Published in final edited form as:

ACS Nano. 2019 May 28; 13(5): 5077–5090. doi:10.1021/acsnano.8b07982.

Cross-Correlative Single-Cell Analysis Reveals Biological Mechanisms of Nanoparticle Radiosensitization

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Abstract

Nanoparticle radiosensitization has been well demonstrated to enhance effects of radiotherapy, motivated to improve therapeutic ratios and decrease morbidity in cancer treatment. A significant challenge exists in optimizing formulations and translation due to insufficient knowledge of the associated mechanisms which have historically been limited to physical concepts. Here we investigated a concept for the role of biological mechanisms. The mere presence of gold nanoparticles led to a down regulation of thymidylate synthase, important for DNA damage repair

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Author Contributions:

T.T. sample preparation, data acquisition, analysis, image processing, figure preparation, interpretation; M.D. irradiation, dosimetry and image processing; N.H.W. distribution fitting; R.B. and D.H. flow cytometry analysis and nanoparticle characterization; M.L. γ H2AX protocols; D.P. XRF imaging; E.B. radiotherapy context, dosimetry quality assurance and interpretation; B.T. discussion and interpretation; I.K. conception, data acquisition, interpretation, figure preparation. All authors contributed to manuscript preparation.

Data Availability.

The data that support the findings of this study are available upon reasonable request.

Code Availability.

Custom scripts used in this manuscript can be provided by authors upon request. These available custom scripts were utilised in this work to perform the multivariate analysis, classification of a cell's phase in the cell cycle as well as quantification and cross-correlation of γ H2AX foci and Au content per cell.

ASSOCIATED CONTENT

Supporting Information Available: The supporting information document includes confocal microscopy, XRF and TEM images. Data on AuNP characterization and stability; DAPI histogram distribution; curve fitting parameters; cell-population statistical comparisons; and further data on foci as a function of gold content are included. Supporting Information is available free of charge via the Internet at <http://pubs.acs.org>.

in the radioresistant S phase cells. By developing a cross-correlative methodology to reveal probabilistic gold nanoparticle uptake by cell sub-populations and the associated sensitization as a function of the uptake, a number of revealing observations have been achieved. Surprisingly, for low numbers of nanoparticles a desensitization action was observed. Sensitization was discovered to preferentially impact S phase cells where impairment of the DNA damage response by the homologous recombination pathway dominates. This small but radioresistant cell population correlates with much greater proliferative ability. Thus a paradigm is presented whereby enhanced DNA damage is not necessarily due to an increase in the number of DNA Double Strand Breaks (DSBs) created, but can be from a nanoparticle-induced impairment of the damage response by down regulating repair proteins such as thymidylate synthase.

Graphical Abstract

Keywords

nanoparticles; radiotherapy; radiosensitization; gene regulation; DNA damage repair

Radiotherapy has achieved significant therapeutic improvements by increased sparing of normal tissue with more accurate beam conformation to treatment target volumes. Since the physical capacity to improve X-ray radiotherapy has all but plateaued, recent improvements in radiotherapy have been dominated by optimisation of concurrent chemoradiotherapy. Significant, but incremental, advances are achieved with optimisation of adjuvant and concurrent treatments, but improvements in tumour control have generally been marginal and come at the expense of systemic toxicities. The most anticipated course for therapeutic improvements is through manipulation of the cancer cells' susceptibility to be damaged by radiation. One promising avenue is through use of nanoparticles which have advanced to a number of clinical trials, principally driven by concepts of locally increased physical absorption cross-sections associated with high atomic number (Z) elements. However, current literature is contradictory: Nanodiamonds¹ can achieve a sensitization effect comparable to high-Z gold nanoparticles; nanoparticles induce sensitization in energetic proton beams² although negligible sensitization is predicted by physical concepts;^{3, 4} and physical theories only partially explain significant macroscale effects observed for

megavoltage X-ray sources.⁵ While probabilities of the physical sensitization mechanisms are well described mathematically, their probabilities do not correlate with the magnitude of radiobiological response observed, particularly for MV photon sources.⁵

The primary cellular target in radiotherapy is DNA. DNA damage from ionising radiation occurs either by direct damage, but predominantly indirectly by the formation of reactive oxygen species. Nanoparticles most often accumulate within the cytoplasm or cytoplasmic vesicles yet impart a radiosensitization effect even though the spatial range of dose enhancement by physical mechanisms is insufficient to reach nuclear DNA. While there is perhaps some oversight of the roles of pair production, X-ray fluorescence⁶ and gamma production in current models, other mechanisms are expected. More recently, concepts around the localised dose deposition and interfacial catalysis of reactive oxygen species (ROS) formation are emerging.^{7, 8} However, species such as H₂O₂ require diffusion, transport *via* aquaporin's (for example), dissociation into hydroxyl radicals and to then undergo reactions with DNA to form DNA double strand breaks (DSBs). There is limited investigation either experimentally or theoretically as to the extent localised enhancement of ROS generated in the cytoplasm and compartments, can damage DNA in the nucleus. While DNA DSBs are traditionally taken as the primary indicator of radiotherapy efficacy, some studies have shown enhanced killing of cells by nanoparticle sensitization while no increase in the number of DSBs has been observed.^{9, 10} These observations have led to a recent shift away from the conventional theory of DNA acting as the primary target; with other damage pathways being proposed as instigators of cell death. Alternatively, or additionally, we hypothesized that the biological impact of altering cellular expression by the mere presence of nanoparticles could induce a sensitization effect *via* biological mechanisms. Literature is increasingly demonstrating that nanoparticles can alter cell regulation of numerous genes and proteins^{11, 12} which we hypothesized could indicate that down regulation of key genes involved in DNA damage repair could contribute to a biological mechanism of sensitizing cells.

TS was chosen as a test for impacting cell response to DNA insult (conceptually represented in Figure 1a). The greatest impact of TS on disease progression is specifically attributed to the production of nucleotides for repairing DNA in the Homologous Recombination (HR) repair pathway. Resistance of cells to radiotherapy and some chemotherapies (and hence the poorer patient outcomes reported¹³) is contributed to by the cell population that repairs DNA by the HR pathway. The HR pathway exists, and is most efficient, in cells in the DNA synthesis phase (known as the S phase) where an uncondensed sister chromatid acts as an effective repair template. If nanoparticles can down regulate TS expression then we believed this would correspond with impairing DNA damage specifically in S phase cells. However, a major limitation and challenge in many nanoparticle studies is to compare “like-with-like”, *i.e.* to compare cell populations with statistically equivalent nanoparticle uptake, as opposed to simply being exposed to the same co-culture conditions. Different nanoparticles are taken up differently by the same, or different cells, and needs to be accounted for to accurately compare inter-cellular behaviour, or nanoparticle parameters. Similarly, many challenges exist in studying cell sub-populations to examine their role in larger ensembles of a cell population to compare biological measures under identical conditions. Inherently large degrees of heterogeneity in cell behaviour confound many efforts in relating variables to

macroscale effects, or elucidating the mechanisms for inducing these observed effects. A further challenge is that specific cell sub-populations can be correlated with poor prognosis and therapeutic failure such as polymorphs,¹⁴ degree of stemness¹⁵ or phase.^{16, 17} Thus macroscale measures can be dominated by a small sub-population of cells.¹⁸

In this context, quantitative cross-correlation of cells' biological response to the uptake of nanoparticles is of great importance to the nanomedicine field. Here, we correlate label-free, transferrin conjugated gold nanoparticle uptake in prostate cancer (PC-3) cells determined by synchrotron X-ray Fluorescence (XRF) with radiobiological response in terms of DNA DSBs for large cell-populations (schematically represented in Figure 1b). Cells were co-cultured with a low, clinically relevant concentration (6 nM) of nanoparticles for 2 hrs (approximately 10 % of the cells' doubling time), a sufficiently short time-frame to minimise apparent inter-phase differences in cell nanoparticle content,¹⁹ potential arrest²⁰ or phase redistribution.²¹ However, within this time we observed significant down regulation of thymidylate synthase. Rinsed cells were irradiated in fresh media with ~4 Gy from an Ir¹⁹² high dose rate (HDR) brachytherapy source. We chose an Ir¹⁹² source, a commonly used radioisotope, both for its relevance with regard to clinical utility and the energy of emissions that theoretically utilise the physical dose enhancement effects of gold nanoparticles, *i.e.* predominantly being above the Au-K absorption edge. The entire spectrum consists of multiple emission energies with an average photon energy emission of ~370 keV.

Cells were stained 1 hr after irradiation for γ H2AX foci as a quantitative marker of DNA DSBs and DAPI for quantitation of DNA content within cell nuclei (Figure 3a,b and Supplementary Figure 1a,b). Imaging for these markers was performed with laser scanning confocal microscopy. Identical cell populations were scanned with synchrotron X-ray fluorescence (S-XRF) microscopy to quantify Au content. Cross-correlative image analysis provided quantification of nanoparticle uptake, DNA content and the number of DNA DSBs in each cell. Nanoparticle-dose response relationships revealed several interesting observations of importance to the radiosensitization phenomenon which so far have not been previously reported. A desensitization of cells occurred for low numbers of nanoparticles, however for greater numbers of nanoparticles the number of DNA DSBs increased with specific preference for radioresistant S phase cells. It is proposed the radiosensitization observed with the nanoparticles is dominated by a biological mechanism *via* down regulating thymidylate synthase expression and predominantly due to preferential sensitization of the small, but critical, S-phase sub-population

RESULTS AND DISCUSSION

Thymidylate Synthase is Down Regulated in PC-3 Cells Co-Cultured with AuNPs.

An important candidate with regard to cancer progression and control is Thymidylate Synthase (TS). TS is an enzyme that acts as a catalyst in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) which is subsequently phosphorylated to deoxythymidine triphosphate (dTTP).²² dTTP is an essential precursor utilised in the synthesis and repair of DNA. Importantly, TS represents the sole intracellular source of TMP and thus inhibition of TS allows exploitation of one of the rare metabolic bottlenecks in the synthesis of DNA, making it a therapeutic target for some of the most

successful drugs used in treating cancer.²³ TS expression has been shown to be elevated in numerous cancer tissues relative to their healthy counterparts with high levels being correlated with poor clinical outcomes^{24–26} along with poor response to radiation.²⁷

Thymidylate Synthase (TS) is an enzyme representing one of the few ‘bottle-necks’ in DNA replication and repair. TS expression, along with other DNA synthesis proteins, can correlate with poorer cancer related prognosis and disease progression.¹³ TS remains as a key target for many chemotherapeutics. Current chemotherapeutics that target TS include anti-metabolites designed to interfere with folate metabolism by essentially outcompeting upregulated biological processes. However, down-regulation of TS (rather than merely competing with it *via* anti-metabolites) is a potential alternative approach we wished to explore.

To investigate the possible effect of AuNPs on TS expression, PC-3 cells were co-cultured with AuNPs at a concentration of ~0.3 nM nanoparticle concentration. After a 2 hour co-culture, cells were fixed, stained with a fluorescent anti-TS antibody and analysed *via* imaging flow cytometry. As seen in Figure 2a, cells co-cultured with AuNPs showed a statistically significant ($p < 0.0001$) decrease in mean TS pixel intensity per cell compared to non-treated cells. In both conditions the distribution of mean TS pixel intensities were well represented by lognormal distributions (Figure 2c,d) with respective Cumulative Distribution Functions (CDFs) plotted in Figure 2b showing the shift in data. A convenient use of the CDF plots (Figure 2b) is to consider the differences in cumulative probability at key values such as the mean TS intensity of the control population and draw comparisons to the NP treated cells. From Figure 2b it can be seen that nearly 82% of the NP treated cells had TS expression lower than the mean for the control population (Supplementary Table 1).

This result poses interesting questions as to the mechanisms of altered gene regulation induced by nanoparticle internalisation^{12, 28} and how these may be exploited for targeting TS as per chemotherapeutic drugs such as 5-Fluorouracil (5-FU).^{23, 29, 30} Although an important distinction is that active compounds such as 5-FU interfere and disrupt TS action compared to AuNPs which are actually impairing TS expression. In addition, nanoparticle toxicities generally appear much less severe than chemotherapeutic agents. How the physical presence of nanoparticles can alter cell regulation is currently unknown but is the topic of continuing research, and is likely highly dependent on physico-chemical properties of the nanoparticles themselves, along with the protein corona they acquire.¹¹

Mean DNA Double Strand Breaks (γ H2AX foci) Per Cell Show No Increase in DNA Damage with AuNPs.

Cell populations co-cultured with AuNPs for 2 hours were then irradiated with a dose of ~4 Gy from an Ir¹⁹² source. DNA DSBs are highly cytotoxic, which (if unrepaired) can lead to cell death, mutation or cell cycle arrest. γ H2AX foci were measured in cells after exposure to 0 or 4 Gy with and without nanoparticles (Figure 3 c,d). There was no evidence of exposure to nanoparticles inducing apoptosis that would be indicated by ‘pan’ staining of cell nuclei. No statistical difference in mean γ H2AX foci counts per cell was measured in cell populations co-cultured with nanoparticles compared to cells without nanoparticles after exposure to 0 ($p=0.18$) and 4 Gy irradiation ($p=0.77$) (Supplementary Table 3).

This finding is in agreement with published *in vitro* results showing no increase in γ H2AX foci counts for cells co-cultured with AuNPs⁹ and gadolinium-based NPs¹⁰ prior to radiation treatment, although increases in γ H2AX foci have been reported with targeted gold nanorods³¹ (AuNRs) in the same cell line used in this report. In that work the authors attributed the observed increases in foci count after irradiation on the enhanced uptake achieved with the targeted AuNRs compared to non-targeted AuNRs. Non-targeted AuNRs showed a significantly lower uptake efficiency and consequently minimal increases in foci counts compared to radiation alone. Those data are also consistent in that a statistical increase in foci when nanoparticles are present is only achieved with a sufficiently high number of nanoparticles. The dependence on uptake has also been reported when comparing the radiosensitizing properties of different shape nanostructures³² whereas once data were normalised to cellular uptake all nanostructures had comparable sensitization properties. However, it must be noted that the aforementioned reports which conclude no increase in γ H2AX foci counts still observe radiosensitization effects when evaluated by clonogenic assays. Such results have led the authors to propose that DNA DSBs are not involved in the mechanism of nanoparticle radiosensitization and other cellular damage mechanisms must exist, such as mitochondrial oxidative stress.³³ While not verified, Stefanciková *et al.*¹⁰ proposed a mechanism of damage to cytoplasmic vesicles and independence from nuclear DNA damage. In such cases, the radiobiological response relies on assumptions with regard to nanoparticle uptake and may in-fact vary significantly from one formulation to another and from one cell to another, obscuring correlations that may elude to sensitization mechanisms.

Low Numbers of AuNPs Desensitize Cells to Radiation.

Given the conflicting reports in the literature regarding the impact of internalised AuNPs on radiation-induced DNA damage, we sought to statistically compare the number of γ H2AX foci between sub-populations of cells by comparing groups of cells with similar nanoparticle uptake. This capability enabled by our analytical approach to discern population nanoparticle- dose responses. The population of cells with nanoparticles shown in Figure 3d was broken into 4 quartiles with equal numbers of cells in each quartile. γ H2AX foci data were compared across the groups (Figure 3e). Surprisingly, low quantities of AuNPs, represented by the first quartile averaging 4.31 pg/cell, induced a desensitization effect compared to cells receiving radiation alone, although the decrease was found to be marginally insignificant ($p=0.057$) from a 1-way ANOVA analysis. However, by further separating the bottom quartile data into 2 equal halves (Supplementary Figure 3) a statistically significant decrease in foci count ($p=0.046$) existed between cells in the lower half of this group (mean Au content = 2.89 pg/cell) and cells with no NPs. Considering only the physical mechanisms of sensitization, this observation would suggest a decrease in the physical absorption of the gamma rays and hence radiation dose for low numbers of nanoparticles. This concept contradicts basic radiation physics and current physical models. A possible explanation would be preferential uptake of nanoparticles in the radiosensitive cells compared to radioresistant cells (discounted later in the current work), or that small numbers of nanoparticles condition the cells with a stress-response, desensitizing them to the subsequent irradiation; suggesting an underlying biological response.

Mean γ H2AX Foci Counts Per Cell Increase for Cells with High Numbers of Internalised AuNPs.

In contrast to the groups with low Au content, statistically significant increases in mean γ H2AX foci per cell were found when comparing the bottom quartile with the 3rd and 4th quartile (p-values of 0.003 and 0.0001 respectively), as well as for comparing the 2nd and 4th quartiles (p= 0.014). Importantly, statistically significant differences were found between the 3rd and 4th quartiles and the control population. A summary of mean γ H2AX foci values is given in Supplementary Table 4 and a table of *p*-values for subpopulation comparisons is included in Supplementary Table 5.

This finding is highly noteworthy. Despite that no significant difference in the mean foci per cell was found for the whole cell-population comparing cells with and without AuNPs (Figure 1d), significant differences in γ H2AX foci counts were found between subpopulations defined by Au content with the control group as well as between cell groupings with differing Au content. In other words, we observed no difference in foci between control and AuNP exposed cells, yet we do observe a correlation of increasing numbers of DSBs for increasing Au content. The significance of this sensitization at the whole population level, however, was obscured by the simultaneous decrease in mean foci counts for cells with low quantities of AuNPs. This highlights the importance of measuring multiple parameters simultaneously for individual cells, rather than bulk assays. Furthermore, this provides a possible explanation as to why some reports observe radiosensitization in bulk assays, such as clonogenic assays, but fail to detect increases in DNA damage. Without the single cell correlated data, our current report would concur with the conclusion by Jain *et al.*⁹ and Štefancikova *et al.*,¹⁰ however we show here that the DNA DSBs continue to remain as a potential mediator in cell response contrary to the “provoking hypothesis”¹⁰ that NPs do not impact DNA damage and/or repair. Based on our data, for our system, we expect to only see a statistical increase in the DNA DSBs of the total cell population under the condition where a greater number of nanoparticles are internalised by cells, or at least internalised more homogeneously above the lowest quartile data in Figure 3e (*i.e.* greater than roughly 10 pg/cell). Given these findings, parameters which influence NP uptake levels *in-vitro* such as cell line, particle size, treatment concentration and surface functionalization must be carefully considered when interpreting radiosensitization effects. Along with these NP parameters the inherent heterogeneity in NP uptake³⁴ is also a factor demanding significant consideration.

To assess the correlation between Au content and γ H2AX foci for individual cell data, the cross-correlated data pairs (*i.e.* Au and γ H2AX foci count) for each cell were fitted using a bivariate normal distribution (BVN) (Figure 4a). A condition for fitting the BVN is that the distributions of both data sets be normally distributed. This condition was satisfied for the γ H2AX foci data as well as the log₁₀ transformed Au data (Figures 4b,c). A chi-squared goodness of fit test confirmed that the log₁₀ Au data fits a normal distribution (p=0.35, meaning there is no evidence to reject the null hypothesis that the data fits a normal distribution). The fitting of the BVN gives a correlation coefficient (giving the degree of correlation) that ranges between -1 (perfect negative correlation) and 1 (perfect positive correlation) between γ H2AX foci count and Au content. The corresponding *p*-value tests the

null hypothesis of no correlation against the alternative that the correlation is statistically significant from zero. In this analysis, this translates to the null hypothesis being the presence of zero correlation between measured γ H2AX foci count and Au content. A p -value < 0.05 rejects the null hypotheses, implying a statistically significant correlation between measured γ H2AX foci count and Au content.

Figure 4a presents the correlated Au content and γ H2AX foci data sets with each data point representing an individual cell. The calculated correlation coefficient of 0.309 and associated p -value being < 0.0001 indicates that there exists a positive, non-zero correlation between AuNP content and γ H2AX foci. It is important to note at this point that this correlation is for the entire cell population. Later, we identify the correlation depends strongly on cell cycle phase, peaking at 0.54 for late S-phase cells. The finding of a positive correlation between AuNP content and γ H2AX foci supports the findings in Figure 3e and highlights the potential of cross-correlated microscopy approaches in mechanistic evaluations of NP mediated radiosensitization. Not only does this approach provide evidence of a correlation between AuNP uptake and γ H2AX foci counts, the two dimensional data measures the correlation (or lack thereof) directly. Inherently correlated data at the single cell level allows for differences in correlations to be measured between cell populations with similar marginal distributions; *i.e.* the effects of heterogeneity and cell sub-populations are observable within the correlated data and two sample sets with the same mean, but different distribution can be differentiated.

AuNP Uptake is Independent of Cell Phase.

Figure 3e revealed that cells with low numbers of internalised nanoparticles correlated with a decrease in γ H2AX foci. It is well established that cells' radiosensitivity varies with progression through the cell cycle phases. We wished to test the possibility that the most radioresistant cells also internalised fewer nanoparticles on average, which could explain the observation across the sub-populations in Figure 3e. Our cross-correlative approach allows separation of cell sub-populations based on cell cycle using DAPI as a marker of DNA content. This is pertinent given the non-uniform cellular response to radiation throughout the cell cycle.¹⁶ Cells were co-cultured with AuNPs for 2 hrs. This duration corresponds to approximately 10% of the doubling time for this cell line and was chosen to limit the effects of dilution of AuNP content as cells pass through mitosis. Along with mitigating the effects of mitosis, rates of NP uptake have been shown to be independent of cell phase¹⁹ and therefore for short incubation times comparable levels of NP uptake can be expected for cells regardless of phase. The specific stage of the cell cycle was determined by analysis of the normalised histogram of integrated DAPI intensity (Supplementary Figure 4), as is well established in the literature.³⁵ While this protocol does not provide resolution of the DNA content comparable to methods such as flow cytometry, it is excellent for facilitating cross-correlative imaging.

For our conditions, AuNP uptake was also found to be independent of the specific stage of the cell cycle (Figure 3a) and is consistent with co-culturing for periods of time that are small compared to the cells' doubling time.¹⁹ Statistically comparable uptake between phases was confirmed from 1-way ANOVA analysis with means and standard deviations of

AuNP uptake presented in Supplementary Table 7. The probability and cumulative distributions of AuNP uptake in cells for each phase were well described by a lognormal distribution (Figures 5b–d). This distribution of cellular NP uptake is consistent with previous reports for both AuNPs³⁶ and Zinc Oxide NPs.³⁷ We believe such heterogeneity is reflective of the natural heterogeneity of receptors that mediate the internalization of the nanoparticles. The expression of receptors and proteins such as TS (observed in Figure 2) thus appear to originate from a multiplicative function of the variables involved in driving cellular expression and hence leading to a log-normal distribution.³⁸ This is in comparison to scenarios where the variables are additive in their effect which leads to a normal distribution. This concept is supported by log-normal/skewed distributions of protein expression observed in cells in literature.^{39, 40} Further work will apply this methodology to correlating nanoparticle uptake with cellular expression. The average uptake per cell determined by XRF across the whole cell population was 21.95 pg ranging from below 1 pg up to ~300 pg. Bulk analysis by ICP-MS (using a separate batch of nanoparticles to those used in the XRF study) determined a mean gold association per cell of 28 ± 4 pg per cell (n=10).

S-phase Cells are Preferentially Sensitized by AuNPs.

Radiosensitivity varies through the cell phase. The proportion of radioresistant S-phase cells has been shown to correlate with poorer prognosis⁴¹ as well as increased chance of relapse,⁴² and as such we extracted data points from Figure 4a based on cell phase to investigate the degree of radiosensitization achieved for each sub-population. Based on our observation that the NPs down regulate TS, we had predicted that a biological mechanism of radiosensitization would have greatest impact on the S-phase cells. Cells have the greatest resistance to radiation in the later part of the S phase and early G2 and are most radiation sensitive in the G2/M phase.^{16, 17} Resistance is attributed to DNA DSBs in late S and G2 phases being readily repaired *via* homologous recombination (HR), this repair process is highly efficient and less prone to DNA misrepair due to the utilisation of the sister chromatid as a repair template.⁴³ Given the HR pathway requires the presence of the sister chromatid it is only possible in S and G2,^{44, 45} but relies on TS for the synthesis of the thymine nucleobase in DNA. The alternate major repair process of DNA DSBs is the Non-Homologous End Joining (NHEJ) repair pathway. While this process is present throughout the cell cycle it dominates repair in G0, G1 and early S-phase cells.⁴⁶

The BVN fitting process was applied to the data sub-populations determined by cell phase according to the normalised DAPI integrated intensity (Supplementary Figure 4). The scatterplots (Figures 6a–c) along with the associated correlation coefficients and the corresponding p-values for the correlations reveal statistically significant non-zero correlations between γ H2AX foci counts and cellular Au content for each cell phase. Interestingly, the increase in foci count as a function of gold varies with cells phase and is non-linear with respect to the quantity of nanoparticles, an observation identifiable due to the methodology used here. The data and associated fits show correlation between foci count and Au content was highest in the radioresistant S-phase cells. The conditional expectation function, which in this case is the conditional expectation of the number of γ H2AX foci given a certain quantity of AuNPs in the cell, for each phase are reproduced on a linear scale

in Figure 6d. This plot further demonstrates the increased propensity to sensitization observed for S phase cells. Importantly, as the Au content approaches zero, the S phase cells measured fewer γ H2AX foci compared to the other phases, consistent with conventional radiobiology observations.

At a AuNP content of 12 pg, the S phase cells' expected foci counts surpass those expected in the radiosensitive G2/M phase. The S phase AuNP uptake CDF (Figure 5c) indicates that 60% of the S-phase cell population contains greater than 12 pg of gold, therefore the majority of S phase cells have an expected sensitization greater than the radiosensitive G2/M cells. The mean Au uptake across all cells considered regardless of phase was approximately 22 pg (Supplementary Table 7), thus indicating that the vast majority of S phase cells are expected to be sensitized more than cells in other phases. Normal distribution fits to the γ H2AX foci data CDFs for each phase are shown in Supplementary Figure 5 with fitting parameters included in Supplementary Table 9.

To illustrate the change in correlation of foci with Au content through the cell cycle the slope values of the conditional expectation function were calculated for 16 sub-populations defined from the DAPI histogram. This calculation was performed by defining a 'window' that was incrementally stepped across the normalised DAPI histogram used to categorise the cell phase data and the conditional expectation functions were then calculated for each window position along with the slope of the function from the parameters of the BVN fit. The slope is the expected increase in foci count with increasing Au and can be considered as a relative measure of the sensitization of cells as a function of AuNP content.

The propensity of S-phase cells to be sensitized could explain minimal differences in DSBs after irradiation comparing cells with and without nanoparticles, while proliferation assays such as the clonogenic assay show significant impact of nanoparticles on survival. Even though the S phase population makes up the smallest proportion of cells it has been shown to play a significant part in colony survival. This has been shown by Sinclair and Morton¹⁸ in observations of cell colony survival curves which significantly differ when S phase cells are removed. Given the disproportionate influence that S phase cells had on colony formation in their observations, any preferential killing of this cell population will lead to greater macroscale effects than what may be expected without the consideration of the influence of this cell subpopulation. This is likely to be one factor contributing to the large macroscale effects in radiosensitization not accounted for by current theoretical models. Clonogenic assays are valuable for determining the α/β ratios of tissues determined by fitting the linear-quadratic model to the radiation dose response. The alpha and beta components describe fundamental tissue response to radiation and are used in defining the optimal delivery of radiotherapy fractionation schedules. In the linear-quadratic function fitting of the clonogenic assays published by Sinclair and Morton¹⁸ a highly linear response was obtained on a log scale for G2/M and only slightly less so for G1 cells. Late S phase cells showed a much greater quadratic component when considering the linear-quadratic model fitting, resulting in a lower α/β ratio.¹⁸ Thus preferential removal of the S phase cells will alter the α/β ratio to produce cell survival curves reminiscent with those published in the literature from radiosensitization studies.^{7, 47} Furthermore, the β component has been linked to radiation damage repair. Thus impairing a cell-population DNA damage repair response is

expected to remove the shoulder in data fitted with the linear-quadratic model. This observation is consistent with many published cell survival curves in relation to nanoparticle sensitization and is consistent with nanoparticles impairing DNA repair and/or preferential removal of the S phase cell population.

The maximum degree of sensitization by AuNP content is observed for late S-phase cells suggesting that the down regulation of TS leads to an impaired ability to repair DSBs and consequently that nanoparticles themselves can alter DNA damage response and reduce repair. Repair mechanisms, their pathways and coordination are intricately linked to cell cycle progression and depend on expression of repair proteins, chromatic compaction and presence of the sister chromatid.⁴⁴ DNA DSBs in S and G2 phases are readily repaired, predominantly by the homologous recombination pathway. However the homology search in the sister chromatid is difficult in G2-M since the chromosomes have condensed. HR repair mostly relies on information available from the sister chromatid, however in G1 only a single chromatid exists and this pathway is essentially negligible, and the NHEJ pathway dominates. Furthermore, intracellular levels of the radical scavenger glutathione increase in the S phase and can further contribute to these cells' radioresistance.⁴⁸ It is expected that the AuNP's alter regulation of other proteins, and that the overall biological effect is confounded by alteration of many other pathways. The role of TS however, particularly for S-phase cells, is a critical determinant in macro-scale radiobiological response.

Data from the work of Sinclair and Morton¹⁸ is also plotted in Figure 6e who determined inherent radiosensitivity/radioresistance of cells and subsequent survival as a function of phase. An intriguing correlation exists between our data and that of Sinclair and Morton. As described, the observed cell cycle dependence on the correlation between γ H2AX foci and Au content are consistent with a biological mechanism(s) contributing to nanoparticle radiosensitization. The highest correlation coefficient was found in the late S-phase cell population (0.54), a population considered the most radiation resistant. As this resistance is primarily attributed to factors involved in DNA repair, such as the dominance of the efficient HR pathway, these data indicate AuNPs can interfere with the HR pathway.

CONCLUSIONS

We present cross-correlated single cell data sets describing DNA content, AuNP uptake and radiation induced γ H2AX foci, correlating DNA damage with quantitative measurement of nanoparticles in phase sub-populations. We show that NPs down regulating proteins associated with DNA damage repair mechanisms can specifically sensitize S-phase sub-populations. Surprisingly, NP's induced mechanisms of de-sensitization as well as sensitization in the same cell-population dependant on NP concentration. Biological, rather than physical mechanisms appear to dominate this process.

These data also indicate a paradigm in that the AuNPs may not increase the number of DSB's induced during irradiation, but rather, impair the cells' ability to recover from such lesions and hence the appearance of 'radiosensitization'. The use of NPs as radiosensitizers has traditionally been thought of as increasing the absolute amount of DNA damage *via* enhancing physical and, more recently, chemical processes. We demonstrate that significant

biological mechanisms impairing cells' DNA damage response are likely to play a significant role in radiosensitization for these AuNPs, and thus the 'sensitized' cells may have experienced an equivalent amount of initial radiation damage but have repaired fewer DSBs compared to control cells. The preferential impact on S phase cells is highly notable with regard to clinical implications as the proportion of these cells can correlate with poorer prognosis.

This paradigm along with the preferential radiosensitization of a small population of S phase cells having a disproportionately large influence on cell-population proliferation assists in linking many observations that are in apparent contradiction in literature. For example: sensitization with low-Z NPs being comparable to AuNPs; sensitization of cells to both MV X-ray sources and protons where the physical interactions are theoretically negligible; and why DNA DSBs do not necessarily correlate with macroscale proliferation assays. It is important to note still that the data we report are specifically for the system of AuNPs, cells and irradiation conditions used here. It is unlikely that statements with regard to exact mechanisms of radiosensitization can be made in a general sense. However, we suspect that the biological mechanisms that nanoparticles can instigate are likely to be driven by surface chemistry as opposed to any unique property of gold in our case. Ongoing research will investigate how broadly biological mechanisms can apply across cancer types and for varying nanoparticle parameters.

These findings highlight a need for careful consideration of intercellular heterogeneity and the advantages of cross-correlative analytical methodologies, important for dose response measures in optimizing formulations and studying mechanistic variables. The cross-correlative technique presented here is an ideal approach to answering many of the problems that arise when studying radiosensitization mechanisms whilst utilising varying cell lines, differing NP formulations, whether those formulations differ by atomic number of surface functionalization. It could also be extended to the study of different biological mechanisms involved in the radiosensitization process such as the quantification of the effects NP formulations have on specific repair pathways such as HR and NHEJ. By acquiring single cell data, 'like for like' comparisons can be made, for example, regardless of overall uptake at the total cell population level, subpopulations based on a defined range of intercellular NP content can be compared. Likewise the effects of a given NP uptake quantity on sensitization can be evaluated as a function of cell line or cell cycle phase by extracting statistical descriptions of cellular response to nanoparticles for populations with statistically identical uptake of nanoparticles (*i.e.* selecting cells from different scenarios with a defined quantity of nanoparticles defined by number, surface area, mass *etc*). In this way, data can be normalized for meaningful comparisons.

Overall, the mechanisms of radiosensitization with metallic nanoparticles is a complex combination of physical increases in dose deposition, chemical and biological mechanisms. We show here that the mechanisms can work both antagonistically (reducing DNA damage for low concentrations) and synergistically. This research has identified a greater significance of biological variables than previously considered and stands as a paradigm shift in the utility and understanding of nanoparticle 'sensitizer' mechanisms.

EXPERIMENTAL SECTION

Cell Culture and AuNP Treatment.

The human prostate cancer cell line, PC-3, was purchased from ECACC. Cells were cultured in RPMI-1640 culture media (Sigma-Aldrich); media was supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2% penicillin/streptomycin (Sigma-Aldrich) and 1% L-Glutamine (Sigma-Aldrich). Cultures were grown in a humidified chamber at 37° C with CO₂ levels maintained at 5%. Cells plated for experiments were at passage 9 and removed from tissue culture flasks at 80% confluence.

For γ H2AX quantification cells were seeded at passage 9 and cultured on tissue culture treated polymer coverslips (Ibidi, Germany) at a density of 20,000 cells per well in removable silicon wells (Sarstedt, Germany). Cells were incubated in a humidified chamber at 37°C in 5% CO₂ overnight to facilitate maximum cell adhesion after which the media was removed and replaced by serum free media containing the transferrin conjugated AuNPs at a AuNP concentration of 6 nM. Cells were incubated for 2 hours in the NP media after which the media was removed and replaced with fresh media and placed back in the incubator for a further 1 hour prior to transport for irradiation.

Cells were incubated for 1 h post irradiation prior to fixation and staining for γ H2AX foci. Cell nuclei were identified and imaged using DAPI. After images were acquired, samples were rinsed thoroughly with Milli-Q (MQ) water and dried in preparation for XRF analysis.

For measurement of TS protein expression, cells were plated in 6 well plates (Corning) at a density of 500,000 cells/well (passage 18). Following overnight adhesion cells were co-cultured with transferrin conjugated AuNPs at a concentration of ~0.3nM for 2 hours. After co-culture cells were washed, fixed and stained for TS expression measured by imaging flow cytometry.

Cell Irradiation Conditions.

Cells were irradiated at the Royal Adelaide Hospital (RAH) Radiation Oncology Department with a microSelectron Iridium-192 source (Nucletron B-V., Veenendaal, the Netherlands) used for high dose rate brachytherapy treatments.

The radiation dose was delivered to the cells by sending the Iridium source to a known position using the departmental source calibration “jig”. The cells in the wells were positioned at a distance of 4 cm from the source position (Supplementary Figure 6). There are no well-established protocols for calculating dose to the cells under these irradiation conditions (irradiation of a cell flask with suspension medium in air). An estimation of the irradiation time necessary to deliver 4.4 Gy to the cells was made using the current air kerma strength of the Ir-192 source and AAPM TG-43 formalism.⁴⁹

An estimation for the irradiation time can be obtained simply by applying an inverse square law correction to the air kerma strength at 1 m (assuming kerma is equal to dose in medium) and converting air kerma to dose in water at the cell layer:

$$\text{Dose /Kerma} \approx S_k \times \left(\frac{100 \text{ cm}}{d}\right)^2 \times T \times \left(\frac{\mu_{ab}}{\rho}\right)_{\text{water, air}}$$

Where S_k is the air kerma strength, d is the distance from the source to the cells

$\left(\frac{\mu_{ab}}{\rho}\right)_{\text{water, air}}$ is the ratio of the mass energy absorption coefficients for water to air and T is the irradiation time. The air kerma strength at the time of irradiation was 18.78 mGy²/h. The ratio of the mass energy absorption coefficients was taken to be 1.11,⁵⁰ and assuming a mean photon energy of 300 keV for an Ir-192 source. The irradiation time required to deliver 4.4 Gy at a distance of 4 cm from the source using this method is 1224 seconds.

The dose delivered for this irradiation time was then calculated using AAPM TG-43 formalism (this is an approximation, as the protocol assumes the source is entirely within a water medium):

$$D(r, \theta) = S_k \times \Lambda \times \frac{G_P(r, \theta)}{G_P(r_0, \theta_0)} \times g_L(r) \times F(r, \theta)$$

$$D(r, 90^\circ) = S_k \times \Lambda \times r^{-2} \times g_L(r)$$

Where $D(r, \theta)$ is the dose rate at the point of interest, S_k is the air kerma strength, Λ is the dose rate constant, G_P is the point source approximation to the geometry function, g_L is the radial dose function, F is the anisotropy function, r is the distance from the source centre to the cells and θ is the angle between the axis of the source and the cells.

The anisotropy function reduces to unity under the conditions used to irradiate the cells. The dose rate constant was assumed to be 1.108 (based on “Dose Calculation for Photon-Emitting Brachytherapy Sources with Average Energy Higher than 50 keV: Full Report of the AAPM and ESTRO”) and a radial dose value of 1.004 was used at a distance of $r=4$ cm.

$$D(4 \text{ cm}, 90^\circ) = 18788 \text{ U} \times 1.108 \times 4^{-2} \times 1.004 \text{ cGy/h}$$

For a treatment time of 1224 seconds

$$D(4 \text{ cm}, 90^\circ, 1224 \text{ s}) = 4.4 \text{ Gy}$$

This dose calculation was verified using Gafchromic EBT3 radiochromic film (International Specialty Products (ISP, Wayne, NJ)). A calibration curve for the EBT3 film was obtained by irradiating 4 calibration films using a 6 MV beam from a clinical Varian 600 C/D linear accelerator (Varian® Medical System, Palo Alto, CA) at the Royal Adelaide Hospital under reference dosimetry conditions. Doses of 0 (control), 1 Gy, 2 Gy and 4 Gy were delivered to the calibration films. A trial run was performed prior to cell irradiation to verify this method of dose calculation.

Once verified, thin sheets of EBT3 film with dimensions approximately (2.6×7.5 cm) were placed above and below the cell wells in order to estimate the delivered dose to the cells as a function of distance from the iridium source. The cells were irradiated for 1224 seconds with a 170.3 GBq source activity.

All films were analysed using Ashland Film QA Pro™ 3 software using a three-channel calibration curve. Film measurements are presented in Supplementary Figure 7. Sources of potential uncertainty in the delivered dose measured include: accurately estimating the distance of the source to the cells, correlating the position of the film with respect to the cell wells, lack of scatter medium (and thus lack of charged particle equilibrium) within the wells. Some variation in dose was expected from the proximal to distal sides of the analysed region of cells, however the difference was less than other sources of variation and no observable trend could be detected across the cells.

NP Synthesis and Characterisation.

A spherical gold nanoparticle solution (0.6 nM) of ~14nm diameter was prepared by the standard sodium citrate reduction method proposed by Turkevich *et al*⁷¹ and widely published in the literature.^{7, 52, 53} AuNPs were first treated with a polyethylene glycol (PEG) solution consisting of a mix of short chain (458.6 Da) to long chain (2,000 Da) PEG (Rapp Polymere) at a volume ratio of 4:1 based on a previously established protocol.⁵⁴ The PEGylated AuNPs were then conjugated with human transferrin (Sigma Aldrich) after activation of terminal carboxylic acid groups using standard carbodiimide chemistry to increase cell uptake.

Seed particle size (14 nm) was confirmed with Dynamic light scattering and Transmission Electron Microscopy (Supplementary Figure 8 and 9 respectively). TEM imaging was performed with a JEOL JEM-2100F-HR system. PEG and Transferrin conjugation was confirmed with UV-Vis measurements (Supplementary Figure 10). Stability of pegylated nanoparticles and transferrin conjugated nanoparticles are provided in Supplementary Figure 11 and 12 respectively. The conjugation of nanoparticle with transferrin was conducted immediately prior to use due to their poor stability beyond ~24 hours. Once conjugated, the zeta potential of the nanoparticles was roughly neutral.

Immunofluorescence Staining for γ H2AX Foci.

Post irradiation cells were fixed and stained for γ H2AX foci to evaluate DNA DSB formation and DAPI for nuclei masking. Briefly, cells were washed with PBS and fixed 1hr post irradiation with an ice cold solution consisting of 95% Ethanol (Chem-Supply) and 5% Acetic acid (Chem-Supply) for 10 mins. Following fixation cells were permeabilised for 15 mins using a PBS solution containing 0.5% Triton X-100 and then blocked using a buffer solution consisting of 5% Goat serum (Sigma-Aldrich) in PBS for 1 hr in a humidified incubator at 37° and 5% CO₂. After blocking cells were incubated for a further 1 hour in a humidified incubator at 37° and 5% CO₂ with 1/500 mouse anti- γ H2AX (Millipore) antibody in PBS + 1% Goat serum. Fluorescent secondary antibody staining was performed by incubating the cells with Goat anti-mouse Alexa 488 (Abcam) at a 1/500 dilution in 1% Goat serum for 1 hr in the same conditions as the primary antibody step. Cells were then

stained for nuclei identification and DNA content analysis using a DAPI solution (1 µg/ml) (Sigma-Aldrich) for 15 mins at room temperature. Finally cells were washed with MQ water for imaging.

Immunofluorescence Staining for Thymidylate Synthase.

Cells were fixed and stained for TS protein for analysis of TS expression *via* Imaging flow Cytometry. Briefly, cells were detached from the wells with trypsin (Sigma-Aldrich) which was then deactivated with RPMI. Cells were then concentrated *via* centrifugation and resuspended in ice cold PBS at a concentration of approximately $1-5 \times 10^6$ cells/ml. Cells were fixed in 100 µL of formalin solution (Sigma-Aldrich) comprised of 10% formalin (approx. 4% formaldehyde). After further washing cells were permeabilised in a solution of 0.05% Triton X-100. Following permeabilisation cells were blocked for 30 mins with 5% BSA. The sample was then incubated with primary antibody (anti-Thymidylate synthase, rabbit polyclonal, Abcam) diluted in 1% BSA (1/1000) for 1 hour at 4°C. After further washing in PBS cells were incubated for 1 hour in the dark with secondary antibody (goat anti-rabbit IgG H&L (Alexa Fluor® 647) (Abcam), washed in PBS and stained with DAPI (1µg/ml) (Sigma Aldrich) for cell nuclei identification.

Confocal Image Acquisition.

Fluorescent images were acquired using a ZEISS LSM 710 laser scanning confocal microscope. (Carl Zeiss, Germany). A 20x objective was utilised with the 488nm laser used for excitation of the γH2AX signal and 405nm laser for the DAPI channel. Images dimensions were 7168×1024 pixels corresponding to approximate image size of 2.9×0.42 mm. These settings resulted in x and y resolutions of 0.415 µm. All images were acquired as z-stacks with a slice thickness of 2 µm and were 48 µm thick.

X-Ray Fluorescence Microscopy Imaging.

XRF elemental distributions were acquired at the Australian Synchrotron X-ray fluorescence microscopy beamline using methods described previously,^{55, 56} which is able to quantitatively measure inorganic composition for any element with K- or L-shell emissions above 2.0keV.

Imaging Flow Cytometry.

Cells stained for TS expression were imaged using an ImageStream^X Mark II multispectral imaging flow cytometer (AMNIS). Approximately 5,000 cells were analysed for each condition with cell images acquired at 40× magnification. Preliminary data analysis was performed to define individual cells using IDEAS image-analysis software (Version 6.2; AMNIS). Both control and treated data sets were merged for gating into relevant cell populations. Firstly, a single cell population was defined by excluding speed beads, cell doublet and triplets, debris or overly small or large cells. This population was further sorted by selection of only Hoechst positive cells for TS analysis. Once defined, population data was imported into MATLAB (2017a, Mathworks) for all further analysis. Compensation was performed to ensure accurate fluorescence intensity (a matrix was created based on single colour compensation files using the IDEAS software).

Inductively Couple Plasma Mass Spectrometry.

AuNPs were added to PC-3 cells in a 6-well plate and incubated for 2 hours before being washed with PBS. After 1 further hour, the cells are trypsinized, centrifuged and the supernatant discarded. Cells were redispersed in 100uL of PBS, added to a prepared solution of 900uL aqua regia (3:1 HCl:HNO₃) and digested overnight. Samples were diluted with thiourea for analysis. ICP-MS was performed using an Agilent 8900 Triple Quad ICP-MS. Varying known concentrations of gold solutions were used for calibration.

Data Analysis and Image Processing.

Maximum intensity projections of the raw confocal images were obtained using Image J software (National Institutes of Health, version 1.47t). Maximum projections were aligned and overlaid with the XRF elemental maps using Adobe Photoshop CC (2015 Adobe Systems Incorporated). Once aligned, the 3 layers (γ H2AX, DAPI and Au) were exported as separate TIF files for quantification of γ H2AX foci and Au content. Briefly, cell nuclei were identified by applying a minimum pixel intensity threshold to the DAPI channel along with in built MATLAB filters to define discrete cell nuclei. Following identification of the cell nuclei γ H2AX foci were defined by grouping pixels of high intensity using a combination of thresholds, specifically, maximum and minimum pixel size of the groupings of pixels as well as a minimum pixel intensity requirement. We defined a foci as 4 connected pixels all with 125 or greater intensity in 8-bit scale. Lastly, the number of discrete foci present in each nuclei were counted and recorded for each cell. Along with these quantification steps, thresholds have been included within the analysis process to exclude misleading features, for example, clusters of cells being counted as a single cell. This quantification was performed with a custom script written in MATLAB 2017a. A more in-depth discussion of this script has been described previously.⁵⁷ All post image processing data analysis was performed in MATLAB (2017a, Mathworks).

DNA content was quantified by integrating the total DAPI pixel values through the Z-projection using custom analysis script in MATLAB (2017a, Mathworks).

Data Fitting.

One dimensional distributions were fitted with the inbuilt distribution fitting application in MATLAB. Fitting was described by equations for a probability distribution function:

$$PDF = \frac{1}{x\sigma\sqrt{2\pi}e} - \frac{(\ln(x) - \mu)^2}{2\sigma^2}$$

Or, cumulative distribution function:

$$CDF = \frac{1}{2} + \frac{1}{2} \operatorname{erf} \left[\frac{\ln(x) - \mu}{\sqrt{2\sigma^2}} \right]$$

Multivariate Analysis

At each condition, the correlated data pairs, $\mathbf{x} = (x_1, x_2)$, were modeled using a bivariate normal (BVN) distribution

$$f(\mathbf{x}) = \frac{1}{(2\pi)^{1/2} |\boldsymbol{\Sigma}|^{1/2}} \exp\left(-\frac{(\mathbf{x} - \boldsymbol{\mu})' \boldsymbol{\Sigma}^{-1} (\mathbf{x} - \boldsymbol{\mu})}{2}\right)$$

with mean vector, $\boldsymbol{\mu}$, and covariance matrix, $\boldsymbol{\Sigma}$. A condition of the multivariate normal distribution is that the marginal distributions of the data be normally distributed. Confidence regions containing $1-\alpha$ fraction of the probability of the BVN distribution are ellipsoids described by

$$(\mathbf{x} - \boldsymbol{\mu})' \boldsymbol{\Sigma}^{-1} (\mathbf{x} - \boldsymbol{\mu}) = \chi^2(\alpha).$$

For the BVN distribution, the conditional expectation of x_1 given x_2 is a line described by

$$E_{x_1|x_2} = \mu_1 + \rho \frac{\sigma_1}{\sigma_2} (x_2 - \mu_2)$$

Where ρ is the correlation coefficient between x_1 and x_2 . Values of μ_1 , μ_2 , σ_1 , and σ_2 , the means and standard deviations of the marginal distributions, were estimated by fits of normal distribution to the 1-D data. The MATLAB built-in function `corr` was used to find ρ as well as to return a p value, testing the hypothesis of no correlation against the alternative that there is a non-zero correlation. If the p value is small, say less than 0.05, the correlation is defined as being significantly different from zero. The conditional expectation function is equivalent to a least squares fit of a linear function to the data.

Statistical Analysis.

All statistical analysis was performed with MATLAB (2017a, Mathworks). Choice on test was determined based on suitability of data. All t tests were 2 sided and multiple comparison corrections were applied as required. Significance was defined for p -values < 0.05 unless otherwise specified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

XRF analysis was performed on the X-Ray Fluorescence Microscopy beamline at the Australian Synchrotron, part of ANSTO. T.T. is a recipient of a UniSA APA scholarship. Discussions and assistance from Magnus Röding (RISE Bioscience and Materials, Sweden), Puthenparampil Wilson (Lyell McEwin Hospital, Australia), Mohammed Pourhassan-Moghaddam (Tabriz University of Medical Sciences, Iran), Marnie Winter (University of South Australia) and John Lawson (Royal Adelaide Hospital, Australia) are greatly appreciated. This research was supported by the Australian Government through the Australian Research Council's Discovery Projects funding scheme (project DP190102119).

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Figure 1.

a. A schematic representation of the concept that: i) Internalization of nanoparticles by cells can lead to down regulation of proteins, including thymidylate synthase (TS), important for DNA damage repair response; ii) Due to the down regulation of TS, conversion of dUMP to dTMP is inhibited; iii) Subsequently, when the DNA is subjected to insult by ionizing radiation causing double strand breaks; iv), the normally effective homologous recombination pathway for repairing DSB's in S-phase cells is also inhibited, leading to a biological mechanism of radiosensitization. **b.** A cross-correlative methodology developed provides a 3-dimensional data set to compare cell populations, and sub-populations, with regard to nano-particle dose-response at the single cell level. Correlating biological markers imaged with laser scanning confocal microscopy with elemental content from synchrotron X-ray fluorescence microscopy for cell populations provides statistically significant, descriptive analysis of cell populations with regard to biological response for a quantified number of nanoparticles. For example, only cells with comparable numbers of nanoparticles are compared, or only cells in a certain phase are compared. The population behaviour can be described by fitting functions and any individual cell from a population can be characterized by its biological markers coupled with its nanoparticle content.

Figure 2 |. Down regulation of Thymidylate Synthase in PC-3 cells exposed to AuNPs.

a, Box and whisker plots of log transformed mean TS pixel intensity for non-treated cells ($n = 1,570$) and cells co-cultured with AuNPs ($n = 483$) determined by imaging flow cytometry. Statistical significance testing was performed by student's t -test. Boxes contain 25–75 percentile data with whiskers spanning $\pm 2.7\sigma$ (σ = standard deviation) corresponding to 0.7 to 99.3 percentile coverage of the data. Red plus signs indicate data that fall outside of this range. The red horizontal line in each box represents the median value of each data set. The 'x' signifies the mean for each dataset. **b,** CDF plots of the lognormal fits to the mean TS pixel intensity data presented in Figure 2c,d. **c,d** Histograms and lognormal PDF fits of the mean TS pixel intensity per cell without and with AuNPs respectively. Lognormal fitting parameters are presented in Supplementary Table 2.

Figure 3 | γ H2AX foci in PC-3 Cells.

a, Cells treated with 6nM AuNP receiving no radiation treatment. L→R: 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI); γ H2AX staining of DNA DSBs; merged image. Field of view shown is $\sim 150 \times 150 \mu\text{m}$. **b**, Cells treated with 6nM AuNP receiving radiation dose of 4 Gy. Field of view shown in images is approximately $70 \times 70 \mu\text{m}$. The full images consisting of a merged confocal and S-XRF Au map is included in Supplementary Figure 1. **c**, Foci measured in cells co-cultured with ($n=79$) or without ($n=156$) 6nM AuNPs receiving no radiation; $p=0.18$, as determined by the student's t-test. **d**, Foci measured in cells exposed to

4 Gy of gamma rays after co-culturing with (n=344) or without (n=154) 6nM AuNPs; p=0.77. Box and whisker parameters are as per previous plots. **e**, Foci from Figure 1d are compared between cells with no AuNPs and quartile sub-populations of cells with AuNPs (n=86 for each quartile). Statistical testing was performed using 1-way ANOVA with post hoc analysis conducted using the Tukey-Kramer method. * represents statistically significant differences at the $p < 0.05$ level. Summary of mean γ H2AX foci counts and p-values for the comparisons in (e) are presented in Supplementary Tables 4&5 respectively.

Figure 4 | γ H2AX foci are correlated with AuNP content in individual cells.

a, Data points represent individual cells, plotted as a function of the number of γ H2AX foci dependent upon AuNP content. The correlation coefficient of the BVN fit was 0.309 (p-value < 0.0001; indicating the correlation coefficient is statistically different to zero). The solid green line is the conditional expectation function, showing the expected value of foci given a value of log Au. The slope of this function is the correlation coefficient multiplied by the ratio of the standard deviations from the (marginal) normal distribution fits. **b**, Histogram of γ H2AX foci data with a normal distribution fit overlaid. **c**, Histogram of log Au data

with a normal distribution fit overlaid. **d**, The cumulative distribution of γ H2AX foci fitted with a normal distribution. **e**, The cumulative distribution of log Au data fitted with a normal distribution. Fitting parameters from Figures 4b–d included in Supplementary Table 6.

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Figure 5 |. AuNP uptake is independent of cell phase.

a, Box and whisker plots of log transformed AuNP uptake data across the cell phases. Given a lognormal NP uptake (Figure 5b,c,d), log transformed Au data is normally distributed and therefore boxes represent the 25th – 75th percentile data with whiskers spanning $\pm 2.7\sigma$ of the data respectively. Red plus signs signify data outside this range. The red horizontal line in each box represents the median value. Each cycle contains the following number of cells: G1 = 180; S = 33; and G2/M = 84. Forty-seven cells could not be accurately classified into a specific phase and were omitted from this component of the analysis. **b**, **c**, **d**, Cumulative data of AuNP uptake fitted with log-normal distributions for G1, S and G2/M phase populations respectively. Lognormal distribution fitting parameters are provided in Supplementary Table 8.

Figure 6 | Cell phase determines degree of radiosensitization.

a,b,c. Scatterplots of γ H2AX foci count versus log Au with expectation values plotted for cells in G1 (correlation coefficient = 0.361; p-value < 0.0001), S (correlation coefficient = 0.426; p = 0.013), and G2/M (correlation coefficient = 0.257; p = 0.018) respectively. Linear functions on each scatterplot represent the conditional expectation function for γ H2AX foci counts. **d.** Conditional expectation functions of γ H2AX foci count with Au content calculated from the BVN fits for each phase plotted on a linear scale and highlighting the increased propensity to sensitization of S-phase cells compared to either G1 or G2/M

phases. **e**, Slope of the expectation value represented by degree of sensitization (left y-axis) and cell radiosensitivity, represented by clonogenic survival data adapted from¹⁸ (right y-axis), plotted against cell cycle. **f**, Colour schematic visualising the change in radiation response through the cell cycle.

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