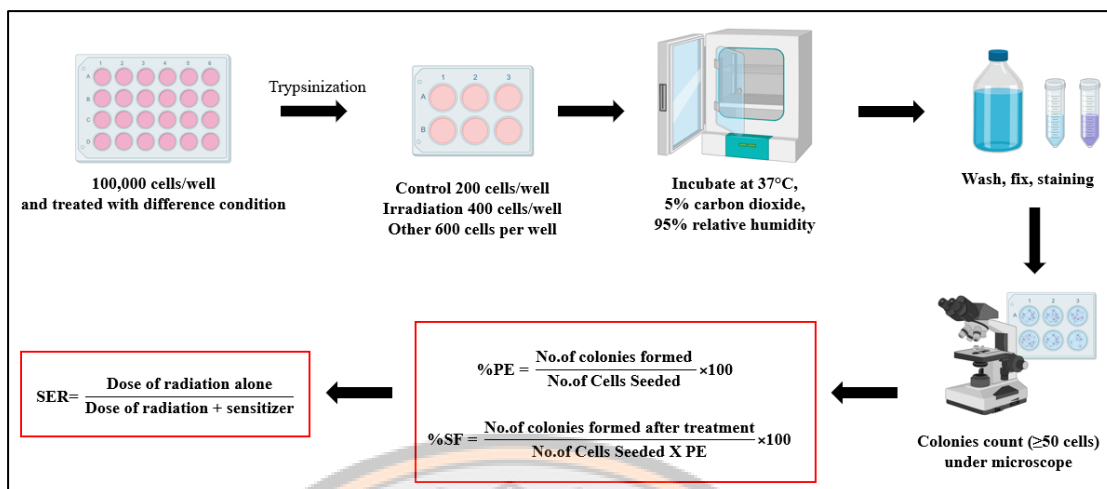


Figure 11 Schematic illustration of a clonogenic assay for the determination of the radiosensitization of Pt-NPs in glioblastoma U87 cells

3.6.2 Immunofluorescence staining for γ -H2AX

The detection of γ -H2AX foci is performed using immunofluorescence staining to quantify DSB in irradiated cells, which form γ H2AX, an early cellular response to the induction of DNA double-strand breaks. Immunofluorescence staining is performed using the following protocol:

1. Glioblastoma U87 cells were seeded into 24-well plates at a density of 2×10^5 cells per well in 2 mL of complete culture medium for four experimental groups:
 - Control group
 - IC₁₀ of Pt-NPs
 - Irradiation at LD₂₀
 - IC₁₀ of Pt-NPs and irradiation at LD₂₀
2. Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell attachment.
3. Irradiation was performed by placing a 24-well plate in a 2 cm-thick bolus, which was positioned on a 30x30x15 cm³ solid phantom and covered with another 1.5 cm-thick bolus. Radiation was delivered using a 6 MV linear accelerator (Clinac 2100 CD, serial number

5515; Varian Medical Systems) at the Department of Radiology, Naresuan University Hospital.

4. The irradiation parameters included a source-to-axis distance (SAD) of 100 cm, a depth of 3.5 cm, a dose rate of 400 MU/min, and a field size of $20 \times 15 \text{ cm}^2$. The gantry angle was set to 0° , and the isocenter was positioned at the midpoint of the bottom of the 24-well plate.

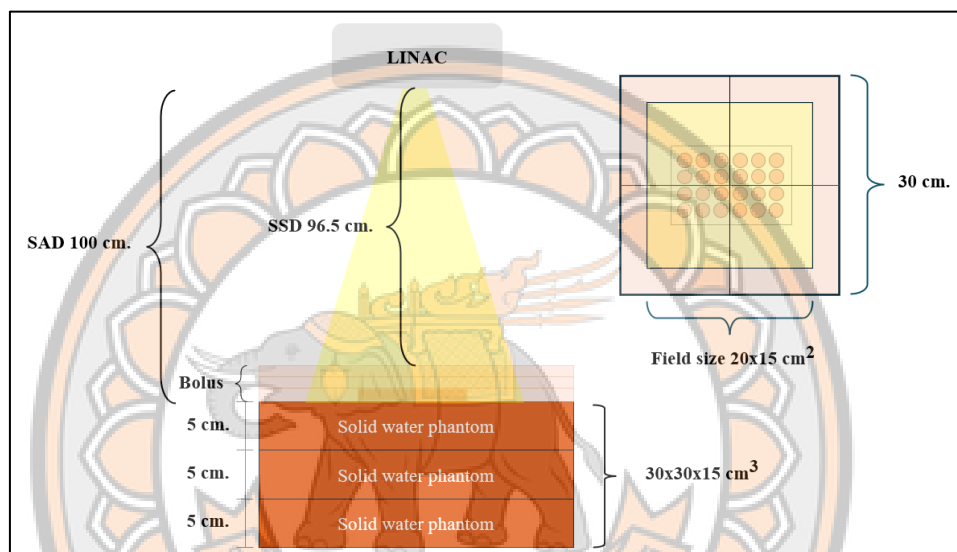


Figure 12 Schematic illustration of irradiation condition

5. After irradiation, the culture medium was removed, and the cells were washed twice with PBS.
6. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by two washes with PBS for 2 min each.
7. The cells were then permeabilized with 0.5% Triton X-100 prepared in PBS for 5 min. After permeabilization, the Triton X-100 solution was removed and replaced with a blocking solution containing PBS and 2% bovine serum albumin (BSA), and the sample was further incubated at room temperature for 1 h to block nonspecific binding.
8. Subsequently, the blocking solution was removed, and cells were incubated overnight at 4°C with a phospho-histone H2AX (Ser139) monoclonal primary antibody (1:800 dilution; Thermo Fisher

Scientific, Rockford, IL, USA) prepared in PBS containing 1% BSA.

9. After incubation, the cells were washed twice with PBS for 5 min each, then incubated with Alexa Fluor 488–conjugated goat anti-mouse IgG secondary antibody (1:1000; Life Technologies, Eugene, OR, USA) for 1 h at room temperature in the dark.
10. Following secondary antibody incubation, the cells were washed three times with PBS for 5 min each and counterstained with DAPI (4',6-diamidino-2-phenylindole; Life Technologies, Eugene, OR, USA) for nuclear staining for 5 min at room temperature.
11. Fluorescence images were acquired using a Carl Zeiss fluorescence microscope.
12. For quantitative analysis, γ -H2AX–positive cells were identified based on the presence of distinct nuclear γ -H2AX fluorescence signals. The number of γ -H2AX–positive cells was counted manually from multiple randomly selected microscopic fields, and the percentage of positive cells was calculated relative to the total number of DAPI-stained nuclei.

3.6.3 Cell Cycle Analysis

Cell cycle analysis is one of the applications of Muse Cell Analyzer (Merck, Darmstadt, Germany). In addition to detecting cell populations at different stages of the cell cycle after being treated with various conditions, such as G0/G1, S, and G2/M, which cells are most radiosensitive in the G2/M phase, less sensitive in the G1 phase, and least sensitive during the latter part of the S phase (55). Therefore, the radiosensitization effect of nanoparticles can be investigated by evaluating the number of cells in the G2/M phase after being treated with Pt-NPs and irradiation, shifting it to a phase that is more sensitive to radiation, thus enhancing the response to irradiation. Cell cycle analysis is performed using the following protocol:

1. Glioblastoma U87 cells were seeded into 24-well plates at a density of 2×10^5 cells per well in 2 mL of complete culture medium for four experimental groups:
 - Control group
 - IC₁₀ of Pt-NPs
 - Irradiation at LD₂₀
 - IC₁₀ of Pt-NPs and irradiation at LD₂₀
2. Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell attachment.
3. Irradiation was performed by placing a 24-well plate in a 2 cm thick bolus, which is positioned on a 30x30x15 cm³ solid phantom and covered with another 1.5 cm thick bolus. Radiation was delivered using a 6 MV linear accelerator (Clinac 2100 CD, serial number 5515; Varian Medical Systems) at the Department of Radiology, Naresuan University Hospital.
4. The irradiation parameters included a source-to-axis distance (SAD) of 100 cm, a depth of 3.5 cm, a dose rate of 400 MU/min, and a field size of 20 × 15 cm². The gantry angle was set to 0°, and the isocenter was positioned at the midpoint at the bottom of the 24-well plate.

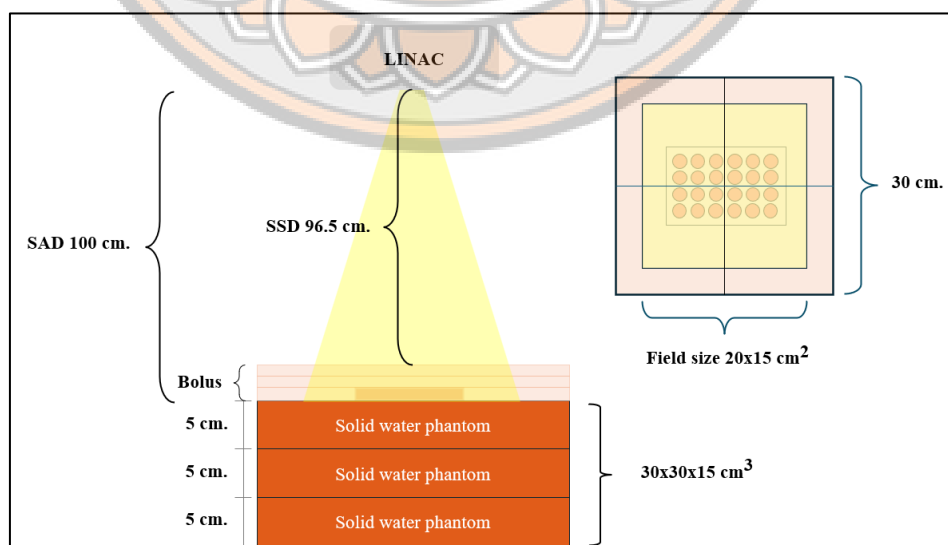


Figure 13 Schematic illustration of irradiation condition

5. Following irradiation, the cells were returned to the incubator and cultured for an additional 24 h.
6. The culture medium was then removed, and the cells were washed with phosphate-buffered saline (PBS). Cells were detached by adding 300 μ L of 0.25% trypsin–EDTA to each well and incubating for 3–5 min.
7. Trypsin activity was neutralized by adding twice the volume of complete culture medium, and the cell suspension was collected into 15-mL Falcon tubes, centrifuged at 1500 rpm for 5 min, then resuspended in 1 mL of medium.
8. Cells were then prepared at a density of 1×10^5 cells in 500 μ L and transferred into 1.5-mL microcentrifuge tubes. The samples were centrifuged at 7000 rpm for 7 min, after which the supernatant was removed, and the cells were fixed with 500 μ L of pre-chilled ethanol and incubated at -20°C for 3 h.
9. Following fixation, 200 μ L of the fixed cell suspension was transferred into a new 1.5-mL microcentrifuge tube, centrifuged at $300 \times g$ for 5 min, and washed once with PBS.
10. Subsequently, 200 μ L of Muse® Cell Cycle Reagent (Merck, Darmstadt, Germany) was added to each tube, and the samples were incubated for 30 min at room temperature in the dark.
11. Cell cycle distribution was analyzed using a Muse® Cell Analyzer (Merck, Darmstadt, Germany).
12. All experiments were performed in triplicate ($n = 3$).

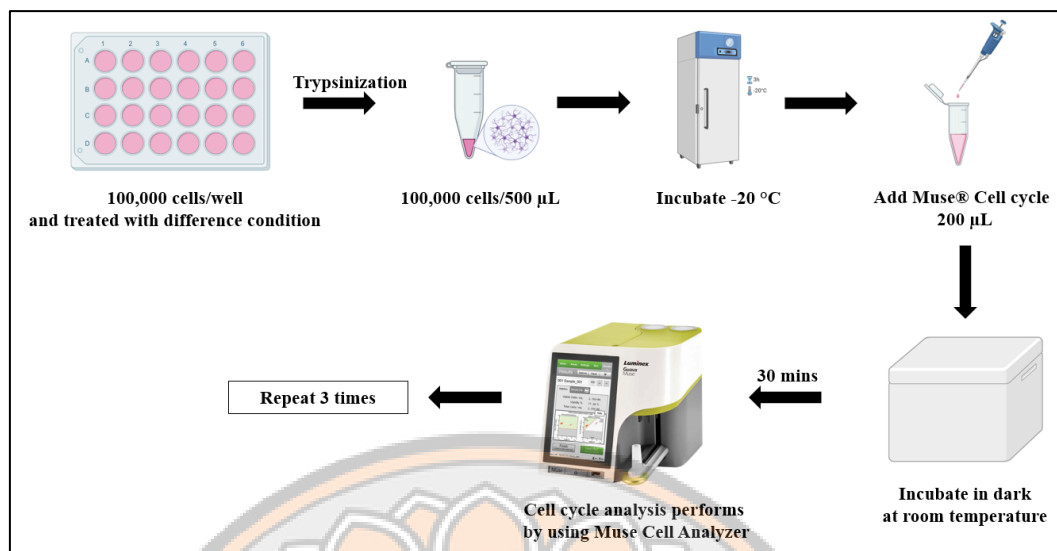


Figure 14 Schematic illustration of cell cycle analysis of Pt-NPs

3.6.4 Apoptotic Analysis

Apoptotic analysis was performed to study the mode of cell death induced by differences in treatment conditions through Annexin V Apoptosis Assay, which can be done using the following protocol:

1. Glioblastoma U87 cells were seeded into 24-well plates at a density of 2×10^5 cells per well in 2 mL of complete culture medium for four experimental groups:
 - Control group
 - IC₁₀ of Pt-NPs
 - Irradiation at LD₂₀
 - IC₁₀ of Pt-NPs and irradiation at LD₂₀
2. Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell attachment.
3. Irradiation was performed by placing a 24-well plate in a 2 cm-thick bolus, which was positioned on a 30x30x15 cm³ solid phantom and covered with another 1.5 cm-thick bolus. Radiation was delivered using a 6 MV linear accelerator (Clinac 2100 CD, serial number 5515; Varian Medical Systems) at the Department of Radiology, Naresuan University Hospital.

4. The irradiation parameters included a source-to-axis distance (SAD) of 100 cm, a depth of 3.5 cm, a dose rate of 400 MU/min, and a field size of $20 \times 15 \text{ cm}^2$. The gantry angle was set to 0° , and the isocenter was positioned at the midpoint of the bottom of the 24-well plate.

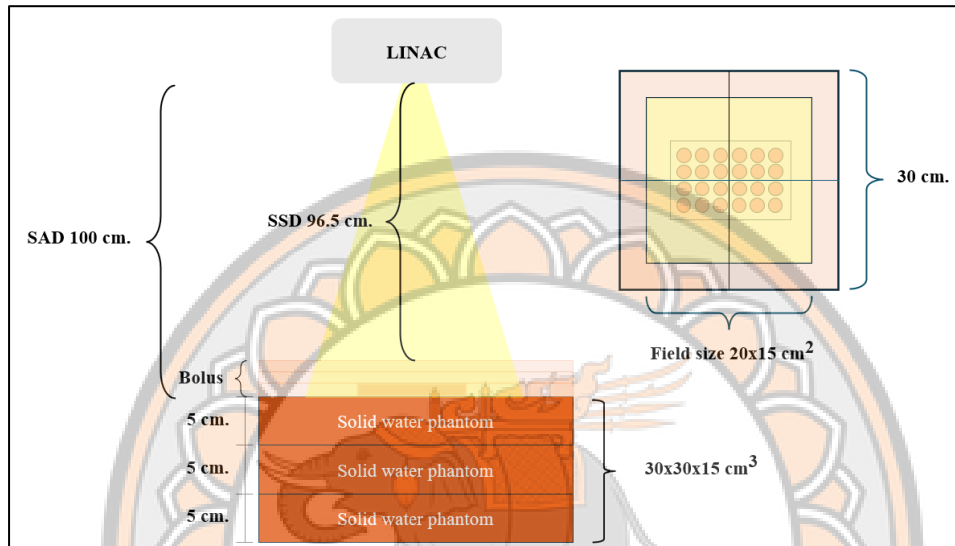


Figure 15 Schematic illustration of irradiation conditions

5. Following irradiation, the cells were returned to the incubator and cultured for an additional 24 h.
6. The culture medium was then removed, and the cells were washed with phosphate-buffered saline (PBS). Cells were detached by adding 300 μL of 0.25% trypsin–EDTA to each well and incubating for 3–5 min.
7. Trypsin activity was neutralized by adding twice the volume of complete culture medium, and the cell suspension was collected into 15-mL Falcon tubes, centrifuged at 1500 rpm for 5 min, then resuspended in 1 mL of medium.
8. 100 μL of Muse® Annexin V reagent was added to each cell suspension, and the mixture was incubated for 20–30 min at room temperature in the dark.

9. Apoptotic cell populations, including viable, early apoptotic, late apoptotic, and dead cells, were analyzed using a Muse® Cell Analyzer (Merck, Darmstadt, Germany).
10. All experiments were performed in triplicate (n = 3).

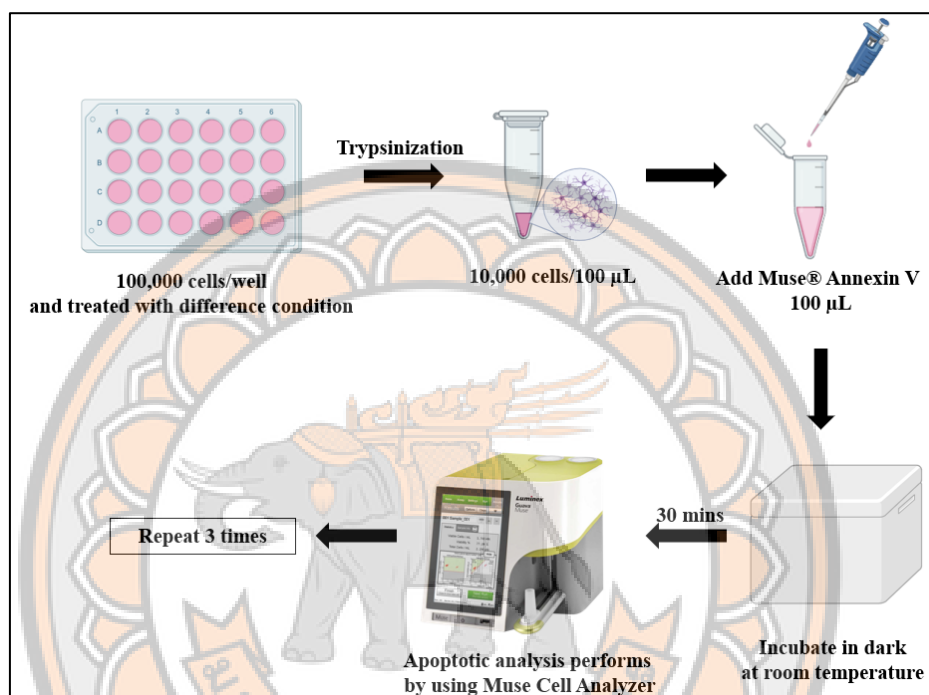


Figure 16 Schematic illustration of apoptosis analysis of Pt-NPs

3.6.5 ROS Detection Assay

A ROS generation assay was performed to evaluate the impact of NPs on the production of reactive oxygen species (ROS) via indirect effects with ionizing radiation. ROS generation assay can be done using the following protocol:

1. Glioblastoma U87 cells were seeded into 96-well plates at a density of 2×10^4 cells per well in 200 µL of complete culture medium and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell attachment.
2. After incubation, the culture medium was removed, and the cells were washed with PBS, then incubated with 200 µL of 20 µM 2',7'-

dichlorodihydrofluorescein diacetate ($H_2DCFH-DA$) for 45 min in the dark

3. Following dye loading, the cells were washed with PBS and treated with Pt-NPs at the IC_{10} concentration for 6 h, which doxorubicin ($2\ \mu M$) was used as a positive control.
4. For irradiation groups, cells were exposed to X-ray irradiation ($2\ Gy$) after the 6-h incubation with Pt-NPs.
5. After irradiation, the cells were returned to the incubator for 2 h prior to ROS measurement.
6. Intracellular ROS levels were quantified by measuring fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microplate reader.

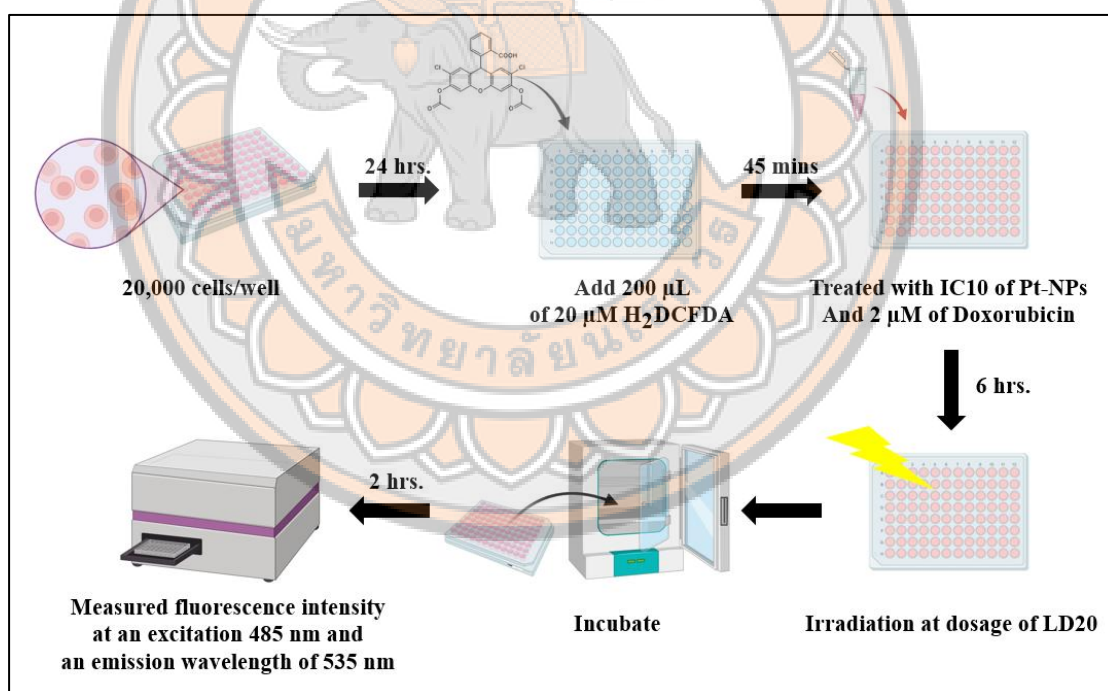


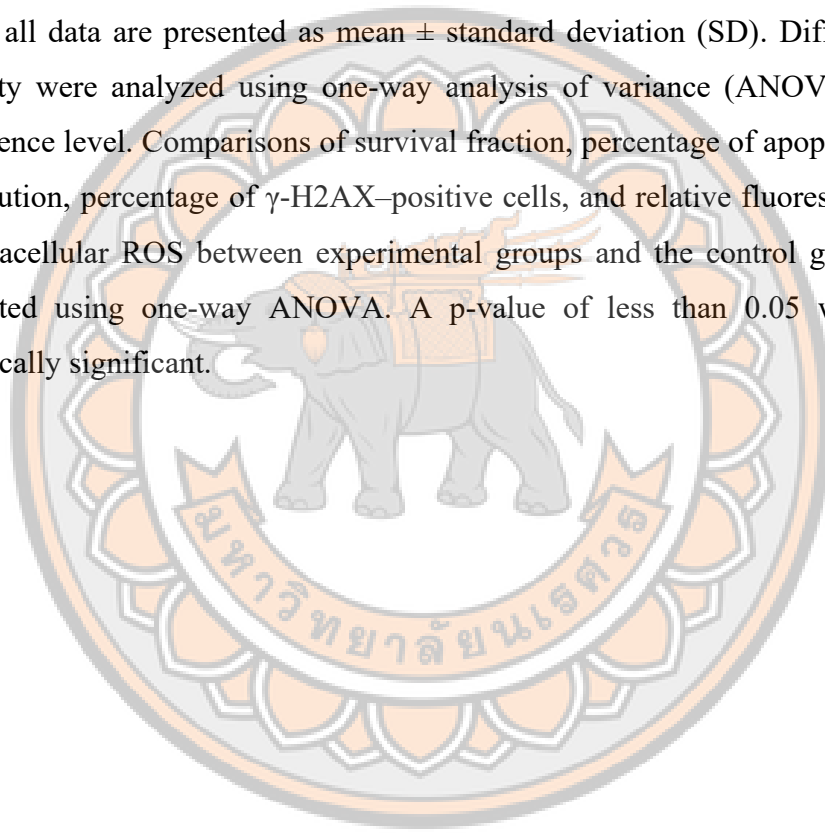
Figure 17 Schematic illustration of the ROS detection assay of Pt-NPs

7. Relative fluorescence intensity (RFI) was calculated using the following equation:

$$\text{RFI} = \frac{\text{Fluorescence intensity of treated groups}}{\text{Fluorescence intensity of control group}} \quad (7)$$

3.6.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism software, which all data are presented as mean $\pm$ standard deviation (SD). Differences in cell viability were analyzed using one-way analysis of variance (ANOVA) at the 95% confidence level. Comparisons of survival fraction, percentage of apoptosis, cell cycle distribution, percentage of γ -H2AX-positive cells, and relative fluorescence intensity of intracellular ROS between experimental groups and the control group were also evaluated using one-way ANOVA. A p-value of less than 0.05 was considered statistically significant.



CHAPTER IV

RESULTS AND DISCUSSION

4.1 Result

4.1.1 Physicochemical characterization of Pt-NPs

After synthesizing the Pt-NPs, their physicochemical properties were characterized to confirm their size, morphology, and successful fabrication. The morphology was examined using SEM, which revealed that the Pt-NPs exhibited a spherical shape with a spiky surface texture (Fig. 18A). The DLS analysis (Fig. 18C-E) showed that Pt-NPs had a hydrodynamic size of 121 ± 13 nm and a PDI of 0.1331 when dispersed in DI water. The zeta potential was measured to be -42 ± 1.4 mV, indicating good colloidal stability. In contrast, when the Pt-NPs were dispersed in PBS, their colloidal stability decreased, as evidenced by an increase in hydrodynamic size and PDI, along with a reduction in the magnitude of the negative zeta potential. In addition, the EDX analysis (Fig. 18B) confirmed the high purity of the nanoparticles, with platinum accounting for approximately 80 wt%.

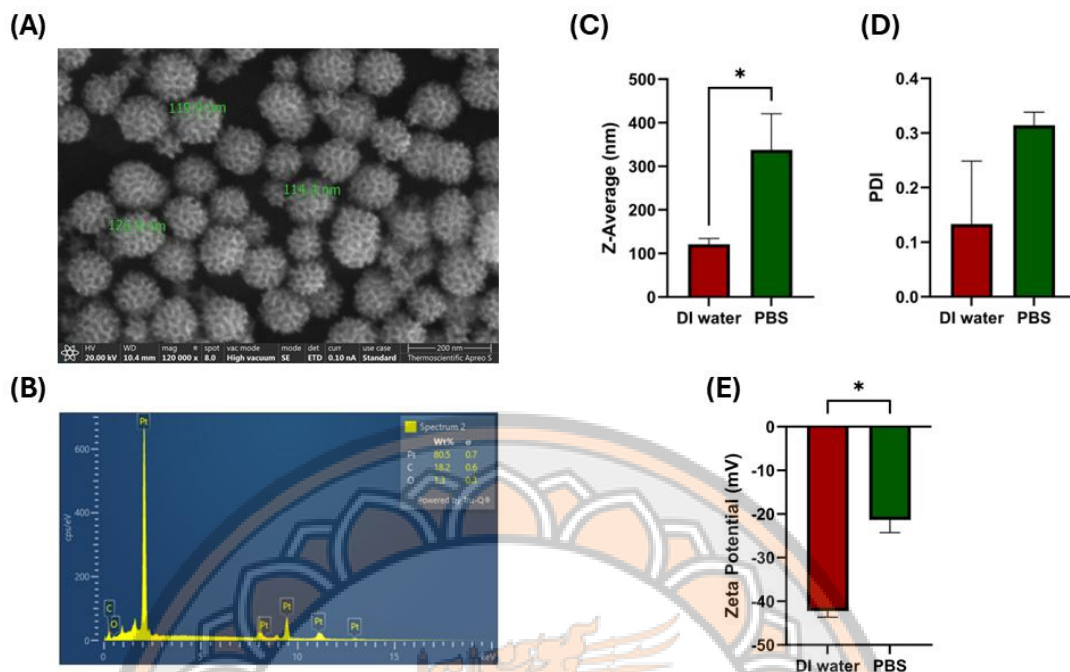


Figure 18 The synthesized Pt-NPs were characterized using SEM, EDX, and DLS. The SEM image shows well-dispersed, uniformly spherical particles (A). EDX analysis confirmed platinum as the primary element (80 wt%) (B). DLS results indicate that particle size increases in PBS compared with DI water (C). Analysis of the polydispersity index (PDI) revealed low values in DI water, which increased significantly in PBS (D). The zeta potential is highly negative in DI water and becomes less negative in PBS (E). The data are presented as the means $\pm$ SDs from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis; $p < 0.05$ was considered significant (*)

4.1.2 Cytotoxicity of Pt-NPs on glioblastoma U87 cells

The cytotoxicity of Pt-NPs toward the glioblastoma U87 cells was evaluated using the MTT assay at 24, 48, and 72 hours across a concentration range of 5–320 $\mu\text{g/mL}$. At all-time points, glioblastoma U87 cells exhibited a concentration-dependent decrease in cell viability in response to Pt-NP treatment. At the 24-hour treatment interval (Fig. 19A), significant reductions in cell viability were observed at 160 $\mu\text{g/mL}$ ($p = 0.0021$) and 320 $\mu\text{g/mL}$ ($p = 0.0002$) compared with the control group. At 48 hours (Fig. 19B), the overall pattern of cell viability was similar to that observed at 24 hours, although a slightly greater reduction was noted across the higher concentrations. Compared with the control group, significant decreases in cell viability were observed at 80 $\mu\text{g/mL}$ ($p = 0.0078$), 160 $\mu\text{g/mL}$ ($p = 0.000022$), and 320 $\mu\text{g/mL}$ ($p < 0.000001$). Finally, at 72 hours (Fig. 19C), the U87 cells exhibited higher overall viability than at earlier time points, which may be attributed to cellular self-recovery or adaptive responses over time. At this interval, only the highest concentration (320 $\mu\text{g/mL}$) resulted in a statistically significant ($p = 0.0006$) reduction in cell viability relative to the control group. In addition, the calculated IC_{10} value at the 24-hour time point was approximately 55 $\mu\text{g/mL}$, and this concentration was selected for use in the subsequent experiments.

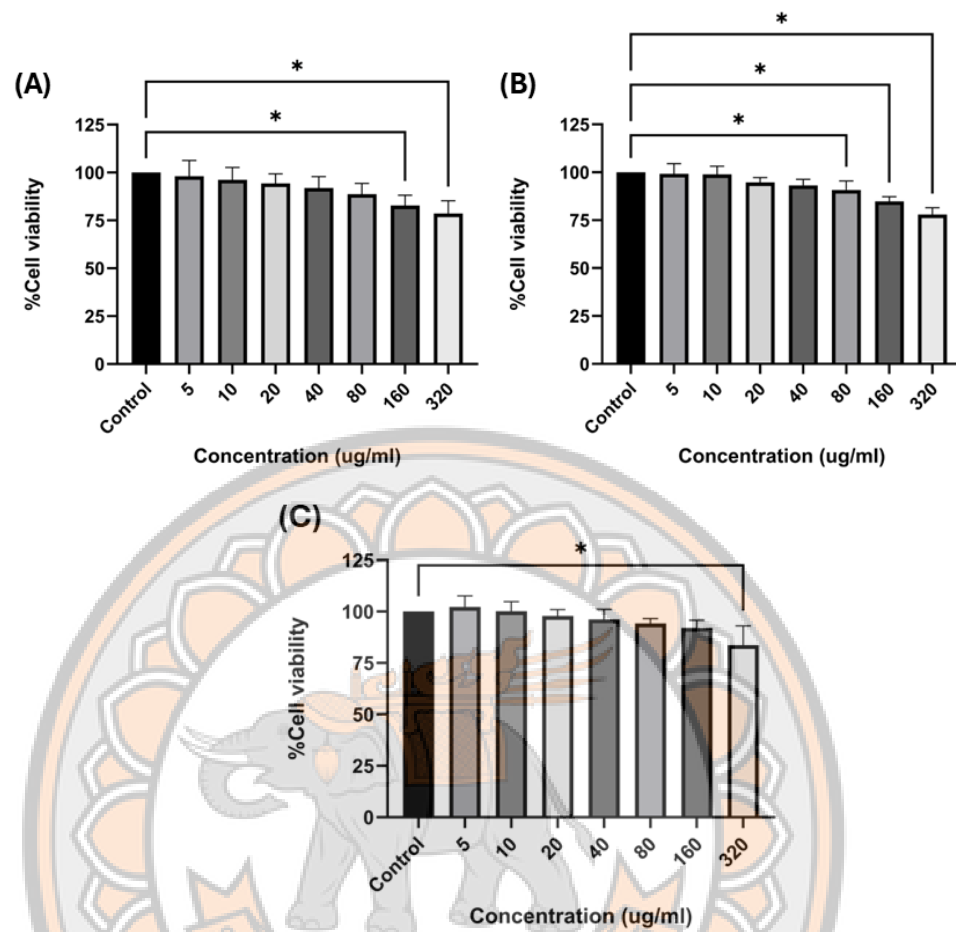


Figure 19 Cytotoxicity of Pt-NPs on U87 glioblastoma cells assessed using the MTT assay at different time points, 24 h.(A), 48 h.(B), and 72 h.(C). The data are presented as the means $\pm$ SDs from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis; $p < 0.05$ was considered significant (*)

4.1.3 Determination of LD₂₀ of glioblastoma U87 cells

Clonogenic survival analysis was performed to determine the radiosensitivity of U87 glioblastoma cells following X-ray irradiation. As shown in Fig. 20, the survival fraction (SF) decreased gradually in a dose-dependent manner across the tested radiation doses. At 1 Gy, the cells exhibited a slight increase in survival (SF = 1.09), as the dose increased to 2 Gy, the survival fraction declined to 0.81, and a further reduction was observed at 4 Gy (SF = 0.53). Marked decreases in clonogenic

survival were evident at higher doses, with SF values of 0.23 at 6 Gy and 0.19 at 8 Gy. Based on the survival fraction (SF), the dose corresponding to a 20% reduction in survival (LD_{20}) was estimated to be approximately 2 Gy. This value was subsequently used as a reference dose for further experiments evaluating the radiosensitizing effects of Pt-NPs.

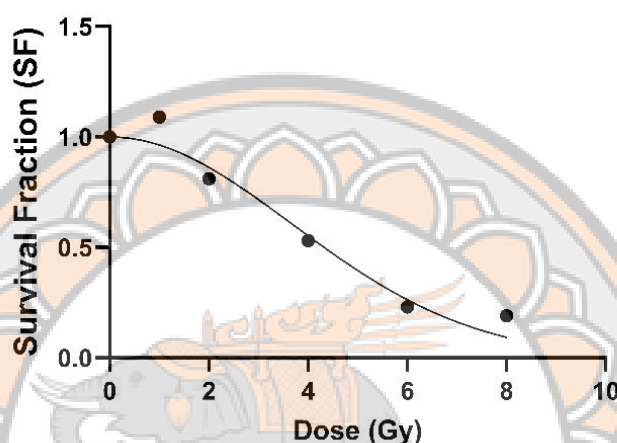


Figure 20 Clonogenic survival analysis was performed to determine of LD_{20} on glioblastoma U87 cells

4.1.4 Determination of the radiosensitization of Pt-NPs on glioblastoma U87 cells

To evaluate the radiosensitization effect of Pt-NPs on U87 glioblastoma cells, clonogenic assays were performed following irradiation with 2 Gy, with or without pretreatment using Pt-NPs at the IC10. As shown in Fig. 21, the control group maintained full colony-forming ability (SF = 1.00). When treated with Pt-NPs alone (SF = 0.72), the surviving fraction showed a significant reduction ($p = 0.006$) compared with the untreated control group, and a similar trend was observed following 2 Gy irradiation (SF = 0.68) ($p = 0.0026$). However, there was no statistically significant difference detected between these two treatment conditions. Upon combining Pt-NPs with 2 Gy irradiation, the surviving fraction decreased to 0.35, which was substantially lower than that observed with either Pt-NPs alone or radiation alone. This combined treatment resulted in a significantly reduced survival rate compared with control ($p =$

0.000002), Pt-NPs alone ($p = 0.0007$) and 2 Gy irradiation alone ($p = 0.0038$), indicating a clear and statistically significant enhancement of radiosensitivity. Furthermore, the radiosensitizing effect of Pt-NPs was quantified by calculating the sensitizer enhancement ratio (SER), which was defined as the ratio of radiation doses required to achieve the same survival fraction in the absence and presence of Pt-NPs. The survival fraction (SF) for cells treated with Pt-NPs in combination with 2 Gy irradiation was 0.35 and based on interpolation from the previously obtained survival curve, a radiation dose of 5.32 Gy was required to achieve the same SF (0.35) under irradiation alone. Therefore, the calculated SER value was 2.66, indicating a pronounced enhancement of radiation-induced cytotoxicity in the presence of Pt-NPs. This result demonstrates that Pt-NPs significantly increased the radiosensitivity of U87 glioblastoma cells under the applied irradiation conditions.

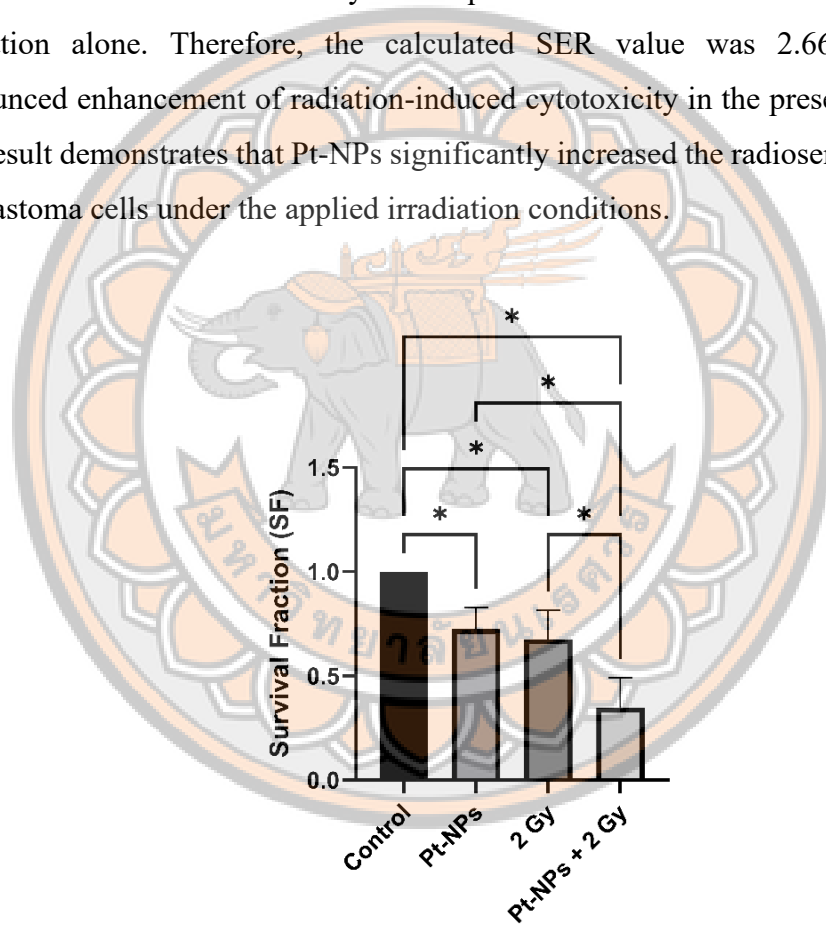


Figure 21 Radiosensitizing effects of Pt-NPs in U87 glioblastoma cells were performed using a clonogenic survival assay, showing significantly reduced survival fractions after combined treatments compared with single. The data are presented as the means $\pm$ SDs from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis; $p < 0.05$ was considered significant (*)

4.1.5 Immunofluorescence staining for γ -H2AX

Immunofluorescence analysis revealed distinct differences in the levels of γ -H2AX-positive cells across the treatment groups (Fig. 22A-B). The control cells presented very low γ -H2AX positivity, with a value of $4.4 \pm 3.8\%$. When cells were treated with Pt-NPs alone, an increase in γ -H2AX signal was observed, with the percentage of γ -H2AX-positive cells rising to $23.92 \pm 2.93\%$, which was significantly higher compared with the control group ($p = 0.001$). Similarly, irradiation alone (2 Gy) induced an elevation in γ -H2AX levels, with the percentage of γ -H2AX-positive cells increasing to $27.02 \pm 7.18\%$, which was significantly higher than in the control group ($p = 0.0001$). Moreover, when Pt-NPs were combined with 2 Gy irradiation, the percentage of γ -H2AX-positive cells increased to $37.89 \pm 6.24\%$, showing a statistically significant elevation compared with either control ($p = 0.000009$) or Pt-NPs ($p = 0.028$). However, this increase was not significantly different from irradiation alone. This result is consistent with the clonogenic assay findings, in which the combination of Pt-NPs and 2 Gy irradiation produced the most significant reduction in surviving fraction. Together, these data further support the enhanced radiosensitizing effect of Pt-NPs under irradiation.

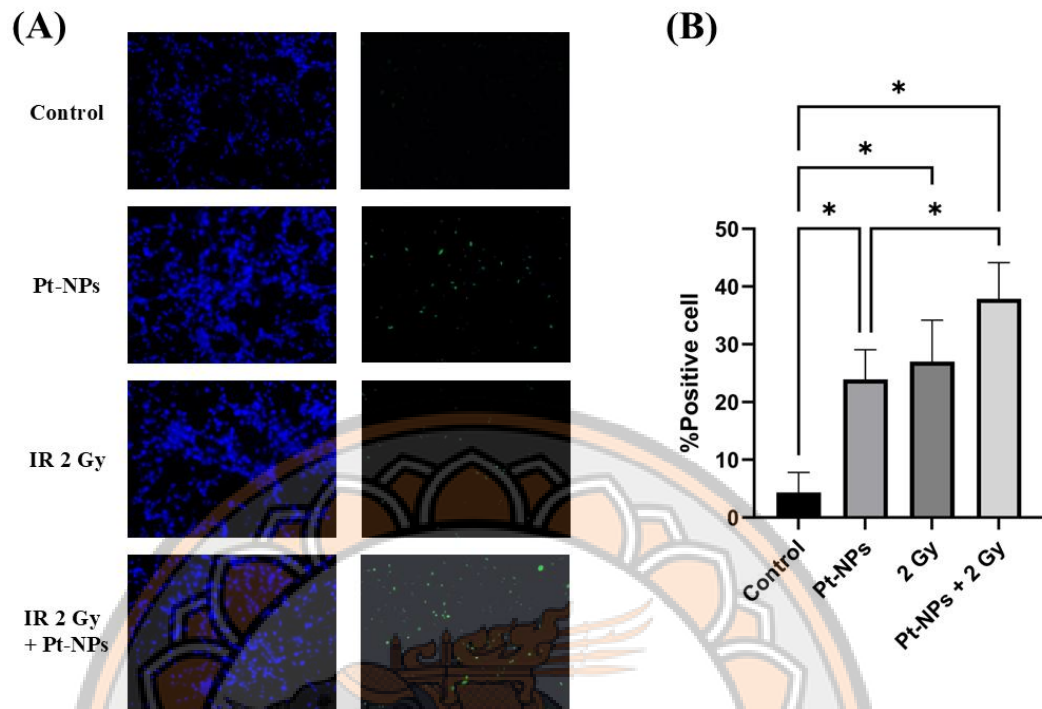


Figure 22 (A) Representative immunofluorescence images displaying γ -H2AX foci (green) and nuclei stained with DAPI (blue), acquired at 20x magnification. **(B)** Percentage of γ -H2AX-positive cells indicating an increased number of DNA double-strand breaks following combined treatment. The data are presented as the means $\pm$ SDs from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis; $p < 0.05$ was considered significant (*)

4.1.6 Cell Cycle Analysis

The effects of Pt-NPs on the cell cycle distribution of glioblastoma U87 cells were analyzed using Muse cell cycle analysis. Compared with the control group, treatment with Pt-NPs alone did not result in a statistically significant change in the G0/G1 phase. In contrast, the combined treatment of Pt-NPs with irradiation led to a statistically significant increase in the proportion of cells in the G0/G1 phase ($p = 0.0348$) (Fig. 23A). In addition, Pt-NPs also induced a significant increase in the accumulation of cells in the S phase (Fig. 23B). In contrast, both Pt-NPs treatment alone and in combination with irradiation resulted in a statistically significant decrease in the accumulation of cells in the G2/M phase (Fig. 23C). Moreover, across all phases of the cell cycle, irradiation at 2 Gy alone did not produce any statistically significant differences compared with the control group.

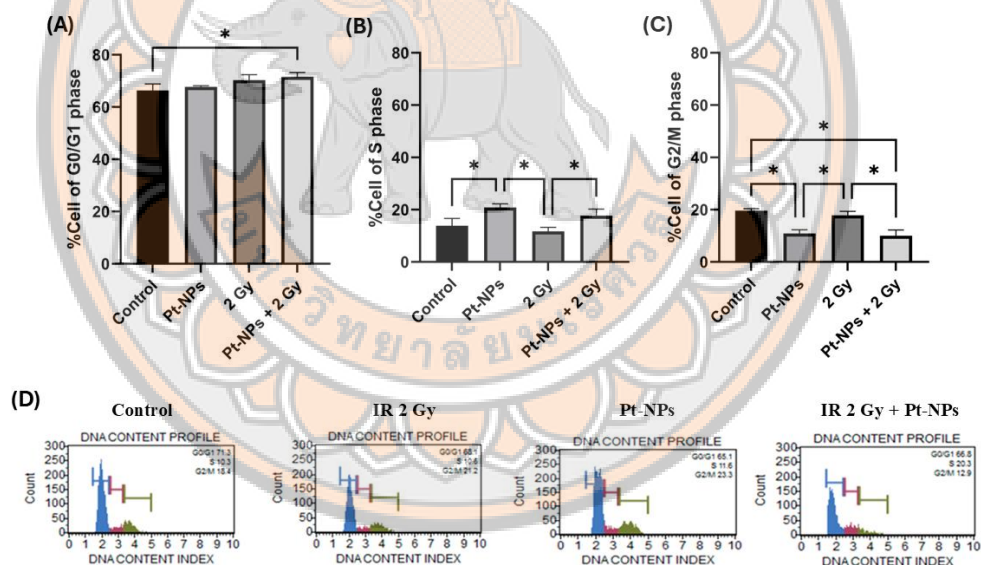
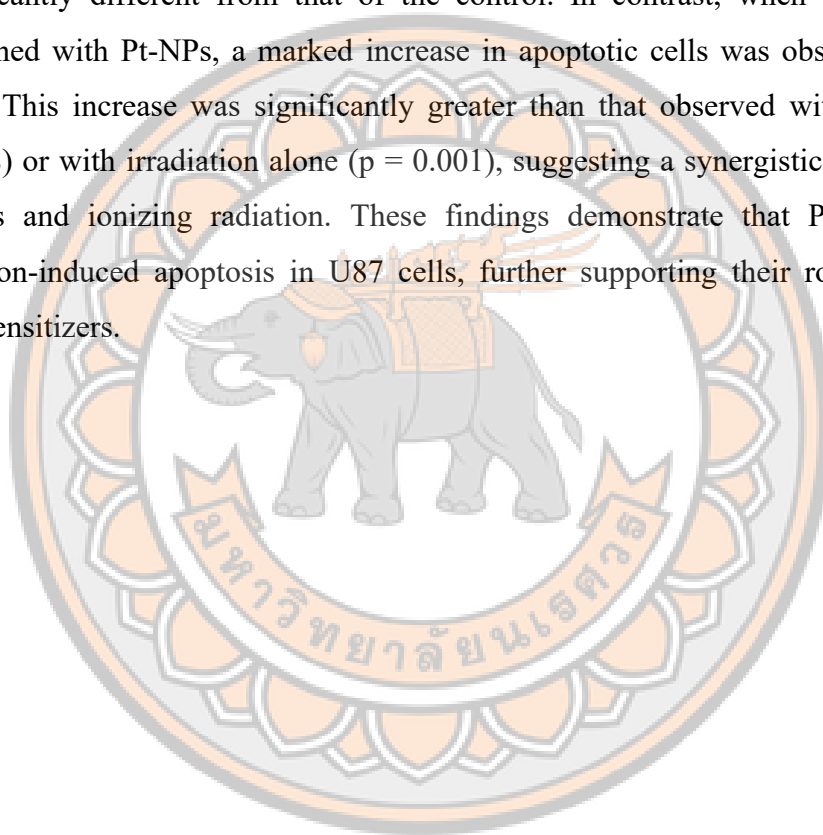


Figure 23 Cell cycle distribution of U87 glioblastoma cells following treatment with Pt-NPs, ionizing radiation (IR), or their combination. (A–C) Bar graphs represent the percentage of cells in (A) the G0/G1 phase, (B) the S phase, and (C) the G2/M phase. (D) Representative histograms of DNA content profiles from Muse cell cycle analysis for each treatment condition. The data are presented as the means $\pm$ SDs from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis; $p < 0.05$ was considered significant (*)

4.1.7 Apoptosis Analysis

Apoptosis induction in U87 glioblastoma cells was analyzed using the Muse apoptosis assay to evaluate the effects of Pt-NPs, irradiation (2 Gy), and their combination. As shown in figure 24, the control group presented a baseline apoptosis rate of $6.1 \pm 4.2\%$. Treatment with Pt-NPs alone resulted in a slight increase in apoptotic cells to $13.5 \pm 0.77\%$, indicating limited induction of apoptosis. Exposure to IR alone (2 Gy) resulted in a relatively low level of apoptosis ($4.7 \pm 5.8\%$), which was not significantly different from that of the control. In contrast, when irradiation was combined with Pt-NPs, a marked increase in apoptotic cells was observed (23.66 ± 0.76). This increase was significantly greater than that observed with Pt-NPs ($p = 0.0368$) or with irradiation alone ($p = 0.001$), suggesting a synergistic effect between Pt-NPs and ionizing radiation. These findings demonstrate that Pt-NPs enhance radiation-induced apoptosis in U87 cells, further supporting their role as effective radiosensitizers.



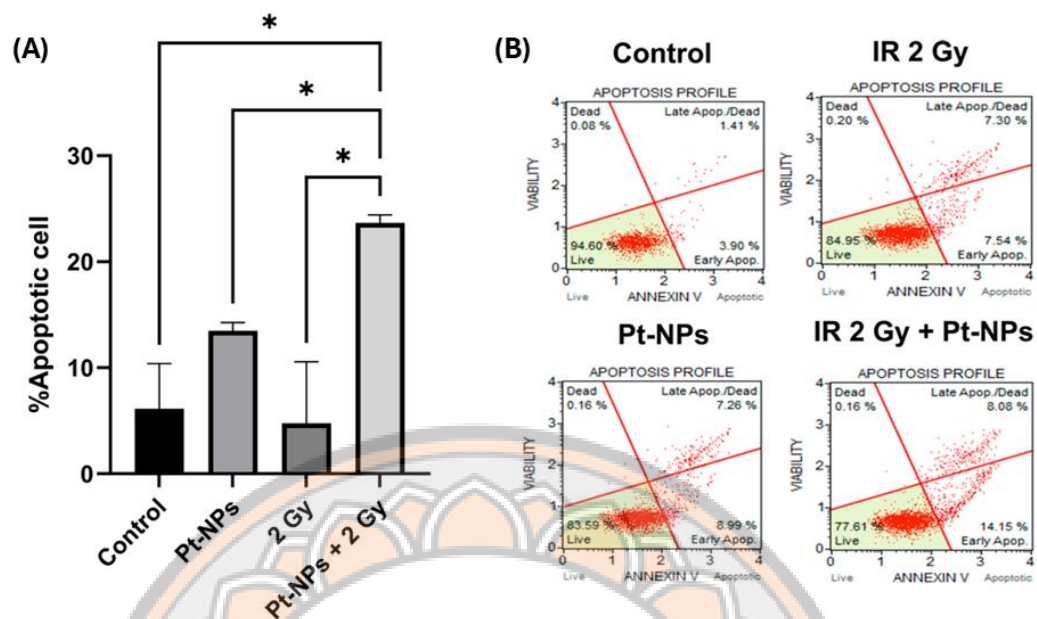


Figure 24 Apoptosis analysis of U87 glioblastoma cells following treatment with Pt-NPs, radiation (IR), or their combination. (A) The percentage of total apoptotic cells was assessed with a Muse cell analyzer after 24 h. (B) Flow cytometry dot plots from one representative replicate showing the distribution of apoptotic cells in each treatment group. The data are presented as the means $\pm$ SDs from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis; $p < 0.05$ was considered significant (*)

4.1.8 ROS Detection Assay

Intracellular ROS generation induced by Pt-NPs in U87 glioblastoma cells was quantified using the H₂DCFDA ROS detection assay, with doxorubicin included as a positive control. As shown in figure 25, treatment with Pt-NPs alone resulted in a 2.4-fold increase in ROS levels compared with the untreated control, however, this difference was not statistically significant. Exposure to 2 Gy irradiation led to a more pronounced elevation, with ROS levels increasing by 4.9-fold. Notably, the combined treatment of Pt-NPs with 2 Gy irradiation resulting in an 11.2-fold increase relative to the control group. This substantial enhancement of ROS production under combined treatment conditions suggests a synergistic effect between Pt-NPs and ionizing radiation, which may contribute to the observed increase in DNA damage, apoptosis, and radiosensitization in U87 cells.

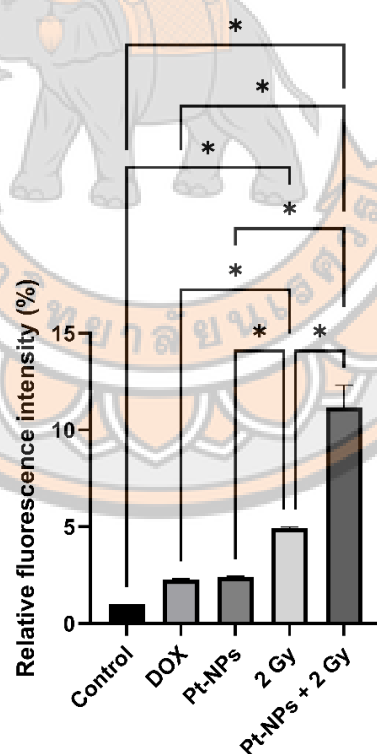


Figure 25 H₂DCFDA ROS detection assay of U87 glioblastoma cells following treatment with Pt-NPs, ionizing radiation (IR), or their combination. The data are presented as the means $\pm$ SDs from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis; $p < 0.05$ was considered significant (*)

4.2 Discussion

In this study, we successfully synthesized Pt-NPs by the chemical reduction method, with the formation of Pt-NPs driven by the synergistic roles of the reagents involved in the synthesis process. H_2PtCl_6 serves as the platinum precursor, providing Pt^{4+} ions that are subsequently reduced to metallic Pt^0 by ascorbic acid, a mild reducing agent that enables slow reduction kinetics. This slow reduction is beneficial for the formation of uniformly sized Pt-NPs, as it gives sufficient time for the surfactant (Pluronic F127) to adhere to the metal seeds and to avoid aggregation. Furthermore, Pluronic F127 also acts as a pore-directing agent, which causes Pt-NPs to be mesoporous. In contrast, the combination of bromide ions and dioxygen allows the porous structure to become interconnected. Together, these components enable the formation of uniform Pt-NPs with controlled morphology and suitable properties for biological applications (56).

Comprehensive physicochemical characterization confirmed the successful synthesis of Pt-NPs with well-defined morphology, size distribution, surface charge, and elemental composition. SEM analysis demonstrated that the synthesized Pt-NPs exhibited a predominantly spherical shape with a distinctive spiky surface. Such surface roughness is advantageous for biomedical applications, as it increases the effective surface area and enhances interactions with biological interfaces, including improved binding and cellular uptake of nanoparticles (57). Moreover, Pt-NPs demonstrated excellent colloidal stability in DI water, as evidenced by a consistent hydrodynamic diameter (121 ± 13 nm), a low PDI (0.1331 ± 0.1155), reflecting a narrow size distribution, and a high negative zeta potential (-42 ± 1.4 mV). This high surface charge suggests strong electrostatic repulsion, excellent colloidal stability and minimal aggregation (58, 59). However, when dispersed in PBS, the Pt-NPs exhibited reduced colloidal stability, as evidenced by an increase in hydrodynamic size (337.6 ± 82.96 nm) and PDI (0.3092 ± 0.024) together with a reduction in the magnitude of the negative zeta potential (-22.76 ± 2.88). The reduced colloidal stability of Pt-NPs in PBS can be attributed to the medium's high ionic strength, which reduces electrostatic repulsion and promotes nanoparticle aggregation (60-62). In addition, elemental analysis by EDX confirmed the high purity of the synthesized Pt-NPs, with platinum accounting for approximately 80 wt% of the total composition. These findings align

with other reports, where EDX has been used to confirm the elemental composition and purity of synthesized nanoparticles (63-65). Overall, these characterization results demonstrate that the synthesized Pt-NPs possess appropriate size, morphology, stability, and compositional purity, making them suitable for subsequent biological evaluations and radiosensitization applications.

The cytotoxicity of Pt-NPs toward U87 glioblastoma cells was evaluated using the MTT assay to determine a suitable non-lethal concentration for subsequent radiosensitization studies. Overall, Pt-NPs induced a concentration-dependent reduction in cell viability across all time points. Interestingly, cell viability at 72 h appeared higher than at earlier time points, which may reflect adaptive cellular responses or partial recovery following prolonged exposure (66). Importantly, the IC₁₀ value at 24 h was determined to be approximately 55 µg/mL and was selected for subsequent experiments, as it minimizes direct cytotoxic effects from nanoparticles alone while allowing the assessment of their ability to enhance radiation-induced cell damage (67). Although data on the concentration of Pt-NPs in U87 cells are limited, research on HeLa cells has revealed that platinum nanodendrites (PtNDs) exhibit cytotoxicity that varies with concentration, with effects noted at concentrations of up to 0.1 mM (approximately 58–60 µg/mL) (68). Since the desired sublethal effect was achieved at 24 h, extending the incubation period was not required for IC₁₀ determination. Nevertheless, it is essential to note that some nanoparticles do not exhibit pronounced cytotoxicity at early time points. Therefore, additional assessments at 48 and 72 h were performed to capture potential delayed or time-dependent cytotoxic effects.

The clonogenic assay was employed to evaluate the long-term survival and reproductive integrity of U87 glioblastoma cells following treatment with Pt-NPs, irradiation, and their combination. In the present study, the combination of Pt-NPs and 2 Gy irradiation resulted in the most pronounced reduction in the surviving fraction (SF $\approx$ 0.35), which was significantly lower than that observed with either Pt-NPs or irradiation alone. Quantitative analysis further revealed a sensitizer enhancement ratio (SER) of 2.66, confirming that Pt-NPs markedly enhanced the radiosensitivity of U87 cells (69). This substantial enhancement supports that Pt-NPs act as potent radiosensitizers rather than merely exerting additive cytotoxic effects. Furthermore, the

γ -H2AX assay revealed that the combination of Pt-NPs with 2 Gy irradiation enhanced the number of radiation-induced DNA double-strand breaks (DSBs) and was the highest percentage of γ -H2AX-positive nuclei. This pattern aligns with the clonogenic survival data presented, supporting the notion that increased DNA damage plays a role in radiosensitization. This enhancement of γ -H2AX formation can be mechanistically linked to the increase in intracellular ROS levels induced under these conditions, which resulted in an 11.2-fold increase relative to the control group. The marked increase in ROS generation observed following the combined treatment of Pt-NPs and 2 Gy irradiation can be explained by several mechanisms. As a high-Z metal, platinum possesses a large photoelectric cross-section, enabling Pt-NPs to absorb incident radiation more efficiently than surrounding biological tissues. Upon irradiation, this enhanced energy absorption leads to the emission of secondary electrons, including photoelectrons, Compton electrons, and Auger electrons (70-72). These low-energy electrons subsequently interact with surrounding water and oxygen molecules, promoting radiolysis reactions that generate highly reactive oxygen species (ROS) (54, 73). These ROS can directly attack the DNA backbone and nucleobases, inducing oxidative base lesions and single-strand breaks (SSBs). When two SSBs occur in proximity on opposite DNA strands these lesions can be converted into DSBs. Furthermore, ROS can also promote DSB formation indirectly by interfering with DNA replication and repair processes (41). However, in this study, the increase in ROS did not result in a statistically significant increase in γ -H2AX levels in the Pt-NPs + 2 Gy group compared to irradiation at 2 Gy alone. In contrast, the clonogenic assay demonstrated a statistically significant difference between these two groups. This suggests that the elevated ROS levels may contribute to cell death by inducing damage to other cellular components in addition to DNA, rather than affecting DNA alone. ROS can induce cell death not only through DNA damage but also via lipid peroxidation, protein oxidation, and mitochondrial dysfunction. These processes lead to membrane destabilization, loss of protein function, and activation of intrinsic apoptotic pathways (74-76). Collectively, these effects lead to the accumulation of unrepaired or misrepaired DSBs, activation of the DNA damage response, and subsequent cell cycle arrest or apoptosis.

This study specifically investigated apoptosis as the primary mechanism of cell death. The observed increase in apoptosis following individual treatments with Pt-NPs or radiation is consistent with previously reported effects of these agents. Radiation primarily causes DNA double-strand breaks, which can also activate apoptotic pathways (77, 78), whereas Pt-NPs have been associated with oxidative stress and disruption of DNA repair in earlier studies (79). In our experiments, the combination treatments further increased apoptosis, with the combination of Pt-NPs and irradiation producing the most substantial effect, indicating that Pt-NPs enhance radiation-induced apoptotic signaling. This increase in apoptosis is consistent with the elevated levels of ROS and DNA double-strand breaks observed under combined treatment conditions, suggesting that excessive DNA damage caused by ROS surpasses the cellular repair capacity and triggers programmed cell death pathways.

Analysis of cell cycle distribution demonstrated that treatment with Pt-NPs, either alone or in combination with 2 Gy irradiation, led to a significant increase in S-phase cell accumulation, accompanied by a reduction in the G2/M population. S-phase arrest is commonly associated with replication stress and DNA damage occurring during DNA synthesis (80, 81), which the observed accumulation of cells in S phase suggests that Pt-NPs may interfere with DNA replication processes, potentially through ROS-mediated oxidative damage to DNA (41). Furthermore, the reduction in the G2/M population observed following Pt-NPs treatment, both alone and in combination with irradiation, may reflect impaired progression through later stages of the cell cycle due to unresolved DNA damage. Cells experiencing replication-associated DNA lesions are often prevented from entering G2/M to avoid mitotic catastrophe (82). Although the G2/M phase is generally regarded as the most radiosensitive stage of the cell cycle, the observed shift away from the G2/M population does not diminish the radiosensitizing efficacy of Pt-NPs. Instead, Pt-NPs continue to exhibit strong radiosensitization through alternative and complementary mechanisms. In particular, the high-Z properties of Pt-NPs promote local energy deposition and secondary electron emission during irradiation, further amplifying DNA damage independently of cell cycle phase and markedly enhancing radiation-induced ROS generation, leading to increased oxidative stress and DNA damage. Overall, these findings demonstrate that Pt-NPs effectively enhance the radiosensitivity of U87 glioblastoma cells through a

multifaceted mechanism involving amplified ROS generation, increased DNA double-strand breaks, cell cycle perturbation, and apoptosis, ultimately leading to a pronounced reduction in clonogenic survival. This integrated response highlights the potential of Pt-NPs as promising radiosensitizing agents for improving the therapeutic efficacy of radiation therapy in glioblastoma.



CHAPTER V

CONCLUSIONS

In this study, platinum nanoparticles (Pt-NPs) were successfully synthesized and thoroughly characterized, confirming their uniform size, mesoporous structure, high purity, and good colloidal stability under appropriate conditions. Biological evaluation in U87 glioblastoma cells demonstrated that Pt-NPs exhibited minimal intrinsic cytotoxicity at low concentrations while effectively enhancing radiation-induced cell damage. The combination of Pt-NPs with 2 Gy irradiation significantly increased intracellular ROS levels, promoted DNA double-strand break formation, induced cell cycle arrest, and enhanced apoptotic cell death, ultimately resulting in a marked reduction in clonogenic survival. These findings collectively demonstrate that Pt-NPs function as effective radiosensitizers through ROS-mediated and DNA-damage-associated mechanisms. Overall, this work highlights the potential of Pt-NPs as promising nanomaterial-based adjuvants to improve the efficacy of radiation therapy for glioblastoma treatment.



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APPENDIX A

1. Characterization of Pt-NPs

Table 4 Results of DLS on Pt-NPs in DI water

Replicate	Z-Average (nm)	PDI	Zeta potential (mV)
1	129.3	0.03607	-40.57
2	105.9	0.1024	-43.25
3	128.2	0.2609	-42.81
Average	121.1	0.1331	-42.21
SD	13.2	0.1155	1.4

Table 5 Results of DLS on Pt-NPs in PBS

Replicate	Z-Average (nm)	PDI	Zeta potential (mV)
1	366	0.3189	-23.02
2	423	0.2789	-23.56
3	335.8	0.3298	-21.7
4	225.6	0.3291	-17.21
Average	337.6	0.3092	-22.76
SD	82.96	0.024	2.883

2. In vitro assessment of Pt-NPs

2.1 Cytotoxicity of Pt-NPs on glioblastoma U87 cells using MTT assay

Table 6 24 hours cell viability by MTT assay of Pt-NPs on Glioblastoma U87 cell

Replicate	Concentration ($\mu\text{g/mL}$)							
	0	5	10	20	40	80	160	320
1	100	92.59	91.13	91.70	88.02	82.23	76.95	75.43
2	100	92.59	96.03	93.16	92.22	93.40	89.98	88.60
3	100	110.03	105.30	101.68	100.17	93.29	82.32	75.65
4	100	97.05	92.34	90.75	87.50	86.06	81.97	74.82
Average	100	98.40	97.49	95.52	93.47	89.64	83.08	79.89
SD	0	10.07	7.20	5.39	6.17	6.42	6.55	7.54

Table 7 48 hours cell viability by MTT assay of Pt-NPs on Glioblastoma U87 cell

Replicate	Concentration ($\mu\text{g/mL}$)							
	0	5	10	20	40	80	160	320
1	100	96.17	98.29	94.99	93.91	87.68	83.93	74.33
2	100	102.43	102.76	97.32	89.07	96.71	88.18	81.80
3	100	104.85	101.46	91.21	96.54	92.33	82.41	80.01
4	100	93.31	93.31	95.32	93.15	86.61	84.80	75.70
Average	100	99.19	98.96	94.71	93.17	90.83	84.83	77.96
SD	0	5.36	4.21	2.55	3.10	4.63	2.44	3.52

Table 8 72 hours cell viability by MTT assay of Pt-NPs on Glioblastoma U87 cell

Replicate	Concentration ($\mu\text{g/mL}$)							
	0	5	10	20	40	80	160	320
1	100	96.10	94.688	94.642	90.060	91.101	88.889	78.033
2	100	106.26	100.34	102.07	102.04	96.42	97.34	92.94
3	100	107.33	106.18	97.77	95.85	95.41	89.90	73.61
4	100	99.42	99.42	97.09	96.69	94.12	91.92	90.26
Average	100	102.28	100.16	97.89	96.16	94.26	92.01	83.71
SD	0	5.41	4.72	3.09	4.90	2.31	3.77	9.35

2.2 Determination of LD₂₀ of glioblastoma U87 cells

Table 9 Number of colonies count, PE and SF after irradiation with 1, 2, 4, 6, 8 Gy

Conditions	Number of colonies count (colonies/well)						Total	Av.	PE	SF
	1	2	3	4	5	6				
Control	38	36	37	35	41	42	229	209.5	23.28	1
	43	35	27	30	26	29	190			
1 Gy	54	50	49	47	51	49	300	304.5	25.38	1.09
	52	56	58	43	52	48	309			
2 Gy	41	40	45	45	41	38	250	227	18.92	0.81
	34	20	30	40	43	37	204			
4 Gy	18	19	20	18	16	18	109	108	9	0.53
	16	20	17	16	20	18	107			
6 Gy	18	14	17	17	16	12	94	64.5	5.38	0.23
	5	10	6	4	4	6	35			
8 Gy	8	8	8	9	10	9	52	53.5	4.46	0.19
	8	9	7	11	10	10	55			

2.3 Determination of the radiosensitization of Pt-NPs

Table 10 SF of Glioblastoma U87 cell after being treated with Pt-NPs, 2 Gy and Pt-NPs + 2 Gy

Replicate	Survival fraction (SF)			
	Control	Pt-NPs	2 Gy	Pt-NPs + 2 Gy
1	1	0.76	0.81	0.47
2	1	0.89	0.75	0.40
3	1	0.68	0.49	0.14
4	1	0.64	0.65	0.38
5	1	0.65		
Average	1	0.72	0.68	0.35
SD	0	0.10	0.14	0.14

2.4 Immunofluorescence staining for γ -H2AX

Table 11 Number of positive cells of Glioblastoma U87 cell after treated with Pt-NPs, 2 Gy and Pt-NPs + 2 Gy

Condition	Area	Positive cell	DAPI	%Positive cell	Average	SD
Control	1	29	410	7.07	4.36	3.77
	2	0	320	0.00		
	3	5	387	1.29		
	4	18	252	7.14		
	5	15	238	6.30		
Pt-NPs	1	94	362	25.97	23.92	2.93
	2	117	500	23.40		
	3	112	383	29.24		
	4	77	451	17.07		
2 Gy	1	124	326	38.04	27.02	7.18
	2	97	435	22.30		
	3	87	451	19.29		
	4	144	536	26.87		
Pt-NPs + 2 Gy	1	162	418	38.76	37.89	6.24
	2	121	387	31.27		
	3	155	355	43.66		

2.4 Cell Cycle Analysis

Table 12 Percentage of cells in each phase in cell cycle after treatment with Pt-NPs, 2 Gy and Pt-NP + 2 Gy

Condition	Replicate	% of cell (cells/ml)			
		G0/G1	S	G2/M	Debris
Control	1	63.9	16.3	19.5	64.7
	2	66.4	14.4	19	54.6
	3	68.8	10.8	20.4	48.9
	Average	66.37	13.83	19.63	67.05
	SD	2.45	2.79	0.71	8.00
Pt-NPs	1	67.9	19.5	12.1	86.6
	2	68	22.2	9.4	86.2
	3	67.1	20.9	11.3	81.8
	Average	67.67	20.87	10.93	84.87
	SD	0.49	1.35	1.39	2.66
2 Gy	1	68.6	13.5	17.7	73
	2	69.7	10.7	19.4	74.7
	3	72.6	10.9	16.3	72.3
	Average	70.30	11.70	17.80	73.33
	SD	2.07	1.56	1.55	1.23
Pt-NP + 2 Gy	1	73.4	15.2	11.1	79.1
	2	70.1	17.7	11.5	86.8
	3	71.1	20.2	7.4	91.2
	Average	71.53	17.70	10.00	85.70
	SD	1.69	2.50	2.26	6.12

2.5 Apoptosis analysis

Table 13 Percentage of apoptotic cells after treatment with Pt-NPs, 2 Gy and Pt-NP + 2 Gy

Condition	Replicate	% Apoptotic cells				
		Live	Early apoptotic	Late apoptotic	Dead	Total apoptotic
Control	1	94.07	0.19	1.07	4.66	1.26
	2	90.8	8.04	1.05	0.11	9.09
	3	91.69	5.54	2.53	0.24	8.07
	Average	92.19	4.59	1.55	1.67	6.14
	SD	1.69	4.01	0.85	2.59	4.26
Pt-NPs	1	86.46	12.51	0.95	0.07	13.46
	2	85.72	13.08	1.2	0	14.28
	3	87.24	11.9	0.85	0	12.75
	Average	86.47	12.50	1.00	0.02	13.50
	SD	0.76	0.59	0.18	0.04	0.77
2 Gy	1	94.73	0.46	0.96	3.85	1.42
	2	94.73	0.46	0.96	3.85	1.42
	3	88.35	7.13	4.34	0.18	11.47
	Average	92.60	2.68	2.09	2.63	4.77
	SD	3.68	3.85	1.95	2.12	5.80
Pt-NP + 2 Gy	1	75.47	23.58	0.96	0	24.54
	2	76.78	22.56	0.66	0	23.22
	3	76.78	22.56	0.66	0	23.22
	Average	76.34	22.90	0.76	0.00	23.66
	SD	0.76	0.59	0.17	0.00	0.76

APPENDIX B

Certificate on approval of biosafety and biosecurity

 ใบรับรองด้านความปลอดภัยทางชีวภาพ	
ชื่อโครงการ (ภาษาไทย)	การสร้าง การหาคู่ลักษณะ และผลความเป็นสารเพิ่มความไวต่อรังสี ของอนุภาคนาโนทองคำที่มีโมเลกุลของเรตินอยด์ไฮดรอกซีโดม
ชื่อโครงการ (ภาษาอังกฤษ)	Fabrication, Characterization, and Radiosensitization Effect of Platinum Nanoparticles on Glioblastoma U87 Cells
ชื่อโครงการย่อย/กิจกรรมที่เกี่ยวข้อง กับความปลอดภัยทางชีวภาพ (กรณี เป็นเพียงส่วนหนึ่งของชุดโครงการ/ โครงการ)	
ชื่อผู้ขอรับการรับรอง	นายพิริชญ์ สุทธิบุญเกียรติ
ชื่ออาจารย์ที่ปรึกษา	ผู้ช่วยศาสตราจารย์ ดร.อภัย ประพันธ์
สังกัดหน่วยงาน/คณะ	คณะสัตวแพทยศาสตร์
เลขคำพิพาทโครงการ	NUBC OT 68-04-21
เลขที่รับรองโครงการ	68-33
ประเภทการรับรอง	งานวิจัยประเภทที่ 2
การรับรองครั้งที่ 1	แนบเสนอเพื่อขอรับการพิจารณาใบรับรองด้านความปลอดภัยทางชีวภาพ ได้ผ่านการพิจารณา และรับรองจากคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มหาวิทยาลัยนครสวรรค์ ครั้งที่ 11/2568 เมื่อวันที่ 6 สิงหาคม 2568 เห็นว่ามีความสอดคล้องกับแนวทางปฏิบัติ เพื่อความปลอดภัยทางชีวภาพ จึงเห็นควรให้ดำเนินการวิจัยด้านความปลอดภัยทางชีวภาพ ตามข้อเสนอการวิจัยได้
วันหมดอายุครั้งที่ 1	5 สิงหาคม 2569
ลงนาม	 (ดร.วิรัช สอนธรรมโพธิ์)
	 ประธานคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มหาวิทยาลัยนครสวรรค์
หมายเหตุ ใบรับรองฉบับนี้ใช้ควบคู่กับหนังสือราชการ เลขที่ อว 0603.01.13(1)/ร 2309 ลงวันที่ 13 สิงหาคม 2568	

Figure 26 Certificate on approval of biosafety and biosecurity