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Ultra-small platinum-based coordination nanoparticles for radiotherapy†‡

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Radiation therapy is an effective and localised method for cancer treatment and its efficacy is considerably improved by using radioenhancing agents. Nanoparticles containing high atomic number (*Z*) elements have emerged as an appealing class of enhancers. This work reports the first example of radioenhancers based on ultrasmall platinum (Pt)-polycyanometallate nanoparticles. Importantly, both novel nano-enhancers (Pt[Pt(CN)₄] and Pt[Fe(CN)₆]) are prepared in fairly large quantities using a water-based, surfactant-free, green route. The post-coating of the inorganic cores by different biocompatible polymers is straightforward and allows probing of the influence of the coating on the radiotherapeutic efficiency of the nanosystems. The structural/chemical integrity is maintained after high doses of γ -radiation and high colloidal stability is obtained in biological media. At 250 μ M total metal concentration, these nanoparticles are non-toxic to cells, but when coupled with γ -radiation, they amplify apoptosis of HeLa cells by 20% at 4.5 Gray, which rises to 60% at larger doses. Furthermore, irradiation studies conducted on a model DNA probe evidence that the amount of lethal Double Strand Breaks (DSB) is significantly enhanced in the presence of these novel Pt-based radioenhancers. The radiotherapeutic efficiency of these versatile coordination nanoparticles obtained using a cheap, fast and green process opens interesting possibilities in the field of nanomedicine.

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Introduction

Even in the 21st century, cancer remains one of the biggest healthcare challenges with 19.3 million new cases detected in 2020, resulting in 10 million deaths all across the globe.¹ There is widespread ongoing research to develop targeted, safe, and patient-specific treatment methods. Radiation therapy is applied to more than 50% of cancer patients to eliminate cancerous tissues in a localised way while minimizing the chances of recurrence in the affected area. A major advantage of radiotherapy is that tumours located deep within the body could also be targeted using high energy X-rays, γ -rays or ion beams as their penetration depth is greater than 10 cm.² However, a high dose of these ionizing radiations can damage the healthy tissues and organs adjacent to the tumour, and cause adverse side-effects like severe fatigue, radiodermatitis and in some cases, even second

cancers due to mutations generated during the process.³ Therefore, several radio-enhancers have been developed over the past two decades to maximize cancer cell killing while working within a safe range of therapeutic doses of radiation. These agents need to be stable, non-toxic to normal tissues and fairly resistant to metabolic breakdown.⁴

Irradiating cells internalized with high-*Z* elements generate a cascade of secondary electrons.⁵ These electrons are brought about by the ionization of heavy metal atoms, which induce a significant increase in the amount of reactive oxygen species (ROS) produced upon water radiolysis. When these radicals are produced in close proximity to the cancer cells, their proliferation is stalled, causing apoptosis and subsequent elimination. In 2004, J. F. Hainfield *et al.* provided the first proof that gold nanoparticles (NPs) significantly improved the *in vivo* efficacy of radiation therapy. They demonstrated that the ultra-small size of these particles proves beneficial for bio-distribution and relatively high concentrations of the heavy metal could be incorporated without toxicity.⁶ The potential of metallic NPs in enhancing the biological efficiency of radiation was realised, and this resulted in an exponential increase in the development of varied particles that can improve the performance of conventional radiation therapy. Additionally, nanoparticles provide a relatively large surface area and are flexible towards size, shape, and charge modifications, which proves to be

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At the end of the incubation period, the culture medium was removed; the cells were rinsed with PBS solution and harvested after trypsinization. The cells were then counted using LUNA counter and centrifuged for 7 min at $200 \times g$. The supernatant was removed and the cell pellets were stored in glass tubes. Before analysis, the pellets were dissolved in aqua regia and diluted using ultrapure water. The quantification of platinum internalized within the cells was performed at UT2A (Pau, France) by inductively coupled plasma (ICP) analysis.

Radio-sensitizing effect on cells

The impact of radiation on cell survival in the presence of prepared NPs was evaluated using a colony formation assay. HeLa cells were cultured in T25 flasks and maintained at 37°C overnight, in a humidified 95% air–5% CO_2 atmosphere. They were then incubated for 6 hours with an enriched culture medium containing PtFe and PtPt NPs at $2.5 \times 10^{-4} \text{ mol L}^{-1}$ total metal concentration. The cells with the nanoparticles, as well as the control containing an equivalent amount of water, were irradiated using the Cs-137 (0.63 MeV) source at Institute Curie. The dose supplied ranges from 0–7.5 Gy. After the exposure, the cells were seeded onto Petri dishes to start the standard 14 day culture time. Colonies formed were fixed, stained, and counted to determine survival fractions at each dose and the data obtained was normalized against the corresponding control. Each measurement was performed in triplicate and the average was used to calculate dose enhancement and amplification factors in the presence of high-Z nanoparticles.

Results and discussion

Synthesis and characterization of Pt-based NPs

The synthesis of $(\text{Pt}[\text{Fe}(\text{CN})_6])$ and $(\text{Pt}[\text{Pt}(\text{CN})_4])$ self-standing nanoparticles is described in the experimental section.

Hereafter, for the sake of simplicity, they will be referred to as PtFe and PtPt NPs. A few studies have reported on colloids of Pt/polycyanometallate-based particles mainly for catalysis purposes, but stabilizers were used to stop their evolution to cyanogels.^{20,30}

In our case, the one-step reaction was carried out in pure water under easily reproducible conditions. A Pt(II) salt was used in the experimental procedure to incorporate a high-Z metal in the NP. Once the activation temperature was reached, the addition of K_2PtCl_4 to the corresponding polycyanometallate solution (either $\text{K}_4[\text{Fe}(\text{CN})_6]$ or $\text{K}_2[\text{Pt}(\text{CN})_4]$) triggered the gradual substitution of chloro (–Cl) ligands by the cyano (–CN) ligands. This was followed by a change in the colour of the reaction mixture depending on the precursors used. The reaction time, temperature, and ratio of precursors added play a crucial role in the final structure and composition of the NPs. The colloidal solutions were fully stable over several months.

TEM micrographs reveal homogenous PtFe and PtPt nanoparticles. The size distribution histograms (Fig. 1) calculated by averaging the sizes of 200 NPs reveal that PtFe particles have a core average diameter of $5.2 \pm 1.0 \text{ nm}$ and PtPt is much smaller at $1.2 \pm 0.4 \text{ nm}$. Due to the intense absorption of the green-coloured PtFe colloidal solution in the laser absorption region, the hydrodynamic diameter obtained was not reliable. DLS measurements indicated that the hydrodynamic diameter of synthesized PtPt NPs was $9.9 \pm 3.9 \text{ nm}$ which appeared to increase by $\sim 3 \text{ nm}$ in three days. However, the addition of PVP after the synthesis, stops the growth and the sub-10 nm size is maintained (ESI,† Fig. S1). The zeta potential of PVP-coated PtFe and PtPt in water was around $-23 \pm 5 \text{ mV}$ and $-27 \pm 6 \text{ mV}$ respectively (ESI,† Fig. S2). The presence of polycyanometallate complex at the surface of the NPs explains the negative charge and their long-term stability in the colloidal solution. The strong electrostatic repulsions prevent aggregation of the particles and

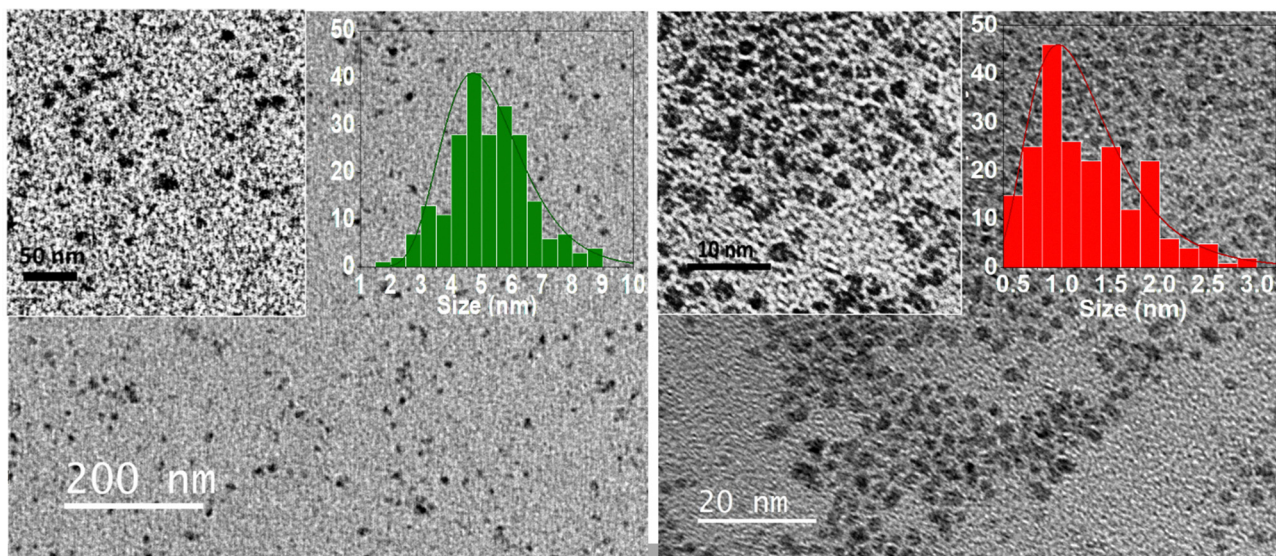


Fig. 1 TEM micrographs of PtFe NPs (left) and PtPt NPs (right) at different magnifications, with their corresponding size distribution charts.



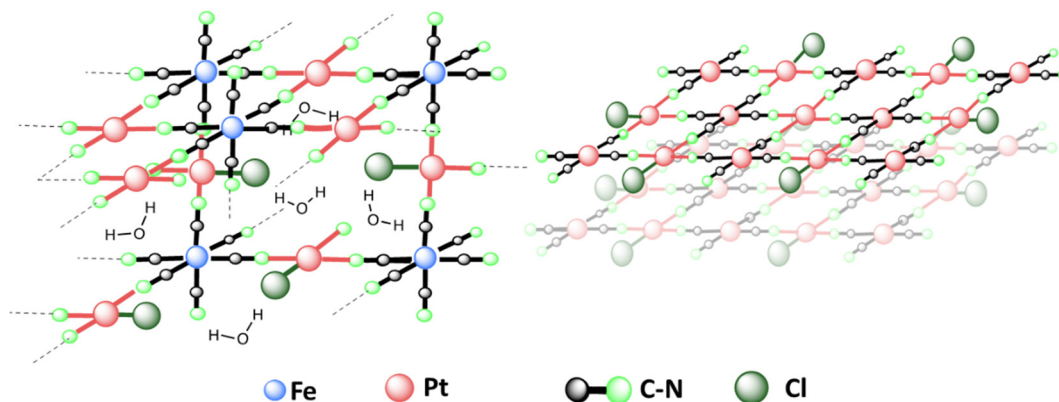


Fig. 3 Proposed structure of PtFe (left) and PtPt (right) NPs based on spectroscopic analysis results. PtFe has a 3D structure similar to Prussian Blue, with large number of vacancies due to incomplete replacement of Cl ligands with CN. PtPt has a 2D stacked structure due to tetracyanoplatinate coordination with 4 other Pt(II).

in Foetal Bovine Serum (FBS). The evolution was studied using UV-visible spectroscopy at the normal body temperature of 37 °C. PtFe NPs underwent very minimal change over the course of 15 hours in FBS (ESI,† Fig. S9). The minor peaks at 414 nm and 772 nm which corresponds to a small amount of Fe^{III} present in the NP, experience a slight decline over time. This is due to the high affinity of phosphate present in the serum towards ferric ions. However, the major band representing Fe^{II}-CN-Pt^{II} that is integral for the nanoparticle structure undergoes no degradation. Similarly, the Pt^{II}-CN-Pt^{II} band around 306 nm in PtPt NPs remains unaffected over 15 hrs at 37 °C. These studies provide an indication that these polycyanometallate-based coordination NPs remain stable during their transport within the human body.

Similar tests were run in artificial lysosomal fluid (ALF) and the evolution of the absorption spectra was studied for a duration of 6 hours (ESI,† Fig. S10). The major peaks of both NPs did not change even in this acidic medium. A small increase in intensity was seen in the peaks corresponding to Fe^{III}, which is attributed to the slight oxidation of Fe^{II} under such conditions. Altogether, it was concluded that these novel nanoparticles are not damaged by the cellular medium and could be used for further tests.

In order to evaluate the viability of the NPs as radio-enhancing agents, their stability under irradiation was investigated. Both the solutions with NPs and the controls (precursors), in the presence of PVP and dextran polymers were exposed to a gamma-radiation dose of 4 kGy. Upon irradiation, reactive oxygen species (ROS) are generated in solution. The ROS play a crucial role in enhancing the effect of radiation in living cells. They are capable of oxidising Fe^{II} to Fe^{III} and it is substantiated by the appearance of a new band around 425 nm after irradiation of ferrocyanide control solution due to the LMCT band of ferricyanide moiety (ESI,† Fig. S11). However, in the presence of dextran polymer, this evolution was not observed indicating no oxidation had taken place. This evidenced that dextran is capable of scavenging the ROS formed and therefore is not appropriate for coating nanoparticles developed for radiation therapy. Since PtFe and PtPt nanoparticles could easily be post-coated, the synthesis was optimised to

include PVP polymer. The presence of PVP does not hinder ROS generation as evidenced by the LMCT band in the irradiated ferrocyanide + PVP sample (ESI,† Fig. S11b). DLS, TEM and UV-visible spectral analysis performed before and after radiation showed that PtFe and PtPt nanostructures are not significantly altered. Thus, they remain stable during the course of radiation treatment. Due to the ROS-scavenging nature of the polymer, the NPs coated with dextran were discarded and further experiments were only performed using PVP-coated NPs.

Radio-induction of nanoscale damage

It has been hypothesised that high-energy radiation beams induce irreparable damage to cell organelles, proteins and nucleic acids by producing ROS, during water radiolysis. These nanometric damages on biomolecules lead to mutations, cell death, and subsequent elimination, and is the most commonly reported working principle behind radiotherapy. In order to study the impact of these new NPs to induce such complex damage on biomolecules, plasmids were used as bio-nanoprobes. They were incubated with PtFe and PtPt NPs ($[M] = 5.3 \times 10^{-6} \text{ mol L}^{-1}$) and subjected to varying doses of radiation. The intact form of the plasmid exists in a supercoiled conformation. Breaks in this circular double-stranded model-DNA diminishes the constraints in the structure and leads to the formation of relaxed (*R'*) and linear conformations (*L'*). The three forms of DNA were separated by gel electrophoresis and the detected amount of each enables quantification of single and double-strand breaks. As described in the experimental section, the amount of DSBs were calculated using the amount of *L'* that rose out of irradiating plasmid pBR322.

The number of lethal, nanometric DSBs generated as a function of radiation dose (0–160 Gy) is presented in Fig. 4. As expected, the number of nanosize breaks rises with an increase in the dose of radiation applied to the samples. But, in the presence of PtFe and PtPt NPs, the amount of damage is significantly enlarged and the sensitizer enhancing ratio (SER) was used to quantify the efficiency of the NPs. It was calculated using the following equation:

$$\text{SER} = \text{DSB}_{\text{NP}} / \text{DSB}_{\text{control}} \quad (2)$$



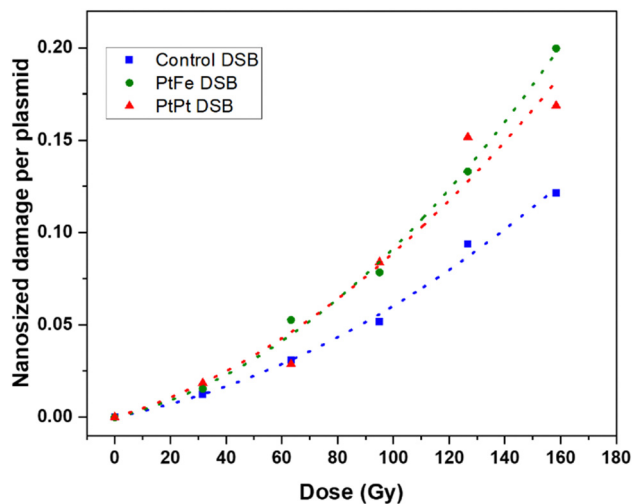


Fig. 4 Nanometric damages induced by gamma radiation on plasmid nanoprobe without NPs (blue) and in the presence of PtFe (green) and PtPt (red) NPs and their respective fitted curves.

where DSB_{NP} and $DSB_{control}$ correspond to the number of nanosize damages per plasmid induced by a certain radiation dose when NPs are present and absent in the solution respectively.

Table 1 Sensitizer enhancer ratio (SER) of PtFe and PtPt NPs at different radiation doses

Dose (Gy)	SER FePt	SER PtPt
30	1.34	1.49
65	1.44	1.48
95	1.51	1.48
125	1.55	1.47
160	1.59	1.46

It is interesting to see that with PtPt, irrespective of the dose supplied, an almost constant enhancement in double-strand breaks in the range of 46–49% was obtained (Table 1). On the other hand, PtFe NPs produce a comparatively smaller effect at low doses (SER 1.34 at 30 Gy) and sequentially intensifies the breaks to the model DNA with increasing dose of radiation (1.59 at 160 Gy). The aim of adding radio-enhancers is to maximize the amount of damage to the cancerous cells, instigate quick apoptosis and minimize the possibility of regeneration while applying a therapeutic dose of radiation. Therefore, even an amplification of 30% in DSBs, in the presence of these porous coordination nanoparticles is very significant. It is important to note that the plasmid used for the experiment is a representative model for different organelles present in the cell and not specifically for DNA in the nucleus. As shown by several groups most NPs do not penetrate or enter the cell nucleus, but complex damage to cytoplasmic organelles also proves detrimental to the cell.^{31,32} Here, we have demonstrated that at a minimal concentration of $5.3 \times 10^{-6} \text{ mol L}^{-1}$, both nanoparticles amplified the effect of radiation, indicating clearly that they could act as radiation-enhancing agents. However, in contrast to cells, once the strand breaks occur after irradiation, no repair mechanisms happen within the plasmid. Therefore as a next step, the effect of NP and radiation on living cells needed to be understood.

Cytotoxicity, uptake and radiation effects of nanoparticles in cells

The primary fibroblasts are used as a model of healthy cells with normal metabolism, while HeLa cells provide an indication of the effect of NPs towards cancerous cells. The effect of a range of different metal concentrations ($[M]$) in PtFe and PtPt NPs was tested in both cell lines with an incubation time of 6 h (Fig. 5). The columns represent average survival fractions with

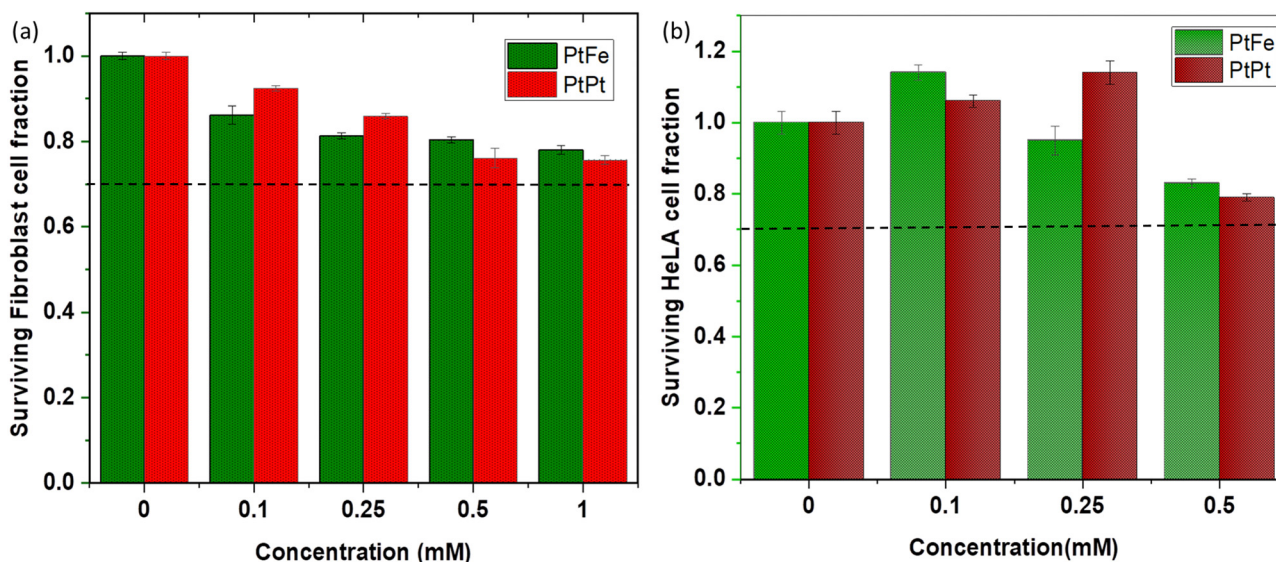


Fig. 5 *In vitro* toxicity results of PtFe and PtPt NPs evaluated by a clonogenic assay using (a) fibroblast cells and (b) HeLa cells after 6 h incubation. The total metal concentration: $[Pt + Fe]/[Pt]$, was considered for the experiment and it lies in the range of $0-1 \times 10^{-3} \text{ M}$. The black dotted line corresponds to the safety limit according to ISO-10933.



respective average deviation values. According to the international standard for biological evaluation of medical devices, a sample is considered non-cytotoxic when even the highest concentration provides 70% cell survival.³³ The fibroblast cell viability is close to 80% at the highest metal concentration ($[M]$) of 1×10^{-3} M for both nanoparticles indicating their safety for medical applications, towards healthy cells. HeLa cells appear more sensitive to the presence of nanoparticles as they reach the same limit at 5.0×10^{-4} M concentration. Therefore, a lower and safer total metal concentration of 2.5×10^{-4} mol L⁻¹ was chosen for further experiments with HeLa cells, as the cancer cell-killing effect rising specifically from radiation enhancement needed to be quantified.

ICP-OES analysis of HeLa (*ca.* 1.3×10^6) cells incubated with PtFe NPs for 6 h showed that a total of 73 ng of Pt was internalized. Therefore, 0.28 fmol of this high-Z element was taken up by one cell. In the case of PtPt NPs, 182 ng of platinum was detected in *ca.* 1.8×10^6 HeLa cells, meaning the amount of Pt internalized was approximately 0.52 fmol per cell. The impact of these particles in cell killing was studied using γ -rays from a Cesium-137 source (0.63 meV). The cancer cells incubated with NPs were irradiated for different time intervals at a dose rate of 0.75 Gy min⁻¹. The colonies formed at the end of the standard 14 day incubation period helped determine the survival fraction (SF) at each dose.

The colony count data were fitted using a linear-quadratic model (LQM) (SF vs. dose) using eqn (3):

$$SF(D) = e^{-(\alpha D + \beta D^2)} \quad (3)$$

D corresponds to the dose supplied, while the parameters α and β describe the cell's radiosensitivity. α represents the induction of directly lethal damages whereas β term corresponds to additive sub-lethal lesions leading to cell death. The α/β provides the dose (Gy) at which 50% of the damages caused to the cells are due to the directly lethal effect of radiation while the rest are a result of additive damages triggering a lethal consequence.

The percentage of surviving cells sequentially decreased with increasing the radiation dose but as presented in Fig. 6, this decline is significantly amplified in the presence of PtFe and PtPt NPs. This result proves the radioenhancing property of these novel polycyanometallate-based NPs. The effectiveness of NPs as radioenhancers was quantified using Dose Enhancement Factor (DEF) which gives a measure of the efficiency of *in vitro* cell killing at a given dose point (D). It was calculated using the following equation:

$$DEF = SF_{\text{control,fit}}/SF_{\text{NP,fit}} \quad (4)$$

SF_{control} and SF_{cyanosol} correspond to the survival fractions of the dose of radiation. Until a radiation dose of 4.5 Gy, both NPs behave similarly and a DEF of ~ 1.25 was obtained. As the dose supplied to the sample increased, high DEF values up to 1.92 for PtPt and 2.45 for PtFe were observed. This corresponds to a 48% and 59% rise in the cancer cell-killing rate in the presence of 2.5×10^{-4} mol L⁻¹ PtFe and PtPt NPs. As reported in Table 2, the α in all three cases are quite similar, indicating that the number of directly lethal damages produced as an effect of

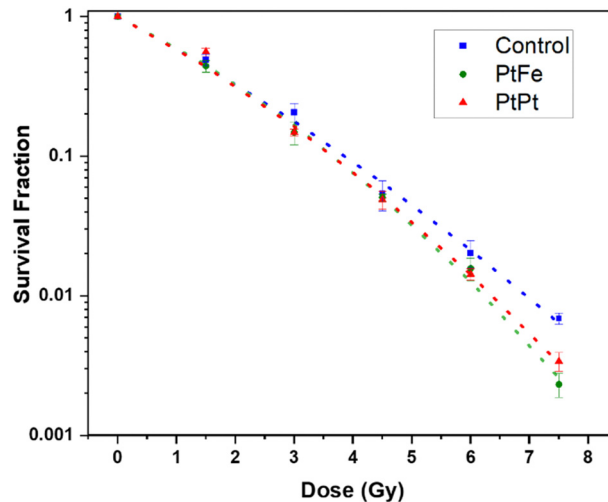


Fig. 6 Survival fraction of HeLa cells after Cs-137 irradiation without NPs (blue), in the presence of 2.5×10^{-4} M PtFe (green) and PtPt (red) NPs using LQM (dotted lines) as a function of dose.

Table 2 Values of α and β obtained from the fitting curve for NPs and control experiment in HeLa cells in the presence of radiation

Sample	α (Gy ⁻¹)	β (Gy ⁻²)	α/β (Gy)
Control	0.515 ± 0.050	0.021 ± 0.007	24.30 ± 9.44
PtFe	0.464 ± 0.050	0.044 ± 0.008	10.57 ± 2.21
PtPt	0.500 ± 0.049	0.034 ± 0.007	14.38 ± 3.56

radiation does not change much in the presence of these NPs. However, the β value shifts from 2.1×10^{-2} Gy⁻² in the control (no NPs) experiment to 3.4×10^{-2} Gy⁻² for PtPt. In the case of PtFe NPs, the β is more than double that of the control, at 4.4×10^{-2} Gy⁻². This significant increase in β value indicates that the radioenhancing mechanisms of such polycyanometallate NPs are unique as they first produce a high amount of sub-lethal damages, which then add up and lead to cancer cell death.

Compared to the control, the presence of Pt increases the number of radicals formed in the solution. It is possible that, due to the microporosity of the NP, the generated ROS quickly disperses through the biological system causing a markedly high amount of sub-lethal damages that is transformed into a final lethal effect in the cell. It has to be noted that as seen with plasmid experiments earlier, despite the presence of a lower amount of high-Z element in PtFe NP, they produce similar and sometimes even better amplification of radiation damage when compared to PtPt NPs.

This could be due to two reasons. The γ -radiation interacts with Fe atoms as well and following a combination of various phenomena (activation, de-excitation and emission of electrons) also leads to the production of radicals that help with cell killing.²⁷ Secondly, as compared to the 2D sheets that form PtPt NP, the PtFe nanostructure is a Prussian Blue-like structure full of defects and vacancies that can be occupied by water molecules. Therefore, more reactive radicals are formed within and around PtFe NP and they have higher freedom of mobility to



