ウェブサイト公開用

（様式 10）
（海外特別研究員事業）

令和 2 年 4 月 30 日

海外特別研究員最終報告書

独立行政法人日本学術振興会 理事長 殿

採用年度　平成 30 年度
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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地： ロイヤルメルボルン工科大学（ オーストラリア ）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
無機ナノ粒子の放射線治療増感効果に関与する因子の解明

3. 派遣期間：平成 30 年 4 月 1 日 ～ 令和 2 年 3 月 31 日

4. 受入機関名及び部局名
Discipline of Medical Radiations, School of Health & Biomedical Sciences, RMIT University

5. 所期の目的の遂行状況及び成果…書式任意 書式任意（A4 判相当 3 ページ以上、英語で記入も可）
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

Report of JSPS Overseas Research

Aims/Objectives:

The aim of our project was to evaluate potential abilities of metal-based nanoparticles (NPs) as radiosensitisers against cancer cells and find the optimal factors to make them more efficient for radiation therapy by performing comprehensive studies using various types of NPs.

Metal-based NPs are currently being studied for their potential applications as dose enhancing agents, i.e., as radiosensitisers in radiation therapy. Upon the interaction of NPs with radiation, the atoms of NPs can emit photoelectrons, Compton electrons, Auger electrons, and fluorescent X-rays through physical processes such as photoelectric absorption of X-rays and the Compton effect. These electrons and photons can generate reactive oxygen species (ROS) by radiolysis of water molecules, which causes more cellular damage and enhances the radiation effects in cancer cells. The dose enhancement effects of NPs against cancer on delivered radiation have been documented in many *in vitro* and *in vivo* studies over the last two decades. However, it is insufficient to determine the optimal factors of NPs including the nanoparticle types, sizes, concentrations, and surface modifications to maximise the radiation effects. Biochemical mechanisms of NPs on the dose enhancement effects are also unclear.

In this project, we performed physical analysis and biological experiments using various NPs including new types of NPs which we originally synthesised. The cellular interactions with NPs and X-rays were evaluated *in vitro* using several cancer cell lines. The achievement of this project lies in five sub-studies:

1. Enhancement of radiosensitising effects by doping samarium
2. Identification of highly ROS induced by metal NPs
3. Measurement of the phantom-based dose enhancement using chemiluminescence technique
4. Effects of NPs on cell migration and adhesion
5. Development of novel NPs conjugated with photosensitiser agent

The key results are shown below. The details of each study should be found in the published papers.

Key results:

1. Enhancement of radiosensitising effects by doping samarium

Titanium dioxide nanoparticles (TiO_2 NPs) have been investigated in their roles as radiosensitisers for radiation therapy. However, unlike high atomic number (Z) elements such as gold, TiO_2 NPs have a low Z which does not enhance radiation effects by an increase in X-ray absorption, but by other biochemical methods. The intent of this study was to make these NPs more efficient by synthesising them with a rare earth element, samarium (Z = 62).

Samarium-doped titanium dioxide nanoparticles ($Ti(Sm)O_2$ NPs) were synthesised by a solvothermal method. The synthesised $Ti(Sm)O_2$ NPs were ~13 nm in diameter as determined by transmission electron microscopy. The X-ray diffraction pattern of $Ti(Sm)O_2$ NPs was consistent with that of anatase-type TiO_2 (Fig. 1a). Energy-dispersive X-ray spectroscopy confirmed the presence of samarium in the nanoparticles (Fig. 1b).

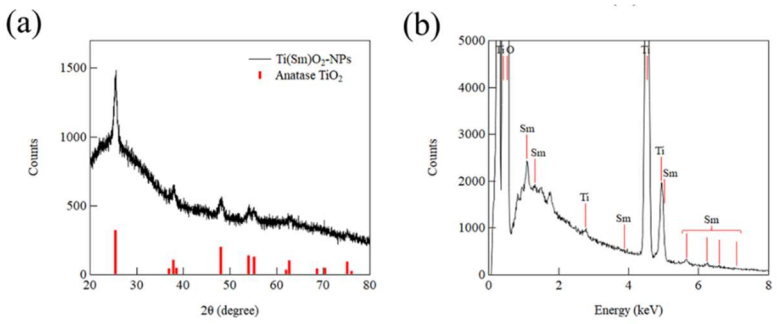

Figure 1. Characteristics of $Ti(Sm)O_2$ NPs. (a) X-ray diffraction spectrum of $Ti(Sm)O_2$ NPs. The bottom bars indicate the peaks of anatase type TiO_2. (d) Energy-dispersive X-ray spectroscopy spectrum of $Ti(Sm)O_2$ NPs.

The surfaces of NPs were modified to prevent the aggregation of NPs in cell experiments. Two types of surface modification were applied to investigate the effect of these surface modifications on the radiation enhancement; aminopropyl trimethoxysilane (APTS) functionalised NPs and polyethylene glycol trimethoxysilane (PEGTS) functionalised NPs. The intracellular uptake and cytotoxicity were assessed *in vitro*

using A549 human lung adenocarcinoma cells and DU145 human prostate cancer cells. At 200 μg/ml, no differences in cellular uptake and cytotoxicity were observed between TiO_2 NPs and $Ti(Sm)O_2$ NPs for both A549 and DU145 cells (Fig. 2). Next, the dose enhancement effects and generation of reactive oxygen species (ROS) in response to 6 MV X-rays were evaluated. The combination of $Ti(Sm)O_2$ NPs and X-rays elicited a greater cytotoxic effect on these cells compared to that of either TiO_2 NPs and X-rays or X-rays alone (Fig. 3a and 3b). $Ti(Sm)O_2$ NPs enhanced ROS produced following X-ray irradiation compared to that elicited in the presence of TiO_2 NPs (Fig. 3c).

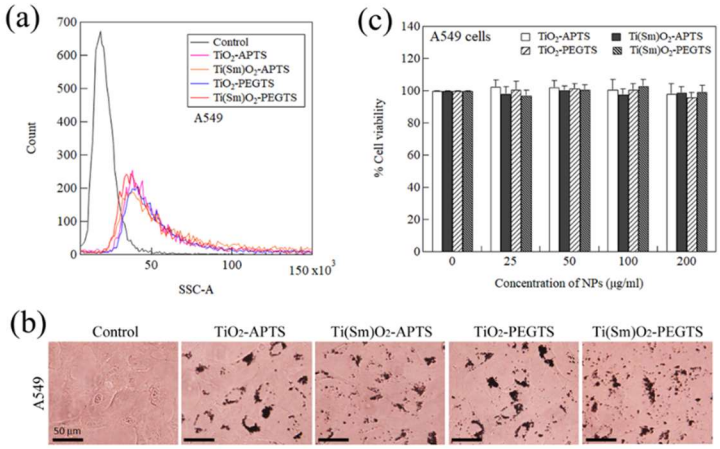

Figure 2. (a) Detection of NPs in cells. SSC area histograms measured by flow cytometry for A549 cells. (b) Representative light microscopy images of A549 cells after 24 h incubation with these NPs. All scale bars present 50 μm. (c) Effect of APTS and PEGTS functionalised TiO_2 NPs and $Ti(Sm)O_2$ NPs on the viability of A549 cells. Similar results were also obtained for DU145 cells.

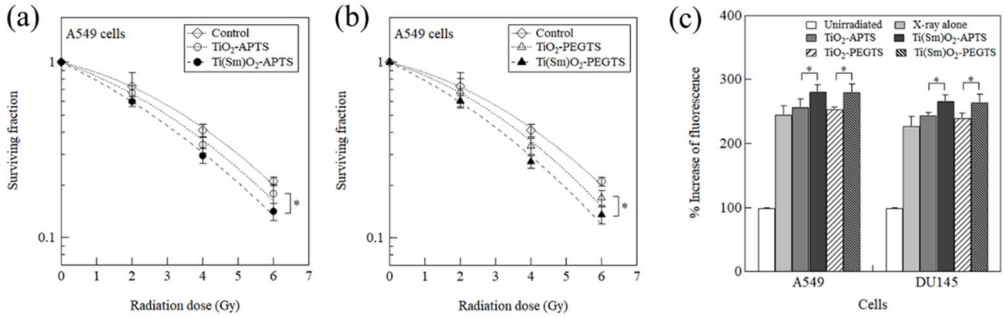

Figure 3. The combination effects of NPs with X-rays. (a) The surviving fraction of cells treated NPs and X-ray irradiation for A549 cells with APTS modified NPs, and (b) with PEGTS modified NPs. Similar results were also obtained for DU145 cells. (c) Intracellular ROS generation by the TiO_2 NPs and $Ti(Sm)O_2$ NPs after 6 Gy of X-ray irradiation. *$p < 0.05$.

Our results show that the doping of TiO_2 NPs with samarium can make them more efficient for radiation therapy. The X-ray absorbance of TiO_2 NPs was increased when these NPs were doped with samarium. This resulted in higher cellular ROS generation by $Ti(Sm)O_2$ NPs upon X-ray irradiation compared to that of TiO_2 NPs. While the cellular uptake and cytotoxicity levels were consistent for both TiO_2 NPs and $Ti(Sm)O_2$ NPs, the combination of the latter with X-rays were more cytotoxic compared to that of the former with X-rays. As no differences were observed between the APTS and PEGTS functionalised NPs, these surface modifications may not affect the enhancement processes of the samarium-doped NPs.

This work was performed in collaboration with many researchers of Australian Radiation Protection and Nuclear Safety Agency, University of South Australia, RMIT Microscopy and Microanalysis Facility, The Alfred hospital, and Kobe University. The part of this research was presented at the Engineering and Physical Sciences in Medicine (EPSM) & Asia-Oceania Congress on Medical Physics (AOCMP) conference 2019 and now in the process of publication.

2. Identification of highly ROS induced by metal NPs

The aim of this study was to identify highly ROS induced by NPs which can enhance radiation effects. The ROS levels induced by gold nanoparticles (Au NPs) and titanium peroxide nanoparticles (TiOx NPs) upon X-ray irradiation were evaluated using three different chemical probes without cells for detecting different ROS; hydroxyl radical (·OH), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2). TiOx NPs showed a distinct ability to generate both H_2O_2 and ·OH, while Au NPs increased only ·OH (Fig. 4). The generation of O_2^- was not increased by TiOx NPs or Au NPs.

Subsequently, the cytotoxic effects of Au NPs and TiOx NPs with X-rays *in vitro* were evaluated using MIA Paca-2 human pancreatic cancer cells. TiOx NPs at high concentrations showed significantly enhanced

radiation effects, while Au NPs had no significant effect at any concentration (Fig. 5). Our results in this study suggest that H_2O_2 induced by NPs could play more important roles to enhance radiation effects than ·OH which has been considered to be the main ROS that causes cellular damage upon X-ray irradiation with NPs. The released H_2O_2 is assumed to be acted as a strong radiosensitising agent of TiOx NPs.

This work was conducted with a PhD student of Kobe University. The part of this research was presented at the Engineering and Physical Sciences in Medicine (EPSM) conference 2018 and now in the process of publication.

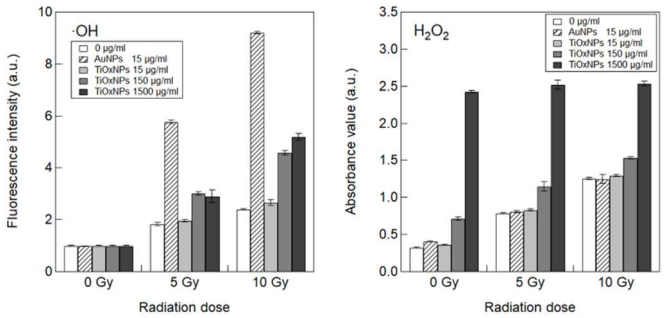

Figure 4. Generation of ·OH and H_2O_2 by different concentrations of Au NPs and TiOx NPs with X-ray irradiation.

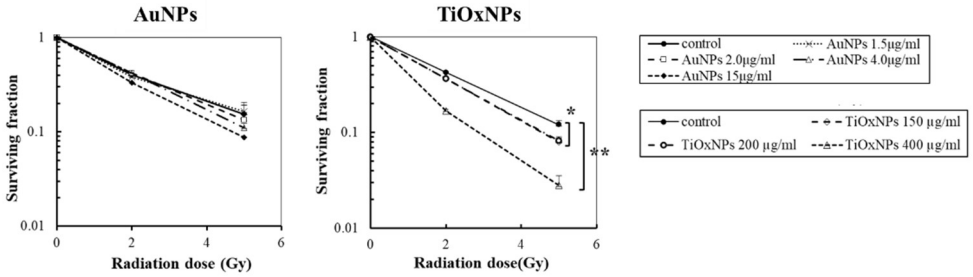

Figure 5. The surviving fraction of cells treated Au NPs and TiOx NPs with X-ray for MIA PaCa-2 cells. *$p < 0.05$.

3. Measurement of the phantom-based dose enhancement using chemiluminescence technique

The dose enhancement using NPs with X-rays has been evaluated in biological experiments which are important but complicated and time-consuming. In this study, we established a new technique to measure the dose enhancement induced by NPs using a chemiluminescence method with 3'-(p-aminophenyl) fluorescein (APF) in a phantom.

The ability of APF to measure dose enhancement induced by Au NPs was confirmed using two different qualities of 6 MV photon beams; the flattening filter (FF) and flattening filter-free (FFF) beams. The fluorescence intensity of APF linearly increased in a dose-dependent manner, showing that there was no saturation of APF signals (Fig. 6). The dose enhancement factors (DEFs) obtained using 30 μg/ml Au NPs was 6.24 ± 0.21 and 6.83 ± 0.32 for FF and FFF beams, respectively. The dose enhancement ratios using the FFF beam were significantly higher than that obtained using the FF beam for each irradiated dose (Fig. 7). This study shows that APF can be easily and reliably used in non-cell based experiments to evaluate the dose enhancement as a result of nanoparticle radiosensitisers. It also shows that the FFF beam has higher dose enhancement with nanoparticles than that of the FF beam.

This work has been published in Radiation Measurements (Nakayama M et al., in press, 2020).

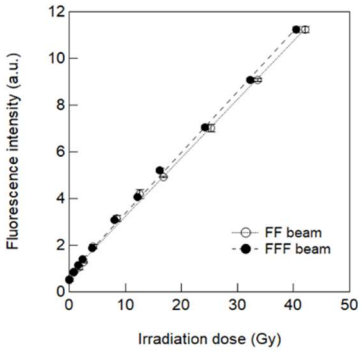

Figure 6. Dose response of APF fluorescence intensity for 6 MV photon beams with FF and FFF modes.

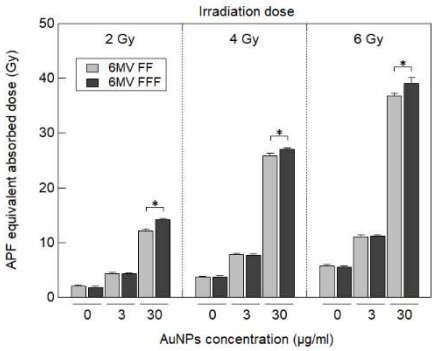

Figure 7. The dose enhancement effects of AuNPs for FF and FFF photon beams. The obtained APF fluorescence intensities were converted to the equivalent absorbed radiation dose. *$p < 0.05$.

4. Effects of NPs on cell migration and adhesion

Cancer metastases are formed when cells migrate from the primary tumour site to other regions. Due to the importance of cell migration and its role in metastasis, the effect of radiation on this process has been studied under both *in vitro* and *in vivo* conditions. In this study, the effects of Au NPs and ionizing radiations on the migration and adhesion of human prostate (DU145) and lung (A549) cancer cell lines were investigated. As shown in Fig. 8, the relative migration of cells irradiated with 5 Gy was found to be 89% and 86% for DU145 and A549 cells, respectively. When the cells were treated with Au NPs this fell to ~75% for both cell lines. However, when the cells were treated with both Au NPs and X-rays an additive effect was seen, as the relative migration rate fell to ~60%. Of interest was that when the cells were exposed to either 2 or 5 Gy X-rays, their ability to adhere to the surface of a polystyrene culture plate was significantly enhanced unlike that seen for Au NPs (Fig. 9). The delays in gap filling (cell migration) in cells treated with X-rays and/or Au NPs can be attributed to various reasons which affects cell motility. In addition, cancer cell's response to X-rays could cause changes to their cytoskeleton which could enhance their adhesiveness.

This work was performed with a PhD student of RMIT University. It was presented at Nano Korea 2019and has been published in International Journal of Molecular Sciences (Shahhoseini E et al., 20, 448, 2019).

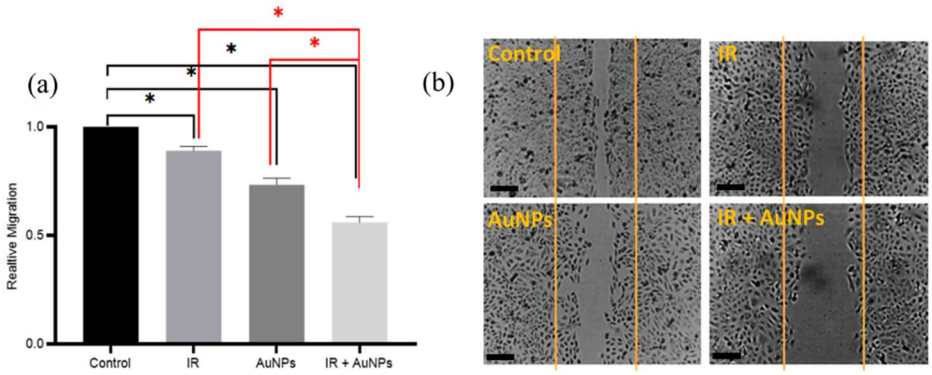

Figure 8. Effects of X-ray irradiation (IR) and/or Au NPs on DU145 cell motility. Cells were treated with 5 Gy X-rays and 1 mM Au NPs. (a) After 24 hr, the relative migration was calculated as a ratio of the gap area at 0 hr to that at 24 hr. (b) Representative microscopy images of DU145 cell gap 24 hr after the scratch. Orange lines present the original gap at 0 hr. All scale bars present 200 μm. Similar results were also obtained for A549 cells. *$p < 0.05$.

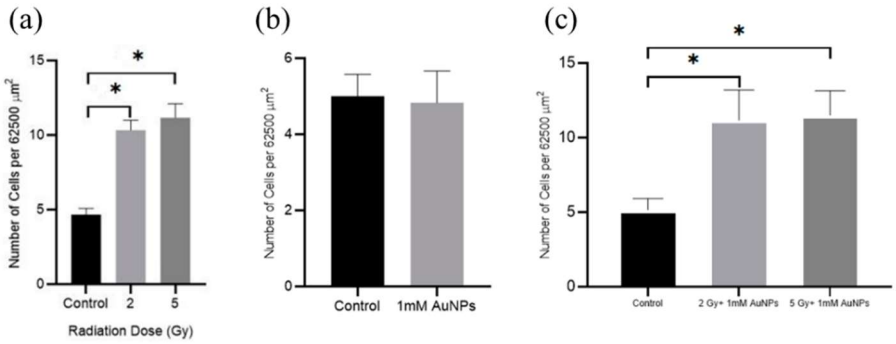

Figure 9. Effects of X-ray irradiation (IR) and/or Au NPs on the adhesion of DU145 cells. Cells were treated with (a) IR (2 or 5 Gy), (b) 1 mM Au NPs, and (c) IR (2 or 5 Gy) and 1 m MAu NPs. After 4 hr, the number of adhered cells were counted. Similar results were also obtained for A549 cells. *$p < 0.05$.

Furthermore, we investigated the effects of Au NPs in combination with X-ray on cellular migration of normal and cancer cells. Human colon normal (CCD841), colon cancer (SW48), skin normal (HEM) and skin cancer (MM481) cells were used in this study. The relative migration rate of cells irradiated with 5 Gy was found to be 85% and 80% for SW48 and MM418 cells respectively (Fig. 10). The treatment with Au NPs induced a similar suppuration in both cell types and when the cells were treated with the combination of Au NPs and X-rays an additive effect was determined, and the migration rate decreased to ~60%. Conversely, the combined treatments on normal cell lines (CCD841 and HEM cells) had a promotion effects on the migration to more than 120%. The further experiments are now ongoing to explain these differences that were found between normal and cancer cells. We also performed these experiments using synchrotron-generated X-ray beams, which was supported by Australian Synchrotron. The completed data are now in the process of publication.

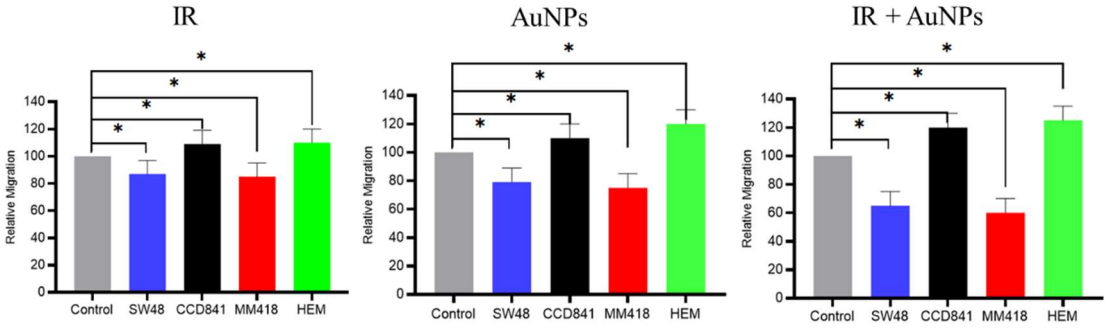

Figure 10. Effects of 5 Gy X-ray irradiation (IR) and/or 1 mM Au NPs on cellular motilities of SW48, CCD841, MM418, and HEM cell lines. Control represents untreated cells. *$p < 0.05$.

5. Development of novel NPs conjugated with photosensitiser agent

The aims of this study was to investigate an innovative way of expanding application of photodynamic therapy (PDT) technique. PDT involves the delivery of a photosensitiser to the tumour, followed by irradiation with specific wavelength of light, which activates the photosensitiser leading to the generation of highly ROS. However, the major drawback of PDT is the low penetration abilities of light and its scattering through tissues, making treatment of deeply seated tumours difficult if not impossible. In this study, we conjugated a photosensitiser agent with metal based NPs which could generate additional ROS when the scintillating lights activate the agent through the same process of PDT.

We synthesised iridium (Ir) based NPs with poly(vinylcarbazole) (PVK) and an amphiphilic polystyrene-graft-PEG (PS-g-PEG) as light scintillating and surface-coating compounds, and porphyrins as photosensitiser agent. The size of synthesised Ir-PVK NPs was about 200 - 300 nm which was much larger than we expected and the shape was not spherical (Fig. 11). The cytotoxicity of these NPs was assessed *in vitro* using DU145 human prostate cancer cells. The result shows that there was no cytotoxicity at up to 250 μg/ml (Fig. 12). The combination of Ir-PVK NPs and 6 MV X-rays elicited a greater cytotoxic effect on DU145 cells compared to that of X-rays alone (Fig. 13). Although these results are preliminary, the synthesised Ir-PVK NPs showed the ability to enhance radiation effects. The ongoing research is to optimise NP size and shape, to detect ROS generated by these NPs, and to evaluate dose enhancement effects using various cell lines.

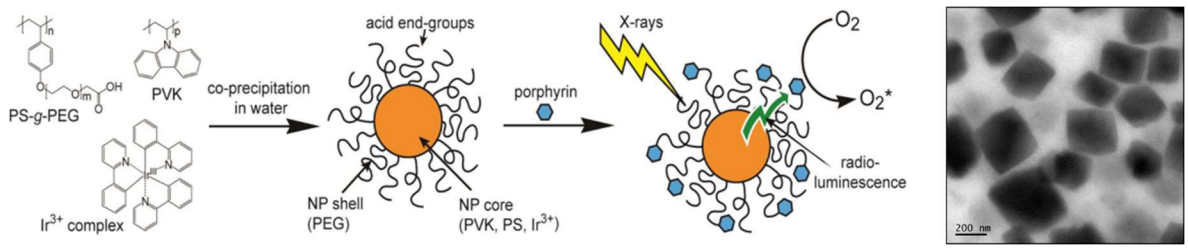

Figure 11. Scheme of the synthesis of photosensitiser-conjugated nanoparticles. A right picture shows a representative transmission electron microscope image of the synthesised nanoparticles.

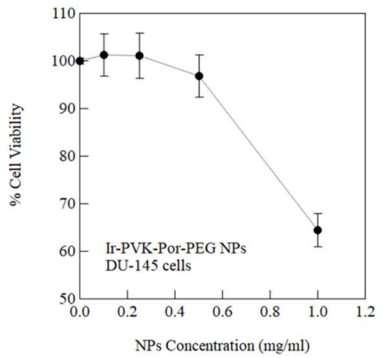

Figure 12. Effects of Ir-PVK NPs with the different concentrations on the viability of DU145 cells.

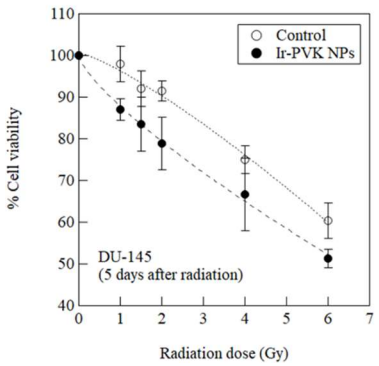

Figure 13. The surviving fraction of DU145 cells treated Ir-PVK NPs and X-ray irradiation. Control represents cells treated without NPs.

This work has been supported by Geelong hospital, and University of South Australia. The part of this research was presented at American Society of Radiation Oncology (ASTRO) conference 2019.