Nanoparticle enhanced radiotherapy

Reem Hussain Ali Ahmad

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I, Reem Hussain Ali Ahmad, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the work.
Abstract

Nanoparticles have been shown to create a localised increase in dose deposition when combined with ionising radiation. Although this has been shown in the literature, there are several factors that can alter the level of enhancement, which need to be investigated before translating the use of nanoparticles for clinical treatments. This thesis aims to investigate three different aspects of this effect: (i) effect of nanoparticles when combined with proton therapy, (ii) study the combined effect of nanoparticle material, size and beam energy with photon irradiation, (iii) consider the biological impact with different cell lines, nanoparticle parameters and radiation types.

To consider the effect of nanoparticles with protons, Monte Carlo simulations were developed to model the effects of nanoparticle concentrations. The use of nanoparticles at clinically relevant concentrations was shown to cause an effect on the Bragg peak, where changes were quantified in the model and validated experimentally. Both simulation and experiment demonstrated a shift in the distal edge of the Bragg peak, with a simulated shift of 4.5 mm compared to a measured shift of 2.2 mm with a beam of 226 MeV protons.

To study the combined effect, another model was developed, studying the effect on dose deposition around a single nanoparticle with photon irradiation. Here the geometry could be altered such that the nanoparticle size and material were studied, as well as the effect of different incident beam energies. These simulations considered the effects on multiple scales to determine the extent of the enhancement, where it is then possible to inform where nanoparticles need to be localised to within a cell to observe the most beneficial effect. The highest level of
Abstract

enhancement was found with 2 nm gold nanoparticles and 90 keV photons.

Finally to investigate the biological impact, an *in vitro* model was used with different cell lines, nanoparticles and radiation types, to gain an understanding of the biological effects. This was able to show differences in cell survival when comparing different cell lines, with different levels of radiosensitivity. As well as this, differences in DNA damage were shown when comparing X-ray radiotherapy and proton therapy. In terms of enhancement, gold nanoparticles were shown to be more effective with MCF-7 cells, whereas gadolinium based nanoparticles caused more cell kill for U87 cells.
Impact

Within radiotherapy it is necessary to deliver sufficient dose to the tumour, whilst sparing healthy surrounding tissues. It has been shown that the use of high-Z nanoparticles, such as gold, can create a localized increase in dose deposition, however a significant amount of work needs to be conducted before this can be translated to clinical use. The effect needs to be optimised, where several nanoparticle characteristics need to be investigated. As well as this, the underpinning mechanisms need to be understood to offer the greatest benefit to more precise radiotherapy techniques.

The work presented here reports on various considerations that need to be made with nanoparticles in proton and x-ray radiotherapy. Parametric studies were conducted through both computational simulations and biological experiments with different cancer cells. Both the models and experiments show how different nanoparticle characteristics can affect the enhancement effect. The implications of these results demonstrate the differences between physical (simulations) and biological (experiments) mechanisms. From this, further studies were highlighted to further understand the reasons for the differences demonstrated between the two.

In terms of the wider impact of these findings, they could provide a new combination therapy for cancer patients. The use of nanoparticles clinically can mean fewer treatment fractions are needed due to the enhanced dose deposition or a more localised dose profile that encompasses the tumour microenvironment leading to complete tumour kill. This in turn will result in an economic benefit as fewer resources are then used in our healthcare system to offer the same result of treating patients with cancer.
The work was a collaborative effort comprised of expertise from different disciplines including radiotherapy physics, computer science, engineering and radiobiology. It spanned across five institutions, University College London, UK, National Physical Laboratory (NPL), UK, Azienda Provinciale per i Servizi Sanitari, Italy, The Clatterbridge Cancer Centre, UK and the Joint Research Centre (JRC), Italy.
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Dedicated to Abdul Wahab Alkasab
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Glossary

APSS  Azienda Provinciale per i Servizi Sanitari.

AuNP  gold nanoparticle.

BP  Bragg peak.

BPW  Bragg peak width.

CDEF  cellular dose enhancement factor.

CTAB  cetyltrimethylammonium bromide.

DEF  dose enhancement factor.

DLS  dynamic light scattering.

DNA  deoxyribonucleic acid.

DSB  double strand break.

EPR  enhanced permeability and retention.

FBS  fetal bovine serum.

GBM  glioblastoma multiforme.

GdNP  gadolinium nanoparticle.

Glu-AuNP  thio-glucose-bound gold nanoparticle.
Glossary

**ICP-AES** inductively coupled plasma atomic emission spectroscopy.

**ICRP** International Commission on Radiological Protection.

**ICRU** International Commission on Radiation Units and Measurements.

**IONP** iron oxide nanoparticle.

**JRC** Joint Research Centre.

**LEE** low energy electron.

**LET** linear energy transfer.

**LQ** linear-quadratic.

**MC** Monte Carlo.

**MCS** multiple Coulomb scattering.

**MEM** Minimum Essential Medium.

**MTT** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

**NDEF** nuclear dose enhancement factor.

**NIST** National Institute of Standards and Technology.

**NP** nanoparticle.

**NPL** National Physical Laboratory.

**NTCP** normal tissue complication probability.

**OAR** organs at risk.

**OER** oxygen enhancement ratio.

**PBS** phosphate buffered saline.
PE  plating efficiency.
PEG  poly(ethylene glycol).
PMMA  polymethyl methacrylate.
RBE  relative biological effectiveness.
RNA  ribonucleic acid.
ROS  reactive oxygen species.
SEM  scanning electron microscope.
SER  sensitiser enhancement ratio.
SF  surviving fraction.
SOBP  spread-out Bragg Peak.
SSB  single strand break.
TCP  tumour control probability.
TEM  transmission electron microscopy.
WET  water equivalent thickness.
Outputs

Journal publications


Conference publications

Outputs


Ahmad R, Royle G, Ricketts K. Novel method to quantify physical dose enhancement due to gold nanoparticles in proton therapy, PTCOG 55, Prague, Czech Republic, May 2016, poster presented.

Ahmad R, Royle G, Ricketts K. Implications of gold nanoparticles used for dose enhancement in proton radiotherapy, ESTRO 35, Turin, Italy, April 2016, poster presented.

Ahmad R, Royle G, Ricketts K. High-Z nanofilms in proton therapy, NPL PPRIG Proton Therapy Physics Workshop, Teddington, UK, September 2015, poster presented.


Ahmad R, Royle G, Ricketts K. A Monte Carlo study investigating the effects of high-Z nanofilms in proton therapy, COST SYRA3 First Training School on radiation therapy, biology and dosimetry, Grenoble, France, May 2014, poster presented.


Prizes

First prize: Eleanor-Davies Colley Prize 2017 for best PhD presentation in the Division of Surgery and Interventional Science

Short-listed to final 6 for Faculty of Medicine 3 minute thesis (3MT) competition 2017
Grants

UCL MRC Pathway to Discovery secondment 2017

GI-CoRE Summer School Travel Fellowship Award, 4th GI-CoRE Summer School for Medical Physics, Sapporo, Japan, August 2017

PTCOG Travel Fellowship Award, PTCOG 54, San Diego, CA, USA, May 2015

UCL studentship 2014-2015

COST SYRA3 travel grant, First Training School on radiation therapy, biology and dosimetry, Grenoble, France, May 2014
Chapter 1

Introduction

1.1 Rationale

Over the past few decades significant progress has been made in the field of cancer therapy with advances in early detection and curative treatment. Treatment modalities vary depending on the type of cancer, location and stage at which it is detected. Examples of treatments include, radiotherapy, surgery and chemotherapy. These can be used alone or in combination, where reports have shown approximately 50 % of patients will receive radiotherapy as part of their treatment.

In current radiotherapy, ionising radiation is used to kill cancerous cells. The success of treatment is somewhat dependent on the stage of development at which the tumour is found. Early malignant tumours are generally localised and therefore treatment becomes more straightforward. The treatment prescribes a certain dose, to be delivered to the tumour, whilst minimising the dose to the surrounding healthy tissue.

Ionising radiation causes damage to the cells by damaging the deoxyribonucleic acid (DNA), where the level of damage caused is dependent on the dose of radiation used. The damage however, is not limited to the cancerous cells. Therefore there needs to be a balance between tumour cure and sparing injury to healthy cells. In order to minimise the damage caused to healthy tissue, the prescribed dose is delivered over multiple smaller doses, known as fractionation. This reduces damage
to healthy tissues, as they proliferate at a slower rate compared to cancerous cells and therefore can repair before replicating.

It has been shown that the insertion of metals, into the body, creates a comparative increase to dose. This was first demonstrated by Castillo et al [12] where they showed that there was a dose increase at the metal-tissue interface in patients with mandibular reconstruction plates. Since then, there has been an interest in finding an efficient way to utilise this enhancement to advance current radiotherapy techniques. This is one of the main reasons why there has been interest in using metal-based nanoparticles (NPs) in radiotherapy. If targeted to the tumour, they have the potential to increase the dose to the tumour specifically, further minimising damage to the surrounding healthy tissue.
1.2 Current radiation therapy techniques

In order to treat cancer, there are many options including surgery, chemotherapy and radiation therapy. Approximately 50% of all cancer patients receive radiotherapy as either a stand alone treatment or as part of a multi treatment strategy [13]. In the UK, most centres offer X-ray radiotherapy, a technique which uses photons to treat the cancer. Currently there is a growing interest in the use of proton therapy, a technique where high energy protons are used instead of photons. The interactions of the two techniques differ greatly, leading to a difference in dose deposition to the tumour site, as can be seen in figure 1.1.

![Comparison of dose profiles between photon and proton irradiations](image)

Figure 1.1: Comparison of dose profiles between photon and proton irradiations

1.2.1 Dose

In all forms of radiation therapy, dose is used as a measure for the amount of radiation that is administered to the patient. More specifically, there are different definitions of dose, such as the physical absorbed dose, equivalent dose and effective dose. The physical absorbed dose as shown in equation 1.1 is defined as the energy absorbed per unit mass of a material where the unit is the gray (Gy).

\[ D = \frac{\Delta E}{\Delta m} \]  \hspace{1cm} (1.1)
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The equivalent dose takes into account the radiation that produced the dose, where the dose is weighted according to radiation weighting factors quoted in the relevant International Commission on Radiological Protection (ICRP) reports (60 and 103) [14, 15]. This is given by equation 1.2, where D is the mean absorbed dose, from a specific radiation, in an organ or tissue and W is the relevant radiation weighting factor. The unit of equivalent dose is the sievert (Sv).

\[ H = \sum_{R} W_{R} D_{T,R} \] (1.2)

Finally the effective dose is the tissue weighted sum of the equivalent doses, as shown in equation 1.3, also with units of Sv.

\[ E = \sum_{T} \omega_{R} H_{T} \] (1.3)

In terms of this work, the dose definition of interest is the physical absorbed dose, as this is what defines the prescribed dose for treatment in radiotherapy.

1.2.2 Killing cancer

DNA is the genetic material making up all the cells in the human body and therefore the most important target in radiotherapy when targeting cancer cells. Ribonucleic acid (RNA) is another biological macromolecule. It is in charge of regulating gene production, which in turn will regulate when, and how, proteins are produced, where RNA damage can lead to apoptosis. Proteins along with DNA and RNA are essential within all living cells [3].

When incident radiation interacts with matter, it can damage the cells through direct or indirect effects. Direct effects describe ionisation of the DNA itself, where the DNA can be partially damaged in the form of a single strand break (SSB). This form of damage can be repaired, however there may be problems associated with the repair. Direct effects can also cause a double strand break (DSB), in this case repair is more complex and therefore less probable. With direct effects, the likelihood of occurrence increases, with the linear energy transfer (LET) of the incident radiation. This is a term used in dosimetry to describe the amount of energy transferred to a
1.2. Radiation therapy

material per unit distance, with units of keV/µm. This concept will be explained in more detail in section 1.4. Ionisations from X-rays are sparse and therefore the likelihood of causing a direct effect is less probable than an alpha particle which has densely ionising tracks [16]. Other direct effects include isolated DNA lesions on single bases and clustered DNA damage [17]. Clustered DNA damage is where two or more elemental DNA lesions may be produced close to each other on opposite DNA strands. This type of damage can cause DSBs as well as SSBs of different complexity due to additional breaks or base lesions. [18, 19]

Indirect effects, such as those caused through photon interactions, induce radiolysis of water, whereby free radicals are produced. These are very reactive and therefore can damage the DNA through SSB or DSBs [20]. It is now understood that the biological effectiveness of radiation depends on several factors, which include the LET, the dose delivered, number of fractions and the sensitivity of the targeted tissue to radiation [21].

It should be noted that when cells are exposed to ionising radiation certain cell cycle check points are activated. The cell cycle is often described as being formed of four phases, G₁, the gap before DNA replication, S, the DNA synthetic phase, G₂, the gap after DNA replication and M, the mitotic phase for cell division [22]. The check points impose temporary arrest at specific stages to allow the cell to correct for defects [23]. In terms of cell sensitivity to radiation, it has been reported that cells are most sensitive in the G₂-M phase, less in the G₁ phase and least sensitive at the late S phase. Ionising radiation has been shown to slow the rate of progression of proliferating cells through different phases of the cell cycle, causing cells to accumulate in the G₂ phase, preventing them from dividing. Radiotherapy doses are usually delivered as fractions over several weeks. With this change in cell cycle, fractionation effects can be demonstrated, where, as the cells are in a more sensitive part of the cell cycle, the next fraction is delivered [24].

In the subsequent sections, the physics describing physical interactions that lead to dose deposition will be explained. It should be noted that, in practice, biological interactions also have a great contribution to the dose [11]. When
considering enhancing the dose using NPs, we must consider the differences in these mechanisms, as will be explored in section 1.5.5.

1.2.3 Radiation therapy

Historically, when using radiation in the treatment of cancer, the most common modality used is X-ray radiotherapy. However, in more recent years charged particle therapy has been more extensively explored due to higher local control of the beam. It is known that with particle therapy there is an increase in the biological effect. This is highlighted by the relative biological effectiveness (RBE), defined as the ratio of dose delivered by a reference radiation, usually 250 kVp X-rays or Cobalt-60 ($^{60}$Co) gamma rays, to the dose delivered by the particle being considered (proton, carbon, etc.) [25]. This concept will be explained in more detail in section 1.4. Both X-ray radiotherapy and proton therapy are investigated within this project.

1.2.3.1 X-ray radiotherapy

In terms of current cancer treatments, external beam X-ray radiotherapy is commonly used as a stand alone treatment or in combination with surgery and systemic treatments such as chemotherapy. This is dependent on the tumour site and the level of progression. X-ray radiotherapy utilises high energy photons in order to irradiate the tumour, ionising the matter they interact with. More specifically this entails the removal of outer shell electrons, which go on to cause further ionisations. When a photon interacts with matter, it is characterised by three separate interactions: photoelectric, Compton and pair production.

The photoelectric effect is where an incident photon (Energy $h\nu$) interacts with a tightly bound electron, transferring all of its energy, whereby a photoelectron is ejected from the bound shell. The energy of the photoelectron ($E_{e-}$) is then given by equation 1.4,

$$E_{e-} = h\nu - E_b$$  \hspace{1cm} (1.4)

where $E_b$ is the binding energy of the photoelectron. Electrons ejected from higher shells will cascade down to fill the inner shell vacancy, releasing a characteristic X-ray. The probability of the photoelectric effect is approximately proportional to
Z^4/E^3, meaning that it is most probable for low energy photons and materials with a high atomic number.

The Compton effect occurs, where the incident photon interacts with a free, loosely bound electron. In this interaction, the photon, with energy \( hv \), is scattered and transfers some of its energy, \( hv' \), to the electron. This electron, known as a recoil electron, propagates and ionises with the energy given to it. The energy given to the recoil electron \( (E_k) \) can be determined using equation 1.5,

\[
E_k = hv - hv' = hv - hv \frac{1}{1 + \alpha(1 - \cos \theta)} = hv \frac{\alpha(1 - \cos \theta)}{1 + \alpha(1 - \cos \theta)}
\]

where \( \theta \) is the photon scattering angle, \( \alpha = \frac{hv}{m_0c^2} \) and \( m_0c^2 \) represents the electron rest mass energy. The probability of this effect is independent of the atomic number and is inversely proportional to the photon energy.

Pair production can occur, only if the incident photon has an energy that is at least 1.022 MeV, the sum of the rest mass energies of an electron and a positron. If the photon has sufficient energy, it can then interact with the strong electric field surrounding the nucleus, where the photon undergoes a change of state, creating an electron-positron pair. The probability of this effect increases with increasing photon energy and is related to atomic number by approximately \( Z^2 \).

In terms of radiotherapy, the most dominant effect is the Compton effect, as this is the prevailing interaction at the energies commonly used in X-ray radiotherapy (6 MV). This is highlighted in figure 1.2, which demonstrates the relative importance of all three interactions.
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1.2.3.2 Proton therapy

Proton therapy exploits the use of protons, rather than photons to kill cancerous cells. From figure 1.1 it was shown that the characteristics of protons allow for a more localised dose profile. To date, there have been many studies comparing the two, however more clinical cases using protons are needed to demonstrate any true physical benefit, as these are currently limited [26]. As well as this, a greater number of long term toxicity studies are needed to determine any differences in toxicity with radiations of a higher LET and RBE.

Proton therapy is generally the preferred irradiation therapy for treating paediatric cancers, due to the sparing of normal healthy tissues. However, even in these cases limited follow up data exists to study the long term effectiveness and toxicities associated with using proton therapy [27]. As of the end of 2016, over 140,000 patients across the world were treated using proton therapy [28]. Whilst this is a substantial number, it is necessary to acquire follow-up data from these patients to determine the long-term effects.
1.2. Radiation therapy

Protons are heavy charged particles, meaning their interactions with matter differ to those of photons. Protons interact with matter in three separate processes: Coulombic interactions with the atomic electrons, Coulombic interactions with the atomic nucleus and nuclear reactions.

The first of these interactions occurs in the form of inelastic Coulombic interactions with atomic electrons. Protons continuously lose energy through these frequent interactions and then ‘slow down’, whereby the proton continues to lose energy as it slows, due to more momentum being transferred to the electron through a proton-electron collision. Due to the energy transferred to the electron, the electron is removed from the orbital of a target atom (ionisation). In order for this to occur, the energy transferred needs to be greater than the binding energy, which in the case of the outermost electron, is of the order of $10 \text{ eV}$ [29].

The second interaction is scattering, more specifically multiple Coulomb scattering (MCS), where the incident proton passes near an atomic nucleus and interacts through a repulsive elastic Coulombic interaction, causing the proton to be deflected. Although the proton loses a negligible amount of energy, this change in trajectory needs to be accounted for in the calculation of dose distributions [30].

Finally, protons can undergo collisions with a nucleus, where a particle is ejected from the nucleus, introducing secondary particles into the area [2]. These secondaries have lower energies and larger angles than the primary proton. Through these ionisations, damage is caused to the molecules of the cell and the DNA, where DNA damage can kill the cell or hinder its ability to proliferate.

These characteristics together form what is known as the Bragg peak (BP), which describes the dose profile observed. Considering the interactions previously mentioned, it is possible to describe the origins of different parts of the dose profile. Figure 1.3 highlights the different regions of the dose profile, where initially the range itself is related to the initial energy of the proton. As the proton travels through matter, there is a build-up region of dose, caused by the emission of secondary particles due to nuclear interactions [31]. Following the build-up region, there is the peak showing the maximum energy deposited. As was previously
explained, protons slow down as they traverse through matter, causing them to deposit great amounts of energy after a certain depth, giving the characteristic shape of the BP. The width of the BP is caused by range straggling, where even considering a mono-energetic beam, where protons would most likely have the same energy, there would still be some uncertainties in the range travelled by the protons. Uncertainties in the range mean that the protons would not all stop at the same point, creating the width of the BP. The BP itself, is one of the benefits of proton therapy compared to X-ray radiotherapy, as it allows for a more localised dose to the tumour.

When considering the use of protons, it is necessary to be able to describe the rate at which they slow down and lose energy. The amount of energy lost per interactions is approximately proportional to $1/v^2$, where $v$ is the particle velocity. For a beam of protons, the energy loss is given by the stopping power as described by equation 1.6,

$$S = \frac{-dE}{dx}$$

(1.6)

where the units are MeV/cm. A more commonly used equation is the mass stopping power, which corrects for the material density as shown in equation 1.7,
\[
\frac{S}{\rho} = -\frac{1}{\rho} \frac{dE}{dx}
\]

(1.7)

where \( \rho \) is the density of the stopping medium [32]. The mean range of protons can be determined by integrating the reciprocal of the stopping power with respect to energy. Due to this relationship, it is possible to use a specific energy to achieve the range required to treat the location of the tumour.

Although the BP offers a sharp dose profile, in practice it is necessary to alter the profile to ensure full tumour coverage. This is carried out by superimposing several energies, in order to form what is known as the spread-out Bragg Peak (SOBP), as shown in figure 1.4. With the SOBP, it is possible to ensure tumour coverage, whilst still maintaining a sharp fall off in dose, protecting possible organs at risk (OAR).

![Figure 1.4: Plot demonstrating how, by superimposing several beamlets, it is possible to form a spread-out Bragg peak (SOBP) able to cover a tumour area [2].](image-url)
1.3 Therapeutic ratio

Within radiotherapy, the aim is to deliver enough radiation to kill the tumour, without irradiating normal tissues to a level that will cause complications. This is highlighted by the concept of both tumour control probability (TCP) and normal tissue complication probability (NTCP) as depicted in figure 1.5. When determining the dose to be delivered to a tumour, considerations are given to maximise the TCP, whilst minimising the NTCP.

![Figure 1.5: Curves demonstrating the probability associated with both TCP and NTCP, leading to a comparison known as the therapeutic ratio.](image)

When considering these curves, a comparison can be made between the two, known as the therapeutic ratio. This ratio compares the amount of dose needed to have a therapeutic effect to the amount that causes adverse effects. The greater the distance between the two curves, the larger the ratio, meaning a lower probability of the treatment causing complications.
1.4 Linear energy transfer, relative biological effectiveness and oxygen enhancement ratio

As stated in section 1.2, there is a difference in the biological effectiveness of radiation on cells when utilising protons and heavier ions compared to photons. This can be described by three different concepts, linear energy transfer (LET), relative biological effectiveness (RBE) and oxygen enhancement ratio (OER) [3].

LET describes the ionisation density, quantified by the average amount of energy absorbed by the media per unit distance travelled by the incident ionising radiation, with units of keV/µm. With proton therapy, the LET is greater than that of photons, it is also more complex, as it varies with depth.

RBE considers the ratio of doses between the radiation being considered and a reference radiation, typically 250 kV<sub>p</sub> X-rays, to give the same biological effect [33]. It should be noted that gamma rays from a 60<sup>Co</sup> unit have also been used as a reference radiation source. As well as being dependent on the radiation considered, the RBE varies with the tissue type, fractionation, dose, dose rate and LET.

OER refers to the enhancement of damage due to the presence of oxygen. This is defined as a ratio of dose in a hypoxic state compared to a normoxic state for the same biological effect. This decreases with increasing LET, which is one of the reasons for using higher LET radiation, as the presence of oxygen is less important for effective cell kill, therefore it may be more beneficial for hypoxic tumours [34].

All three quantities are closely related as depicted in figure 1.6, where as the LET increases, the OER decreases until it reaches unity where there is no oxygen effect. As well as this, the RBE increases with increasing LET until a point of 100 keV/µm, where it then decreases with higher LET. At an LET of 100 keV/µm, the average separation between ionising events correlates with the diameter of the DNA double helix, approximately 2 nm [34]. Therefore radiation with this LET is more likely to cause a DSB from a single track. At a greater LET this decreases due to what is known as the ‘overkill effect’, where the ionisations are too close together to cause an increase in RBE, therefore the energy is wasted [3].
1.5 High-Z nanoparticle aided radiation therapy

Radiation therapy has improved over the years, however there are still limitations associated with using radiation alone. These include factors such as the tumour’s sensitivity to radiation, where hypoxic tumours tend to be more radioresistant. It has been shown that the presence of oxygen during irradiation causes radiation sensitivity, as the oxygen interacts with free radicals, fixing the damage caused into a permanent irreparable state [35]. Another factor is the level of dose that can be prescribed for treatment. If a high enough dose is given any tumour cell can be killed. However, in practice the dose used is limited, due to the possible radiotoxicity of healthy surrounding tissues.

Over the past few decades, much work has been conducted to investigate the use of high-Z nanoparticles (NPs) within radiation therapy, to determine the effectiveness of NPs as radiosensitisers. By combining NPs with radiation, it is then possible to increase the therapeutic ratio, which was described in section 1.3. Here the TCP curve would be shifted to lower doses to achieve the same effect, widening the gap between the curves and increasing the ratio.

In terms of the NPs used for medical applications, various forms have been used within the literature, where advancements in the synthesis of NPs has allowed...
1.5. Enhanced radiation therapy

for modifications to the size, shape and surface functionality. Commonly used methods of creating colloidal gold nanoparticles (AuNPs) include treating hydrogen tetrachloroaurate (HAuCl$_4$) with citric acid in boiling water. Others have extended this to use a two-phase synthesis process where a phase-transfer reagent is applied along with a reducing agent and the solution is then stabilised by thiols [36, 37].

Studies using NPs in radiotherapy varied between experimental work, both in vivo and in vitro, to Monte Carlo (MC) simulations predicting damage levels and optimising the radiosensitisation effect by altering different variables.

As many factors need to be considered to demonstrate the effectiveness of NPs in radiation therapy, it is necessary to review the work previously carried out by others. The knowledge gained on specific mechanisms can be collated, to exploit the radiosensitisation effect and enhance the therapeutic effect. This section summarises the literature and highlights key findings for various variables, as well as the enhancement mechanisms to help understand how NPs can benefit radiation therapy.

### 1.5.1 Literature for nanoparticle enhanced radiation therapy

The majority of current research investigating NPs as radiosensitisers has been carried out using X-ray radiation, ranging from experiments to MC studies.

#### 1.5.1.1 Experiments

One of the first experiments to demonstrate the potential of NPs as radiosensitisers was conducted by Hainfeld et al, where they demonstrated the use of NPs in an in vivo experiment [38]. They reported significant improvement in tumour control using AuroVist™ 1.9 nm AuNPs and kilovoltage photon irradiation.

Others such as Chithrani et al, carried out an in vitro study to investigate radiosensitisation as a function of both NP size and dose using HeLa cells [39].

Taggart et al, carried out in vitro experiments, also using the AuroVist™ AuNPs, whilst varying the cell line used, to investigate the cellular responses of various cancer types, where the highest enhancement ratio shown was 1.90, with T98G, a glioblastoma cell line [40].
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Jain et al, looked at the cellular uptake, toxicity and radiosensitisation effect of AuroVist™ AuNPs on three cell lines under hypoxic and oxic conditions [41]. Using a concentration of 12 µM (24 hour incubation), they showed lower cellular uptake for all cell lines under hypoxic conditions, with significance only demonstrated for one cell line (DU145). They also showed a significant reduction in cell proliferation for hypoxic MDA-MB-231 cells. Finally, with the MDA-MB-231 cells, they showed a sensitiser enhancement ratio (SER) of 1.41 with 21% oxygen, compared to 1.39 and 1.1 with 1% and 0.1% respectively.

Taupin et al, considered the use of gadolinium nanoparticles (GdNPs) with F98 glioma cells, where they showed a sensitisation enhancement ratio of 2.44, for an energy of 65 keV photons [42].

Following on from these studies with photon irradiation, groups extended the work to consider the use of different incident beams. Kim et al [43] investigated the use of gold and iron NPs in a mouse model at an incident proton energy of 45 MeV; a one-year survival of 58 - 100% was found with NPs compared to 11 - 13% with irradiation alone. Polf et al investigated NPs of diameter 44 nm located in a tissue equivalent phantom at a concentration of 1 ng/cell [44]. An increase in the effectiveness of tumour cell killing by 15 - 20% was found at an incident proton energy of 160 MeV.

Others also considered the use of heavier ions, such as carbon ion beams. Kaur et al demonstrated this using HeLa cells with glucose capped AuNPs (size between 5 and 9 nm), irradiated by a 62 MeV beam [45]. They firstly showed that the AuNPs were not cytotoxic (concentration of 5.5 µmoles/ml incubated for 6 hours), then through cell survival experiments, reported an approximate 29% reduction in dose with NPs, to achieve a 90% cell kill, compared to no NPs. This corresponded to an increase of approximately 41% in RBE. Similar results were demonstrated by Liu et al using HeLa cells and carbon beams, but with 15 nm citrate-capped AuNPs [46, 47]. They demonstrated a maximum dose enhancement factor (DEF) of 1.44 (samples incubated for 24 hours with 15 µg/ml), which they attributed to an increase in production of hydroxyl radicals.
1.5.1.2 Simulations

Following on from Hainfeld et al’s study, groups attempted to demonstrate the effects of NPs combined with photons through simulations. One of the first groups to attempt to model the dose enhancement effect was Cho et al [48]. Here they modelled three different concentrations of AuNPs, where concentrations were simulated by weight rather than single-sized NPs. From this they were able to demonstrate that the enhancement effect increased with concentration. As well as this, they showed an energy dependence, whereby energies in the orthovoltage range were most effective.

Others such as Lechtman et al, developed a MC based radiosensitisation predictive model for AuNPs, which provided comparable predictions to what was observed though experimental cell survival [49]. Their model predicted macroscopic dose enhancement, which was compared to cell survival data with PC-3 prostate cancer cells. From their experiment with AuNPs (30 nm at a concentration of 2 mg/ml incubated for 24 hours), they showed a SER of $1.21 \pm 0.13$, compared to $1.20 \pm 0.12$ from their predictive model.

Few NP studies have been carried out experimentally using proton beams, including a number of MC simulations. Wälzlein et al conducted a simulation of a single nanosphere of controllable material, surrounded by water, with proton dose enhancement up to a factor of 2 for gold and platinum at 80 MeV [50]. Another MC study conducted by Gao and Zheng simulated a single AuNP within a water phantom; they concluded that the production of secondary electrons increased with decreasing proton energy, whereas the average kinetic energy of secondary electrons stemming from AuNP interaction increased with proton energy [51]. Kwon et al also simulated a single AuNP within water [52]. Here however, they considered the radial dose distribution due to secondary electrons, where they found that the effect due to AuNPs extended over several micrometers in the longitudinal direction and several nanometres in the radial direction. Lin et al, demonstrated a difference in enhancement mechanisms between proton and photon NP-interactions through MC simulations. With protons, the highest enhancement was found closest to
the NP [53]. A biological model by Lin et al, showed that for protons, a higher NP concentration was needed to achieve the same effect compared to photons for extra-nuclear cell-internalised AuNPs [54].

1.5.2 Nanoparticle toxicity

When recommending the use of NPs for clinical use, the toxicity is an important factor that needs to be considered, as NPs have a large surface to volume ratio, which leads to different biological activations compared to the bulk material. Within the literature, the factors that are deemed to affect the toxicity are the physiochemical parameters, such as surface charge, shape, size, chemical composition and NP stability. Other factors such as the route of administration can affect biodistribution of NPs and in turn, affect where they accumulate and are metabolised, leading to differences in toxicity [55].

Several groups have conducted in vitro studies to look at the cytotoxicity of NPs, one of which was by Connor et al, where they looked at different NP concentrations studying toxicity effects in human leukaemia cells [56]. By studying the cell viability, they showed that 18 nm NPs with citrate and biotin surface modifications, did not appear to be toxic at concentrations up to 250 µM. In contrast, Li et al demonstrated oxidative stress when they studied human lung fibroblast cells with AuNPs (1 nM), which could lead to oxidative DNA damage [57]. A study by Qiu et al investigated three different coatings and reported that with cetyltrimethylammonium bromide (CTAB) coatings, they found shape independent, but coating dependent cytotoxicity [58].

At a high enough concentration, many substances become toxic, therefore it is always important to consider the concentration being used. It should be noted that although in vitro toxicological studies can offer an insight into the dose of NPs that can be used without hindering cell proliferation, these effects do not directly translate into in vivo or human studies [59].

A study demonstrating this difference was carried out by Feng et al, where they investigated toxicity in vitro with SKOV-3 ovarian cancer cells and in vivo with SKOV-3 tumour bearing nude mice, using iron oxide nanoparticles (IONPs)
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[60]. Different formulations were tested, where one demonstrated cytotoxic effects at the concentrations tested (3.125 to 100 µg/mL). With the same NPs, the *in vivo* work showed for a concentration of 5 mg/kg, all mice died and a quarter died with 2.5 mg/kg within 24 hours post-injection. No cytotoxicity or mouse death was found with other formulations, which highlights the need for thorough toxicity tests when manipulating NP properties.

1.5.3 Variables affecting enhancement

From the literature, it was evident that many variables could affect the level of enhancement observed [61, 62, 63]. Each of these will be summarised in sections 1.5.3.1-1.5.3.4.

1.5.3.1 Material

The first variable considered is the NP material. In order to recommend the use of NPs with radiotherapy, it would firstly be appropriate to use a material that is readily used for medical applications. One consideration is that it is necessary for the material to have a relatively high atomic number, compared to that of water, in order to observe an enhancement effect [61].

McMahon et al conducted a study investigating various materials through MC simulations, looking specifically at the macro and micro scale dose enhancement [7]. Although at clinical beam energies (MV), they found minimal energy dependence, it was shown that elements such as Europium, Gadolinium and Ytterbium offered a higher effect. This was hypothesised to be due to the low density of these materials, whereby there would be a reduction in self-absorption, leading to a higher dose deposition.

More recently groups have experimented with multiple materials for the NP, where the core and outer shell are comprised of different materials to allow for a multifunctional NP that can be used for theranostic (therapy and diagnostic) applications. A study by Ahn et al considered the use of an iron oxide core with a tantalum oxide shell in MC simulations irradiated with 70 and 150 MeV protons [64]. From this they found a lower enhancement with their multifunctional NPs.
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compared to AuNPs (15.76 for Au compared to 7.82 for multifunctional NP for 70 MeV). They concluded however that these NPs may still be beneficial due to the reduced cost of the NP compared to an AuNP and the possibility of tumour targeting with the use of a magnetic field. This would exploit the superparamagnetic property of the iron oxide core to improve NP uptake within the tumour by using a well-designed, externally applied magnetic field to focus the targeting of the NPs.

1.5.3.2 Concentration

The next variable is the concentration. This firstly affects the level of uptake within the cell, however it also affects the toxicity and enhancement. Chithrani et al looked at the effect of concentration on cellular uptake using HeLa cells and AuNPs of different sizes [65]. They used inductively coupled plasma atomic emission spectroscopy (ICP-AES) to determine the the concentration of AuNPs taken up by the cells, where they showed an increase in the number of NPs per cell with increasing concentration. They also considered the cell viability and demonstrated no toxicity due to the presence of AuNPs.

Note, it is necessary to distinguish between the concentrations that are injected and those that are typically taken up by the cells. From the literature it is known that even when high concentrations are injected, the actual uptake within the cells is only a few percent of that injected [66, 67].

1.5.3.3 Size

Another factor is the NP size. This is of particular importance as it affects both enhancement and uptake. Tumour vasculature is known to be ‘leaky’, whereby the size could affect the number of particles that can pass through into the tumour [68].

Studies such as that of Chithrani et al, demonstrated the dependence of the enhancement effect on the NP size. From their in vitro work with AuNPs and HeLa cells, they found the most enhancement with 50 nm AuNPs. This was attributed to these NPs being able to enter cells more efficiently through receptor-mediated endocytosis [65].

Another consideration is the chemical coating as this can greatly alter the size of the NP. Ma et al demonstrated significant changes in AuNP sizes
when functionalising with poly(ethylene glycol) (PEG). Using scanning electron microscope (SEM) they found the average diameter to be approximately 53 nm, whereas the hydrodynamic diameter was approximately 108 nm [69]. They also considered different nano-material shapes, all coated with the same amount of PEG, demonstrating significant differences in cellular uptake, where spherical NPs offered the highest level of uptake in KB cancer cells, compared to nanospikes and nanorods. Considering the radiosensitisation effect, they showed that this correlated with their findings for cellular uptake, where spherical AuNPs also had the highest SER.

1.5.3.4 Beam characteristics

Finally, the irradiation type and beam energy can alter the enhancement. From studies carried out with photons, it was evident that the beam energy had a considerable effect on the level of enhancement observed both experimentally and through simulations. Many groups attributed the observed dose enhancement, to the difference in mass energy absorption coefficient between the high-Z material and soft tissue, as described by the International Commission on Radiation Units and Measurements (ICRU), where the differences are shown below in figure 1.7 (data from the National Institute of Standards and Technology (NIST)) database. It is evident from this plot, that by targeting the NPs such that they are localised to the tumour, an enhancement of physical dose would be expected in the orthovoltage range (< 500 keV), due to the NPs having a higher mass attenuation coefficient than soft tissue [70]. This difference greatly decreases at higher energies, those used in clinical radiotherapy.
Studies have compared two energies, one around the k-edge of gold (80.7 keV), and the other, a clinically relevant energy, such as 6 MV photons.

McMahon et al demonstrated the sensitisation effect of AuNPs on plasmid DNA, for a range of energies from 1.8 to 80 keV [71]. From this work, it was evident that simulations could not compare to what was observed experimentally, where they showed a fluctuation in the enhancement effect with incident beam energy. This fluctuation in enhancement was also demonstrated by Rahman et al, where they considered a similar energy and reported cell survival [72].

In terms of work carried out with proton irradiation, Jeynes et al conducted both simulations and an in vitro study [73]. Using a free radical scavenger they showed that the death associated with photons is partly caused by reactive oxygen species (ROS), which is increased with AuNPs. As well as this they showed that the ROS species have little effect on the cell death when using protons (3 MeV). They concluded that there would be less dose enhancement with protons compared to photons and that free radicals were the main cause for the radiosensitisation observed.

Li et al considered the use of AuNPs in A431 cells using a proton beam at different LET values [74]. They showed an LET dependent radiosensitisation effect, where they explain that the interaction probability between AuNPs and protons is
due to different factors. The first is related to the NPs internalised within the cell, which they showed was size dependent. The second was the number of projectiles, where higher-LETs require fewer protons to achieve the same dose as a lower LET protons. In short AuNPs that are localised on a proton track, will interact more with high-LET protons.

1.5.4 Nanoparticle uptake

All of the NP characteristics previously discussed in section 1.5.3 will affect the level of cellular uptake. As previously explained, the tumour vasculature is known to be ‘leaky’ which allows for NPs to permeate the angiogenic endothelium [75]. As well as this defective vasculature structure, tumours have impaired lymphatic drainage [76]. Both of these tumour characteristics allow for preferential uptake within the tumour, without the use of specific active targeting methods, through the enhanced permeability and retention (EPR) effect. In terms of the mechanism for NPs to enter the cell, it has been theorised that this occurs through endocytosis, as depicted in figure 1.8, which is dependent on various NP characteristics discussed in section 1.5.3 [65, 5].

![Figure 1.8: Hypothesised mechanism of NP cellular uptake is endocytosis, which is shown to depend on many NP characteristics such as size, shape and surface properties [5].](image-url)
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1.5.5 Enhancement mechanisms

When considering the use of radiation for the treatment of cancer it is important to consider all the mechanisms that contribute to tumour cell death. In general these are split into physical, chemical and biological mechanisms [62].

Firstly we will consider the physical mechanisms which occur when exposing matter to ionising radiation. Ionising radiation has sufficient energy to eject bound electrons. If given sufficient energy, the electrons that are ejected can travel further causing a cascade of ionisations. These electrons can undergo several interactions, losing kinetic energy during each, until all their energy has been deposited (section 1.2.3.1). When these electrons interact and liberate electrons they create free radicals, which are atoms with an unpaired electron.

Following on from physical mechanisms, we consider chemical mechanisms. This is where the highly reactive radicals that were created undergo their own reactions to restore cellular charge equilibrium. The radicals can undergo reactions making the damage permanent, rendering the deoxyribonucleic acid (DNA) damage to be irreparable.

Finally, the biological mechanisms occur which involves the cellular repair processes. The damage caused by the incident radiation can lead to a failure to repair which in turn will lead to cell death.

These are the basic mechanisms that biological systems experience when irradiated. With regards to the introduction of NPs there are distinct mechanisms that occur to cause an increase in this damaging effect. In sections 1.5.5.1-1.5.5.3 the literature describing these mechanism in relation to NP enhanced radiation therapy will be summarised.

1.5.5.1 Physical mechanisms

When introducing NPs into a material, a level of physical enhancement can be observed. This was theorised to be due to the differences in energy absorption of Au (Z = 79) compared to soft tissues (comprised of materials with low Z) as was discussed in section 1.5.3.4. This difference therefore causes a greater energy deposition per unit mass, increasing the local dose deposition to the
1.5. Enhanced radiation therapy

tumour. Generally the enhancement effect is due to an increased production of photoelectrons, Auger electrons and low energy secondary electrons.

Zheng et al carried out a study using plasmid DNA, with and without AuNPs and irradiated with 60 keV electrons [77]. Through this they could assess the damage caused due to the radiation, without the effect of biological responses. From this they concluded that energy released was due to low energy electrons (LEEs) (<100 eV), responsible for most of the additional damage caused. Carter et al measured hydroxyl radical-induced DNA strand breaks [78]. They reported that these electrons have effective ranges of 1 - 10 nm.

1.5.5.2 Chemical mechanisms

Of all the mechanisms, this is the one least investigated, however from the reports in the literature it is evident that these mechanisms have an effect on the overall sensitisation.

In terms of the chemical mechanisms, two have been suggested within the literature.

The first of these is chemical sensitisation. Zheng et al worked again with plasmid DNA, here however they used different electron energies (1, 10, 100, 60,000 eV) [79]. They showed an enhancement with 1 eV electrons, however no ionisations occur at this energy, therefore this increase in damage is not related to producing more electrons. It was theorised to be causing a higher chemical sensitivity due to negative ions weakening the bonds within the DNA, sensitising the DNA to damage from LEEs that are emitted from the NPs. This was later extended by Yao et al, where they demonstrated a size dependence in this effect, whereby smaller particles (5 nm compared to 15 nm) were more effective at chemical enhancement [80].

The second mechanism is an increase in radical formation. It has been reported by many groups that the surface of NPs is electronically active and capable of causing chemical reactions [81, 82].

In terms of AuNPs, their catalytic properties were attributed to their small size and high curvature which disrupts the highly organized crystal structure of
bulk gold. Altering the electronic configuration of surface atoms allows for radical production at the reactive surface of the AuNPs [83].

Although both of these mechanisms are plausible, it should be noted that for the chemical sensitisation of DNA to radiation damage, to occur, the NPs need to be located within the nucleus, such that they can bind to the DNA. This is due to the higher chemical sensitivity to fragmentation by LEEs due to the electrostatic binding of NPs DNA. Therefore damage to the DNA occurs through dissociative electron attachment [79].

### 1.5.5.3 Biological mechanisms

Higher beam energies (6 MV), such as those normally used to treat tumours, were predicted to have minimal enhancement effects, as interaction probabilities decrease at these higher energies, compared to orthovoltage energies. Wang et al carried out an *in vitro* study with A549 cells and thio-glucose-bound gold nanoparticles (Glu-AuNPs), where they showed a SER of 1.49 with 6 MV photons [84]. Similar results were found by other groups, where the enhancement decreased with increasing beam energy, but were higher than those predicted from the physical mechanisms [39, 85, 86, 87].

Due to the discrepancies between simulations and what was observed experimentally, it lead to theories of a biological enhancement. This was supported by the findings of Jain et al, where they showed an enhancement effect with MDA-MB-231 cells, but demonstrated no increase in DNA double strand breaks (DSBs) [88]. They also showed that AuNPs enhanced the effect of the radiomimetic agent, bleomycin, without radiation, suggesting biological interactions with cells.

In terms of the biological mechanisms associated with radiosensitisation, they have been classed as three separate pathways.

The first of these is oxidative stress, whereby NPs have been shown to induce the formation of ROS. With radiation, the free radicals formed, along with the ROS can cause damage by either interacting with the biological molecules or by causing oxidative stress, which in turn leads to cell death [89, 90, 91]. Oxidative stress has been hypothesised to be caused by mitochondrial dysfunction due to an increase in
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ROS [92, 93].

Another biological mechanism, is the impact on the cell-cycle. In general, the cell cycle phase determines how sensitive the cell is to radiation. The most sensitive cells are those in the $G_2$ - M phase, decreasing in sensitivity in the $G_1$ phase and least sensitive in the S phase [24].

In this case, NPs have been shown to disrupt the cell-cycle, where the effect is dependent on various factors including the NP characteristics. It should be noted that the cell-cycle effects are also cell line dependent, where some groups have reported no influence on the cell cycle. An example of this was demonstrated by Butterworth et al [94], where they found significant levels of apoptosis in DU-145 cells but not in MDA-231-MB cells. In conjunction with this they also showed increased levels of oxidative stress and number of DNA damage markers.

The third pathway is the inhibition of DNA repair. It was previously explained in section 1.2.2 that ionising radiation can cause damage to the DNA in the form of SSBs and DSBs, where DSBs are considered more damaging, as failure to repair these results in cell death.

One study that demonstrated this was conducted by Chithrani et al [39], where they demonstrated an increased number of DNA damage markers ($\gamma$-H2AX and 53BP1). The increased residual damage that they showed, was thought to be indicative of delayed DNA repair, which in turn demonstrates a radiosensitisation effect.

As with the cell cycle effect, some groups demonstrated no effect on the inhibition of DNA repair in the presence of NPs combined with radiation therapy [88].

Overall these differences, demonstrated by different groups, highlight a need for parametric studies to be considered, whereby various NP properties are compared, across different cell lines and irradiation types to determine the biological effect of NP radiosensitisation.
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1.6 Research questions, aims and hypotheses

Although the proof of concept had already been conducted by different groups to demonstrate a radiosensitisation effect through *in vivo* and *in vitro* studies, there was still a significant amount of work needed before recommending the use of NPs in a clinical setting.

The following aims were then set for this work, in order to gain an understanding of the optimised setup for radiosensitisation with NPs:

1. Determine the macroscale implications of introducing concentrations of NPs with proton therapy. Proton therapy is considered specifically here due to the highly localised dose deposition forming the Bragg peak (BP). It was hypothesised that by introducing clinically relevant concentrations of high-Z NPs, there would be a level of dose enhancement as well as changes to the dose deposition profile. This aim is broken down into the following technical objectives:

   • Create a suitable MC simulation capable of simulating concentrations of NPs
   • Investigate the effect of NP material, concentration and incident proton beam energy to quantify both changes in dose distribution and physical dose enhancement
   • Develop a method to validate simulated findings
   • Carry out experimental measurements to quantify the distribution of physical dose

2. Determine the nanoscale dose enhancement due to the presence of a single NP. It was hypothesised that the enhancement would differ depending on the NP material and size, as well as incident beam characteristics. This aim is broken down into the following technical objectives:

   • Create a MC simulation that is able to model nanoscale dose depositions to determine dose enhancement effect due to the presence of a NP
1.6. Aims

- Investigate impact of NP variables including NP size, material and incident beam energy with photons, on nanoscale dose enhancement
- Quantify the extent of the enhancement
- Extend the model to demonstrate the effect of a chemical coating

3. Determine the biological effect of irradiating cells loaded with NPs. It was hypothesised that differences in biological effects would be observed when comparing different NP materials and cell types. This aim is broken down into the following technical objectives:

- Determine the most suitable biological experiments to gain an understanding of the overall effect of NPs in cells combined with radiation treatment
- Investigate different NP variables including cell type, NP material and incident radiation to demonstrate the effect on cell survival and level of DNA damage

From these aims, the following hypotheses were formed for this thesis:

1. Nanoparticles will modify the shape of the Bragg peak in proton therapy, in a concentration dependent manner

2. Changes to the Bragg peak due to nanoparticles in proton therapy will be dependent on the density of the nanoparticle material

3. Smaller NPs will provide a greater dose enhancement effect for photon radiotherapy

4. Dose enhancement will decrease with increasing incident photon beam energy

5. Decrease in cell survival following combined therapy of nanoparticles and radiation treatment compared to radiation alone
6. Differences in cell survival will occur between cell lines for the same nanoparticle

7. Increase in DNA damage with the combined therapy of nanoparticles and radiation treatment compared to radiation alone

1.7 My contribution to this work

This project required an interdisciplinary approach to meet its aims, where several collaborations were formed in the process. The following departments and institutes were involved in this project:

- Division of Surgery and Interventional Science, University College London, London, UK

- Department of Medical Physics & Biomedical Engineering, University College London, London, UK

- Metrology for Medical Physics Group, National Physical Laboratory (NPL), Teddington, UK

- Proton therapy Department, Azienda Provinciale per i Servizi Sanitari (APSS), Trento, Italy

- The National Eye Proton Therapy Centre, The Clatterbridge Cancer Centre NHS Foundation Trust, Bebington, UK

- Department of Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool, UK

- Consumer Products Safety Unit, European Commission’s Joint Research Centre (JRC). Ispra, Italy

Below, each of the different research areas is listed, along with my contribution to each one:
1.7. Contribution to the work

• **Computational modelling:** As a student within the Medical Physics and Biomedical Engineering department, UCL, I was able to develop my Monte Carlo models. Here I wrote the code to simulate both my macro and nano scale models, along with MATLAB scripts to analyse the results. These were run on both a workstation based in the department and the UCL Legion High Performance Computing Facility (Legion@UCL).

• **Physical dose enhancement measurements:** The gafchromic film phantom technical design was created by a summer student within the Medical Physics and Biomedical Engineering department, UCL. The phantom was then made by our collaborator at UCLH. I was involved in the design process to ensure the design would be suitable for the planned experiment. The experiment also needed an L-shape holder to keep the phantom in place during irradiation. I designed and built the holder at the Institute of Making, UCL.

The experiment was carried out at the Proton therapy Department, APSS, Trento, Italy. I planned and prepared the experimental design for this work. I was part of a team consisting of members from UCL and the Proton therapy Department, APSS which operated the experiment by firstly ensuring the water equivalent path length of the setup used, to then carry out the gafchromic film experiment with and without NPs. I then carried out the post-processing of the results to produce the depth dose profiles.

• **Cell culture:** As a student within the Division of Surgery and Interventional Science, UCL, I was able to spend time in the lab learning cell culture techniques under the supervision of Stephanie Bogan and Carolina Ramos from the Division.

• **Photon radiobiology measurements:** I was successful in a proposal for a secondment to work at NPL considering the biological effects of NPs. As part of the secondment, I was based part-time at the NPL for a year, from September 2017, where I was trained in various radiobiological tests. As part of this work, I prepared all the samples and NPs before irradiation,
Chapter 1. Introduction

where I then treated some samples with NPs. As part of a team consisting of members from UCL and NPL, we arranged the setup for photon irradiation and irradiated all the relevant samples. I then worked on removing, counting and seeding the relevant densities for the clonogenic work. As well as this, I also fixed the DNA damage samples at two timepoints. Following on from the irradiation, I carried out the staining and counting for all samples at NPL, then used both MATLAB and SPSS to produce the results presented in this thesis.

• **Proton radiobiology measurements:** These measurements were carried out at The Clatterbridge Cancer Centre. I was part of a team consisting of members from UCL, Clatterbridge Cancer Centre and University of Liverpool. I prepared the samples and treated with NPs before irradiation. The facility at Clatterbridge had a setup in place, developed by colleagues at the University of Liverpool, which allowed for radiobiological experiments to be carried out. The team worked on preparing the setup to irradiate our biological samples. I then worked on removing cells from the dishes, while a colleague counted the cells, such that I could then seed the relevant densities for clonogenic work. I also worked to fix the DNA damage samples at two time points. I then carried out the staining and counting for all samples at NPL and then used both MATLAB and SPSS to produce the results presented in this thesis.

1.8 Novelty of this work

Research efforts are being focused to increase the understanding of NP enhanced radiation therapy. Currently a complete understanding of the mechanisms associated with NP enhanced therapy does not exist, therefore novel solutions are needed to advance the field in order to translate this work to clinical applications. Several aspects of the work presented in this thesis are novel:

• This thesis reports the first quantification of BP changes due to the addition of NPs.
• Many groups offered either simulations to determine dose enhancement, or biological experiments to show decrease in survival. The use of gafchromic films in a custom made phantom to measure the depth dose profile with protons was novel as it was the first experimental demonstration of physical dose enhancement due to NPs.

• The use of a parametric study on the nanoscale to encompass the combined effects of a number of variables (NP size, material, beam energy and chemical coating) on dose deposition was novel.

• The parametric in vitro study, comparing the effect of cell line, different commercial NPs and irradiation types was novel.

• Gene expression measurements had not been carried out with NPs combined with radiation. This thesis reports the sample preparation that was carried out, which would then be analysed for gene expression of cells treated with NPs and radiation. Within the scope of this work, only the RNA quantities are reported.

1.9 Structure of this thesis

The current chapter consisted of a brief introduction into current radiotherapy techniques and highlights how NPs can be used to improve these techniques. Details of the research questions, novelty and personal contribution to the work has also been given.

Chapter two will demonstrates simulating the macro-scale effects of NPs in proton therapy. The use of high-Z NPs at clinically relevant concentrations was hypothesized to cause an effect on the Bragg peak. These changes were quantified through simulations and validated though a physics experiment using gafchromic films at a proton therapy facility.

Chapter three demonstrates a parametric study considering different parameters known from the literature to influence the enhancement effect, such as the NP size and material. These simulations considered the nano-scale effects and demonstrated
changes in dose deposition surrounding a single NP. Simulations at this scale can help inform experimental design, because by understanding the extent of the enhancement effect, it is possible to recommend where NPs need to be localised to within a cell to observe the most beneficial effect.

Chapter four used an *in vitro* model to demonstrate the differences in radiosensitisation effect between two cell lines, two NP types and two types of incident radiation. Initially clonogenic assays were carried out for all the variables considered, demonstrating the overall effect on the level of cell survival. Another measure was the level of DNA damage, which was carried out through immunostaining for the antibody 53BP1.

Chapter five summaries the overall findings from this thesis and concludes on the impact of these on the field.
Chapter 2

Impact of heavy metal NPs on macroscale dose enhancement

This chapter introduces the macro scale model that was developed to investigate the effect of heavy metal nanoparticles (NPs) on dose enhancement in proton therapy. The work described here allowed for quantification of changes to the Bragg peak through simulations along with an experiment to validate the model.

The work in this chapter resulted in the following outputs:


• Ahmad R, Royle G, Ricketts K. Novel method to quantify physical dose enhancement due to gold nanoparticles in proton therapy, PTCOG 55, Prague, Czech Republic, May 2016, poster presented.

• Ahmad R, Royle G, Ricketts K. Implications of gold nanoparticles used for dose enhancement in proton radiotherapy, ESTRO 35, Turin, Italy, April 2016, poster presented.

• Ahmad R, Royle G, Ricketts K. Effects of heavy metal nanofilms in proton therapy, PTCOG 54, San Diego, CA, USA, May 2015, poster presented.
Chapter 2. Impact of heavy metal NPs on macroscale dose enhancement

2.1 Rationale for use of Monte Carlo models

Monte Carlo (MC) methods allow for simulations to be formed, whereby the transportation of radiation through matter is modelled. The main benefit of utilising MC simulations for particle transport is that they take into account the probabilistic nature of particle interactions across a wide range of scientific applications. The use of MC simulations is widely accepted within the field of radiotherapy, where during treatment planning medical physicists have the option to run MC simulations to compute the dose distribution of a treatment plan.

For the purposes of this work, it was necessary to use MC simulations as it allowed for many relevant quantities to be modelled, ranging from secondary particle production to tracking and energy depositions. Although it is possible to conduct similar simulations with an analytical model, these would produce different results as these models only consider energy depositions from primary photons and photoelectrons [95, 96]. Another aspect is that analytical models assume a linear trajectory and do not take into account particle interactions, but consider the probability of an interaction based on the number of incident photons. MC models however would consider the particle trajectory, and changes in direction due to an interaction and the likelihood of creating a secondary particle, which would then also be tracked. An example of this was demonstrated by Paro et al, where they carried out both MC and analytical simulations to investigate dose enhancement in the presence of NPs [97]. For the analytical model they used various equations to determine the total energy deposited in a cell due to photoelectrons, which was then translated into a DEF. With the MC model they simulated a cell irradiated by a photon beam with a Gaussian distribution, where they tracked all particles and determined any depositions of energy. They concluded that MC simulations offered a more comprehensive approach to calculating DEFs.

Analytical models are therefore useful for a proof of principle study, as the run time for these simulations is considerably less than MC simulations. Here however, the effects of utilising NPs in proton therapy are investigated, where modelling particle transport is crucial to determine the overall effects.
2.2 Rationale for modelling NP concentrations in proton beam therapy

From the literature, reviewed in section 1.5.1, it was evident that there was potential for proton dose enhancement from the introduction of NPs into tissue; however, the optimum NP parameters needed to be determined for proton therapy. To summarise, photon studies demonstrated that the level of enhancement is influenced by the size, concentration and material of the NPs, as well as the beam energy. It is necessary to consider the work from photon studies, as in comparison, fewer studies have been carried out with protons.

In terms of cellular uptake of NPs, it was reported by Chithrani et al [65], that NP cellular uptake through endocytosis is NP size dependent with an optimum size of 50 nm.

As explained in sections 1.5.1.2, groups successfully demonstrated the beneficial effects of NPs in proton therapy on the nano-scale [50]. However, these studies modelled a single NP and therefore do not demonstrate the macro scale effects or bulk effect of clinically relevant concentrations of NPs on the BP position and shape. With protons it is particularly important to model the depth dose profile due to the sharp fall off. If this is not sufficiently characterised then it could lead to under-dosing the tumour site and no cure, or overdosing surrounding healthy tissue with associated side effects.

Here, a nano-film model able to simulate NP concentrations in a non computationally expensive manner is presented, where it was possible to alter the material, concentration and beam energy.

2.3 Validation of macro scale model

Although simulations are extensively used within the literature, they have limitations in geometry design due to the computational demand of MC simulations. It was therefore necessary to design an experiment to validate the results and determine, firstly, if a physical dose enhancement could be measured, and secondly if any changes to the BP could be observed experimentally.
In terms of radiation therapy, there are several ways to determine the dose, such as using ionisation chambers, thermoluminescent dosimeters (TLDs) or Gafchromic film measurements [98, 99, 100, 101]. For the purposes of this work, Gafchromic films were selected to carry out the dose measurements. Some have reported an underestimation of dose with films within the BP region [102]. This was not an issue for the work, as a relative measurement was needed to compare with and without NPs, so the overall enhancement effects along with changes to the BP would still be observed.

2.4 Materials and methods

2.4.1 Simulations

Geant4 is an open source, Monte Carlo simulation toolkit that can be used to simulate the passage of particles through matter [103]. Although it was originally created for particle physics applications, it has been expanded over the years to be applicable to medical applications. With this work, Geant4 version 10.0p02 was used to investigate the effects of concentrations of high-Z nano-materials using incident protons. The simulations investigated three clinically relevant materials, (Gold (Au), Silver (Ag) and Platinum (Pt)), all of which have been used for in vivo and in vitro experiments ([104, 105, 106]). Three clinically relevant concentrations were considered (0.01 mg/ml, 0.1 mg/ml and 6.5 mg/ml), where the highest concentration was shown in the in vivo study by Hainfeld et al, which showed this level of uptake in the periphery of a tumour [38]. Smaller concentrations were found in the in vitro studies where Nativo et al showed an uptake of 1.05 mg/ml with HeLa cells and 16 nm AuNPs [107]. Chithrani et al also used HeLa cells, but smaller 14 nm AuNPs and found an uptake of 0.06 mg/ml [65].

Two clinical proton energies were considered, one at 60 MeV, commonly used for ocular tumours [108], the other at 226 MeV, used predominately for deep-seated tumours. The beam sizes had diameters of 7 mm and 2.7 mm for energies of 60 and 226 MeV respectively, representative of clinical beam sizes. A mono-energetic proton beam was used with \(8 \times 10^6\) incident protons, to achieve Poisson statistical
2.4. Materials and methods

errors less than 2\%. The physics list used for these simulations was the reference physics list ‘QGSP\_BIC’, where the production cuts were set to 1 nm. Simulations were run on a workstation within the Medical Physics and Biomedical Engineering department, UCL, where for each simulation the approximate CPU time was 100 hours.

2.4.1.1 Nano-films

Using a similar idea to that of Li et al \[109\] where they explored the use of thin foils, a geometry that used nano-films was created. Nano-films were used to model different concentrations of NPs and limit the computational time for a 1D nano-distributions.

2.4.1.2 Geometry setup

In order to model the effects of NP concentrations in proton therapy it was decided that, with the nano-film model, the films would be contained within the width of the Bragg peak, simulating NPs being localised to the tumour region. As was explained in section 1.2.3.2, the BP is used to give a more localised dose to the tumour, therefore treatments will localise the BP to cover the tumour region. Within proton therapy the Bragg peak width (BPW) of a pristine Bragg peak is defined as the distance between the 80\% proximal and 80\% distal dose (figure 2.1 A) \[32\]. As the dose deposition profile differs depending on the incident beam energy, it was necessary to initially determine the width for each of the respective energies considered. This was carried out through preliminary simulations, comprised of a basic water phantom, without nano-films, for each energy considered, where dose readings were recorded to determine the lateral dose profile and the BPW.

2.4.1.2.1 Concentration calculation

Each film had a width and length equal to those of the simulated water phantom, and thickness in dimensions of nanometers. Using this setup, it was possible to simulate a NP concentration by modelling the average inter-nanoparticle separation for the concentration being considered, where this separation would represent the thickness of water contained between subsequent films. The
Chapter 2. Impact of heavy metal NPs on macroscale dose enhancement

Figure 2.1: (A) Nano-film simulation model, with dimensions of 4 cm x 4 cm x 4 cm for 60 MeV and 35 cm x 35 cm x 35 cm for 226 MeV. Nano-films were contained within the 80% to 80% width of the BP within a water phantom. Nano-films had length and width equal to that of the water volume and thickness 50 nm. (B) Enhancement range shows the distance over which the nano-films enhance the dose (DEF > 1).

separation was given by equation 2.1,

\[ r = \frac{1}{n^{\frac{1}{3}}} \]  

where \( r \) is the inter-particle separation and \( n \) is the particle density \( N/V \). This has been summarised for all the materials, concentrations and energies considered in table 2.1. It should be noted, that as the nano-films were contained within the BPW (80% - 80%), the number of films would differ between beam energies. The BPW for 60 MeV was 0.078 cm and 0.78 cm for 226 MeV. Finally, table 2.1 also summarises the differences due to the approximating the particle distance, demonstrated by the percentage difference in number of nano-films. The reasons for the approximation are explained in section 2.4.1.5.

Calculating nano-film numbers in such a manner allowed us to also account for differences in density, where the denser materials would have fewer nano-films for the same concentration. The average inter-particle distance was approximated, such
that the value was divisible into nano-film layers 50 nm thick. This was the selected thickness of the nano-film corresponding to the optimum NP diameter quoted for cellular uptake by Chithrani et al [65]. In this study they investigated colloidal AuNP sizes ranging between 14 and 100 nm, where the surface was not modified and the NPs were stabilised by citric acid ligand. The AuNPs were incubated in HeLa cells for 6 hours, where they reported the highest level of uptake with 50 nm AuNPs.
Table 2.1: Number of nano-films needed to model each material, concentration and energy combination. The percentage difference has been quoted to demonstrate the differences between number of films simulated to number of films needed to model each concentration.

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
<th>60 MeV</th>
<th>226 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mg/ml</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Ag</td>
<td>3 mg/ml</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Au</td>
<td>2 mg/ml</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Pt</td>
<td>1 mg/ml</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/ml</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Ag</td>
<td>0.1 mg/ml</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Au</td>
<td>0.05 mg/ml</td>
<td>2.35</td>
<td>2.35</td>
</tr>
<tr>
<td>Pt</td>
<td>0.01 mg/ml</td>
<td>2.45</td>
<td>2.45</td>
</tr>
</tbody>
</table>

*Note: 42*
2.4.1.3 Simulation validation

As many different MC codes could have been used, it was necessary to determine that Geant4 results compared well with other codes available. To determine this, a simple water phantom was simulated in each respective code modelling a 62 MeV proton beam, where the dose was recorded. From this, a depth dose profile was acquired which showed comparable results between the codes. Simulations were carried out on Geant4, TOPAS and FLUKA. The results from TOPAS match those of Geant4 as expected, as this code acts as an overlay on Geant4, therefore the physics list used would be the same. With the TOPAS simulations however, the dose recording was carried out within the code rather than using a scoring mesh, therefore this was used to determine if there were any differences in the two ways of recording dose. With FLUKA, some differences were shown in the dose deposition (figure 2.2), due to differences in the physics list used by FLUKA.

![Depth dose plot demonstrating the validation carried out with a basic water phantom comparing the Monte Carlo codes Geant4, TOPAS and FLUKA](image)

**Figure 2.2:** Depth dose plot demonstrating the validation carried out with a basic water phantom comparing the Monte Carlo codes Geant4, TOPAS and FLUKA

2.4.1.4 Dose distribution analysis

The main assumption of the nano-film model was that the nanoscale was simulated in the longitudinal beam direction only. For this assumption to be valid a correction was applied to exclude secondary particles which travelled laterally. This was
carried out by recording the coordinates of the secondary particles, to firstly determine those that travelled within the nano-films a distance greater than 50 nm and secondly to find those that deposited energy within the nano-film. Energy deposition was recorded using a scoring mesh across the entire phantom. It was then possible to calculate the dose to water, allowing the depth dose to be plotted, to investigate BP changes. In order to quote energy deposition to water, energy deposited within the nano-films was excluded. This was similarly carried out for the secondary electrons recorded within the nano-films.

By using the nano-film model it was possible to investigate many different aspects of dose enhancement and energy distribution: (i) BPW was calculated, related to changes in the BP shape. Due to changes in shape, measurements were carried out at two points. The first was between the proximal 80 % and distal 80 % defined as the width of the BP, the other between the proximal 80 % and distal 10 % demonstrating changes that occur towards the end of range. (ii) Longitudinal shift, related to potential changes in tumour coverage, defined at the end of range, using the depth where the dose had fallen to 0 Gy for each material, relative to water. For the 226 MeV simulations a dose value was obtained every 0.1 mm within this region, whereas for 60 MeV this was at every 0.01 mm. (iii) DEF, related to physical dose and defined as the ratio of the dose to water with nano-films compared to the dose to water alone. DEF was quoted within specific regions; (a) comparing peak dose values of water with nano-films to water alone, termed DEF of peak dose, (b) considering the dose depositions within the volume, up to the peak dose for water with nano-films, to water alone, termed DEF for volume to peak dose. These measures allowed for any changes in the shape of the BP to be taken into account. (iv) Enhancement range, related to BP shape changes and, defined as the distance spanning the beginning of the nano-film region, where the DEF is 1, and ending at the last point where the DEF is 1, (figure 2.1(B)). This allowed for longitudinal shifts or narrowing of the BP to be quantified. (v) The number of secondary electrons produced, to investigate the cause of the predicted enhancement.
2.4. Materials and methods

2.4.1.5 Limitations

To a first approximation, this model allows the determination of how the use of NP concentrations can have an effect on proton therapy. However, the use of nano-films to simulate NPs within the model fundamentally overestimated the level of change to the BP. In practice, NPs would be dispersed in all directions, and the incident proton would not necessarily encounter neighbouring NPs at the frequency it did by passing through the consecutive nano-films. Another limitation of the model was that it only demonstrated changes in the longitudinal direction, whereas in practice a more accurate model would consider changes in all directions. Finally, within the model itself, the number of nano-films was approximated, so the total depth of spacing was divisible by 50 nm, the thickness chosen to model a NP with the same diameter. This was necessary so that recordings in the films could be excluded.

2.4.2 Experimental validation

In terms of the experimental design, it was initially necessary to decide what proton energy would be used for the experiment, as this would determine the phantom size. Changes to the BP were predicted to be small, where a high clinical beam energy was needed to make the effect measurable. Therefore, it was decided that the validation would be carried out at the Trento Proton Therapy Center, Italy, which had a maximum beam energy of 226 MeV. The phantom needed to be designed to be placed at the BP, covering measurements of the BPW, whilst containing water and NPs and providing a way for the dose to be measured.

For the validation, concentrations of 5.5 and 1.1 mg/ml of spherical AuNPs with a diameter of 50.7 ± 7.1 nm (nanoComposix, San Diego), suspended in de-ionised water were used to assess the effect of concentration on dose and confirm the patterns of change in proton dose distribution estimated by the simulation model. A concentration of 6.5 mg/ml, equal to the highest simulated concentration, was not commercially available. This concentration was simulated as it was representative of \textit{in vivo} AuNP uptake [38]. The concentrations of 5.5 and 1.1 mg/ml were sufficient, as the experiment was used to verify the macro scale changes, rather than as a direct quantitative comparison. In order to carry out a direct comparison,
additional simulations were carried out, for Au, to demonstrate the simulated effects with concentrations of 5.5 and 1.1 mg/ml.

2.4.2.1 Phantom design

A phantom was designed such that it could hold Gafchromic film pieces at different depths through the phantom along the beam direction (figure 2.3). The phantom was developed to provide dose measurements at sufficient spatial resolution, for a range of NP concentrations. The phantom was made from polymethyl methacrylate (PMMA), as this material is a suitable water-equivalent plastic for high energy proton beams [110].

As it had been decided that measurements would be carried out at the Trento Proton Therapy Center, Italy, where protons had an average range of approximately 32 cm and width of approximately 8 mm, the phantom needed dimensions to capture measurements within the width. Therefore the phantom had external dimensions of $6.1 \times 5 \times 4 \text{ cm}^3$ and internal dimensions of $4.1 \times 3 \times 3 \text{ cm}^3$, where solid water slabs would be used for the build-up region before the phantom.

Dose measurements were obtained at 1 mm intervals, where each slit, for film placement, was 0.5 mm thick. The technical drawing (figure 2.3, left) for the phantom was carried out by Pierre Béguerie as part of a summer placement at UCL, where the phantom was then made by Denzil Booth at UCLH (figure 2.3, right).
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2.4.2.1.1 Phantom holder

After ensuring the film phantom was placed in the correct position along the BP, it was necessary to have a holder to keep the phantom in place and ensure reproducible positioning. An L–shaped holder was designed (figure 2.4, left) and created using an Ultimaker 2 3D printer at the Institute of Making, UCL. A lid was also created at the Institute of Making, formed of PMMA, to ensure there was no spillage of the NP solution (figure 2.4, right).

Figure 2.3: CAD drawing of custom made film phantom (left). Phantom is able to hold Gafchromic films and take measurements 1 mm apart and was made from PMMA (right).

Figure 2.4: Design of the L-shape holder that was printed (left). Image of the L-shape and phantom being used during the experiment (right).
2.4.2.2 Experimental setup

As shown in figure 2.5 the setup consisted of several blocks of solid water (Gammex, Middleton, WI) and an entrance window comprised of PMMA, with a total water equivalent thickness (WET) of 29.1 cm. This created a sufficient build-up of dose, to allow the BPW to be contained within the phantom. The main compartment of the film phantom had a lateral depth of 4.1 cm, filled with a solution of either distilled water or de-ionised water and AuNPs of known concentration. During irradiation, the phantom was aligned with the beam so that the width and depth of the BP were in the phantom. This was achieved using a large diameter multi layer ionization chamber, Giraffe (IBA Dosimetry), which allowed confirmation of the WET of the setup.

![Figure 2.5](image)

**Figure 2.5:** Setup used for the experiment in Trento comprised of solid water slabs, used to provide sufficient build-up before the film phantom. The water equivalent thickness (WET) of the slabs was 29.1 cm allowing the width of the BP to fall within the film phantom.

EBT3 Gafchromic films (Ashland, USA) were used for the experiments, which had a dynamic range of 0.2 to 10 Gy. The films were marked for orientation and
2.4. Materials and methods

labelled for scanning post-irradiation, because the polymers of the active layer have an orientation dependent response, therefore this needed to be kept constant [111]. A beam energy of 226 MeV was used where 20 MU was delivered, corresponding to a dose range of 6 – 37 cGy. Gafchromic films were calibrated covering a range from 0 – 250 cGy, using a clinical procedure [102]. This involved the use of ion chamber measurements to determine the dose delivered to the film, where a calibration curve was produced to convert values of Gafchromic film optical density to the known dose. Reference water measurements were carried out initially using Gafchromic films at depths of 0 and 17 cm outside the film phantom and nine measurements in the phantom between 30.3 and 33.2 cm. This corresponded to a net peak water dose of 37 cGy, such that a depth dose plot could be obtained. Figure 2.6 demonstrates the approximate positions of the points considered for the nine measurements in the width of the BP. The film phantom was then filled with the two NP concentrations and irradiated. When Gafchromic films were used in the film phantom they were only exposed to the fluid for a few minutes. It has been shown in the literature that prolonged exposure can lead to fluid ingress, where León-Marroquín et al demonstrated that after an hour of EBT3 films being exposed to water, the liquid penetrated approximately 1 mm into the edge of the film [112]. For the purposes of this work this was not an issues as the films were exposed for a short period of time.

2.4.2.2.1 Read out

After irradiation each film was contained in a light-tight envelope and kept at room temperature. Films were read out after 24 h to allow for the polymerisation of the active layer in the film [113]. An EPSON perfection V750 pro scanner (transmission mode with 72 dpi scanning resolution) was used for the read out, where the pixel values from the red channel and optical density was determined for each measured point. With the depth dose plot, it was possible to determine the DEF for each concentration. The plot was also used to calculate the expected shift of the distal edge, where the end of range was used to compare water containing AuNPs with water alone as explained in section 2.4.1.4.
Chapter 2. Impact of heavy metal NPs on macroscale dose enhancement

Figure 2.6: Positions of the measurements taken for the experiment where the film phantom was used along the BPW at 30.95, 31.15, 31.45, 31.6, 31.75, 31.9, 32.35, 32.8, 34.9 cm respectively. The measurements for 0 and 20 cm were taken with the films on the solid water slabs.

2.5 Results

2.5.1 Changes to the Bragg peak due to high-Z nano-materials

2.5.1.1 Simulation results

Nano-films demonstrated the effects on energy distribution in the longitudinal beam direction, where it was shown that high concentrations caused changes to Bragg peak shape through both a shift in the distal edge and a narrowing of the overall BP width. Depth dose plots were used to show the effect of nano-films on energy deposition.

2.5.1.1.1 Percentage depth dose plot changes

High concentrations resulted in significant changes to the shape of the BP, where changes decreased with decreasing concentration (figure 2.7). Changes were also material dependent, where Au and Pt caused higher changes than Ag. These changes were demonstrated for both 60 and 226 MeV.
2.5. Results

Figure 2.7: Depth dose plots shown of simulated nano-film results demonstrating the enhancement and changes high-Z materials cause to the shape of the Bragg peak. Nano-film regions begin at depths of 31.31 and 3.02 for 226 and 60 MeV respectively. All materials normalised to peak water dose for each concentration.

2.5.1.1.2 Longitudinal shift

The longitudinal shift, defined in section 2.4.1.4, for both energies are presented in table 2.2. The highest shift was found to be 5.3 mm with Pt at 226 MeV with a concentration of 6.5 mg/ml. The shift decreased with decreasing concentration and decreasing material density for both energies. Differences in the magnitude of the shift were demonstrated between the two energies used due to the differences in proton range between them.
### 2.5.1.1.3 Bragg peak narrowing

The BPW had been narrowed due to the nano-films. As narrowing causes changes to the shape of the BP, measurements were carried out at two different points. The level of narrowing increased with material density and concentration, where the results have been summarised in Table 2.3. The greatest level of narrowing can be seen for the 80% - 10% BP dose line at 226 MeV, where Pt with a concentration of 6.5 mg/ml shows a narrowing of approximately 40% compared to reference simulations with water alone. Similarly, for the 80% - 80% BP dose line at 60 MeV, Pt also showed the greatest change with 6.5 mg/ml, where a narrowing of approximately 39% was observed.

<table>
<thead>
<tr>
<th>Material</th>
<th>6.5 mg/ml</th>
<th>0.1 mg/ml</th>
<th>0.01 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 MeV</td>
<td>226 MeV</td>
<td>60 MeV</td>
</tr>
<tr>
<td>Ag</td>
<td>-0.34 ± 0.01</td>
<td>-3.7 ± 0.1</td>
<td>-0.09 ± 0.01</td>
</tr>
<tr>
<td>Au</td>
<td>-0.40 ± 0.01</td>
<td>-4.7 ± 0.1</td>
<td>-0.11 ± 0.01</td>
</tr>
<tr>
<td>Pt</td>
<td>-0.43 ± 0.01</td>
<td>-5.3 ± 0.1</td>
<td>-0.12 ± 0.01</td>
</tr>
</tbody>
</table>
Table 2.3: Narrowing of the Bragg peak width as a percentage for all materials, concentrations and energies being considered. Note: Quoted errors correspond to the error propagation associated with the measured BP widths between the material and water values.

<table>
<thead>
<tr>
<th>Material</th>
<th>60 MeV</th>
<th>226 MeV</th>
<th>60 MeV</th>
<th>226 MeV</th>
<th>60 MeV</th>
<th>226 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>$-31.72 \pm 0.78$</td>
<td>$-24.26 \pm 0.53$</td>
<td>$-12.64 \pm 0.25$</td>
<td>$-13.24 \pm 0.29$</td>
<td>$-6.73 \pm 0.13$</td>
<td>$-10.29 \pm 0.22$</td>
</tr>
<tr>
<td>Au</td>
<td>$-36.97 \pm 0.91$</td>
<td>$-27.94 \pm 0.62$</td>
<td>$-14.12 \pm 0.28$</td>
<td>$-25.73 \pm 0.57$</td>
<td>$-8.98 \pm 0.17$</td>
<td>$-14.71 \pm 0.32$</td>
</tr>
<tr>
<td>Pt</td>
<td>$-39.38 \pm 0.96$</td>
<td>$-35.29 \pm 0.78$</td>
<td>$-15.27 \pm 0.30$</td>
<td>$-19.12 \pm 0.42$</td>
<td>$-8.17 \pm 0.15$</td>
<td>$-17.65 \pm 0.39$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>60 MeV</th>
<th>226 MeV</th>
<th>60 MeV</th>
<th>226 MeV</th>
<th>60 MeV</th>
<th>226 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>$-26.46 \pm 0.36$</td>
<td>$-27.91 \pm 0.33$</td>
<td>$-7.99 \pm 0.09$</td>
<td>$-14.73 \pm 0.17$</td>
<td>$-3.50 \pm 0.04$</td>
<td>$-13.18 \pm 0.15$</td>
</tr>
<tr>
<td>Au</td>
<td>$-32.12 \pm 0.43$</td>
<td>$-33.33 \pm 0.49$</td>
<td>$-9.62 \pm 0.10$</td>
<td>$-14.34 \pm 0.17$</td>
<td>$-5.16 \pm 0.05$</td>
<td>$-11.63 \pm 0.14$</td>
</tr>
<tr>
<td>Pt</td>
<td>$-35.66 \pm 0.48$</td>
<td>$-40.31 \pm 0.53$</td>
<td>$-10.56 \pm 0.12$</td>
<td>$-12.40 \pm 0.15$</td>
<td>$-5.41 \pm 0.06$</td>
<td>$-10.85 \pm 0.13$</td>
</tr>
</tbody>
</table>
2.5.1.2 Experimental results

Nanoparticles were shown to cause measurable changes to the BP.

2.5.1.2.1 Validation of AuNPs narrowing effects

Figure 2.8 demonstrates the experimental results of the phantom study, where a 14% narrowing was measured (80-10%), along with a 2.2 mm shift at a concentration of 5.5 mg/ml. This effect was found to be concentration dependent, as no narrowing was observed with 1.1 mg/ml. These two concentrations were also simulated, where the results in figure 2.8 show a BP shape change with a shift of 4.5 mm and narrowing of approximately 32% (80-10%) for 5.5 mg/ml.
2.5. Results

(a) Dose enhancement Gafchromic film dose measurement in water and a watergold NP solution at concentrations of 5.5 and 1.1 mg Au/ml.

(b) Depth dose plot showing the changes to the shape of the Bragg peak by normalising each to the respective peak dose.

**Figure 2.8:** Comparison of experimental findings to those simulated with a concentration of 1.1 and 5.5 mg/ml for Au with a beam energy of 226 MeV.
2.5.2 Simulated enhancement due to high-Z nano-materials

As well as quantifying the changes in energy distribution, the model also quantified the level of dose enhancement and range of the enhancement.

2.5.2.1 Dose enhancement

Table 2.4 summarises the simulated dose enhancement factor (DEF) findings, where as expected there was an increase in enhancement with increasing nano-film concentration and material density. As Pt has the highest density, it showed DEFs of 1.069 (6.9 %) and 1.060 (6 %) at 6.5 mg/ml for 60 and 226 MeV respectively. The difference in DEF between Pt and the other materials for this concentration was less than 2 %, where considering the errors was deemed to be statistically significant.

In terms of the effect of incident beam energy, it was shown that higher enhancements were found with an energy of 60 MeV. The enhancement was also shown to not increase linearly with concentration, which was expected, as higher concentrations increase the likelihood of energy depositions within the nano-films.
Table 2.4: DEF for peak doses and volume to the peak dose, comparing all materials for all concentrations at energies of 60 and 226 MeV.

<table>
<thead>
<tr>
<th>Material</th>
<th>60 MeV</th>
<th>226 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>1.038 ± 0.001</td>
<td>1.027 ± 0.002</td>
</tr>
<tr>
<td>Au</td>
<td>1.062 ± 0.001</td>
<td>1.053 ± 0.002</td>
</tr>
<tr>
<td>Pt</td>
<td>1.066 ± 0.001</td>
<td>1.042 ± 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>60 MeV</th>
<th>226 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>1.018 ± 0.001</td>
<td>1.012 ± 0.002</td>
</tr>
<tr>
<td>Au</td>
<td>1.020 ± 0.001</td>
<td>1.020 ± 0.002</td>
</tr>
<tr>
<td>Pt</td>
<td>1.021 ± 0.001</td>
<td>1.016 ± 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>60 MeV</th>
<th>226 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>1.009 ± 0.001</td>
<td>1.012 ± 0.002</td>
</tr>
<tr>
<td>Au</td>
<td>1.014 ± 0.001</td>
<td>1.017 ± 0.002</td>
</tr>
<tr>
<td>Pt</td>
<td>1.010 ± 0.001</td>
<td>1.016 ± 0.001</td>
</tr>
</tbody>
</table>

2.5.2.1.1 Validation of AuNPs enhancement effects

Table 2.5 summarises the additional simulations carried out to match the concentrations used experimentally. The highest enhancement was found experimentally as 1.26 with a concentration of 1.1 mg/ml, whereas simulations showed the highest enhancement with 5.5 mg/ml where the DEF was 1.04.
Simulations underestimated the DEF for both concentrations, with differences of 19 % and 14 % for 1.1 and 5.5 mg/ml respectively.

### Table 2.5: DEF for peak doses, comparing experimental to simulated findings for concentrations of 1.1 and 5.5 mg/ml at an energy of 226 MeV, with the percentage difference between the two.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Experimental</th>
<th>Simulated</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>1.26</td>
<td>1.02</td>
<td>-19 %</td>
</tr>
<tr>
<td>5.5</td>
<td>1.21</td>
<td>1.04</td>
<td>-14 %</td>
</tr>
</tbody>
</table>

#### 2.5.2.2 Enhancement range

Table 2.6 demonstrates the enhancement range for the DEF from simulations defined as the distance spanning the beginning of the nano-film region, where the DEF is 1, and ending at the last point where the DEF is 1, (figure 2.1(B)). It was found that at 226 MeV, both Au and Pt offered the highest enhancement range with 5.6 mm at 0.01 mg/ml, compared to 6.5 mg/ml where Au and Pt were 4.1 mm and 4.0 mm respectively. At 60 MeV, Ag had the highest enhancement range with 0.58 mm at a concentration of 0.01 mg/ml, compared to 0.46 mm at 6.5 mg/ml.

The main benefit of this measure was to show that although higher concentrations offer a higher enhancement in terms of peak dose value, the range in which they enhance the dose is reduced due to prematurely attenuating the beam. High-Z materials scatter protons through larger angles, where an increase in the level of scatter is observed with decreasing concentration, due to the larger separation between subsequent nano-films at low concentrations. This is because the concentration increases the range over which enhancement occurs is reduced. An example of this is with Au at a beam energy of 226 MeV, where the enhancement range is 5.6 for 0.01 mg/ml compared to 4.1 for 6.5 mg/ml. This is due to the fact that the incident beam has been attenuated by a greater amount of material with the higher concentrations and materials of a higher density.
2.5. Results

Table 2.6: Enhancement range for all materials and for all materials, concentrations and energies being considered

<table>
<thead>
<tr>
<th>Material</th>
<th>0.01 mg/ml 60 MeV</th>
<th>0.01 mg/ml 226 MeV</th>
<th>0.1 mg/ml 60 MeV</th>
<th>0.1 mg/ml 226 MeV</th>
<th>6.5 mg/ml 60 MeV</th>
<th>6.5 mg/ml 226 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>0.58 ± 0.01 5.2 ± 0.1</td>
<td>0.50 ± 0.01 5.6 ± 0.1</td>
<td>0.46 ± 0.01 4.1 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Au</td>
<td>0.57 ± 0.01 5.6 ± 0.1</td>
<td>0.51 ± 0.01 5.1 ± 0.1</td>
<td>0.45 ± 0.01 4.1 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt</td>
<td>0.52 ± 0.01 5.6 ± 0.1</td>
<td>0.54 ± 0.01 5.1 ± 0.1</td>
<td>0.44 ± 0.01 4.0 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5.2.3 Secondary electrons

It is commonly understood within the literature that the main factor causing NP-induced physical dose enhancement is the resulting increase in number of secondary electrons [114, 52]. Our model corroborates this as shown in figure 2.9, where an increase in the number of secondary electrons could be observed with an increase in nano-film concentration for both energies. The differences in electron yields are due to the different beam energies, where higher energies propagate through more material, therefore increasing the number of secondary electrons.

![Graph showing number of secondary electrons vs concentration for different materials at 60 MeV](image)
Figure 2.9: Plot showing the increase in number of secondary electrons with increasing concentration for all materials for both beam energies

2.6 Discussion

The introduction of high-Z nano materials has been shown to cause a number of changes to the Bragg peak when introduced in proton therapy. The first of these is an increase in the level of dose deposition, demonstrated by the DEF. The greatest enhancement was produced by Pt, at the highest tested nano-film concentration of 6.5 mg/ml with a simulated DEF of $1.069 \pm 5 \times 10^{-4}$ and $1.060 \pm 1.1 \times 10^{-3}$ at 60 and 226 MeV respectively. As expected the level of enhancement increased with concentration, where three orders of magnitude of concentrations were presented. The percentage increase in DEF from the lowest to highest concentration for Pt was approximately 6 % for 60 MeV and 5 % for 226 MeV. The level of enhancement was shown to not increase linearly with concentration. This was expected, as at higher concentrations, dose depositions are more likely to occur within the nano-films due to the reduced spacing between subsequent films. From table 2.1, it can be seen that the spacing between films for Pt at an energy of 226 MeV with a concentration of 6.5 mg/ml is 0.6 µm, compared to 5.15 µm for 0.01 mg/ml.

From the phantom experiment, a DEF of approximately 1.26 was observed with a concentration of 1.1 mg Au/ml and 1.21 with 5.5 mg Au/ml. This was in
disagreement with simulations which showed an increased DEF with concentration; however, it is thought that Gafchromic films were prone to quenching effects which may lead to lower observed DEF at higher concentrations. It has been shown in the literature that quenching occurs with Gafchromic films in regions of higher linear energy transfer (LET). This is due to a higher density of events occurring, whereby a fraction are not recorded and therefore there is a loss of signal, which underestimates the dose in the Bragg peak [100]. The DEF observed experimentally however was greater than simulation; experiment showed a DEF of 1.21 for AuNPs at 5.5 mg/ml, compared to the simulated DEF of 1.039 for the same concentration. The reason for this is thought to be due to the setup of the simulations, where the nano-films are equally spaced within a specified region (BPW), whereas an incident proton would not necessarily encounter neighbouring NPs at this frequency. In our simulations protons are forced to interact with consecutive nano-films, whereby the enhancement would have been underestimated, as in practice a concentration of NPs would be dispersed in all directions, whereas here they are localised to the longitudinal direction. Also, the electrons that travelled laterally were excluded in order for the main assumption of the model where the nano-scale was simulated only in the longitudinal beam direction.

The range of enhancement however decreased with increasing concentration, where at 60 MeV the range for Pt reduced by 15 % from 6.5 mg/ml to 0.01 mg/ml and by 29 % for 226 MeV. Due to the changes demonstrated with the shape of the BP, this was expected as lower concentrations caused less significant changes, whereby they could enhance the dose across a longer range. However, higher concentrations would prematurely attenuate the beam and therefore lower the range of enhancement.

Importantly, both simulation and measurement found a narrowing of the BP width for NPs and water compared to water alone. This is of clinical importance as changes to the dose deposition must be accounted for to ensure treatment plans remain accurate. Simulations showed a narrowing of 23 % from the 80 % − 80 % BP dose line and 32 % from the 80 % − 10 % BP dose line at 226 MeV with Au at
a concentration of 5.5 mg/ml. Experimental findings demonstrated a narrowing of approximately 7% (80% – 80%) and 14% (80% – 10%) at an AuNP concentration of 5.5 mg/ml and no observable narrowing at 1.1 mg/ml. It is clinically known that high-Z materials cause a level of broadening in the beam, when introduced before the patient. Here, however, the high-Z materials were located within the BPW, towards the end of range, causing a reduction in the level of range straggling and narrowing of the BPW. This resembles the clinical use of NPs in proton beam therapy, where NPs would be expected to fall within the BPW, being targeted and localised within the tumour. As incident protons have a finite amount of energy, their interactions with high-Z materials cause a reduction in the range straggling normally observed with water, where protons reached their end of range closer together due to the barrier caused by the high-Z material. The BP changes were energy dependent; for high energy simulations the effects were more significant due to increased proton range at high energies, where those with longer ranges have more measurable effects. It can be seen that this effect still occurred at the lower energy of 60 MeV. Differences between simulated and measured results were expected due to different set-ups, where simulations did not take beam divergence into account. Another reason for the expected differences was that experimentally the interactions that occur on the nanoscale differ greatly to what can be predicted on the macro scale. Further advancements in Monte Carlo physics lists are needed, for a variety of materials at the nanoscale, in order to improve the accuracy. Importantly, the use of nano-films to simulate NPs within the model fundamentally overestimated the level of change to the BP; in practice NPs would be dispersed in all directions, and the incident proton would not necessarily encounter neighbouring NPs at the frequency of the consecutive nano-films. Both BP width narrowing and distal edge shift were overestimated in the simulation, where narrowing was 39% and shift was 51% lower experimentally.

Both simulations and experiment demonstrated a shift in the distal edge. It was found from the simulations that although Pt did not have the highest Z of the materials tested, it had the highest physical density, causing the highest level
of change; a 5 mm shift in the distal edge compared to water alone was shown for Pt of concentration 6.5 mg/ml at 226 MeV. At the same energy, experimental findings demonstrated a 2.2 mm shift in the distal edge for Au at a concentration of 5.5 mg/ml. This shift was expected and is observed clinically, where high density materials are known to cause a range shift in proton beam therapy [115]. With high density materials there are differences in the stopping power for the incident proton. This can be demonstrated with the NIST database PSTAR which calculates the stopping power and range of protons in different materials [116]. From this it can be seen that the stopping power for 226 MeV protons incident on water is 4.159 MeV/cm and is 41.63 and 46.03 MeV/cm for Au and Pt respectively. This shows that Pt, the densest material considered in this study, loses the greatest amount of energy per unit length, which relates to the shift observed in our findings. In terms of clinical translation, this shift in dose deposition will need to be quantified, as in practice a SOBP is used to cover the tumour volume, which consists of several beamlets as explained in section 1.2.3.2. Therefore if a normal dose deposition profile is used for a single energy, rather than the shifted one due to the NPs, the tumour coverage will be insufficient.

In terms of the causes of dose enhancement, the percentage increase in number of secondary electrons with nano-films and water compared with water alone was found to be greater at 60 MeV than 226 MeV, with an increase of 41 % for Au at concentration 6.5 mg/ml at 60 MeV compared to 3 % for 226 MeV. This is in agreement with the findings of Gao and Zheng [51], which showed a higher number of secondary electrons produced at lower proton beam energies after interactions with an AuNP. This correlates with our findings for the DEF, where 60 MeV offered a higher level of enhancement than 226 MeV with a concentration of 6.5 mg/ml, therefore we expect a higher increase in secondary electrons. It should be noted that with the study from Gao and Zheng, they considered the electrons on the nanoscale, from a single AuNP, therefore a direct comparison of our findings could not be carried out, as our model considered concentrations of nanoparticles on the macroscale.
With the level of dose enhancement demonstrated experimentally, it would be beneficial to use AuNPs clinically to increase the level of dose deposition to the tumour in proton treatments. It should also be noted however, that the enhancements presented here demonstrate physical dose enhancement alone. In practice, the enhancement is attributed to different mechanisms as explained in section 1.5.5, where the literature highlights discrepancies between simulations and biological experiments due to factors not being accounted for in simulations. As few studies have been carried out with protons, we can consider these discrepancies with photon irradiation. An example of this is with simulations carried out with MV photons, where little or no physical dose enhancement was predicted, however experimentally, cellular studies show significant cell kill enhancement for this energy, demonstrating the need for more complex simulations accounting for factors other than physical enhancement alone [117, 118].

Butterworth et al summarised various in vitro studies, including a prediction of the enhancement effect from the concentration of AuNPs used, where the values for those that used 6 MV photons has been reproduced in table 2.7 [11].

**Table 2.7:** Summary of review tabulated by Butterworth et al considering in vitro studies using 6 MV photons [11].

In terms of the NP concentrations considered, they are achievable in practice as demonstrated by Hainfeld et al [38]. This study quantified in vivo AuNP uptake levels, where an uptake concentration of 6.5 mg Au/g AuNPs was shown in mice through a single intravenous injection. Just as importantly, a 90 % lower concentration was achieved in healthy surrounding tissue. Therefore, the findings presented have the potential to be observed clinically. As such, if NPs are used clinically and this localisation is achieved, treatment plans would then need to take these changes into account to ensure an accurate plan. Shifts in
the sharp distal edge of the BP have potential to under-dose or miss irradiate the tumour target [120], where here a shift of 2.2 mm was measured. This is of particular clinical significance as treatment plans have volumes that are specified to account for uncertainties in treatment planning and delivery. Therefore these changes would need to be accounted for as part of these uncertainties [121]. With the introduction of NPs, the cumulative effect on the uncertainties have not been previously evaluated.

It should also be considered that the sharpening of the BP, has the potential to make the treatment more conformal. This is one of the reasons there is interest in the use of carbon ion therapy, as this has a sharper BP with a narrower width. In a study by Mohamad et al, they demonstrate a percentage depth dose plot comparing protons and carbon ions, where there is approximately a 70% reduction in width between protons and carbon ions [6]. Although we have shown a narrowing of only approximately 40%, it can be argued that the use of NPs narrows the beam, but benefits from the absence of the fragmentation tail seen with carbon ion therapy (figure 2.109).
This redistribution of energy deposition can be related back to the LET, which was described in detail in section 1.4. By sharpening the BP, the ionisation density increases, corresponding to an increased energy deposition per unit distance travelled by the incident ionising radiation, therefore a higher LET. As previously explained, the relative biological effectiveness (RBE) varies with depth, where although a value of 1.1 is used for protons, it would be greater at the distal end of the BP. RBE increases with LET until approximately 100 keV/µm, where it then decreases (figure 1.6). By adding NPs, the dose needed to achieve the same biological effect decreases, therefore leading to an increase in RBE.

This study demonstrated experimentally a significant change, where in the case of a high AuNP concentration, a 2.2 mm shift at the end of range should be taken into account. Therefore, with protons, great care must be taken when recommending the use of NPs as the bulk effects on dose distribution must be
2.7. Conclusion

This study demonstrates a flexible working model able to simulate concentrations representative of clinically achievable NP concentrations, in a non-computationally expensive setup. Compared to other models which consider a single NP, this model was able to highlight the macro scale effects and investigate changes to the Bragg peak, whilst varying energy, and NP material and concentration. Both simulation and experiment demonstrated a change in the Bragg peak shape, where a shift of 4.5 mm was simulated for Au with 5.5 mg/ml compared to 2.2 mm measured experimentally. Differences in simulation to experiment were due to differences in the distribution of AuNPs in solution. Another consideration is that with the Gafchromic films the active layer (28 µm thick) is sandwiched between two 125 µm polyester substrates, which may absorb some low energy, short-ranged electrons. The width of the Bragg peak was shown to narrow in the simulations by approximately 23 % compared to 14 % from the experiment for 226 MeV with a concentration of 5.5 mg/ml. Both demonstrated a level of dose enhancement, where experimental findings showed a higher level of 1.21 compared to simulated 1.04 for 5.5 mg/ml. The simulations demonstrated concentration dependent, longitudinal shifts in the Bragg peak, which were more evident at higher beam energies due to the longer proton range. At a concentration of 6.5 mg/ml platinum shifted by 5.3 mm at 226 MeV, compared to 0.43 mm at 60 MeV. These changes were also material dependent, where Pt caused the highest change, followed by Au then Ag, suggesting the effects were dependent on the physical density rather than the atomic number. In terms of the enhancement, simulations showed it to be concentration, material and energy dependent. Overall these results demonstrate the need for quantifying fully understood, and changes to the BP should be accurately quantified such that treatment plans remain valid. In order to conclude on consequences of changes to the BP, a further study has been proposed, which will incorporate the use of a commercial treatment planning system along with Monte Carlo simulations in order to obtain adequate dose distribution calculations.
Chapter 2. Impact of heavy metal NPs on macroscale dose enhancement

the range of protons in a water to water-NP setup if NPs are to be used for clinical proton treatments, due to significant BP longitudinal shifts, and the potential to narrow the BP for more conformal treatments.
Chapter 3

Multiscale parametric study on dose deposition with photon irradiation

This chapter introduces the multi scale model that was developed to conduct a parametric study with a single NP and photon irradiation. The work described here allowed for a number variables to be investigated to determine the optimum setup, as multiple scales were modelled to consider the extent of NP effects.

The work in this chapter resulted in the following outputs:


- Ahmad, R., Di Giovanni, J., Sellin, R., Burke, P., Royle, G., & Ricketts, K. Preliminary investigation into the effects of a PEG nanoparticle coating on dose enhancement in radiotherapy, European Nanomedicine Meeting, London, UK, April 2017, poster presented

3.1 Rationale

In a similar manner to the macroscale simulations (chapter 2), a nanoscale model allows for several vital quantities to be simultaneously investigated to gain an in depth understanding of the physical interactions that could lead to a dose enhancement.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

A model on this scale can also help inform biological experiments, as it allows for an understanding of the physical enhancement, where the extent of enhancement for each variable combination (material, size and beam energy) can be quantified. In turn this can help inform where the NPs need to be localised to within a cell, by quantifying the range of enhancement, to achieve the greatest impact.

Here the investigation of utilising nanoparticles (NPs) in X-ray radiotherapy, by modelling different NP parameters, is used to determine their overall effects. The reason for specifically considering photon irradiation, was that it is the most commonly used form of radiation therapy, therefore in terms of optimising variables for clinical translation, this would be the ideal radiation type.

3.2 Simulating on the nanoscale

From the literature, many groups have offered varying results on the enhancement effect depending on the setup used both experimentally and through simulations. Within these studies, a number of variables were considered, such as the NP material, size, radiation type as well as the incident beam energy, where each variable demonstrated a certain impact on the overall effect [50, 122, 123].

Leung et al looked specifically at secondary electrons produced from interactions with a single AuNP irradiated by photons [124]. They considered multiple diameters and beam energies, where they showed that the majority of dose deposition was outside the NP rather than inside the NP. They quoted the mean effective range of electron tracks for the energies considered to range from 3 \( \mu \text{m} \) to 1 mm. They showed that lower energies (keV) were more effective at generating electrons and increased the number of interactions, with increasing NP size.

Cai et al, modelled cancer cells in varying forms (single cell, monolayer and cluster) and demonstrated the effects of different numbers of different sized AuNPs at keV energies. They calculated the nuclear dose enhancement factor (NDEF) and cellular dose enhancement factor (CDEF) and demonstrated that the amount of AuNPs needed to double the prescribed dose, which decreases with increasing NP size [125].
3.2. Simulating on the nanoscale

Heuskin et al conducted MC simulations looking at different sizes of NPs with different materials, with and without a coating, irradiated by low energy protons and alpha particles. They considered two geometries, one comprised of a single NP, the other of a cell geometry. They reported negligible enhancement on the macroscopic level, however for the single NP, they showed a local increase in LET (5 keV/µm with titanium NPs compared to 2.5 keV/µm with water), suggesting the need for nanoscale assessment [126].

It was therefore evident, that an important aspect to consider when simulating NPs, is the scale of the simulation. From the few studies that have been carried out simulating a single NP, it was evident that enhancement effects differ greatly depending on the scale being considered (differences between macro and nano scaled simulations).

Other factors such as the beam characteristics needed to be evaluated. With an incident radiation source of photons, there has been much debate as to the ideal beam energy to observe the greatest enhancement effect as explained in section 1.5.3.4. With photons, lower energies corresponding to the K-edge were predicted to give the highest enhancement with predictions showing minimal enhancement at higher clinical energies. It should be noted that these predictions only consider the physical enhancement which is attributed to an increase in secondary electrons, as can be expected with energies near the K-edge of the material. In practice, what was observed experimentally combines both physical and biological effects, corresponding to higher levels of enhancement for both low keV, and higher MeV energies [11].

With regards to the NP size, various groups conducted experimental studies to demonstrate the level of NP uptake within cells, where certain sizes, such as 50 nm, can take advantage of the leaky vasculature of tumour cells, leading to passive uptake within tumour cells [68]. It was also reported by Chithrani et al [65], that NP cellular uptake through endocytosis is NP size dependent, with an optimum size of 50 nm. Other groups have used a wide range of NP sizes experimentally, offering varying results, such that it is not clear if the differences in enhancement are due to
Finally, an equally important variable that was considered was the NP material itself. Huang et al demonstrated the effects of gold, platinum, gadolinium and iodine, where they modelled these materials as mixtures of water with concentrations of each respective material. This study demonstrated a local dose enhancement attributed to an increase in the photoelectric effect for low energy X-rays and pair production for higher energy X-rays used in radiotherapy [127]. With this study, the macroscale effects were modelled as they considered the effects of atoms with high atomic numbers and created a mixture with water, rather than modelling concentrations of nanoparticles. To observe the dose enhancement effect, the material needed to have a sufficiently high atomic number, as well as being readily used within current clinical applications.

Another study by McMahon et al, modelled the effect of the atomic number on dose enhancement for various photon energies [7]. By simulating a single NP with a diameter of 20 nm, they demonstrated the complexity of dose enhancement with increasing energy and atomic number. Figure 3.1 from their work shows that at low energies there are variations in the enhancement with atomic number, whereas at higher, clinical energies, exceeding 1 MeV, there is little variation in enhancement with atomic number. In this study they considered the macroscale effects, where they determined the dose enhancement per unit mass of contrast agent by approximating this to the ratio of mass energy absorption coefficients for all materials as a function of energy. They then offered a relative DEF by normalising results to the maximum contrast for the energy being considered.

Although each of these studies demonstrated an effect from each of the variables considered in each respective study, it highlighted the need for a thorough study, considering the combined effect of all variables to determine the most suitable setup.

In terms of conducting the simulations, a suitable choice would be the Monte Carlo toolkit Geant4. This has been extended to offer nanoscale resolution to particle tracking, widening the applications that the toolkit can be used for.
3.2. Simulating on the nanoscale

Figure 3.1: Dependence of physical enhancement on atomic number and incident energy, demonstrated by McMahon et al [7]

Currently this extension, Geant4-DNA [128, 129], is only valid in liquid water, however there are developments underway to introduce other materials into this extension of Geant4. The work conducted by Tran et al, demonstrated the capability of Geant4-DNA to model the increased dose deposition with the introduction of NPs under proton irradiations [130], where they showed significant enhancement close to the NP, decreasing with increasing distance from the NP.

As with the macroscale model, MC simulations allow for the particle interactions to be modelled. Here however, it is important to carefully consider the physics that is being utilised to predict the probability of an interaction occurring. It is reported within the literature that approximately 50 % of all ionisations are produced by electrons with a starting energy less than 1 keV [131].

At low energies, simulation codes rely on theoretical, semi-empirical or scaling
methods to determine event-by-event tracking, as the exact cross-sections are not available from experiments [132, 133].

With the Geant4-DNA extension, it is possible to model physical interactions for specific particles, including the one of interest for this work, the electron. For this, elastic scattering, electronic excitation, vibrational excitation, ionisation and molecular attachment can be simulated in the energy range 7.4 eV to 1 MeV [129]. In order to validate these models, the cross-sections were benchmarked against experimental measurements carried out in water vapour, as measurements in liquid water do not exist [128]. As the electron range for those with energy of a few eV, is in the order of a few nanometres, this physics list was sufficient for the purposes of our work.

With this work, we focus on conducting a parametric study that investigates NP size along with NP material and photon energy, to demonstrate the effects of utilising NPs in radiation therapy. An extension of the study was also applied to offer preliminary results on the impact of a chemical coating. The results demonstrate the effects that these variables collectively have on the predicted physical enhancement effect through an in silico study. More specifically we record the dose deposition on multiple scales, from the nano to the macro scale, to demonstrate the overall effect of these variables and to estimate ranges of secondary electrons to determine if cell location is an important factor to consider. Within the literature the combined effect of NP material, size and beam energy have not been collectively investigated. Therefore this work can help demonstrate the combined effect of all three variables.

3.3 Materials and methods

3.3.1 Geometry setup for multi-scale model

As with the macro scale model, the MC simulation toolkit, Geant4 [103], was used for this work. Simulations were formed to model the effects of different variables on dose depositions surrounding a single NP. The geometry consisted of a single NP placed in the centre of a water phantom with dimensions of $1 \times 1 \times 1 \ cm^3$, to
3.3. Materials and methods

Figure 3.2: Simulated setup consisting of a single NP placed at the centre of a water phantom, where the size and material of the nanoparticle were altered. The photon beam was directed at the middle of the NP and altered to mimic the NP dimensions.

account for full scattering conditions, as shown in figure 3.2. The beam was incident on the centre of the NP and had dimensions that were altered, to mimic those of the NP.

Within Geant4 it is possible to specify a physics list to the users specifications. As the DNA physics list was only applicable in liquid water it was necessary to create a physics list where DNA physics applied to the water phantom region and a standard electromagnetic physics list (Livermore) applied to the NP region. This differed to the physics list used in chapter 2, as the list in that chapter was suited to proton therapy and higher energies, whereas the Livermore physics list is recommended for low energy electromagnetic processes, suited to our multiscale model.

It should be noted that the standard physics lists are capable of modelling interactions of all materials detailed within the National Institute of Standards and Technology (NIST) material database. It should also be noted that the DNA physics only applies to specific particles such as electrons and protons, but applied standard Livermore models for photon interactions. This however was sufficient for the purposes of this project, as the main interest in utilising the DNA physics, was
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

to allow for tracking secondary electrons within the water phantom to nanoscale resolution. The cuts were set to 1 nm, where Geant4-DNA does not use any production cuts as it simulates all interactions, however this cut applied to the electromagnetic physics list.

In order to investigate the effects that NP material could have, we considered three clinically relevant materials, gold, platinum and gadolinium [134, 105, 8]. These all have a sufficiently high atomic number and have been used by other groups within the literature. As well as this, NP size was altered to determine if the electrons produced due to the interactions with the high-Z materials, would have sufficient energy to propagate outside of the NP. NP diameters modelled were 2, 20 and 50 nm respectively [135]. 2 nm was chosen, as this is representative of the 1.9 nm NPs that were used by Hainfeld et al in their initial study with AuNPs and mice [38]. Another study by Zhang et al showed that pegylated AuNPs between 10 and 20 nm had higher levels of uptake than 45 or 60 nm pegylated AuNPs, therefore it as decided that 20 nm would be investigated [136]. Finally, 50 nm was chosen as it was shown by Chithrani et al to be preferentially taken up within cells through endocytosis [65].

Finally, the incident photon energy was altered; the enhancement effect is known to be dominant in the orthovoltage range (<500 keV), however the effect of the other variables along with energy had not been extensively investigated previously. Also, as this method is hoped to be used in clinical radiotherapy, we need to determine the most optimum setup at clinical beam energies. Therefore, modelled beam energies were 90 keV, 150 keV and 1.5 MeV (mean energy of a 6 MV beam) respectively. In order to achieve sufficient statistics at this resolution it was necessary to use $10^{10}$ incident photons to have Poisson statistical errors less than 5%.

Energy deposition was recorded within the phantom on three different scales, the nano, micro and macroscale as depicted in figure 3.3. For each of the regions, a scoring mesh was applied, with different bins to achieve the voxels needed for each region. This method of recording allowed for the nano region to have
3.3. Materials and methods

High resolution voxels of $1 \times 1 \times 1 \, nm^3$. As this is particularly computationally expensive, it was only used for a $500 \times 500 \times 500 \, nm^3$ region, where it was predicted the most variability would be observed. Following on from this, energy depositions were recorded in voxels of $0.1 \times 0.1 \times 0.1 \, \mu m^3$ for the micro region. As this region had dimensions of $10 \times 10 \times 10 \, \mu m^3$, it was representative of a cell and therefore would give an indication as to where would be most beneficial for NPs to be localised to within a cell. Another consideration was the clinical significance of the enhancement range, as the enhancement from NPs in a cell could affect enhancement in a neighbouring cell. This is of particular clinical importance as this could demonstrate considerations that need to be made for healthy tissues surrounding a tumour. Finally, recordings were taken in voxels of $0.1 \times 0.1 \times 0.1 \, mm^3$ for the macro region, which was $1 \times 1 \times 1 \, cm^3$, this was used to fully encompass the tracks of all particles. This is particularly important when considering NPs for clinical use, as this information is used in patient treatment plans.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

Figure 3.3: Energy deposition recording schematic showing the NP region at the centre of the nano region, contained within the micro region, all of which are within the macro region.

Firstly, with the energy depositions, the NP region was excluded to demonstrate the energy deposition in water. The values were then grouped into shells (value every 0.005, 0.1 and 100 µm, for nano, micro and macro regions respectively). Energy depositions were converted to dose, considering the mass of each respective shell. From this, it was then possible to determine the dose enhancement factor (DEF), the ratio of the dose with a NP to the dose without. The
3.3. Materials and methods

DEF was then quoted for each shell to demonstrate the DEF against the distance from the NP surface.

These simulations were particularly computationally demanding where it was necessary to use a large computer cluster. The UCL Legion High Performance Computing Facility (Legion@UCL) was used to run the code across multiple threads, where each simulation took approximately a week to run.

3.3.2 Geometry setup for chemical coatings

The model was extended to also consider the effects of a chemical coating, whereby poly(ethylene glycol) (PEG) was simulated, as this is a commonly use coating for AuNPs, as it stabilises the NPs [137].

In a recent study by Minelli and Shard, they reported quantitative measurements of NP coating thicknesses, showing an increase with increasing NP size [138]. Although they demonstrated a thickness of 12 nm with 50 nm AuNPs for a 2 kDa PEG coating, they explained that the maximum expected thickness for this molecular mass is 16 nm.

It was therefore decided that this maximum thickness would be investigated for all the sizes and materials considered in the previous part of this model. With the maximum thickness, it would be possible to determine if the electrons produced would have sufficient energy to escape both the NP and the coating, to then deposit energy within the water phantom. A homogeneous layer was applied to the exterior of the NP as shown in figure 3.4. In terms of the physics list, the standard physics was also applied to this layer. As well as this, the energy depositions within the PEG layer, were also excluded with the depositions in the NP, to demonstrate energy deposited in water. All other aspects of the simulation were conducted in the same manner as the previous geometry from section 3.3.1.

Within Geant4, it was possible to simulate the PEG layer from its chemical composition, however this is a simplification as the coating does not form a full thickness layer around the NP. In practice the layer is comprised of chains, however modelling this can be computationally expensive. Therefore as a first study, this overestimation can be used to assess the level of change, determining if secondary
3.3.2 Data outputs

In order to demonstrate the effect of NP material and size, it was necessary to also quote the DEF with respect to the mass of the material. This would enable direct comparison of DEFs for NP solutions of the same concentration: accounting for differences in density of the metal and the fact that to have equivalent NP concentrations, smaller NPs would be present in greater numbers than larger NPs. A direct comparison of DEF of smaller NPs with larger NPs is not a fair comparison for this reason. To determine the mass for each respective NP, considering differences in size and material, the volume of the NP was initially...
determined. Then using the density of the NP material and multiplying it by the volume, it was possible to determine the mass per NP. The values for each NP mass were therefore determined for each material and size combination and have been summarised in Table 3.1. Using these values, the DEF was divided by the respective mass of the NP, whereby the DEF per unit mass of NP material could be quoted, where this interpretation of DEF would allow for fair comparisons between different sizes and materials of NPs. For ease of interpretation, an additional table was reported, whereby the relative DEF per unit mass of NP material was quoted. This was calculated by dividing the value for DEF per unit mass by the maximum value reported for each respective size and beam energy combination. This would allow for clearer comparisons to be made.

**Table 3.1:** Mass per NP for each material and NP size combination

<table>
<thead>
<tr>
<th>Material</th>
<th>Density (g/cm³)</th>
<th>Mass per NP (ng) 2 nm</th>
<th>20 nm</th>
<th>50 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>19.3</td>
<td>$8.1 \times 10^{-11}$</td>
<td>$8.1 \times 10^{-8}$</td>
<td>$1.3 \times 10^{-6}$</td>
</tr>
<tr>
<td>Pt</td>
<td>21.45</td>
<td>$9.0 \times 10^{-11}$</td>
<td>$9.0 \times 10^{-8}$</td>
<td>$1.4 \times 10^{-6}$</td>
</tr>
<tr>
<td>Gd</td>
<td>7.9</td>
<td>$3.3 \times 10^{-11}$</td>
<td>$3.3 \times 10^{-8}$</td>
<td>$5.2 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Another measure that was considered was the enhancement range. This demonstrates the distance from the NP surface where an enhancement was observed. To determine the effectiveness of the enhancement, the range was determined for enhancements greater than 5% (DEF > 1.05).

DEF against distance has been plotted for each comparison to show the radial distribution of dose enhancement. DEF/unit mass of NP material has also been plotted to account for the concentration effect, where for the same concentrations, smaller NPs require more NPs to make the same concentration as highlighted in Table 3.1.

These plots were produced comparing all three materials at 50 nm for each beam energy, to show the material dependence. Plots were then produced for Au, looking at the effect of different sizes for each of the energies considered. Finally, a plot for each material with a diameter of 50 nm was produced for all energies.
considered, to demonstrate any energy dependence. This diameter was chosen, as the study by Chithrani et al showed preferential uptake with this NP size [39]. The results were produced in the same manner for the un-coated and coated NP, however only a diameter of 50 nm was investigated, with beam energies of 90 and 150 keV. This combination was chosen as 50 nm, having the largest size considered, was most likely to affect the number of electrons able to escape the NP, to then interact with water. As such this size, along with low energies, predicted to demonstrate the highest level of physical enhancement, was investigated.

For all plots produced, error bars were not included due to the small errors (< 5 %), where on a log scale plot they were not visible. Errors have been included in all numerical tables to demonstrate the errors associated with the quoted results.

3.4 Results

Uncoated NP

3.4.1 Radial dose enhancement

Simulations demonstrated the collective effect of NP material, size and incident beam energy, where the greatest enhancement was found with AuNPs with a diameter of 2 nm at a beam energy of 90 keV (DEF/ per unit mass of NP material = 2.33E+13 ± 1.04E+12 ng⁻¹ ). The DEF has been tabulated for all variables in tables 3.2, 3.4 and 3.6, where here the DEF has been quoted with respect to the mass of each NP in ng. As well as this, tables 3.3, 3.5 and 3.7 demonstrate the relative DEF per unit mass of NP material. As explained previously, this measure normalises to the maximum value for each respective size and beam combination.
### Table 3.2: DEF corrected for differences in number of particles by quoting DEF per unit mass of NP material, for each respective NP size and material combination with a beam energy of 90 keV.

<table>
<thead>
<tr>
<th>Distance from NP surface (μm)</th>
<th>DEF per unit mass of NP material (ng⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90 keV</td>
</tr>
<tr>
<td></td>
<td>2 nm</td>
</tr>
<tr>
<td></td>
<td>Au</td>
</tr>
<tr>
<td>0.01</td>
<td>$2.33 \times 10^{13} \pm 1.04 \times 10^{12}$</td>
</tr>
<tr>
<td>0.1</td>
<td>$8.68 \times 10^{11} \pm 2.18 \times 10^{10}$</td>
</tr>
<tr>
<td>1</td>
<td>$5.28 \times 10^{10} \pm 2.36 \times 10^{9}$</td>
</tr>
<tr>
<td>5</td>
<td>$1.72 \times 10^{10} \pm 5.11 \times 10^{8}$</td>
</tr>
<tr>
<td>10²</td>
<td>$1.25 \times 10^{10} \pm 9.93 \times 10^{8}$</td>
</tr>
<tr>
<td>10³</td>
<td>$1.24 \times 10^{10} \pm 5.50 \times 10^{8}$</td>
</tr>
</tbody>
</table>

#### 3.4. Results
### Table 3.3: Relative DEF per unit mass of NP material, for each respective NP size and material combination with a beam energy of 90 keV. All values have been normalised to the maximum value for each respective size.

<table>
<thead>
<tr>
<th>Distance from NP surface (µm)</th>
<th>Relative DEF per unit mass of NP material</th>
<th>90 keV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 nm normalised to a value of $2.33 \times 10^{13}$</td>
</tr>
<tr>
<td></td>
<td>Au</td>
<td>Pt</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0000</td>
<td>0.8928</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0372</td>
<td>0.0335</td>
</tr>
<tr>
<td>1</td>
<td>0.0023</td>
<td>0.0020</td>
</tr>
<tr>
<td>5</td>
<td>0.0007</td>
<td>0.0007</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0005</td>
<td>0.0005</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.0005</td>
<td>0.0005</td>
</tr>
<tr>
<td>20 nm normalised to a value of $1.35 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>1.0000</td>
<td>0.9485</td>
</tr>
<tr>
<td>0.1</td>
<td>0.2067</td>
<td>0.1816</td>
</tr>
<tr>
<td>1</td>
<td>0.0308</td>
<td>0.0266</td>
</tr>
<tr>
<td>5</td>
<td>0.0045</td>
<td>0.0048</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0010</td>
<td>0.0009</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.0009</td>
<td>0.0008</td>
</tr>
<tr>
<td>50 nm normalised to a value of $8.23 \times 10^8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>1.0000</td>
<td>0.9071</td>
</tr>
<tr>
<td>0.1</td>
<td>0.2847</td>
<td>0.2451</td>
</tr>
<tr>
<td>1</td>
<td>0.0833</td>
<td>0.0704</td>
</tr>
<tr>
<td>5</td>
<td>0.0101</td>
<td>0.0108</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0012</td>
<td>0.0011</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.0010</td>
<td>0.0009</td>
</tr>
</tbody>
</table>
**3.4. Results**

<table>
<thead>
<tr>
<th>Distance from NP surface (μm)</th>
<th>DEF per unit mass of NP material (ng⁻¹)</th>
<th>150 keV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Au</td>
<td>Pt</td>
</tr>
<tr>
<td><strong>2 nm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>$7.56 \times 10^{12} \pm 3.38 \times 10^{11}$</td>
<td>$7.17 \times 10^{12} \pm 3.21 \times 10^{11}$</td>
</tr>
<tr>
<td>0.1</td>
<td>$3.43 \times 10^{11} \pm 8.62 \times 10^{9}$</td>
<td>$3.22 \times 10^{11} \pm 8.10 \times 10^{9}$</td>
</tr>
<tr>
<td>1</td>
<td>$2.33 \times 10^{10} \pm 1.04 \times 10^{9}$</td>
<td>$2.11 \times 10^{10} \pm 9.45 \times 10^{8}$</td>
</tr>
<tr>
<td>5</td>
<td>$1.35 \times 10^{10} \pm 4.01 \times 10^{8}$</td>
<td>$1.23 \times 10^{10} \pm 3.65 \times 10^{8}$</td>
</tr>
<tr>
<td>$10^2$</td>
<td>$1.25 \times 10^{10} \pm 9.91 \times 10^{8}$</td>
<td>$1.12 \times 10^{10} \pm 8.91 \times 10^{8}$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>$1.24 \times 10^{10} \pm 5.51 \times 10^{8}$</td>
<td>$1.11 \times 10^{10} \pm 4.95 \times 10^{8}$</td>
</tr>
<tr>
<td><strong>20 nm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>$5.51 \times 10^{9} \pm 2.15 \times 10^{8}$</td>
<td>$5.41 \times 10^{9} \pm 2.11 \times 10^{8}$</td>
</tr>
<tr>
<td>0.1</td>
<td>$1.06 \times 10^{9} \pm 2.62 \times 10^{7}$</td>
<td>$9.66 \times 10^{8} \pm 2.38 \times 10^{7}$</td>
</tr>
<tr>
<td>1</td>
<td>$1.23 \times 10^{8} \pm 5.51 \times 10^{6}$</td>
<td>$1.18 \times 10^{8} \pm 5.28 \times 10^{6}$</td>
</tr>
<tr>
<td>5</td>
<td>$2.46 \times 10^{7} \pm 7.34 \times 10^{5}$</td>
<td>$2.29 \times 10^{7} \pm 6.82 \times 10^{5}$</td>
</tr>
<tr>
<td>$10^2$</td>
<td>$1.32 \times 10^{7} \pm 1.05 \times 10^{5}$</td>
<td>$1.19 \times 10^{7} \pm 9.48 \times 10^{5}$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>$1.24 \times 10^{7} \pm 5.53 \times 10^{5}$</td>
<td>$1.12 \times 10^{7} \pm 4.98 \times 10^{5}$</td>
</tr>
<tr>
<td><strong>50 nm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>$2.18 \times 10^{8} \pm 1.43 \times 10^{7}$</td>
<td>$2.11 \times 10^{8} \pm 1.38 \times 10^{7}$</td>
</tr>
<tr>
<td>0.1</td>
<td>$7.11 \times 10^{7} \pm 1.70 \times 10^{6}$</td>
<td>$6.52 \times 10^{7} \pm 1.55 \times 10^{6}$</td>
</tr>
<tr>
<td>1</td>
<td>$1.90 \times 10^{7} \pm 8.50 \times 10^{5}$</td>
<td>$1.82 \times 10^{7} \pm 8.14 \times 10^{5}$</td>
</tr>
<tr>
<td>5</td>
<td>$2.71 \times 10^{6} \pm 8.06 \times 10^{4}$</td>
<td>$2.50 \times 10^{6} \pm 7.44 \times 10^{4}$</td>
</tr>
<tr>
<td>$10^2$</td>
<td>$9.21 \times 10^{5} \pm 7.32 \times 10^{4}$</td>
<td>$8.40 \times 10^{5} \pm 6.67 \times 10^{4}$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>$8.00 \times 10^{5} \pm 3.56 \times 10^{4}$</td>
<td>$7.21 \times 10^{5} \pm 3.21 \times 10^{4}$</td>
</tr>
</tbody>
</table>
Table 3.5: Relative DEF per unit mass of NP material, for each respective NP size and material combination with a beam energy of 150 keV. All values have been normalised to the maximum value for each respective size.

<table>
<thead>
<tr>
<th>Distance from NP surface (µm)</th>
<th>Relative DEF per unit mass of NP material</th>
<th>150 keV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 nm normalised to a value of $7.56 \times 10^{12}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Au</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0000</td>
<td>0.9477</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0453</td>
<td>0.0426</td>
</tr>
<tr>
<td>1</td>
<td>0.0031</td>
<td>0.0028</td>
</tr>
<tr>
<td>5</td>
<td>0.0018</td>
<td>0.0016</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0016</td>
<td>0.0015</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.0016</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 nm normalised to a value of $5.51 \times 10^9$</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0000</td>
<td>0.9814</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1928</td>
<td>0.1754</td>
</tr>
<tr>
<td>1</td>
<td>0.0224</td>
<td>0.0214</td>
</tr>
<tr>
<td>5</td>
<td>0.0045</td>
<td>0.0042</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0024</td>
<td>0.0022</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.0023</td>
<td>0.0020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 nm normalised to a value of $2.18 \times 10^8$</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0000</td>
<td>0.9685</td>
</tr>
<tr>
<td>0.1</td>
<td>0.3257</td>
<td>0.2985</td>
</tr>
<tr>
<td>1</td>
<td>0.0870</td>
<td>0.0834</td>
</tr>
<tr>
<td>5</td>
<td>0.0124</td>
<td>0.0114</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0042</td>
<td>0.0038</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.0037</td>
<td>0.0033</td>
</tr>
</tbody>
</table>
### 3.4. Results

Table 3.6: DEF corrected for differences in number of particles by quoting DEF per unit mass of NP material, for each respective NP size and material combination with a beam energy of 1.5 MeV.

<table>
<thead>
<tr>
<th>Distance from NP surface ($\mu$m)</th>
<th>DEF per unit mass of NP material (ng$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Au</td>
</tr>
<tr>
<td></td>
<td>2 nm</td>
</tr>
<tr>
<td>0.01</td>
<td>$8.87 \times 10^{11} \pm 2.80 \times 10^{10}$</td>
</tr>
<tr>
<td>0.1</td>
<td>$3.03 \times 10^{10} \pm 5.38 \times 10^{8}$</td>
</tr>
<tr>
<td>1</td>
<td>$1.33 \times 10^{10} \pm 5.01 \times 10^{8}$</td>
</tr>
<tr>
<td>5</td>
<td>$1.25 \times 10^{10} \pm 3.15 \times 10^{8}$</td>
</tr>
<tr>
<td>$10^2$</td>
<td>$1.24 \times 10^{10} \pm 4.64 \times 10^{8}$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>$1.24 \times 10^{10} \pm 8.26 \times 10^{8}$</td>
</tr>
</tbody>
</table>

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### Table 3.7: Relative DEF per unit mass of NP material, for each respective NP size and material combination with a beam energy of 1.5 MeV. All values have been normalised to the maximum value for each respective size.

<table>
<thead>
<tr>
<th>Distance from NP surface (µm)</th>
<th>Relative DEF per unit mass of NP material</th>
<th>1.5 MeV</th>
<th>2 nm normalised to a value of $8.87 \times 10^{11}$</th>
<th>20 nm normalised to a value of $1.65 \times 10^{8}$</th>
<th>50 nm normalised to a value of $1.19 \times 10^{7}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Au</td>
<td>Pt</td>
<td>Gd</td>
<td>Au</td>
</tr>
<tr>
<td>0.01</td>
<td></td>
<td>1.0000</td>
<td>0.4221</td>
<td>0.0974</td>
<td>0.9269</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>0.0341</td>
<td>0.0236</td>
<td>0.0415</td>
<td>0.4012</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.0150</td>
<td>0.0133</td>
<td>0.0338</td>
<td>0.1401</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.0141</td>
<td>0.0128</td>
<td>0.0344</td>
<td>0.0784</td>
</tr>
<tr>
<td>$10^2$</td>
<td></td>
<td>0.0139</td>
<td>0.0125</td>
<td>0.0340</td>
<td>0.0694</td>
</tr>
<tr>
<td>$10^3$</td>
<td></td>
<td>0.0139</td>
<td>0.0125</td>
<td>0.0340</td>
<td>0.0688</td>
</tr>
</tbody>
</table>
3.4. Results

3.4.1.1 Material dependence

Simulations demonstrated that although Au and Pt offered comparable levels of enhancement for all energies, as shown in figures 3.5 and 3.6, Au offered higher enhancements. From tables 3.2 - 3.6 it can be seen that the values for Au are consistently higher than Pt, where the differences range between 1 and 18% depending on the size, beam energy and distance from the NP surface. An example of this is with an energy of 90 keV, Au had a DEF per unit mass of NP material of $2.33\times10^{13} \pm 1.04\times10^{12}$ ng$^{-1}$ compared to $2.08\times10^{13} \pm 9.31\times10^{11}$ ng$^{-1}$ for Pt, at a distance of 0.01 $\mu$m from the surface of the NP. As was explained in section 1.2.3.1, the photoelectric effect interactions are most probable for low energy photons and materials with a high atomic number. This correlates with our findings, where Au has the highest atomic number and demonstrates the highest level of enhancement.

Considering a size of 2 nm at 10 nm from the surface of the NP, the differences between materials are comparable at 90 and 150 keV. Au is 11% and 5% higher than Pt at 90 and 150 keV, and is 56% and 54% higher than Gd at the same energies. With 1.5 MeV however, the differences are greater, with 58% increase compared to Pt and 90% to Gd. If, however, we consider a distance of 5 $\mu$m, Au is still greater than Pt for all energies, however Gd was between 47% and 59% greater than Au for all energies. These differences in energy deposition, across the distances considered from the surface of the NP, are due to the differences in Auger cascades with the different materials. With all materials it was shown that enhancement decreased with increasing distance from the NP surface.

In terms of the shape of the plot presented in figures 3.5 and 3.6, this is due to the energy deposition from short-ranged low energy electrons ($< 1$ keV), where electrons with approximately 10 eV will travel less than 20 nm in liquid water [140]. At the higher energy of 1.5 MeV, the Compton effect was more dominant, therefore the enhancement was lower, due to the weak dependence on atomic number. At this energy, the enhancement effect was orders of magnitude less than with orthovoltage energies, which will be quantified in section 3.4.1.3.

It should be noted that due to the approximation of the model, there are no
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datapoints between 5 and 100 µm, corresponding to the cross-over between the micro and macro region, where some differences may occur that have not been quantified. Also, due to the multiscale nature of these simulations, it was necessary to plot results on a log log scale to encompass all the results, as such, large decreases are seen between the different regions, as the voxel sizes differed across the regions used.
Figure 3.5: DEF for all materials with a diameter of 50 nm for a beam energy of a) 90 keV, b) 150 keV and c) 1.5 MeV
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

Figure 3.6: DEF/ng of NP material for all materials considered, with a diameter of 50 nm for a beam energy of a) 90 keV, b) 150 keV and c) 1.5 MeV
3.4. Results

3.4.1.2 Size dependence

Results demonstrate that NPs with a diameter of 2 nm give the greatest enhancement for all energies considered. This is due to both less self absorption within the NP and an increased number of NPs to interact with when using smaller NPs for the same concentration.

Considering Au at 90 keV, it can be seen from table 3.2 that at a distance of 0.01 µm from the NP surface 2 nm NPs had a DEF per unit mass of NP material three orders of magnitude higher than 20 nm and 5 orders greater than 50 nm. Although there are differences in atomic number, the same trends were demonstrated for Pt and Gd. Similarly for 150 keV, a difference of three orders of magnitude was shown between 2 and 20 nm, with four orders between 2 and 50 nm. For 1.5 MeV, Au and Pt followed similar trends, however Gd had a difference of two orders of magnitude between 2 and 20 and 4 orders between 2 and 50 nm.

Although similar trends were shown across the different materials considered, the respective energy depositions were greater with Au due to the higher atomic number, which as previously explained, the photoelectric effect is dependent on.

It should be noted that figures 3.7 and 3.8 demonstrate the need for correcting for a relevant concentration when investigating a size relationship in a simulation. From figure 3.7 it can be seen that a single 50 nm NP offers the highest DEF at distances greater than 0.03 µm, whereas figure 3.8 shows greatest enhancement from 2 nm NP across the entire phantom. Therefore by plotting DEF alone, we do not consider that fewer 50 nm NPs will be present for a given concentration of NPs compared to 2 nm NPs. Figure 3.8 accounts for the higher number of particles with smaller NPs to make the same concentration for that material (table 3.1). With these plots the legend quotes the respective mass for each NP size, as this will affect the point where the DEF tends to unity.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

Figure 3.7: DEF for all diameters considered, demonstrating the effect of NP size for Au, with a beam energies of a) 90 keV, b) 150 keV and c) 1.5 MeV
3.4. Results

Figure 3.8: DEF/ng of Au, for all diameters considered, demonstrating the effect of NP size, with a beam energy of a) 90 keV, b) 150 keV and c) 1.5 MeV
3.4.1.3 Energy dependence

As demonstrated in the literature, there is a significant energy dependence with the dose enhancement effect, which decreases with increasing energy. With Au and a diameter of 2 nm the enhancement is 68 % smaller (66 % for Pt and Gd respectively) at 150 keV compared to 90 keV and 96 % (98 % and 99 % for Pt and Gd respectively) smaller with 1.5 MeV at a distance of 0.01 µm from the surface of the NP. Figure 3.9a shows the highest level of enhancement for Au with a diameter of 50 nm is from an incident beam energy of 90 keV \( (8.23 \times 10^8 \pm 2.93 \times 10^7 \text{ ng}^{-1}) \).

The trends shown for all materials are consistent for the energies considered, when comparing the same material and size at different energies (figure 3.9).
3.4. Results

Figure 3.9: DEF/ng demonstrating the effect of energy on NPs with a diameter of 50 nm for a) Au, b) Pt and c) Gd.
3.4.2 Enhancement range

Table 3.8 shows that particles with a larger size, offered the highest enhancement range for all energies. NPs with a size of 50 nm offered the greatest range from the surface of the NP (400 µm), for Au and Pt at 90 keV. Considering an energy of 90 keV, all materials showed greatest enhancement with 50 nm, where enhancement was approximately 75 % greater for Au and Pt and 98 % for Gd comparing 50 nm and 2 nm. Enhancement ranges were shown to decrease with increasing beam energy, where 90 and 150 keV gave comparable ranges. Comparing the different materials, Au offered the highest enhancement range when comparing the same sizes at the same energies. Gd, having the lowest atomic number and density showed the lowest enhancement range. These findings correspond to the effective range of secondary electrons produced, where the ranges differed depending on the NP size, material and incident beam energies. However, the electrons produced were cascades of low energy electrons (LEEs) with ranges of the order of 10 nm.

With this measure we consider only the range of the enhancement, not the magnitude, therefore 50 nm would be expected to have the longest enhancement range due to a larger amount of material to interact with.
Table 3.8: Enhancement range of a single NP for all NP materials, sizes and beam energies considered.

<table>
<thead>
<tr>
<th>Nanoparticle size (nm)</th>
<th>Enhancement range for single NP (µm)</th>
<th>90 keV</th>
<th>150 keV</th>
<th>1.5 MeV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Au</td>
<td>Pt</td>
<td>Gd</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>100±50</td>
<td>100±50</td>
<td>5±0.05</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>200±50</td>
<td>200±50</td>
<td>100±50</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>400±50</td>
<td>400±50</td>
<td>300±50</td>
</tr>
</tbody>
</table>
3.4.3 Energy distribution of emitted electrons

3.4.3.1 Material dependence

Considering figure 3.10, it can be seen that for all energies, Au and Pt offer a comparable amount of energy deposition per unit volume, with Gd less in the nano and micro regions. Interestingly figure 3.11 shows similar numbers of electrons produced per unit volume for all materials across all regions for the three energies considered. This demonstrates the dependence on atomic number, whereby those with a higher atomic number offer higher depositions per electron within the nano and micro regions. This is further highlighted by figure 3.12, where the average energy deposition per electron per unit volume is illustrated, showing similar results between Au and Pt, both of which differ to that of Gd.

It should be noted that with the energy distribution, the results have not been quoted with respect to the mass of each NP material. This was to allow for an understanding of the energy depositions due to the electrons produced, rather than scaling to see the effective dose enhancement effect.
3.4. Results

Figure 3.10: Energy deposited per unit volume, demonstrating the effect of material, on NPs with a diameter of 50 nm, for beam energies of a) 90 keV, b) 150 keV and c) 1.5 MeV
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

![Graphs showing electrons per unit volume for different beam energies and materials](image)

(a) 90 keV 50 nm

(b) 150 keV 50 nm

(c) 1.5 MeV 50 nm

**Figure 3.11**: Electrons per unit volume, demonstrating the effect of material, on NPs with a diameter of 50 nm, for beam energies of a) 90 keV, b) 150 keV and c) 1.5 MeV
Figure 3.12: Average energy deposited per electron per unit volume, demonstrating the effect of material, on NPs with a diameter of 50 nm, for beam energies of a) 90 keV, b) 150 keV and c) 1.5 MeV
3.4.3.2 Size dependence

From figure 3.13, it can be seen that across all energies, a size of 2 nm has the highest energy deposition per unit volume only within the first 10 nm from the surface of the NP (20 and 50 nm were 67 % and 89 % lower respectively at 1.5 MeV). This then becomes lower than both 20 and 50 nm within the nano and micro regions (27 % and 11% lower than 20 and 50 nm respectively at 1.5 MeV, 35 nm from the surface of the NP). Considering figure 3.14 however, the number of electrons per unit volume is consistently higher for 2 nm within the nano region across all energies. The combination of these two correlates with the higher differences between sizes when considering the average energy deposition per electron per unit volume (figure 3.15). From this plot the difference decrease at a distance of 10 nm from the surface of the NP is 72 % and 92 % for 2 nm compared to 20 and 50 nm respectively. Again here it should be noted that differences due to the mass of each size have not been corrected for.
3.4. Results

Figure 3.13: Energy deposited per unit volume of Au, demonstrating the effect of NP size, for beam energies of a) 90 keV, b) 150 keV and c) 1.5 MeV.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

Figure 3.14: Electrons per unit volume, for Au, demonstrating the effect of NP size, for beam energies of a) 90 keV, b) 150 keV and c) 1.5 MeV
3.4. Results

Figure 3.15: Average energy deposited per electron per unit volume, for Au, demonstrating the effect of NP size, for beam energies of a) 90 keV, b) 150 keV and c) 1.5 MeV.
3.4.3.3 Energy dependence

Figure 3.16 shows that for all three materials, across the nano and micro regions the energy deposition per unit volume is more pronounced at an energy of 90 keV. When considering the macro region however, energies of 1.5 MeV then offer higher depositions, with 90 keV giving the least. This correlates with the proposed short ranged electrons that are produced at orthovoltage energies.

Considering figure 3.17, it can be seen that higher energies create more electrons. This is due to ionisation with the NP, whereby Auger electrons are created, where a cascade can occur leading to the emission of a large number of secondary electrons [141]. These electrons, however, have low energies as the average deposition per electron per unit volume is greater at lower energies within the nano and micro regions (3.18).
Figure 3.16: Energy deposited per unit volume, demonstrating the effect of energy, on NPs with a diameter of 50 nm, for a) Au, b) Pt and c) Gd.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

Figure 3.17: Electrons per unit volume, demonstrating the energy dependence for NPs with a diameter of 50 nm, for a) Au, b) Pt and c) Gd.
Figure 3.18: Average energy deposited per electron per unit volume, demonstrating the energy dependence, for NPs with a diameter of 50 nm, for a) Au, b) Pt and c) Gd.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

PEG coated NP

3.4.4 Radial dose enhancement

Simulations including the coating demonstrated the same trends as without the coating. Table 3.9 demonstrates the enhancement with a coating of 16 nm (2 kDa molecular weight), where the highest enhancement for the variables considered, was $2.10 \times 10^8 \pm 8.28 \times 10^6$ ng$^{-1}$ with Au and a beam energy of 90 keV. When comparing to an uncoated NP, it can be seen that there is a 75% decrease in the DEF per unit mass of NP material. The differences have been tabulated in table 3.11, where for the nano and macro regions there is a decrease for both energies. Within the micro region however, there is a change for both 90 and 150 keV. This is thought to be due to both the differences in energies of electrons that can escape the NP and the difference in distance from the surface of the NP. Considering an uncoated NP with a diameter of 50 nm, the first quoted DEF is at a distance of 0.035 µm with respect to the centre of the NP, however the coated one is at 0.051 µm.
Table 3.9: DEF corrected for differences in number of particles by quoting DEF per unit mass of NP material, for each respective NP size and material combination with a size of 50 nm, beam energy of 90 and 150 keV and a PEG coating 16 nm thick.

<table>
<thead>
<tr>
<th>Distance from NP surface (µm)</th>
<th>DEF per unit mass of NP material (ng⁻¹) with a PEG coating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Au</td>
</tr>
<tr>
<td></td>
<td>50 nm 90 keV</td>
</tr>
<tr>
<td>0.01</td>
<td>$2.10 \times 10^8 \pm 8.28 \times 10^6$</td>
</tr>
<tr>
<td>0.1</td>
<td>$1.43 \times 10^8 \pm 3.53 \times 10^6$</td>
</tr>
<tr>
<td>1</td>
<td>$6.70 \times 10^7 \pm 2.99 \times 10^6$</td>
</tr>
<tr>
<td>5</td>
<td>$8.34 \times 10^6 \pm 2.48 \times 10^5$</td>
</tr>
<tr>
<td>$10^2$</td>
<td>$9.59 \times 10^5 \pm 7.62 \times 10^4$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>$8.06 \times 10^5 \pm 3.58 \times 10^4$</td>
</tr>
</tbody>
</table>

3.4. Results
### Table 3.10: Relative DEF per unit mass of NP material, for each respective NP size and material combination with a size of 50 nm, beam energy of 90 and 150 keV and a PEG coating 16 nm thick. All values have been normalised to the maximum value for each respective energy.

<table>
<thead>
<tr>
<th>Distance from NP surface (µm)</th>
<th>Relative DEF per unit mass of NP material with a PEG coating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 nm 90 keV normalised to a value of $2.10 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>Au</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0000</td>
</tr>
<tr>
<td>0.1</td>
<td>0.6837</td>
</tr>
<tr>
<td>1</td>
<td>0.3196</td>
</tr>
<tr>
<td>5</td>
<td>0.0398</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0046</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.0038</td>
</tr>
</tbody>
</table>
Table 3.11: Percentage difference between DEF per unit mass of NP material, with and without a PEG coating, for each material at energies of 90 and 150 keV.

<table>
<thead>
<tr>
<th>Distance from NP surface (µm)</th>
<th>Percentage difference between DEF per unit mass of NP material (ng(^{-1})) of a NP with and without a PEG coating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Au</td>
</tr>
<tr>
<td>50 nm 90 keV</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>(-74.67 \pm 3.96)</td>
</tr>
<tr>
<td>0.1</td>
<td>(-39.16 \pm 1.34)</td>
</tr>
<tr>
<td>1</td>
<td>(-2.76 \pm 0.17)</td>
</tr>
<tr>
<td>5</td>
<td>(0.20 \pm 0.01)</td>
</tr>
<tr>
<td>(10^2)</td>
<td>(-0.47 \pm 0.05)</td>
</tr>
<tr>
<td>(10^3)</td>
<td>(0.08 \pm 0.005)</td>
</tr>
<tr>
<td>50 nm 150 keV</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>(-76.40 \pm 6.05)</td>
</tr>
<tr>
<td>0.1</td>
<td>(-33.08 \pm 1.21)</td>
</tr>
<tr>
<td>1</td>
<td>(-3.11 \pm 0.20)</td>
</tr>
<tr>
<td>5</td>
<td>(1.22 \pm 0.05)</td>
</tr>
<tr>
<td>(10^2)</td>
<td>(0.25 \pm 0.03)</td>
</tr>
<tr>
<td>(10^3)</td>
<td>(-0.44 \pm 0.03)</td>
</tr>
</tbody>
</table>
3.4.4.1 Material dependence

Figure 3.19 shows the uncorrected DEF, where Au is 85% higher than Gd at 10 nm from the surface of the NP at 90 keV (80% at 150 keV). When corrected for differences in mass of NP this becomes 63% for 90 keV (51% at 150 keV) as shown in figure 3.20. From these plots, it can be seen that the significant enhancement at 10 nm, that was evident in figure 3.6 (the case for the uncoated NP) is not observed, due to the PEG coating.

**Figure 3.19:** DEF for all materials with a diameter of 50 nm and a PEG coating 16 nm thick for a beam energy of a) 90 keV and b) 150 keV
3.4. Results

![Graph](image)

(a) 90 keV 50 nm

(b) 150 keV 50 nm

Figure 3.20: DEF/ng of NP material for all materials considered, with a diameter of 50 nm and a PEG coating 16 nm thick for a beam energy of a) 90 keV and b) 150 keV
3.4.4.2 Energy dependence

Considering figure 3.21, as with the uncoated NP, 90 keV offers higher enhancement in the nano region, where 150 keV for Au is 75% lower at 10 nm from the surface of the NP. For all materials considered, it was shown that the enhancement was consistently higher with 90 keV compared to 150 keV.
Figure 3.21: DEF/ng demonstrating the effect of energy on NPs with a diameter of 50 nm and a PEG coating 16 nm thick, for a) Au, b) Pt and c) Gd.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

### 3.4.5 Enhancement range

Table 3.12 demonstrates the enhancement range, where the longest range was 300 $\mu$m with Au and Pt at 90 keV. This was 25% less than with an uncoated particle, where the coating could have altered the number of electrons that could escape the NP and deposit energy within the water. This energy causes damage through indirect effects, where free radicals are produced resulting in chemical reactions that damage the DNA as explained in section 1.2.2. In terms of the simulations, Geant4-DNA has recently been extended to include a chemistry module that simulates the production of chemical species [142]. As this is a recent addition to Geant4-DNA and is still being extensively developed, it was not implemented within this work. In the future however, it could be used to consider the interactions of chemical species and determine the extent of these to gain a deeper insight into the enhancement range.

Table 3.12: Enhancement range of a single NP with a PEG layer for all NP materials, sizes and beam energies considered.

<table>
<thead>
<tr>
<th>Nanoparticle size (nm)</th>
<th>Enhancement range for single NP with a PEG layer ($\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90 keV</td>
</tr>
<tr>
<td>Au</td>
<td>Pt</td>
</tr>
<tr>
<td>50</td>
<td>300 ± 50</td>
</tr>
</tbody>
</table>

### 3.4.6 Energy distribution of emitted electrons

#### 3.4.6.1 Material dependence

As with the uncoated NP, the energy depositions per unit volume were comparable between Au and Pt, where they were both higher than Gd (Gd is 85% and 80% lower than Au and 84% and 80% lower than Pt for 90 and 150 keV respectively) (figure 3.22). Here however, at 10 nm from the surface of the NP, for Au at 90 keV the energy deposited per unit volume is 94% lower than that of the uncoated NP. As with the uncoated NP, the electrons per unit volume were comparable for all materials across all regions for both energies (figure 3.23). For both energies Au and Pt have a higher average energy deposition per electron per unit volume than...
Gd in the nano and micro regions (figure 3.24).

Figure 3.22: Energy deposited per unit volume, for all materials with a diameter of 50 nm and a PEG coating 16 nm thick for a beam energy of a) 90 keV and b) 150 keV.
Figure 3.23: Electrons per unit volume, for all materials with a diameter of 50 nm and a PEG coating 16 nm thick for a beam energy of a) 90 keV and b) 150 keV.
3.4. Results

Figure 3.24: Average energy deposited per electron per unit volume, for all materials with a diameter of 50 nm and a PEG coating 16 nm thick for a beam energy of a) 90 keV and b) 150 keV.

3.4.6.2 Energy dependence

Figure 3.25 demonstrates the energy dependence of the energy deposition per unit volume, where for all materials, 90 keV offered higher deposition in the nano and micro region, but lower depositions in the macro region. The number of electrons are comparable across both energies for all materials (figure 3.26), with a higher average energy deposition per electron per unit volume for 90 keV in the nano and micro regions (figure 3.27). This follows the same trend as the uncoated NP.
Figure 3.25: Energy deposited per unit volume, demonstrating the effect of energy on NPs with a diameter of 50 nm and a PEG coating 16 nm thick, for a) Au, b) Pt and c) Gd.
3.4. Results

Figure 3.26: Electrons per unit volume, demonstrating the energy dependence for NPs with a diameter of 50 nm and a PEG coating 16 nm thick, for a) Au, b) Pt and c) Gd.
Chapter 3. Multiscale parametric study on dose deposition with photon irradiation

Figure 3.27: Average energy deposited per electron per unit volume, demonstrating the effect of energy on NPs with a diameter of 50 nm and a PEG coating 16 nm thick, for a) Au, b) Pt and c) Gd.
3.5 Discussion

By placing a single high-Z NP in the centre of a water phantom, significant levels of dose enhancement could be observed radially around the NP. The work focused on demonstrating the dependence of dose enhancement on beam energy, NP material and size across multiple scales. The first finding was that, as shown by Leung et al [124], if we consider the use of Au, it can be seen that energies closer to the K-edge of Au (80.73 keV) show the most significant enhancement, decreasing with increasing energy. At higher, more clinically relevant energies enhancements are still observed, however, with Au at 50 nm for example, the enhancement at 90 keV is 68 times greater than at 1.5 MeV, 10 nm from the surface of the NP. Leung et al, demonstrated a difference in enhancement of approximately 200% between 50 nm AuNPs irradiated with 50 kVp compared to 6 MV [124]. With their work they calculated the enhancement by taking the ratio of total number of interactions of Au compared to water, rather than the ratio of dose depositions.

As the aim of the study was to consider the variables affecting dose enhancement, the NP material will initially be considered. The material dependence is consistent throughout the simulations, whereby Au offered the highest level of enhancement. In terms of atomic numbers, Au has the highest atomic number, whereas Pt has a higher density. As was explained in section 1.5.5.1, the effect of having a high-Z material with low energy photons is to increase the photoelectric effect, which has a dependence on the atomic number. This correlates with the findings of McMahon et al [7], where they investigated a range of atomic numbers from 14 to 80 for a single 20 nm NP. They showed at low energies there are variations in energy and atomic number dependent changes to the enhancement effect, which corresponds to the K-, L- and M-shell absorption. From this work, their plot of relative DEF with respect to atomic number and photon energy (figure 3.1), demonstrates a relative enhancement per unit mass of approximately 0.7 for Gd compared to 1 for Au at 90 keV. With our work we found a relative enhancement of approximately 0.8 between Au and Gd at 90 keV, 5 µm from the NP surface, for a diameter of 20 nm. This distance was chosen as it is representative of the size of
a typical human cell, where NPs would be localised to. Differences between the two are attributed to the differences in methods, as their plot was created from an analytical model considering the macroscale effects, where a single enhancement is quoted rather than enhancement quoted from the surface of the NP. This study also conducted MC simulations on the nanoscale, where they also demonstrated a higher energy deposition from Au closer to the NP surface compared to Gd, where in the micro region they also showed Gd to then have higher depositions. These differences in energy depositions at distances from the NP surface are attributed to differences in the Auger cascades produced by different materials. In our work this was demonstrated at a higher energy of 1.5 MeV, at distances close to the NP, in the nano region, Au still offered the highest enhancement (Au 58 % higher than Pt and 90 % higher than Gd 10 nm from the surface of the NP for 2 nm NP). At further distances, in the micro region, Gd offered higher enhancement. It should also be noted that these findings will need to be combined with biological studies, as the NP material can offer differences in toxicity (explained in section 1.5.2) that need to be accounted for. Overall, this work has demonstrated that the atomic number does influence the amount of enhancement, as demonstrated by the relative DEF, where in most cases Au offered the highest enhancement. The relationship is complex however, as it changes with both NP size and incident beam energy. The exception to Au offering highest enhancement, was shown with a diameter of 20 nm and beam energy of 1.5 MeV. Here Pt showed the highest enhancement. At this energy, the Compton effect is dominant, which has a dependence on density rather than atomic number, as the effect is proportional to both physical density and the number of outer shell electrons (electron density). When comparing the physical densities of Au and Pt it was shown in table 3.1 that Pt was denser by approximately 11 %. The combination of size and denser material was thought to cause a higher enhancement with Pt rather than Au for this case.

Another important factor is the size, as this can affect the number of electrons that can escape as well as how much material there is to interact with to produce secondary electrons. By modelling the DEF per unit mass of NP material, a
fair comparison could be made between the different NP parameters to determine the enhancement potential of each combination. It was shown that when simply considering the DEF, a size of 50 nm would offer the highest enhancement at distances greater than 0.03 \( \mu \)m. However, for a given concentration there would be fewer particles for this size, whereby the cumulative effect of smaller particles offered a higher enhancement.

For all energies considered, 2 nm offered the highest enhancement, due to both less self absorption and a higher number of particles to interact with if results were scaled to model a given concentration. For an energy of 90 keV, 2 nm NPs showed enhancements three orders of magnitude higher than that of 20 nm and five orders greater than 50 nm. In terms of the enhancement range however, larger NPs had a greater range of enhancement (100 \( \pm \) 50 \( \mu \)m compared to 400 \( \pm \) 50 \( \mu \)m for Au, comparing 2 and 50 nm respectively, at 90 keV). Considering the average energy deposition per electron per unit volume, 2 nm only offered higher depositions within the first 10 nm, where following on from that both 20 and 50 nm deposited more. Due to the finite amount of energy, 2 nm NPs deposit the majority of energy close to the NP, whereas larger particles deposit smaller amounts across a longer range. In terms of clinical relevance, the size is an important factor as it affects the number of NPs that can be taken up in the cell, toxicity, as well as the level of enhancement. In terms of NP uptake, Chithrani et al demonstrated that NPs are taken up within vesicles in the cytoplasm and were not within the nucleus [65]. From our findings, 2 nm may offer the highest enhancement, however due to the short range of enhancement, considerations need to be made as to the target for the damage. Biological studies are needed to complement these results to demonstrate if short ranged enhancement would be sufficient or if larger NPs are needed, such as 50 nm which can enhance for a longer range. As explained in section 1.2.2, indirect effects such as those from photon interactions, induce radiolysis of water, whereby free radicals are produced. The use of high-Z NPs increases the generation of ROS, which in turn induces oxidative stress, damaging cell function, leading to cell death [143]. The majority of DNA damages occurs from ROS, therefore it may be that
the range of energy deposition from physical enhancement is not the leading factor, but more the range of ROS, which has a range of some µm [144].

When considering the energy deposited at distances from the surface, it could be seen that the most significant deposition was always within the first 10 nm from the surface, decreasing with increasing distance from the NP, when considering an uncoated NP. Similar trends were shown with coated NPs, whereby the most significant levels of enhancement were within the nano region. Here, however, the magnitude of the enhancement was reduced (75 % reduction for 50 nm Au NP at 90 keV), as the large number of very low energy electrons found close to the NP surface with an uncoated NP were absorbed in the PEG layer for the coated NP. Interestingly this only reduced the enhancement range by 25 % for Au and Pt at 90 keV. The extension of simulating a PEG layer was only investigated for 50 nm, as this was thought to have the highest impact on the number of electrons able to escape. Having demonstrated a significant difference in both enhancement and energy distribution, it would be of interest to carry out simulations for other NP sizes to determine the magnitude of the effect on smaller NPs. It was shown that the highest impact was closest to the surface of the NP, where low energy electrons would be unable to escape the PEG layer. Although we have explained that in practice the PEG layer is comprised of chains rather than a homogeneous layer surrounding the NP core, it was also highlighted that the protein corona that is formed when the NP comes into contact with physiological media increases the size of the NP. As such, although we have overestimated the number of electrons absorbed due to the PEG layer, in practice, this may still be valid due to the number that could be absorbed within the protein corona. It is therefore important to consider coatings and the protein corona when attempting to optimise NP design based on physical dose enhancement. This can be carried out by simulating different coatings through their chemical compositions, where the protein corona on NPs would be modelled by using experiments to quantify the thickness. Measurements can be carried out with dynamic light scattering (DLS), measuring the hydrodynamic diameter of NPs after incubation in biological media,
which can then be added to the NP geometry [145].

In terms of recommending a NP for clinical use, further studies are needed to determine the optimum target within a cell for the NPs to interact with. The findings from this study, in terms of the enhancement range, can then be used to predict the level of damage. Although we have shown 2 nm to be the most optimum in terms of enhancement for the same concentration, in practice the level of uptake within tumour cells will differ depending on the NP material, shape and size as well as the cell type. A study by Hsiao et al demonstrated the differences in cellular uptake with silica NPs of different sizes with four cell lines [146]. With this they demonstrated differences of approximately 67 % for the same size across the different cell types and 98 % for the same cell line with higher uptake with 15 compared to 60 nm. As such, an extension of this model could be used to recalculate the DEF/number of NPs, where the number of particles taken up by different cell types is used to determine the most optimum setup for physical dose enhancement, whilst taking into account biological uptake.

Further work would be to conduct a parametric study with concentrations to determine the cumulative enhancement effect. This adds an interesting point to consider whereby electrons produced by a single NP will most likely interact with a neighbouring NP if the concentration is sufficiently high. This can increase the overall effect; however, it is thought that this will not simply be a linear relationship between concentration and enhancement effect as some electrons may lose their energy within a subsequent NP. Another important consideration is the chemical coating of NPs, as this can significantly alter the NP diameter [136], which in turn may affect the number of electrons able to escape and deposit energy in the surrounding water. Although we have demonstrated the effect of a PEG coating, in practice there are several coatings that can be used. Also, experimentally when NPs are introduced into the body in contact with physiological media, the size is greatly increased due to the protein corona. Therefore a quantitative assessment is required to determine the implications of this on physical dose enhancement.
3.6 Conclusion

This work demonstrates a flexible model that is able to simulate the effects of a number of variables that are critical in the optimisation of the physical dose enhancement effect with the introduction of high-Z materials. Compared to other models that consider a single nanoparticle, this study investigated three critical variables, nanoparticle size, material and beam energy, across multiple scales to investigate the optimum setup. As well as these, the model was extended to determine if the addition of a PEG coating would greatly affect the level of enhancement.

In agreement with the literature, an energy dependence was demonstrated, whereby lower energies showed more significant enhancements. By taking into account the mass per NP, for each size and material, the model demonstrated that the setup which offered the highest overall enhancement was with a 90 keV beam and gold nanoparticles with a diameter of 2 nm. It was also shown that the addition of a PEG coating altered the level of enhancement as well as the average energy deposition per unit volume. Although this energy is much lower than those used clinically, it was also demonstrated that enhancements would still be observed at clinical energies.
Chapter 4

Measurement of NP enhancement in a biological system

This chapter introduces the radiobiological study that was conducted, investigating various variables to demonstrate the biological impact of NPs combined with radiation treatment.

The work in this chapter resulted in the following outputs:


4.1 Rationale

Having demonstrated varying levels of enhancement through Monte Carlo (MC) simulations and physics experiment, the work was expanded to consider the biological impact of utilising nanoparticles (NPs) with radiotherapy. With an in vitro study, it is possible to show how the radiosensitisation effect may vary with different cell types, radiation properties, as well as NP characteristics. From the literature it was reported that although physical dose enhancement occurs, the
biological contribution to the overall radiosensitisation effect is more significant. Butterworth et al collated experimental findings and calculated the predicted enhancement effect compared to the reported sensitisation effect as was discussed in section 2.6. Simulations were shown to underestimate the biological effect by approximately 20 % for cells irradiated with 6 MV photons [11].

With the use of NPs, various groups have carried out biological assays, where as with simulations, the reported results were varied. In terms of biological experiments the differences are caused by the diversity in cell lines, NPs with their respective coatings, concentrations, irradiation parameters as well as the assays quoted to demonstrate the effects. This has therefore resulted in significant variations in the results, where significant enhancements of a factor of 25 were shown by Rahman et al, with Aurovist™ gold nanoparticles (AuNPs) at a concentration of 50 mg/ml with bovine aortic endothelial cells (BAEC) and 80 kV photons, compared to smaller enhancements shown by Chithrani et al [39], where they synthesised 50 nm AuNPs at a concentration of 50 ng/ml in HeLa cells and found an enhancement factor of 1.17 with 6 MV photons. As well as this, there are also differences in protocols between research groups in the maintenance of their cells, as well as slight variations in the protocols for the assays.

Therefore the aim of this study was to parameterise our cell experiments to investigate the effect of cell line, NP type and radiation type on the observed radiation damage enhancement. It was hypothesised that the addition of NPs would cause an enhancement effect for both photon and proton irradiations. Another was that NPs with a higher Z would show greater enhancement as demonstrated in the MC simulations. Finally, a difference due to cell line was expected due to differences in radiosensitivity, which would then cause differences in the enhancement effect.

### 4.2 Variables

To gain an understanding of the biological impact of introducing NPs into a cell, three different variables were considered. These variables were: irradiation type,
NP material and cell type.

4.2.1 Irradiation type

From the literature it is evident that the radiation type will influence the enhancement effect. When considering a radiobiological study this becomes more significant as particle therapy has a higher relative biological effectiveness (RBE) and linear energy transfer (LET). Considering proton therapy, which offers a more localised dose deposition compared to X-ray radiotherapy, a higher biological effect can be observed compared to X-ray radiotherapy, due to a higher RBE and LET. X-ray radiotherapy has an average LET of approximately 0.2 keV/µm compared to between 1 and 2 keV/µm for protons [147]. In proton therapy it is common practice to adopt an RBE of 1.1 relative to X-ray radiotherapy with photons. It is known that this value changes and is greater than 1.1 at the distal edge of the Bragg peak (BP), however this generic value is still used [148]. When considering heavier ions such as carbon, the RBE becomes more significant. With carbon, this increased RBE is also related to a decrease in radioresistance in hypoxic tumours, leading to a decrease in the oxygen enhancement ratio (OER) [149].

With this difference in biological effect, it is of particular interest to see if the use of NPs with photons can make the biological effect comparable to that observed with protons without NPs. Another factor was difference in beam energy, where two proton energies were considered, representative of high (11 MeV with a LET of approximately 12 keV/µm) and low (60 MeV with a LET of approximately 1 keV/µm) LET, compared to 6 MV photons.

4.2.2 Nanoparticle type

As the NP material can affect the radiosensitisation effect, it was decided that two commercially available NP types would be investigated. The first were spherical AuNPs (Aurovist™) (1.9 nm), with a thiol coating and the second were spherical gadolinium nanoparticles (GdNPs) (AGuIX®) (∼ 3 nm), comprised of a polysiloxane matrix with cyclic chelates of gadolinium as shown in figure 4.1. Both NP materials have a sufficiently high atomic number to observe the predicted
Figure 4.1: Design of AGuIX ® nanoparticle, comprised of a polysiloxane matrix with cyclic chelates of gadolinium [8]

radiosensitisation effect [150, 151].
4.2. Variables

The final variable, was the cell type, which can determine where NPs may be most beneficial. Two cell types were considered, MCF-7, a human breast adenocarcinoma (figure 4.2a) cell line and U87 (figure 4.2b), a human glioblastoma cell line. The reasons for using these cell lines are explained in sections 4.2.3.1 and 4.2.3.2. In terms of our investigation, the cell type influences many other variables. Depending on the cell, the uptake of NPs can differ, as different cell types have different surface to volume ratios, which in turn will lead to differences in NP uptake as previously discussed in section 1.5.4. This was demonstrated by Dos Santos et al, where they investigated the level of uptake in five different cell lines, with different sizes of NPs [152]. With 1 \( \mu \)m NPs at a concentration of 20 \( \mu \)g/ml (24 hour incubation) they used flow cytometry to show the probability of finding a number of particles in a cell, where HCMEC/D3 cells showed a probability of 60% to internalise more than 3 particles compared to 5% with HeLa cells.

Another aspect is that different cells will respond to radiation differently, where
some are more radioresistant, in which case the use of NPs is of particular interest as it may radiosensitise the cells. The reason for differences in radiosensitivity between cells are due to many factors such as differences in ability to repair damage (section 1.5.5.3). Another factor is if the cells are hypoxic, as these are more radioresistant [153]. Finally, the sensitivity also varies with the phase of the cell-cycle [24]. It should also be noted that different cell lines express different surface proteins which may attach to NPs in different amounts [154].

With this work only cancer cell lines were used, where controls were taken to be cells without NPs or without radiation or both. However, an extension of this work would be to include healthy cells. The benefit of this would be to determine the effect of NPs on healthy cells, as well as the effect of a combined treatment of NPs with radiation. Possible cell lines that could have been used were MCF-127 for human breast cells [155] and HCN-2 for human brain cells [156].

4.2.3.1 MCF-7
This cell line was derived from a Caucasian female with metastatic mammary carcinoma [157]. It is a well established cell-line within the field of breast cancer research and has contributed to the improvement of patient outcomes [158]. When studying cancers, specific genes are identified with varying functions. One of these is p53, a tumour suppressor gene, that regulates a range of cellular responses. This is a gene that has been shown to lead to cancer when mutated [159]. Another gene is the wild type p53, where the growing expectation is that tumour cells that express this gene should be more susceptible to cytotoxic treatments than those expressing mutant p53 genes [160, 161]. It has been shown that MCF-7 cells have a wild type p53 gene [162].

Within the literature, this cell line has not been reported as being radioresistant. It is possible however to work with a derivative of this cell line which corresponds to radioresistant cells, known as MCF-IR20 [163].

For the purposes of this work, it was decided that MCF-7 cells would be used, as this has been the most extensively used breast cancer cell line, is well characterised and would be suitable for a parametric in vitro study with NPs.
4.2.3.2 U87

This human glioblastoma multiforme (GBM) cell line was derived from a Caucasian male with a tumour resected intraoperatively. This cell line was characterized by Ponten et al [164] and is now one of the most commonly used cell lines within the neuro-oncology research field [165]. As with the MCF-7, U87 cells also express a wild-type p53 tumour suppressor gene, but not a mutant p53 [166].

For this work it was of particular interest to work with a radioresistant cell line, to determine if NPs could offer a beneficial effect. As such, U87 cells were well suited as they were both radio and chemo-resistant [167, 168], meaning that any demonstrated benefit of treatment with NPs could lead to an improvement in patient outcomes if translated into the clinic. This cell line has also been well documented in the literature, allowing for comparisons to be made between our work and that of others in the field.

4.2.4 NP uptake

Although NP uptake is not considered as part of this investigation, it must be noted that this is influenced by both the NP and cell types, and will therefore impact the level of enhancement. A study by Fang et al, considered the use of iron oxide nanoparticles (IONPs), where they demonstrated the level of uptake of these NPs in both U87 and MCF-7 cells [169]. With this work, they seeded cells onto 12-well plates and incubated with 50 µg/ml of NPs (hydrodynamic size of 44 nm) for 2 hours, then conducted flow cytometry to determine the uptake. From this work they showed a preferential uptake in U87 cells compared to MCF-7 cells (0.6 compared to 0.12 pgFe/cell). With this study fluorophores were conjugated onto the NPs, therefore it is difficult to determine if these results are representative of the uptake expected of NPs used for therapeutic purposes. However, the study does demonstrate differences in uptake with cell line, where for our future work it would be of interest to determine uptake variations with different NP and cell types.
4.3 Clonogenic assay

In terms of biological measurements, the gold standard within radiobiology is the clonogenic assay. This is a cell survival assay which considers the ability of a single cell to grow and form a colony, where a colony is defined as a cluster of cells with a minimum of 50 cells in close proximity to each other [170]. It is general practice to use the clonogenic assay when assessing the usability of a new drug or treatment in the early stages of an investigation, as it quantifies cell survival post treatment. Before the clonogenic assay can be carried out, it is necessary to initially determine the plating efficiency (PE) for each cell line with no radiation dose. This is described by equation 4.1, which shows that PE is the capability of the cell line to produce colonies from a single cell suspension.

\[
PE = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100 \tag{4.1}
\]

In terms of the clonogenic assay, it is based on determining the surviving fraction (SF). In radiobiology this can be described by the linear-quadratic (LQ) model where the SF is defined by equation 4.2,

\[
SF = e^{-(\alpha D + \beta D^2)} \tag{4.2}
\]

where alpha and beta are fitting parameters and D is the dose. Alpha describes the initial slope, the linear part of the curve, whereas beta describes the curvature, the quadratic part. Considerations need to be given to the dose range when planning a cell survival experiment, as the LQ model depends on the range for which the dose response curve is fitted to. Difficulties arise at both low doses (< 1 Gy), due to a low level of cell inactivation and at high doses (>10 Gy), due to low cell survival [171, 172].

From the literature it is possible to calculate a prediction for the SF by finding alpha and beta parameters specific to the cell line being considered. These parameters are then put into equation 4.2, along with the relevant doses being considered to calculate the SF for each respective dose.
Another equation for SF is

\[ SF = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded} \times \text{PE}} \] (4.3)

where by using the predicted SF and PE the equation can be rearranged to determine the approximate number of cells that need to be seeded to form approximately 50 colonies per well.

After conducting the cell experiment it is then possible to use equation 4.3 to determine the SF for each respective dose, such that a cell survival curve can be produced and all relevant parameters can be identified.

4.3.1 Clonogenic assay use in the literature for NP enhanced radiation therapy

With the use of photons, a study by Butterworth et al reported their cell survival findings with MCF-7 cells combined with Aurovist™ 1.9 nm AuNPs and irradiated with 160 kVp photons [94]. They investigated two concentrations (incubated 1 hour prior to irradiation) of NPs and demonstrated DEFs for both. At a concentration of 10 µg/ml the DEF was 1.41 and at 100 µg/ml it was 1.09.

Another study by Kong et al also used MCF-7 cells, here however they used two types of functionalised AuNPs (cysteamine or thio-glucose), at a concentration of 0.75 mg/ml and irradiated with 200 kVp photons [173]. Using the clonogenic assay they found complete cancer cell death after 5 days using thio-glucose bound AuNPs, compared to a 43 % survival in the control.

A study by Stefancikova et al used U87 cells with GdNPs and irradiated with 1.25 MeV photons from a cobalt 60 source. With a concentration of 0.5 mg/ml (6 hour incubation prior to irradiation), they showed an enhancement factor of 22.6 % with NPs [174].

In terms of clonogenic studies carried out with proton irradiation, a study was carried out by Chaudhary et al using U87 cells at 6 different positions along the BP to demonstrate the variations in RBE for both pristine and SOBPs [175]. This study showed an underestimation in the biologically effective dose (physical dose × RBE
Chapter 4. Measurement of NP enhancement in a biological system

of approximately 18% when considering a fixed RBE of 1.1, as explained in section 4.2.1. Although no NPs were used in this study, it demonstrates the variations in RBE when using proton irradiation. This highlights the need for different energies to be investigated with protons, to determine how NPs will impact these variations relative to the control.

In a recent review, Paganetti summarised RBE values for clonogenic cell survival to demonstrate the relationship between RBE, dose and proton beam characteristics [176]. This review highlighted the need for standardisation in reporting parameters for cell survival fittings, such that comparisons can be made between photons and protons. The specific parameters mentioned were $\alpha$ and $\beta$ for both photons and protons, as well as the dose averaged LET at the position of the biological sample, all of which will be tabulated in this work.

### 4.4 53BP1 assay

Another measurement that was considered was the 53BP1 assay, which looks to quantify clustered deoxyribonucleic acid (DNA)-damage through the marker 53BP1 [177, 178]. Initially the number of foci per cell are quantified at 2 time points, one fixed 30 minutes after irradiation, one 24 hours post-irradiation, along with a control, all quoted as averages. Following on from this, histograms were produced to demonstrate the spread of the foci counts, as the average may mask if there are two peaks on the histogram.

In terms of the literature, a study by Stefancikova et al used U87 cells combined with 0.85 mg/ml of GdNPs at multiple incubation times (1, 4, and 24 hours) to investigate the effects of GdNPs on DNA damage. Initially they determined the cellular uptake using confocal microscopy and transmission electron microscopy (TEM), demonstrating the NPs being localised to the cytoplasm, at different time points (1, 4 and 16 hours) where NPs were internalised after 1 hour and longer incubation times had no effect on uptake. They then irradiated their samples with a cobalt 60 source (mean energy of 1.25 MeV) at doses of either 1 or 4 Gy, considering the average foci per cell across 8 hours post-irradiation. They
demonstrated no significant difference on nuclear DNA by using GdNPs with radiation compared to radiation alone. They therefore concluded that GdNPs may amplify radiation-induced cell killing through effects independent of the DNA [179].

In contrast, Chithrani et al looked at HeLa cells with 50 nm AuNPs (50 ng/ml with 24 hour incubation) irradiated with 220 kV and 6 MV photons at 4 Gy [39]. Considering two time points of 4 and 24 hours they demonstrated an increase in the number of double strand breaks (DSBs) for both energies, with a higher increase at the lower energy.

This part of the work looks to quantify the level of DNA damage due to the use of NPs with ionising radiation, with specific consideration to the effects of radiation type (proton at two LET values vs photon), cell line (MCF-7 and U87) and NP type (Gd and Au).

4.5 Gene expression measurements

The final measurement was the extraction of ribonucleic acid (RNA), conducted as part of a larger study investigating the gene expression. The extraction of RNA is conducted as part of the microarray analysis to determine which genes are up or down regulated following treatment [180]. This is of particular interest, as this gives further insight into the effects of NPs on cells, demonstrating the mechanisms involved in NP enhanced radiotherapy, which is not currently understood as explained in section 1.5.5. Considering cell survival alone is insufficient in investigating the effect of NPs, as certain cellular stress can be caused which may not lead to cell death. This needs to be considered for both irradiated and unirradiated samples with and without NPs, as it has not been extensively documented within the literature. Although the analysis of the genes expressed was not conducted as part of this thesis, the sample preparation and treatment was conducted, along with RNA measurements for all the samples.

A study by Stassen et al investigated the effects of radiation on MCF-7 cells after irradiation with 240 kV photons at a dose of 2 and 6 Gy [181]. They
demonstrated a dose-dependent gene expression for six genes due to radiation. Three of these had already been quoted in the literature (GLUT-1, PKCI and WAF-1), whereas three had not been previously shown to be radiation-induced genes (ISGF3G, MRP8 and PSME3).

Another study by Amundson et al extensively characterised the radiation response of over 60 cell lines, comparing both cell survival and gene expression responses. For the gene expression, the measurements were carried out 4 hours after irradiation with 8 Gy from a $^{137}\text{Cs}$ source. They showed a decreased expression of mitosis-associated genes (PLK1, AURKA, CENPA and PPP2R5A) across the different cell lines tested. They also demonstrated specific genes up regulated in cell lines with a wild-type p53. It was suggested that these findings highlight regulatory networks, in response to radiation, for the cells tested, which can be exploited by drugs to enhance radiotherapy [182].

This part of the study aims to produce and treat the samples for gene expression measurements, where the extraction of RNA is carried out. This work is part of a larger study, where future work involves the analysis of genes expressed due to the combination of NPs with ionising radiation. Currently within the literature no study has investigated the specific genes that are overexpressed with the combination of NPs and radiation.

### 4.6 Materials and methods

The full protocols for cell maintenance, subculture, fixing, staining, counting, and assays can be found in the appendices section. Within this section, an overview of the methods needed for the assays will be given.

#### 4.6.1 Cell culture

Cell lines were cultured in Minimum Essential Medium (MEM) (Fisher Scientific, UK), supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, UK) and 1% penicillin-streptomycin (Fisher Scientific, UK). Cells were maintained in a Heracell™ VIOS 160i CO2 incubator (Thermo Fisher Scientific, Inc), at 37 °C with 5% CO$_2$/95% air.
4.6. Nanoparticles

Two types of nanoparticles were investigated, the first were freeze-dried spherical AuNPs (Aurovist™) with a diameter of 1.9 nm (Nanoprobes Inc, NY, USA) [183] that were re-dispersed in phosphate buffered saline (PBS) (Fisher Scientific, UK) and stored at −20 °C as per the manufacturer’s instructions. The second were freeze-dried gadolinium based NPs, comprised of a polysiloxane matrix with cyclic chelates of gadolinium (AGuIX®), making NPs with a diameter of approximately 3 nm (NH TherAguix, France) [184]. These were dispersed in ultrapure water (Fisher Scientific, UK) and stored in a refrigerator at 4 °C. Both of these stock solutions were diluted in culture media to a concentration of 0.5 mg/ml, such that they could be added to the NP samples.

4.6.3 Nanoparticle toxicity

To ensure that the concentration of NPs introduced into the cell culture was non-toxic, it was necessary to seed a fixed number of cells (MCF-7 = 1000 cells per well, U87= 5000 cells per well) onto a six-well plate (Sigma, UK) and introduce different concentrations of the chosen nanoparticle. For each cell line two plates of cells were seeded, one for each nanoparticles. Both were left in the incubator for 24 hours for the cells to attach. From the literature it was reported that a concentration of 0.5 mg/ml (24 hour incubation) was not toxic and offered a good ratio between concentration and enhancement effect for MDA-MB-231 cells with AuNPs [88]. Therefore this and a lower concentration of 0.2 mg/ml were added to the cells to test for toxicity. After 24 hours of introducing the NPs, the medium and nanoparticle mixture was removed. The cells were then washed twice with PBS and then each well was filled with 5 ml of fresh medium. The plates were then incubated for 14 days. Following this, the plates were fixed and counted as will be described within the plating efficiency section 4.6.4.

4.6.4 Plating efficiency

For both cell lines, confluent flasks were trypsinised and counted. This stock cell solution was diluted into different cell densities and seeded onto six-well plates,
where each cell line had six densities plated in size plates, meaning each cell density had six separate wells representing the cell density. For MCF-7 the number of cells seeded per well were, 100, 200, 500, 1000, 2000 and 5000 cells. With U87 it was necessary to seed more cells as few colonies were formed in an initial test. As such the densities seeded per well were 5000, 7500, 10000, 20000 and 25000 cells. Following this, the plates were kept in an incubator for 10−14 days to allow sufficient time for colonies to form. The plates were then removed from the incubator, where the medium was removed and the plates were washed twice with PBS. Following this, colonies were then stained with 0.4 % crystal violet and manually counted using a Nikkon Eclipse Ti-S inverted microscope. The full protocols for staining can be found in appendix E.

4.6.5 Pre-irradiation

As irradiations were planned for photons and protons it was necessary to select a suitable surface to seed cells onto and keep this consistent for comparisons to be made. It had been decided that proton irradiations would be carried out at the Clatterbridge Cancer Centre, where a setup had been devised with a specialised sample holder, capable of irradiating multiple samples by using a translation stage. This holder was designed for 35 mm culture dishes (Corning, UK), which were used in both photon and proton experiments to allow for comparisons to be made. Initial experiments were carried out with these dishes to determine the amount of solution and number of cells needed to achieve near confluence on the day of irradiation. From these experiments it was decided that cells would be seeded at a density of 150,000 cells/ml, where 2 ml of cell solution was added to each dish (figure 4.3).

Cells were cultured onto the dishes 48 hours before irradiation such that they would reach between 80-100% confluence on the day of irradiation. 24 hours before irradiation, the media was changed for the control samples. The NP samples had media containing NPs added, at a concentration of 0.5 mg/ml. A 24 hour incubation was used as groups such as Trono et al have shown an increase in cellular uptake with increasing incubation time, with various cell lines such as PK-45, and Panc-1 and 20 nm AuNPs [185].
4.6. Materials and methods

Figure 4.3: Petri dishes with cells seeded at a cell density of 150,000 cells/ml with 2 ml of cell solution added to each dish

Following a 24 hour incubation, the NP solution was removed, the samples were gently washed with PBS, removing any extracellular NPs and fresh medium was added to each dish.

Before irradiating with photons it was necessary to fill the dishes with medium (figure 4.4 a) for dosimetry purposes, to account for full scattering conditions. These were then sealed using Parafilm ® M (Sigma, UK) immediately before irradiation (figure 4.4 b). It should be noted that some variability in dish fill could have occurred when sealing the dishes, however efforts were made to ensure each dish was completely filled. With proton irradiations this was not necessary due to differences in beam direction, where protons would interact initially with the cells attached the bottom of the dish, whereas with photon irradiation the photons would traverse through the medium before interacting with the cells.
4.6.6 Irradiations

4.6.6.1 Photon irradiations

With the photon irradiations it was decided that for the clonogenic work, 5 dose points would be investigated in triplicate, 0, 1.5, 3, 4.5, and 6 Gy. These dose points were chosen to give a sufficient cell survival curve fitting. Dose points were investigated for each cell line and nanoparticle combination. For both the 53BP1 marker and the gene expression samples, irradiations were carried out with a dose of 2 Gy, as this is typically the amount of dose given in a radiotherapy fraction. The dishes were irradiated at the National Physical Laboratory, Teddington, UK with a 6 MV linac (Elekta Versa HD™), with a dose rate of 6.5 Gy/min. The samples were placed on the patient couch on top of a stack of 10 cm solid water slabs, as can be seen in figure 4.5, to provide adequate scattering conditions. The samples were placed in the centre of a 10 x 10 cm field size to ensure that each dish was covered. The dose calculation was based on reference conditions using depth dose data from ionisation chamber measurements for this field size. The dose output was also confirmed with ionisation chamber measurements.
4.6. Materials and methods

To determine the field uniformity, gafchromic films were used and placed under the dish, between the water slab and the dish. Films were irradiated (figure 4.6) and then analysed to determine the optical density, where film handling and analysis was carried out in a similar manner to the work described in section 2.4.2.2. From multiple measurements the optical density was determined, where the average value across 4 separate experiments was $109 \pm 1.4$. 

*Figure 4.5:* Experimental setup for clonogenic assay irradiations using photons, where the sample was placed on blocks of solid water in a 10 x 10 cm field.
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4.6.6.2 Proton irradiations

Irradiations were carried out at the Clatterbridge Centre for Oncology proton beam line, Bebington, UK, which has an energy of 62 MeV and is a clinically calibrated unit with a well defined monitor unit (MU) to dose calibration for each LET.

With proton irradiation, it is known that different points of the Bragg peak have different values of LET. It was therefore of interest to investigate two points across the BP, one within the build-up region (low LET region using approximately 60 MeV beam giving an LET of $\sim 1 \text{ keV/\mu m}$) and another around the peak (high LET region using approximately 11 MeV beam giving an LET of $\sim 12 \text{ keV/\mu m}$) (dose rate of approximately 5 Gy/min). With regards to the doses for the proton clonogenic work, 5 dose points were considered, 0, 1, 2, 3 and 5 Gy, as from the literature this dose range was shown to be sufficient in fitting the cell survival with proton irradiation. For the 53BP1 marker irradiations were carried out with a dose of 2 Gy.

In terms of dish placement, the setup differed as the beam direction was horizontal compared to the vertical beam used with photons. This horizontal beam is due to the fact that irradiations were carried out at a centre which specialises in

Figure 4.6: Gafchromic film post-irradiation, where the film was placed under the dish being irradiated.
4.6. Materials and methods

Figure 4.7: Experimental setup for clonogenic assay irradiations using protons

treating ocular cancers. This centre had a specialised setup capable of irradiating multiple biological samples due to the use of a translation stage as can be seen in figure 4.7. Here, it was important to ensure that the samples were secured with Parafilm to prevent leakage of medium, as samples would be held vertically whilst being irradiated.

4.6.7 Post-irradiation

Following irradiation the samples were analysed depending on the protocol for the specific assay. Tables 4.1, 4.2 and 4.3 give a summary of samples seeded to account for all the variables that were considered. A complete dataset for each dose point or fixation time point, would have 9 dishes per cell type, 3 controls, 3 with AuNPs and 3 with GdNPs NPs. With the clonogenic and 53BP1 proton irradiations, due to time constraints it was decided that only GdNPs would be considered. When seeding cells for both clonogenic and 53BP1, there were issues with cell confluence, particularly with MCF-7s, therefore the number of samples were reduced as shown in tables 4.1 and 4.2, where no low LET measurements were taken for the clonogenics and no low LET measurements for MCF-7s with the 53BP1, with reduced numbers for both assays with high LET measurements. With the gene expression work, the microarray could hold 8 samples at a time, therefore
Chapter 4. Measurement of NP enhancement in a biological system

Table 4.1: Summary of the dishes that were seeded for clonogenic assays, covering all types of cells, NPs and radiation type. The NP concentration used for both AuNPs and GdNPs was 0.5 mg/ml (24 hour incubation). A complete dataset for each dose point would have 9 dishes per cell type, 3 controls, 3 with AuNPs and 3 with GdNPs. The total number of samples prepared was 123.

<table>
<thead>
<tr>
<th>Radiation type</th>
<th>Dose (Gy)</th>
<th>Dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AuNPs</td>
</tr>
<tr>
<td></td>
<td>MCF-7 U87</td>
<td>MCF-7 U87</td>
</tr>
<tr>
<td>Photon 6 MV</td>
<td>0</td>
<td>3 3</td>
</tr>
<tr>
<td>MV with dose rate of 6.5 Gy/min</td>
<td>1.5</td>
<td>3 3 3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3 3</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>3 3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3 3</td>
</tr>
<tr>
<td>Proton</td>
<td>0</td>
<td>1 2</td>
</tr>
<tr>
<td>High LET ∼ 11 MeV</td>
<td>1</td>
<td>1 1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2 2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2 2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2 1</td>
</tr>
</tbody>
</table>

Table 4.2: Summary of the dishes that were seeded and Immunostained for the DNA damage marker 53BP1. The NP concentration used for both AuNPs and GdNPs was 0.5 mg/ml (24 hour incubation). A complete dataset for each fixation point would have 9 dishes per cell type, 3 controls, 3 with AuNPs and 3 with GdNPs. The total number of samples prepared was 96.

<table>
<thead>
<tr>
<th>Radiation type</th>
<th>Dose (Gy)</th>
<th>Fixation time post-irradiation</th>
<th>Dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AuNPs</td>
<td>GdNPs</td>
</tr>
<tr>
<td></td>
<td>MCF-7 U87</td>
<td>MCF-7 U87</td>
<td>MCF-7 U87</td>
</tr>
<tr>
<td>Photon 6 MV</td>
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<td>24 hours</td>
<td>3 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30 minutes</td>
<td>3 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24 hours</td>
<td>3 3</td>
</tr>
<tr>
<td>Proton</td>
<td>0</td>
<td>24 hours</td>
<td>1 3</td>
</tr>
<tr>
<td>High LET ∼ 11 MeV</td>
<td>2</td>
<td>30 minutes</td>
<td>1 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24 hours</td>
<td>1 3</td>
</tr>
<tr>
<td>Proton Low</td>
<td>0</td>
<td>24 hours</td>
<td>3</td>
</tr>
<tr>
<td>LET ∼ 60 MeV</td>
<td>2</td>
<td>30 minutes</td>
<td>3</td>
</tr>
<tr>
<td>MeV</td>
<td>2</td>
<td>24 hours</td>
<td>3</td>
</tr>
</tbody>
</table>

for our initial investigation, 24 samples were prepared as highlighted in table 4.3. For all samples containing NPs, a 24 hour incubation with NPs+medium was carried out, with a concentration of 0.5 mg/ml.
4.6. Materials and methods

Table 4.3: Summary of the dishes that were seeded for gene expression measurements, covering all types of cells and NPs. The NP concentration used for both AuNPs and GdNPs was 0.5 mg/ml (24 hour incubation). Irradiations were only carried out with photons. The total number of samples prepared was 24.

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Control MCF-7</th>
<th>Control U87</th>
<th>AuNPs MCF-7</th>
<th>AuNPs U87</th>
<th>GdNPs MCF-7</th>
<th>GdNPs U87</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
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<tr>
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<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

4.6.7.1 Clonogenic survival assay

For the clonogenic assay, each flask was washed twice with PBS, trypsinised, counted and then replated onto six-well plates, at cell densities that would give approximately 50 colonies per well. A spreadsheet was created to calculate the dilution needed for each sample, by using equation 4.3 and using the approximation for the SF from equation 4.2. Plates were incubated for 10-14 days. As with the PE, plates were stained with 0.4 % crystal violet and manually counted. The full protocol can be found in appendix E.

Using the counted number of colonies, equation 4.3 could then be used to determine the SF and produce cell survival curves for each variable considered. The curves were then fitted to the LQ model (section 4.3) using MATLAB® R2017a (MathWorks, Natick, MA, USA), to determine the fitting parameters $\alpha$ and $\beta$ from equation 4.2. As with the simulations, it was possible to determine the DEF. Here however, as we are considering cell survival, the ratio is of the doses needed to give the same SF as that of the control group for a dose of 2 Gy was calculated. Ratios were determined using the SF fits, which were fitted using a non-linear least-squares fitting procedure, weighted to the errors associated with each measurement. It was also possible to determine the $\alpha/\beta$ ratio, to demonstrate any changes to survival curves when introducing NPs into cells before irradiating. In both cases, statistical significance was determined using a t-test with the statistical software SPSS (IBM Corp).
4.6.7.2 53BP1 foci formation assay

For the staining of the DNA damage marker 53BP1, the samples were split into their respective groups post-irradiation, as some needed to be fixed 30 minutes after irradiation, whereas others were fixed after 24 hours. Samples that were not fixed 30 minutes after irradiation, were stored in the incubator for 24 hours, where they were fixed in the same manner. To fix the samples (appendix D), a fixing solution was prepared (methanol:acetic acid) and stored in the freezer beforehand. Medium was removed and each dish was washed twice with PBS. Fixing solution was added to each dish and left for 30 minutes in the fridge. Solution was then removed and replaced with PBS to keep samples from drying. Samples were stored in the fridge until the immunofluorescence staining for the DNA damage marker 53BP1 was carried out.

In terms of the staining, initially the samples were washed, then a permeabilisation buffer was added and left for 10 minutes. The buffer was then removed, the dish was washed and the blocking buffer was added, after which the sample was placed in the incubator for 2 hours. This was removed and the primary antibody was added and the sample was incubated for an hour. The sample was then washed three times, 5 minutes for each wash. The secondary antibody was added (sample covered in foil as the antibody is light sensitive) and incubated for an hour. It was washed three times again before adding the DAPI staining. The sample was then imaged with the CellInsight CX5 High Content Screening Platform (Thermo Fisher Scientific, Inc), where one channel would show the DAPI staining, identifying the cells, the other channel shows the anti-body staining, highlighting the foci within the cells. The sample was imaged from multiple fields, where the foci per cell were then manually counted. To achieve statistical significance, 50 cells per sample were analysed and the number of foci in each cell was recorded. As mentioned in 4.6.7, due to a low number of cells, it was not possible to have three samples for each timepoint with protons. Therefore for proton samples with a reduced number of samples, more cells were counted such that all plots represented the foci per cell, where 150 cells were counted for each quoted result. The full
4.7 Results

protocol for fixing and staining can be found in appendix D and F.

Using the counted foci it was possible to plot the average number of foci per cell for each respective timepoint and treatment. Histograms demonstrating the spread of the number of foci were also produced. Statistical significance was determined using a t-test.

4.6.7.3 Gene expression measurements

This part of the work was a collaborative effort, where I carried out the sample preparation, addition of NPs, treatment with photons and harvesting of cells at the National Physical Laboratory, Teddington, UK. The measurement of RNA concentration and purity, as well as the actual microarray, gene expression measurements, were carried out at the Joint Research Centre (JRC), Ispra, Italy.

Post-irradiation (30 minutes), the dishes were gently washed twice with PBS. Once the PBS was removed, RLT plus RNase lysis buffer (Qiagen, USA) was added to each sample to lyse the cells, breaking down the membrane to allow for extraction of the RNA. Samples were then stored at \(-80^\circ C\) until they needed to be shipped to Italy, where they were processed by Alessia Bogni.

4.7 Results

4.7.1 Clonogenic variations due to the addition of NPs

4.7.1.1 Photon irradiation

For U87 it was found that both Gd and AuNPs caused an increase in the level of cell kill, the most significant was with GdNPs showing a DEF of 2.04 ± 0.53 (p = 0.030) as reported in table 4.4 and shown in figure 4.8a. Although an enhancement was found with AuNPs (1.28 ± 0.52), it was determined to not be statistically significant due to the errors (standard deviation) associated with the SF.

With MCF-7s both NPs showed statistically significant changes to the survival curve (figure 4.8b), with DEFs of 1.49 ± 0.08 and 1.28 ± 0.13 for AuNPs and GdNPs respectively.

\(\alpha\) and \(\beta\) values were determined from the curve fittings, whereby the \(\alpha/\beta\) ratio
was calculated for each curve. Significance was tested between the ratios of samples containing NPs to those without NPs as reported in table 4.5. With the U87 cells, it was found that the addition of NPs increase the ratio, with significance shown with both NPs, with an $\alpha/\beta$ ratio of $58.6 \pm 4.5$ (p<0.001) and $14.5 \pm 1.1$ (p=0.002) for GdNPs and AuNPs respectively compared to a ratio of $7.8 \pm 1.2$ for cells alone. For the MCF-7 cells, both NPs had ratios significantly different from NP-free cells, with $\alpha/\beta$ ratios of $77.7 \pm 3.5$ (p<0.001) and $44.2 \pm 2.4$ (p<0.001) for AuNPs and GdNPs respectively compared to a ratio of $11.1 \pm 0.2$ for cells alone. An increase in the $\alpha/\beta$ ratio signifies that tumour response is less dependent on the dose per fraction, therefore a lower dose can be used [186].
4.7. Results

(a) U87

(b) MCF-7

Figure 4.8: Cell survival curves for a) U87 and b) MCF-7, where for both cell lines, cells + AuNPs or cells + GdNPs were assessed, as well as a control of cells alone. Samples were irradiated with photons from a 6 MV linac.
Table 4.4: Dose enhancement factors tabulated for all samples, along with a p-value highlighting significance between NP-free control and NP samples, based on a t-test

<table>
<thead>
<tr>
<th>Sample</th>
<th>DEF</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 Cells + AuNPs</td>
<td>1.28 ± 0.52</td>
<td>0.458</td>
</tr>
<tr>
<td>U87 Cells + GdNPs</td>
<td>2.04 ± 0.53</td>
<td>0.030</td>
</tr>
<tr>
<td>MCF-7 Cells + AuNPs</td>
<td>1.49 ± 0.08</td>
<td>0.004</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs</td>
<td>1.28 ± 0.13</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Table 4.5: Alpha and Beta values used for the survival curve fitting for each sample, along with a p-value highlighting significance between control and NP samples, based on a t-test

<table>
<thead>
<tr>
<th>Sample</th>
<th>α (Gy(^{-1}))</th>
<th>β (Gy(^{-2}))</th>
<th>Ratio (Gy)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 Cells</td>
<td>0.2723 ± 0.1146</td>
<td>0.03495 ± 0.03816</td>
<td>7.8 ± 1.2</td>
<td>0.002</td>
</tr>
<tr>
<td>U87 Cells + AuNPs</td>
<td>0.3792 ± 0.4132</td>
<td>0.02614 ± 0.008</td>
<td>14.5 ± 1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U87 Cells + GdNPs</td>
<td>0.6982 ± 0.256</td>
<td>0.01191 ± 0.054</td>
<td>58.6 ± 4.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells</td>
<td>0.3436 ± 0.0191</td>
<td>0.0308 ± 0.0067</td>
<td>11.2 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells + AuNPs</td>
<td>0.6033 ± 0.0381</td>
<td>0.0078 ± 0.0271</td>
<td>77.7 ± 3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs</td>
<td>0.5187 ± 0.0937</td>
<td>0.01174 ± 0.0282</td>
<td>44.2 ± 2.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

4.7.1.2 Proton irradiation

Although proton irradiations were planned and carried out, no results were obtained from the clonogenic assays with either cell line. Two separate visits to the Clatterbridge Center were carried out, however issues were faced with both.

During the first visit the cells were seeded at the relevant densities and were left in Clatterbridge to incubate for 10 days. Following the staining of the plates, it was evident that the cells had become over-confluent and had peeled away from the surface of the well (figure 4.9a), meaning colonies could not be counted.

During the second visit, fewer cells were seeded to account for the over-confluence, where half the number of cells were seeded for both the wells planned to give approximately 50 colonies (A1 to A3) and those giving approximately 100 colonies (B1 to B3). A week later it was found that the plates were all contaminated and no results could be taken as all the wells in the plates had been affected (figure 4.9b)
4.7. Results

Figure 4.9: Two separate proton experiments were conducted, each of which had issues associated with it. A) Over confluent cells which lead to them growing on top of each other and die before before staining. B) Plates were contaminated and cells were no longer viable.
4.7.2 Change in foci count of DNA damage markers due to the addition of NPs

For U87 it was found that both NPs caused a significant increase in the level of DNA damage in the form of foci counts/cell for each radiation type tested. Figure 4.10 demonstrates the average number of foci for both photon and proton irradiation with and without NPs for both cell lines. From this plot it is evident that the introduction of NPs causes a statistically significant increase in the number of foci/cell when compared to samples without NPs. It was noted however, that by only quoting the average foci/cell, the underlying effects of using NPs or irradiating with protons may be masked. As such, histograms were produced to demonstrate the spread of the number of foci/cell (figure 4.11). It should be noted that only the 30 minute data was used for the histograms, as both the control and 24 hour data had too few foci to demonstrate differences with histograms. These low counts are reported in tables 4.6 and 4.7.
4.7. Results

Figure 4.10: Average foci counts per cell, for cells + AuNPs, cells + GdNPs and cells alone as a control, for a) U87 and b) MCF-7 cell lines, demonstrating both photon and proton irradiations at 2 respective time points. NPs incubated for both cell lines for 24 hours at a concentration of 0.5 mg/ml. Significance demonstrated by *** for p < 0.001, ** for p < 0.01, * for p < 0.05 and no significance for p > 0.05.
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(a) U87 cells alone, comparing photon (6 MV) and high LET proton irradiation (11 MeV)

(b) U87 cells with and without GdNPs irradiated with photons (6 MV)
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(c) U87 cells with and without AuNPs irradiated with photons (6 MV)

(d) U87 cells with and without GdNPs irradiated with high LET protons (11 MeV)
(e) U87 cells with and without GdNPs irradiated with low LET protons (60 MeV)

(f) U87 cells alone, comparing photon (6 MV) and low LET proton irradiation (60 MeV)
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(g) MCF-7 cells alone, comparing photon (6 MV) and high LET proton irradiation (11 MeV)

(h) MCF-7 cells with and without GdNPs irradiated with photons (6 MV)
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(i) MCF-7 cells with and without AuNPs irradiated with photons (6 MV)
(j) MCF-7 cells with and without GdNPs irradiated with high LET protons (11 MeV)

Figure 4.11: Histograms demonstrating the spread of values for the number of foci per cell, for cells + AuNPs, cells + GdNPs and cells alone as a control, irradiated with high and low LET protons and photons for U87 and MCF-7 cell lines, fixed 30 minutes post-irradiation.

The values for foci have been tabulated for U87 and MCF-7 in tables 4.6 and 4.7 respectively. Statistical significance has been summarised in table 4.8 and the effects are discussed in sections 4.7.2.1 to 4.7.2.3.
### Table 4.6: Average foci count per cell for cells + AuNPs, cells + GdNPs and cells alone as a control, for U87

<table>
<thead>
<tr>
<th>Sample</th>
<th>Photon</th>
<th>Proton</th>
<th>Photon</th>
<th>Proton</th>
<th>Photon</th>
<th>Proton</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 Cells</td>
<td>NA</td>
<td>1 ± 1</td>
<td>NA</td>
<td>1 ± 1</td>
<td>NA</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>U87 Cells + AuNPs</td>
<td>2 ± 2</td>
<td>2 ± 2</td>
<td>2 ± 2</td>
<td>2 ± 2</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>U87 Cells + GdNPs</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>4 ± 2</td>
<td>4 ± 2</td>
</tr>
</tbody>
</table>

Summary of the average foci counts per cell for each time point.

Table 4.6: Average foci count per cell for cells + AuNPs, cells + GdNPs and cells alone as a control, for U87.
Table 4.7: Average foci count for cells + AuNPs, cells + GdNPs and cells alone as a control, for MCF-7

<table>
<thead>
<tr>
<th>Sample</th>
<th>0 Gy Photon</th>
<th>0 Gy Proton high LET</th>
<th>2 Gy 30 min Photon</th>
<th>2 Gy 30 min Proton high LET</th>
<th>2 Gy 24 hours Photon</th>
<th>2 Gy 24 hours Proton high LET</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 Cells</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>27 ± 5</td>
<td>30 ± 4</td>
<td>3 ± 2</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>MCF-7 Cells + AuNPs</td>
<td>1 ± 1</td>
<td>NA</td>
<td>32 ± 4</td>
<td>NA</td>
<td>3 ± 2</td>
<td>NA</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>32 ± 4</td>
<td>34 ± 4</td>
<td>2 ± 1</td>
<td>6 ± 4</td>
</tr>
</tbody>
</table>
Table 4.8: Summary of statistical significance for average foci counts, for cells + AuNPs, cells + GdNPs and cells alone as a control, for both U87 and MCF-7 with protons and photons

<table>
<thead>
<tr>
<th>Statistical significance for average foci counts</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 Cells photon vs U87 Cells + AuNPs photon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U87 Cells photon vs U87 Cells + GdNPs photon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U87 Cells proton (high) vs U87 Cells + GdNPs proton (high)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U87 Cells proton (low) vs U87 Cells + GdNPs proton (low)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U87 Cells proton (high) vs U87 Cells proton (low)</td>
<td>1.00</td>
</tr>
<tr>
<td>U87 Cells + GdNPs proton (high) vs U87 Cells + GdNPs proton (low)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U87 Cells + GdNPs photon vs U87 Cells + GdNPs proton (high)</td>
<td>0.413</td>
</tr>
<tr>
<td>U87 Cells photon vs U87 Cells proton (high)</td>
<td>0.073</td>
</tr>
<tr>
<td>U87 Cells photon vs U87 Cells + GdNPs proton (high)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U87 Cells + AuNPs photon vs U87 Cells + AuNPs photon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells photon vs MCF-7 Cells + AuNPs photon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells photon vs MCF-7 Cells + GdNPs photon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells proton (high) vs MCF-7 Cells + GdNPs proton (high)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs photon vs MCF-7 Cells + GdNPs proton (high)</td>
<td>0.014</td>
</tr>
<tr>
<td>MCF-7 Cells photon vs MCF-7 Cells proton (high)</td>
<td>0.003</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs proton vs MCF-7 Cells + GdNPs proton (high)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs photon vs U87 Cells photon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs photon vs U87 Cells + GdNPs photon</td>
<td>0.03</td>
</tr>
<tr>
<td>MCF-7 Cells + AuNPs photon vs U87 Cells + AuNPs photon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7 Cells (high) vs U87 Cells proton (high)</td>
<td>0.010</td>
</tr>
<tr>
<td>MCF-7 Cells + GdNPs proton (high) vs U87 Cells + GdNPs proton (high)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

It should be noted that with the proton irradiated samples, although issues were faced with the clonogenic assay work it was still possible to obtain some samples for the DNA damage work. From our first visit, samples could be used as there was no contamination, but samples became over confluent when seeding the relevant densities for the clonogenic work. With the DNA damage the samples were fixed either 30 minutes or 24 hours post irradiation and were checked to ensure they had not become over confluent. With the samples from the second visit, the clonogenic samples were contaminated, however when fixing the samples there was no contamination present. Therefore it was thought that the contamination occurred whilst seeding the different densities for the clonogenic assay or within the incubator as the cells are stored for a long period of time to allow for colony growth.
4.7. Results

4.7.2.1 Effect of radiation type on enhancement

From table 4.8 it can be seen that differences in incident radiation cause statistically significant differences in the average foci counts. When considering U87 and MCF-7 cells alone, compared to cells with GdNPs irradiated with protons, the p-value was less than 0.001. Interestingly when considering cells alone, comparing photons with high LET protons, significance was only shown with MCF-7 cells (p-value = 0.003). Similar results were found between cells with GdNPs irradiated with photons compared to high LET protons, where no significance was shown with U87s (p-value = 0.413) but was with MCF-7 cells (p-value = 0.014).

If we consider the spread of the number of foci, it can be seen in figure 4.11a, that when we consider the controls for photon and proton (high LET) irradiations with U87 cells, there are differences in the histograms. The median for proton irradiated samples was 23.5 compared to 20 for photon irradiated. Also the mode was 61 % higher with protons (18 compared to 29 foci per cell for photon and higher LET protons respectively). From the plots it can be seen that the shape of the plot is skewed to higher foci numbers per cell with the proton (high LET) irradiated samples whereas photon samples follow normal distribution. Differences were also shown with MCF-7s (figure 4.11g), where the median shifted from 27 foci/cell to 29.5 with proton irradiation.

As expected, when comparing photon irradiation to low LET protons, the spread was comparable as shown in figure 4.11f, where both followed a normal distribution. When considering low LET proton irradiation of cells, compared to those with GdNPs, it can be seen that GdNPs again shift the spread of foci per cell (figure 4.11e). From this figure, it can be seen that the use of NPs shifts the entire distribution when comparing to the control. Here the median was shifted from 19.5 to 32 foci per cell and the mode increased by 83 % from 18 to 33 foci per cell, which is 14 % higher than cells treated with high LET protons alone.
4.7.2.2 Effect of NP type on enhancement

As experiments with AuNPs were only carried out with photons, only this comparison could be made, where for both cell lines there was significance demonstrated between the two NPs, both with p-values less than 0.001. Interestingly, with U87 cells, more foci were found with GdNPs, whereas with MCF-7s more were found with AuNPs as shown in tables 4.6 and 4.7. This agreed with the findings from the clonogenic work, as shown in figure 4.8, where with U87 cells, GdNPs caused the most cell kill, whereas with MCF-7 cells, AuNPs caused the most. From the clonogenic work, the DEF was 2.04 and 1.28 for GdNPs and AuNPs, whereas with the foci the DEF with 2 Gy at 30 minutes post-photon irradiation, was 1.5 and 1.25, a difference of 36 % and 2 % for GdNPs and AuNPs respectively.

Considering the histograms comparing cells alone against GdNPs with photon irradiation (figure 4.11b), a significant shift in the spread of foci per cell can be seen for U87 cells. From this figure it can be seen that as with the low LET proton results, the addition of GdNPs has shifted the entire distribution. Here, however, the distribution has also changed, where it appears that a double Gaussian fit would be more appropriate to describe the distribution. The mode has shifted from 18 with cells alone to 30 with the addition of GdNPs, an increase of 67 %, as well as an increase in the median, from 20 to 34. This is interesting as it demonstrates the ability of GdNPs to make the cells more sensitive to the damaging effects of radiation, whereby the number of foci with GdNPs and photons, was comparable to cells alone with high LET protons as highlighted in section 4.7.2.1. A change is also observed with AuNPs, here however the median is only shifted from 20 to 20.5 and the mode is 17 % less at 15 foci per cell compared to 18 without AuNPs for photon irradiation (figure 4.11c). The less significant change can also be seen in the figure (figure 4.11c compared to 4.11b), where the distribution has not been shifted but is skewed to a higher number of foci per cell with the addition of AuNPs.

With MCF-7 cells, changes are also observed with the addition of NPs with photon irradiation, where a shift in the spread of foci per cell can be seen for both
NPs (figures 4.11h and 4.11i). Here for both NPs the mode increased to 32 foci per cell (20 % increase). This is surprising as AuNPs were shown to have a higher DEF from the clonogenic assays, with an enhancement of 49 % compared to 28 % from GdNPs (table 4.4).

4.7.2.3 Impact of cell line on enhancement

In terms of comparisons of results between cell lines, significance was demonstrated in all cases, comparing cells alone with photons and protons, cells with AuNPs with photons and cells with GdNPs for photons and protons.

With the addition of AuNPs, it could be seen that there were differences between the two cell lines with photon irradiation. From figure 4.11i, it was evident that AuNPs caused a change in the distribution of foci per cell, where the median value is shifted from 27 to 32 foci per cell. If however, we consider U87 cells (figure 4.11c), it could be seen that AuNPs do not cause significant changes to the distribution of foci per cell. When comparing the effect of AuNPs in the two cell lines, the modal number of foci per cell in MCF-7 cells is double that of U87 cells (30 compared to 15).

With the use of GdNPs and high LET protons, differences were also demonstrated between the cell lines. With U87 cells, GdNPs shifted the median from 23.5 to 31 foci per cell (figure 4.11d), whereas with MCF-7 cells, (figure 4.11j), the shift was less significant with 29.5 compared to 33. Interestingly however, the modal value with MCF-7 cells increased by 10 %, whereas with U87 cells, it only increased by 7 %.

4.8 Discussion

Considering the clonogenic assay work, it was evident from the results that the addition of NPs created a significant decrease in cell survival. Although this decrease was observed for both cell lines and both NP types, the rate of decrease differed between the two variables. The $\alpha/\beta$ ratio demonstrates the dose where cell killing from linear and quadratic components from LQ equation (section 4.3) are equal. The alpha part represents the intrinsic radio-sensitivity of the cells,
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describing the number of cells killed per Gy in an unrepairable way, whereas beta represents the repairable portion of the cell damage [187]. From table 4.5, it was evident that the addition of NPs, both increased the alpha value, indicating more cells killed per Gy and decreased the beta value, meaning a smaller proportion of repairable cell damage. Together, these led to higher $\alpha/\beta$ ratios for both cell lines and NPs. For U87 the greatest ratio increase was with GdNPs, with a percentage difference of 651% and with MCF-7 the greatest ratio was with AuNPs, with a percentage difference of 594%.

This demonstrates the effectiveness of the treatment, whereby a low ratio can indicate a resistant tissue, as was seen with the U87 control, which is known to be a radioresistant cell line. Another factor was the radiation type used, as it is known that when irradiating with protons, the survival curve is steeper, which indicates less repaired damage. The linear curve is due to an increasing alpha value with increasing LET [188], indicating a higher ratio.

In terms of the reliability of the findings, the $\alpha/\beta$ ratio found for U87 (no NPs) was comparable to the findings quoted in the literature, which ranged between 5 and 10 Gy with photon irradiation, where our value was $7.8 \pm 1.2$ [189, 190, 191, 192, 193].

For both cell lines the addition of NPs caused a significant increase in the $\alpha/\beta$ ratio. One of the original research questions, was whether the addition of NPs to cells irradiated by photons, can make the cells more sensitive to radiation and have comparable damage to the observed with protons. From the work of Chaudhary et al, the $\alpha/\beta$ ratio for U87 cells irradiated by protons at an LET of 11.9 keV/µm was approximately 10 Gy [175]. The findings show higher values than this for both types of NPs, indicating a more linear curve, similar to that seen with protons, but in our case, a steeper curve for both NPs. This means that the addition of NPs causes the cells to be more sensitive to the effects of radiation.

In terms of the findings, they matched well with Mowat et al, who demonstrated cell survival with U87 cells and the same GdNPs, using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [194].
This assay assesses the cellular metabolic activity and in turn the cell viability [195]. Although this is a different assay, it has been shown that both clonogenic and MTT assays give similar results [196]. From this study they also demonstrated an increase in the level of cell kill with the addition of GdNPs, where at 2 Gy they had an increase of 12.5% in cell kill, when irradiating U87 cells with a concentration of 0.5 mg/ml (1 hour incubation) with 6 MeV photons. The findings showed an enhancement of 2.04 compared to their 1.125 for the same concentration. Although both show an enhancement, the work reported here, used a 24 hour incubation, whereby more particles could have been internalised in the cells leading to a higher enhancement. The difference in irradiation energy, 6 MeV compared to 6 MV could have affected the level of cell kill.

When comparing to the study by Stefancikova et al, where they also used GdNPs and U87 cells irradiated with photons (cobalt source compared to measurements with a linac in this study), at a concentration of 0.4 mg/ml (12 hour incubation), there is a difference in the observed DEF, where they found it to be 1.23, whereas this study found a value of 2.04 [174]. A reason for this difference is that although the NPs and cell line were the same, the incubation time differed. They incubated for 12 hours, whereas this study used a 24 hour incubation. This could mean that a longer incubation time is needed to increase the number of NPs taken up within the cell, improving the DEF. This study also had a higher concentration of 0.5 mg/ml compared to their 0.4 mg/ml, whereby more GdNPs may have been internalised within the cell, causing a higher enhancement effect. Further studies are needed to determine the level of uptake with varying concentrations and incubation times, where spectroscopy techniques could be used to demonstrate the NP uptake. Finally, although this study considers GdNPs from the same research group, the formulation used may have differed, as their study obtained NPs before their commercialisation, whereas this work utilised the commercial product, AGuIX ®.

A study by Butterworth et al, using the same commercial AuNPs and MCF-7 cells could not be compared to as they irradiated with a lower energy of 160 kVp.
Chapter 4. Measurement of NP enhancement in a biological system

photons, which has been predicted to cause more enhancement as explained in section 1.5.3.4 [94]. Another difference was the incubation time, where they used two different concentrations and incubated for 1 hour. Contrary to their findings where they showed more significant enhancement with a lower concentration of 0.01 mg/ml (1.41), compared to 0.1 mg/ml (1.09), this study found higher enhancement (1.49) at a higher concentration of 0.5 mg/ml. As well as this, a longer incubation time (24 hours) and higher beam energy (6 MV photons) were used. Here it is difficult to conclude if the higher concentration is more optimum, as although it gave a higher DEF than that of Butterworth et al, the incident beam energy differed, therefore a comparison can only be made considering the same energy. Also within the literature it is unclear how the enhancement effect is related to the NP, as a higher concentration does not necessarily mean a higher enhancement. From physical dose enhancement, one would expect more self absorption due to a higher number of NPs being present. Although the authors did not comment on the reason for a higher enhancement with a lower concentration, it could have been due to a combination of high concentration and low beam energy. The higher concentration would have allowed more NPs to be internalised within the cells, where due to the significant increase in secondary electron production at this energy, self-absorption could have been higher, leading to a lower enhancement effect.

For the 53BP1 work, the findings differed to those of Stefancikova et al [179], where they found that NPs neither increased induction of DSBs nor affected their repair. This study used U87 cells incubated with GdNPs at a concentration of 1 mg/ml (1 hour incubation) and irradiated with 1 Gy from a cobalt source (mean energy of 1.25 MeV). Although their findings differed to those found in this study, it should be noted that this has been hypothesised to be due to the higher concentration as explained by Rima et al [197]. In this study they considered GdNPs and found that the number of vacuoles per cell containing GdNPs as well as the average size of the vacuoles increased with concentration up to 0.6 mg/ml, but for 2 mg/ml, the average size increased but their number per cell decreased, therefore if the quantity
4.8. Discussion

decreases per cell their effect should also decrease. The number of vacuoles is of interest, as this has been shown to be where NPs are confined to, rather than the nucleus or the mitochondria, as demonstrated by Mironava et al [198]. In this study they considered the use of AuNPs at two sizes (13 and 45 nm) and showed the vacuoles are distributed uniformly across the cytoplasm, with none detected within the nucleus or mitochondria. From our simulations shown in chapter 3, the range of enhancement was quantified, where at a clinical energy of 1.5 MeV, the greatest enhancement range was 100 µm (50 nm diameter NPs). Therefore physical enhancement would still be expected due to the presence of NPs even considering their confinement to vacuoles.

One interesting result was that there was no significance observed in DNA damage between the U87 cells with GdNPs irradiated with photons compared to U87 cells with GdNPs irradiated with high LET protons, although differences were found between cells alone (photon) compared to those with GdNPs (high LET protons). This suggests that the addition of GdNPs to the U87 cells, increases the biological effect, irrespective of the radiation.

With the DNA damage results, it should be noted that our values, 30 minutes post-irradiation, were generally lower than those quoted in the literature. It is generally expected that the foci count is on average, approximately 25 foci per cell per Gy [199]. As we irradiated with 2 Gy the values were all lower than expected. However, this was thought to be due to the single projection used, rather than a z-stack projection. When looking at DNA damage, usually the residual damage is quantified, to demonstrate the level of un-repaired damage. However, due to the low counts this was not carried out, where further work would firstly use a z-stack to quantify more accurately the foci, where the residual damage could then be studied.

When considering the sensitising effect between the two different cell lines, it was shown from the clonogenic DEF that the addition of GdNPs to U87 cells made these cells a lot more sensitive to the damaging effects of radiation (DEF = 2.04). A possible reason for the differences observed when comparing the two cell types is the level of uptake within the cell. With these two cell lines there
were differences in the sizes where from the literature, the average sizes of U87 cells are between 12 and 14 µm [200], whereas MCF-7 cells are between 15 and 17 µm [201] (approximately 21% larger). It should be noted however that U87 cells have a star-like shape, whereas MCF-7s are more circular. This difference in cell membrane surface area can caused differences in the level of uptake when comparing the two cell lines.

4.9 Conclusion

This study was able to demonstrate biological changes encountered by cells due to the presence of NPs combined with ionising radiation. It was possible to quantify differences between both cell lines and NP types.

In terms of the clonogenic assays with U87, an enhancement of dose was observed, whereby a lower dose needed to be delivered (0.98 Gy with GdNPs compared to 2 Gy without NPs) to offer the same level of cell kill as that observed with cells alone at 2 Gy. Compared to the literature we demonstrated a higher level of enhancement (2.04 compared to 1.23), with a longer incubation time (24 hours instead of 12) and higher concentration (0.5 compared to 0.4 mg/ml). It was hypothesised that the longer incubation time would have led to a higher level of uptake, due to longer exposure to the NPs. Future studies should investigate the level of uptake within the cells across different incubation times, to determine the most optimum time, using inductively coupled plasma atomic emission spectroscopy (ICP-AES) and TEM to show the bio-distribution and quantify the intracellular concentration.

Significant differences were demonstrated with the DNA damage comparing protons and photons, through differences in the average number of foci per cell. However, it would be of interest in the future to attempt clonogenic assays with protons again, to determine any differences in $\alpha/\beta$ ratio for both cell lines and NP types with protons. This can then allow for conclusions to be made on the efficacy of NPs to make cells treated with photons experience comparable damage to what is normally seen with particle therapy, or to conclude if the combined effect of NPs
with radiation causes sensitisation irrespective of incident beam type.

4.10  Future work

As mentioned within this chapter, to conduct microarray analysis, it was necessary to produce and treat the samples, then extract and quantify the amount of RNA in each sample. As specific quantities of RNA were required for the microarray, dilutions were carried out prior to the measurements, where both irradiated and un-irradiated samples were processed, profiling the genes expressed. Reporting the genes expressed and their relevance in terms of pathways involved was not within the scope of this thesis. Therefore, this chapter reports the production and treatment of the samples needed for this larger study. Within this thesis, only samples lysed 30 minutes post-irradiation were produced, where further work will involve treatment of samples and lysing cells 24 hours post-irradiation. As this work aims to further our insight into the effects of NPs on cells, two time points are needed, as this shows the differences between genes expressed immediately after radiation treatment and those expressed after cellular repair occurs. From the genes expressed, it is then possible to gain an understanding of the mechanisms involved, both in the presence of NPs and NP enhanced radiotherapy.
Chapter 5

Final remarks

Overall this thesis has added to the breadth of knowledge in the field of nanoparticle enhanced radiation therapy. This was a multidisciplinary effort, where physics, engineering, computational modelling and radiobiology techniques were used to gain a deeper understanding of the effects of nanoparticles when combined with ionising radiation.

The work was split into three distinct projects. The first of which offered one of the first quantitative measures of physical dose enhancement due to the introduction of nanoparticles in a proton beam. Within the literature, the majority of experimental work carried out with nanoparticles offers an insight into the biological effects through cell survival or other biological measures. In terms of physical dose enhancement, this was always quantified through simulations. Here however, both Monte Carlo simulations and experimental measures of the physical dose enhancement were investigated. Gafchromic films at various depths, were used to compare the dose with and without nanoparticles, whilst constructing a depth dose profile. The model offered a simplification, to model concentrations of nanoparticles in a non-computationally expensive manner, whereby lateral changes in dose depositions could be quantified. Comparisons were made between the simulations and experiment, where both showed changes to the overall shape of the Bragg peak, demonstrating both a narrowing (simulated 32 % compared to measured 14 %) in the width and a level of enhancement (simulated 1.04 compared to measured 1.21 in the presence of AuNPs with a diameter of 50 nm). Although
there were discrepancies between the two, it was attributed to differences in the two set-ups, such as simulations not taking into account the beam divergence. Another is that interactions on the nanoscale differ to those on the macroscale, which the simulations would not have taken into account. As the concentrations used were clinically achievable, the work highlighted a need for quantification of proton range if nanoparticles are to be used in a clinical treatment. Further studies would need to be carried out to determine if planning systems can sufficiently account for these changes, or if other modifications need to be made to ensure a valid treatment plan. When carrying out a treatment plan, a CT scan of the patient is used, where the Hounsfield units are converted to electron density. With the use of nanoparticles, it would be necessary to initially determine if the CT scan could account for any changes in electron density due to the addition of nanoparticles. If changes are not shown, additional work would need to be carried out to account for the changes to the Bragg peak demonstrated in this work. A study by Galper studied a range of gold nanoparticle concentrations (10 mM to 1.5 M) and predicted a minimum of 5.8 mM of gold nanoparticles needed within the tissue for contrast to be shown [202].

The second part of this work offered a parametric study to give an insight into the effect of different variables on the enhancement with photon irradiation. Within the literature, studies have either focused on one material and investigated different sizes, or different materials with a specific size. Here the collective effect of these factors with various photon beam energies were studied, showing the dose deposition for each type of nanoparticle. A correction was applied to these simulations to take into account the differences in number of particles between different nanoparticle size and material combinations. This allowed for comparisons to be made between the different sizes, where it was shown that 2 nm gold nanoparticles offered the highest level of enhancement with 90 keV photons. With the extension of a PEG coating it was shown that this decreases the level of enhancement, as well as the average energy deposition per unit volume. These differences were significant (approximately 75 % reduction) within 0.1 μm from
the surface of the NP, where at greater distances the differences were less than 5%. Although this work demonstrates the effects of a number of variables, there were limitations to the model. One of these was the three regions had different voxels size, this was due to the computationally expensive nature of the simulations where it was not possible to have nano scaled voxels across the entire phantom. Another limitation was that the model assumes solid NPs, whereas in practice NP compositions can be more complex, such as the AGuIX ® NPs (figure 4.1) used in our biological assay work, which were comprised of a polysiloxane matrix and cyclic chelates of Gd. It was thought that the model would overestimate the enhancement from Gd, as the assumption of a solid NP would mean more material for the incident radiation to interact with. Whereas in practice, this NP has a large portion of silicone. Although it could also be argued that the model would also underestimate the enhancement, as the structure of this NP could make it more likely for electrons to escape, rather than be absorbed within the NP.

Finally, the last part of this work expanded from considering physical mechanisms, to look at the biological mechanisms. Following on from the nano-scale simulations it was decided that small particles would be investigated (< 5 nm). It was interesting to demonstrate differences from what was found due to physical mechanisms alone. Simulations showed a material dependence, where gold offered a higher enhancement then gadolinium. From the radiobiology study, this was also true for the MCF-7, with the U87 cells, however, cell survival showed a higher cell kill with gadolinium nanoparticles. Future work would investigate the relative level of uptake between the two cell lines and nanoparticles considered, as if one type of nanoparticle had a lower uptake for a specific cell line, it would lead to a lower enhancement.

Preliminary measurements were carried out with the microarray for U87 cells following incubation with nanoparticles (30 minutes post-irradiation). As this forms part of a larger study, future work will look to analyse genes expressed for this time point as well as an additional time point, 24 hours after irradiation. It is thought that this could give a clearer understanding on the biological impact of nanoparticles
combined with radiation. By determining gene expression changes, it is possible to
determine the specific pathways that are activated with nanoparticles, which can in
turn be utilised to optimise the effect.

With the multi scale model and the biological work it was possible to make
comparisons between the two independent studies, as both AuNPs and GdNPs were
studied at a clinical energy of 6 MV photons. For this comparison the DEF quoted
in chapter 4 was divided by the mass of a 2 nm NP for the respective material,
such that comparisons could be made. Also in terms of the DEF/unit mass of NP
material quoted in chapter 3, the enhancement was quoted at a distance of 5 µm, as
this is representative of the size of a typical cell. The results of this comparison are
tabulated in table 5.1. For AuNPs, the experimental findings were 25 % higher with
U87 cells and 46% with MCF-7 cells. With GdNPs however the differences were
103 % for U87 cells and 27 % for MCF-7 cells.

**Table 5.1:** Comparison of the predicted enhancement from simulations with Au and Gd
NPs to experimental findings with clonogenic assays Au and Gd NPs with U87
cells irradiated with 6 MV photons.

<table>
<thead>
<tr>
<th>Material</th>
<th>Predicted DEF (x10^{10}/ng)</th>
<th>Experimental DEF (x10^{10}/ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U87</td>
<td>MCF-7</td>
</tr>
<tr>
<td>Au</td>
<td>1.26 ± 0.07</td>
<td>1.58 ± 0.64</td>
</tr>
<tr>
<td>Gd</td>
<td>3.04± 0.17</td>
<td>6.16 ± 1.60</td>
</tr>
</tbody>
</table>

It can be seen that there are discrepancies between the findings from the
simulations compared to those found in the experiment. Cellular experiments
showed greater enhancement than what was demonstrated by simulations for both
cell lines and NP types. This is in agreement with the literature where it is known
that multiple mechanisms contribute to the enhancement effect, where simulations
only account for physical mechanisms. A possible way to validate simulated
findings, is to carry out measurements of SSB and DSBs with plasmid DNA, as
this gives an indication of damage due to radiation alone.
In terms of the recommendations from this work, our multi scale simulations have investigated several variables of interest, where the effect of NP material, size, coating and beam energy were considered. From this work it was evident that the size had an effect on the level of enhancement, where 2 nm was predicted to show the most enhancement. In vitro studies were also carried out and demonstrated an enhancement effect. This work however only used small NPs around 2 nm, therefore further studies would need to investigate the effect of size, comparing to the simulated predictions. Both of these studies considered the effect of different materials. Simulations showed Au to have the highest enhancement, this however was not true for both cellular experiments. It was therefore highlighted, that cellular uptake may be a more important variable to consider. This is also true for the addition of a coating, where simulations showed a great reduction in enhancement nanometres from the surface of the NP. Cellular experiments would need to be carried out with coated NPs, to determine the effect this has on uptake and enhancement. Finally, the variability in uptake between cell lines needs to be investigated. Therefore recommendations for future studies would be to prioritise measurements of cellular uptake when optimising NP size, coating and material, as this will inevitably affect the level of enhancement observed.

Considering factors such as cell cycle effects and production of ROS, can allow for a more complete understanding of the effect of introducing NPs in radiation therapy. Further work could also consider more cell lines or more complex 3D models to demonstrate any differences. The measurement of gene expression should give some indication on the pathways affected by the introduction of NPs, however the results of this are outside the scope of this thesis. Both simulations and experiments considered the use of a clinical energy of 6 MV, where from the simulations and literature we know that orthovoltage energies have a larger effect. Future work will look into translating the experimental work with the comparison of two NPs and cell lines to demonstrate the biological effect at orthovoltage energies. This will allow for an understanding of the mechanisms, whereby lower energies are predicted to have higher physical enhancement, due to differences in
mass energy absorption coefficients between high-Z materials and soft tissues. It is unclear, however, how this translates experimentally with the combination of different mechanisms.

Each of these projects has contributed to the field of nanoparticle enhanced radiation therapy. However, the mechanisms are still not fully understood, where further multidisciplinary studies need to be proposed, to truly understand and optimise this effect, before translating to clinical use.
Appendix A

Cell maintenance

Technical equipment

- Incubator: 37 °C, 5 % CO₂/95 % air
- Biological safety cabinet
- Water bath: 37 °C
- Inverse phase contrast microscope
- Tissue culture flasks: 75 cm²
- Pipettes
- Pipette gun
- Beaker for waste

Materials

- MEM supplemented with 10 % FBS and 1 % pen/strep
- PBS
- 0.25 % Trypsen-ethylenediaminetetraacetic acid (EDTA)
- 70 % ethanol spray
- Bleach
Appendix A. Cell maintenance

Protocol

1. Warm up reagents in the water bath

2. Clean the hood using ethanol spray

3. Prepare beaker with bleach for waste

4. Add beaker, pipettes, pipette gun and reagents to the hood, spraying each item with ethanol beforehand

5. Remove cell flask from the incubator to view the cell culture using the microscope

6. Determine the confluence and ensure the cells are not contaminated

7. In the hood, remove the medium from the flask into waste beaker

8. Add ~ 12 ml of fresh medium to the flask

9. Return the flask to the incubator
Appendix B

Cell sub-culture

Technical equipment

• Incubator: 37 °C, 5% CO₂/95 % air

• Biological safety cabinet

• Water bath: 37 °C

• Inverse phase contrast microscope

• Tissue culture flasks: 75 cm²

• Pipettes

• Pipette gun

Materials

• MEM supplemented with 10% FBS and 1% pen/strep

• PBS

• 0.25 % Trypsen-ethylenediaminetetraacetic acid (EDTA)

• 70% ethanol spray

• Bleach
Appendix B. Cell sub-culture

Protocol

1. Warm up reagents in the water bath

2. Clean the hood using ethanol spray

3. Prepare beaker with bleach for waste

4. Add beaker, pipettes, pipette gun and reagents to the hood, spraying each item with ethanol beforehand

5. Remove cell flask from the incubator to view the cell culture using the microscope

6. Determine the confluence and ensure the cells are not contaminated

7. In the hood, remove the medium from the flask into waste beaker

8. Wash the cells with 10 ml of PBS. Note: This step is important as it removes any remaining medium that can neutralise the trypsin.

9. Add 3 ml of trypsin-EDTA solution to the 75 cm$^2$ cell culture flask

10. Place the flask in the incubator for 2-5 min

11. Verify with the microscope that the cells have detached. If needed, gentle tapping can be used until detachment is observed.

12. Add 7 ml of medium to the flask to neutralise the trypsin. Note: the ratio of Trypsin:medium should always be at least 2:1

13. Transfer a fraction of the cell solution into a new flask (minimum 10 %)

14. Add 12 ml of fresh media to the new flask

15. Gently pipette the mixture to ensure a single cell suspension

16. Store the flask in the incubator
Appendix C

Cell counting

Technical equipment

• Haemocytometer
• Pipettes
• Pipette gun
• Universal tube

Protocol

1. After adding trypsin to the confluent flask and counteracting it with medium
   transfer the cell solution to a tube

2. Ensure that the solution has been mixed well

3. Remove 10 $\mu$L from the stock cell solution and flood the haemocytometer
   chamber

4. With the microscope, count the number of cells in 4 of the 9 of the large
   squares of the grid

5. Determine the average number of cells counted (N\text{counted}).

6. The total number of cells in suspension is given by

\[ N_{\text{cells/ml}} = N_{\text{count}} \times C_{\text{haem}} \]  

(C.1)
C_{haem} is the conversion factor of the haemocytometer, which in our case was $10^4$. 

Appendix D

Fixing cells

Technical equipment

• Freezer: $-20 \degree C$

• Fridge: $4 \degree C$

• Incubator: $37 \degree C$, 5 % CO$_2$/95 % air

• Glass bottle to store freezing solution

• Aspirator

• Beaker for waste

• Pipettes

• Pipette gun

• Universal tube

Materials

• PBS

• Dishes/plates to be fixed

• Bleach
Appendix D. Fixing cells

Protocol

1. Prepare fixing solution (methanol:acetic acid)
2. Store fixing solution in the freezer
3. Prepare a beaker with bleach for water
4. Remove sample from the incubator
5. Remove media from the sample into waste beaker
6. Wash twice with PBS
7. Aspirate any leftover PBS
8. Remove fixing solution from the freezer
9. Add enough solution to cover the sample (2 ml for dishes)
10. Place sample in the fridge for 30 minutes
11. Remove fixing solution (aspirate if needed to ensure sample does not become cloudy)
12. Add 2 ml to each sample
13. Store in the fridge
14. If stored for more than a week, replace PBS
Appendix E

Staining cells for clonogenic assay

Technical equipment

• Incubator: 37 °C, 5 % CO₂/95 % air

• Pipettes

• Pipette gun

• Aspirator

• Beaker for waste

• Beaker with warm water

• Tray

Materials

• PBS

• Crystal Violet

• Dishes/plates to be stained

• Bleach

• 70 % ethanol spray
Appendix E. Staining cells for clonogenic assay

Protocol

1. Clean the hood using ethanol spray
2. Prepare beaker with bleach for waste
3. Prepare a beaker with warm water to remove excess crystal violet
4. Add beakers, pippettes, pipette gun and reagents to the hood, spraying each item with ethanol beforehand
5. Place beakers and crystal violet in the tray to prevent spillage
6. Take dishes/plates to be fixed out of the incubator, remove media into waste beaker and gently wash each sample twice with PBS
7. Remove PBS and use aspirator to ensure all of it is removed
8. Add 2 mL of crystal violet to each sample
9. Leave this for 10 minutes at room temperature
10. Remove crystal violet and gently wash sample in warm water
11. Leave sample to dry
Appendix F

Staining cells for 53BP1 assay

Technical equipment

- Incubator: 37 °C, 5 % CO$_2$/95 % air
- CellInsight CX5 High Content Screening Platform
- Pipettes
- Pipette gun
- Beaker for waste
- Aluminium foil

Materials

- Tritox X-100
- PBS
- Goat serum
- Bovine serum albumin (BSA)
- Primary Antibody: 53BP1 (Novus Biologicals)
- Secondary Antibody: Alexa Flour ® 488 goat anti-rabbit IgG (H+L)
- DAPI fluorescent stain
Appendix F. Staining cells for 53BP1 assay

Protocol

1. Prepare washing buffer (0.25 % Triton X-100 in PBS)

2. Prepare permealization buffer (0.5 % Triton X-100 in PBS)

3. Prepare blocking buffer (1 % BSA, 10 % goat serum, 0.1 % Triton X-100)

   Note: It is important to use the serum that matches the species in which the secondary antibody was raised

4. Prepare DAPI staining solution, (1:1000) diluted in PBS.

5. Remove stored samples from fridge and leave them at room temperature for 10-15 minutes.

6. Discard PBS and add 2 ml/dish of fresh PBS and rinse at least once

7. Add 2 ml of permeabilisation buffer and leave at room temperature for 10 minutes.

8. Remove permeabilization buffer and wash once with PBS

9. Discard PBS and add 2 ml/dish of blocking buffer

10. Incubate for 1-2 hours at 37 °C in incubator

11. Prepare primary antibody (53BP1 antibody (1:1000) in blocking buffer)

12. Remove the blocking buffer and add 1 ml of primary antibody

13. Incubate for 1 hour at 37 °C in incubator

14. Wash with washing buffer 3 times, 5 minutes for each wash

15. Prepare secondary antibody (Alexa Fluor 488 Goat anti Rabbit (1:1000) in blocking buffer). Note: this is light sensitive so keep it stored in the dark

16. Prepare foil to cover each sample

17. Wash with washing buffer 3 times, 5 minutes for each wash keeping it in the dark
18. Prepare fluorescent stain

19. Add 1 ml of DAPI stain

20. Set up the sample in CX5 and image from multiple fields

21. Count number of foci/cell, with at least 50 cells counted per samples
Appendix G

Lysing cells for gene expression measurements

Technical equipment

• Freezer −80 °C

• Pipettes

• Pipette gun

• Beaker for waste

• DNase/RNase free vials

• Scraper

Materials

• RLT plus RNeasy lysis buffer (store at room temperature)

• Treated/untreated samples (Note: a minimum of 500,000 cells is needed per sample)

• Bleach

• 70 % ethanol spray
Appendix G. Lysing cells for gene expression measurements

Protocol

1. Clean the hood using ethanol spray

2. Prepare beaker with bleach for waste

3. Add beaker, pipettes, pipette gun and reagents to the hood, spraying each item with ethanol beforehand

4. Remove media into waste beaker and gently wash each sample with PBS twice

5. Ensure all PBS has been removed

6. Add 500 µL of lysis buffer to each sample

7. Wait 10 minutes (a gel should form on the surface)

8. Scrape the gel and transfer the contents to a vial

9. Store all vials at −80 °C
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