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Nanoparticle-Mediated X-Ray Radiation Enhancement for Cancer Therapy

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Abstract

Metallic nanoparticles with a high atomic number release Auger electrons in response to external beam X-ray radiation. When these nanoparticles are selectively delivered to tumors, they have the potential to locally enhance the effects of radiation therapy. Optimizing the therapeutic efficacy of these nanoparticles, however, remains a challenging and time-consuming task. Here we describe three different assays that can be used to experimentally quantify and optimize the in vitro therapeutic efficacy of nanoparticle-mediated X-ray radiation enhancement. These include an IC50 extended dose response curve, clonogenic cell survival assay, and immunoblotting. Collectively, these assays provide information about whether a given nanoparticle provides radiosensitization, the extent of the radiosensitization, and the potential mechanism of radiosensitization.

Keywords

Radiosensitization; Metallic nanoparticles; DNA damage; Clonogenic assay; IC50; Immunoblotting; Surviving fraction

1 Introduction

Cancer continues to be a major public health problem in the USA, with 595,690 new deaths anticipated in 2016, making it the second leading cause of death [1]. Radiation therapy, the use of ionizing radiation for cancer treatment, is currently administered to over half of all cancer patients to treat primary tumors, prevent recurrence, or relieve symptoms caused by cancer [2]. Radiation therapy can be used alone or in combination with chemotherapy or surgery, and is often selected for its ability to slow tumor growth and control energy deposition over time [3, 4]. Unfortunately, the use of high-intensity ionizing radiation poses significant risks to surrounding healthy tissue [5].

Several techniques have been used to minimize the radiation dose delivered to normal cells while maximizing that delivered to cancer cells. These methods include fractionating the radiation dose over time, so that cancer cells can enter a more radiosensitive phase in the cell cycle before re-treatment, or fractioning over space by intersecting the tumor with lower-dose beams from multiple directions [6]. Other methods include systemic administration of radioprotective drugs to scavenge free radicals in healthy cells [7], radiosensitizers to

selectively increase radiosensitivity of tumor cells [8], and metallic nanoparticles to locally enhance free radical production [9].

Designed to accumulate in tumors via the enhanced permeation and retention effect, metallic nanoparticles can selectively enhance the effect of X-rays in tumor cells via the localized release of Auger electrons (Fig. 1). Nanoparticles with a high atomic number absorb lower energy X-rays (30 keV–1 MeV), resulting in the displacement of an inner shell electron and its subsequent replacement by the fall of an electron from a higher level. The energy released during filling of the first electron vacancy can eject another electron from the atom, known as an Auger electron [9]. The released Auger electrons travel only very short distances, and thus tend to hydrolyze water molecules within the same cell, creating free radicals that induce DNA damage [11]. When paired with X-ray induced damage, the combination of single- and double-stranded DNA breaks makes DNA repair and replication more difficult, eventually resulting in cell death [10].

Several assays can be used to experimentally quantify and optimize the in vitro therapeutic efficacy of nanoparticle-mediated X-ray radiation enhancement. The IC₅₀ extended nanoparticle dose response curve is used to determine relative toxicity of nanoparticles alone, in order to select a safe concentration range at which nanoparticles can be administered for radiation enhancement. In this assay, cell monolayers are treated with different concentrations of nanoparticles to identify the nanoparticle concentration that results in 50 % cell kill. Unlike conventional cytotoxicity studies, the IC₅₀ assay described here is longer in duration in order to be sensitive to cell growth inhibition as a result of DNA damage [12]. The clonogenic cell survival assay is used to measure the fraction of surviving cells that retain proliferative capacity following X-ray irradiation [13, 14]. In this assay, cells are pretreated with nanoparticles, subjected to X-ray irradiation, and then re-plated at low seeding densities to enable the counting of individual cell colonies. Immunoblotting, or western blotting, is used to identify changes in protein expression following treatment. In this assay, protein expression is sampled at different time-points following X-ray irradiation, both the in presence and absence of nanoparticles, in order to determine how pretreatment with nanoparticles enhances radiation sensitivity [15]. Different primary antibodies can be selected based the putative mechanism of interest, such as DNA damage and repair, apoptosis, and ROS generation. Collectively, these assays provide information about whether a given nanoparticle provides radiosensitization, the extent of the radiosensitization, and the potential mechanism of radiosensitization.

2 Materials

2.1 Cell Culture Reagents for IC₅₀ and Clonogenic Cell Survival Assay

1. Complete cell culture medium containing appropriate supplements for optimal cell growth. Store at 4 °C. Warm to 37 °C immediately before use.
2. Phosphate-buffered saline (PBS). Store at 4 °C.
3. 0.25 % trypsin with 1 mM EDTA. Store at –20 °C.
4. 0.4 % (w/v) trypan blue.

5. 10 % (v/v) neutral-buffered formalin.
6. 0.5 % (w/v) crystal violet solution.

2.2 Immunoblotting

1. Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) or equivalent commercially available protein quantification kit.
2. Mini Protean Tetra Cell with buffer tank, electrode assembly, lid, power cables, and buffer dam (Bio-Rad, Hercules, California, USA) or equivalent commercially available gel electrophoresis system.
3. Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, California, USA) or equivalent commercially available gel transfer system.
4. Trans-Blot Turbo RTA Transfer Kit with transfer stacks and polyvinylidene difluoride (PVDF) membranes.
5. Lysis buffer: 1.0 % (v/v) Nonidet P-40 (NP-40), 50 mM Tris-HCl (pH 8.0), 150 mM NaCl in deionized water. Store at -20°C .
6. 2 $\times$ Laemmli sample buffer: 65.8 mM Tris-HCl (pH 6.8), 26.3 % (w/v) glycerol, 2.1 % SDS, 0.01 % bromophenol blue in deionized water. Store at room temperature.
7. 2 $\times$ Sample buffer: (20 % (v/v) reducing agent (2-mercaptoethanol, dithiothreitol (DTT), or Cleland's reagent) in 2 $\times$ Laemmli sample buffer. Prepare fresh.
8. Running buffer: 25 mM Tris-HCl (pH 8.3), 190 mM glycine, 0.1 % SDS in deionized water. Store at room temperature.
9. Washing buffer: 0.5 % (v/v) Tween 20, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl in deionized water. Store at room temperature.
10. Blocking buffer: 3 % (w/v) bovine serum albumin (BSA) in washing buffer. Prepare fresh.
11. Transfer buffer: 25 mM Tris-HCl (pH 8.3), 190 mM glycine, 20 % (v/v) ethanol in deionized water. Store at 4°C .
12. Western ECL Blotting Substrate Kit containing luminol enhancer and peroxide solution.
13. Precision Plus protein dual color standard (Bio-Rad, Hercules, California, USA). Store at -20°C .
14. Precision Protein Strep Tactin-HRP conjugate (Bio-Rad, Hercules, California, USA). Store at 4°C .
15. Primary antibody against the biomarker of interest. Store according to manufacturer's instructions. Potential biomarkers that may be examined are listed in Table 1.

16. ImageJ software, available from the NIH (<http://imagej.nih.gov>).

3 Methods

3.1 IC50 Extended Nanoparticle Dose Response Curve

Carry out all procedures at room temperature unless otherwise noted.

1. Seed cells (100,000/well) into 6-well cell culture plates and add 2 mL of complete cell culture medium. Prepare a sufficient number of plates to test multiple nanoparticle concentrations and controls in triplicate. Incubate plates at 37 °C in 5 % CO₂ for 24 h.
2. Resuspend the nanoparticles of interest in a small volume of water or saline, and then sonicate or pipette to achieve a monodisperse suspension. To determine the concentration of nanoparticles that produces 50 % cell kill in the absence of radiation, nanoparticles are generally administered across the nM to mM dose range. Prepare several different dilutions of nanoparticles in complete media. Replace the cell supernatant with 2 mL of cell culture medium with or without nanoparticles. Label the lid of each plate with the treatment conditions and incubate for at least 24 h.
3. 24 h after nanoparticle addition, harvest the cells for re-seeding at a lower cell density. Remove the media, wash each well once with PBS, and incubate with 0.5 mL pre-warmed 0.25 % trypsin–EDTA for 3 min or until the cells have detached. Neutralize the trypsin with 1 mL complete cell culture medium (or medium containing soybean trypsin inhibitor), pellet the cells in a 1.5 mL microcentrifuge tube, and resuspend cells in 1 mL media. Mix equal volumes of 0.4 % trypan blue and cells, and count the cells using an automated cell counter or hemocytometer.
4. Seed the cells in new 6-well plates at 500–5000 cells/well. Add 3 mL complete media and incubate for at least 1 week or until colonies form in the control wells.
5. After sufficient colonies have formed, remove the cell culture media, wash three times with 1 mL PBS, and add 500 µL of 10 % (v/v) neutral-buffered formalin (or 6 % (v/v) glutaraldehyde) and 50 µL of crystal violet to each well for 30 min at room temperature. Remove formalin–crystal violet mixture with repeated washing using deionized water. Let the plates dry overnight. Example colonies of cells seeded at three different densities are shown in Fig. 2a.
6. Once the plates are dry, individual colonies can be counted manually using pen and paper or ImageJ software. The plating efficiency, PE, is calculated as

$$PE = \frac{\text{\#colonies formed}}{\text{\#cells seeded}}$$

using the number of colonies counted in the untreated controls. The surviving fraction, SF, for each treatment group is calculated as

$$SF = \frac{\# \text{colonies formed}}{\# \text{cells seeded} \times PE}$$

Once the survival fraction has been calculated for each dose, the survival curve can be plotted as shown in Fig. 2b.

3.2 Clonogenic Cell Survival Assay

1. Seed cells (100,000–300,000) into T25 cell culture flasks and add 3 mL of complete cell culture media. A minimum of two flasks is needed for each radiation dose: one with nanoparticle treatment and one without. Typical radiation doses include: 0, 2, 4, 6, 8, and 10 Gy. Incubate flasks at 37 °C in 5 % CO₂ until they reach ~70 % confluency, changing media as needed (*see* Notes 1 and 2).
2. Resuspend the nanoparticles of interest in a small volume of water or saline, and then sonicate or pipette to achieve a monodisperse suspension. Dilute the nanoparticle concentrate with a sufficient volume of complete media in order to fully replace that in the T25 flasks. Remove media from each T25 flask, add 3 mL of diluted nanoparticles or untreated media, and return the cells to the incubator for at least 24 h (*see* Note 3).
3. Twenty-four hours after nanoparticle treatment, irradiate the cells using a cabinet or small animal X-ray irradiator. For each radiation dose, you should have one plate treated with nanoparticles, and one without. Be sure to also bring the nonirradiated cells to the X-ray irradiator in order to subject the control cells to the same environmental conditions as the treated cells. After irradiation, return the cells to the incubator.
4. Four hours after irradiation, harvest the cells for replating in 6-well plates. One flask at a time, remove media, wash with 1 mL PBS, incubate with 1 mL pre-warmed 0.25 % trypsin-EDTA for 3 min or until the cells have detached. Neutralize the trypsin with 2–3 mL complete media (or media containing soybean trypsin inhibitor), pellet the cells in a 15 mL centrifuge tube, and resuspend cells in 2–3 mL media. Mix equal volumes of 0.4 % (w/v) trypan blue and cells, and count the cells using an automated cell counter or hemocytometer.
5. Seed the cells in triplicate in 6-well plates. The appropriate seeding density is usually on the order of 100–10,000 cells per well. This seeding density is deliberately low so that cells with proliferative capacity will appear as distinct, nonoverlapping colonies following 2 weeks of culture. It may be necessary to

¹As an alternative to T25 flasks, cells can be seeded into 6-well plates.

²Radiation doses are measured in Grey (Gy), where 1 Gy = 1 J/kg. The dose is calculated by the total exposure time multiplied by the energy of the X-ray beam.

³Dilutions are calculated using the equation: $V_{np} = \frac{C_{want}}{C_{stock}} \times V_{total}$, where C_{want} is the concentration desired, C_{stock} is the concentration of the stock nanoparticle solution, V_{total} is the total volume to make, and V_{np} is the volume of nanoparticle stock needed.

plate the irradiated and/or nanoparticle-treated cells at a higher density than the untreated controls. Once the appropriate seeding density is determined (this may take several tries), dilute and seed the cells into 6-well plates, with each well containing 3 mL media. Incubate the colonies for at least 2 weeks, changing media only if needed.

6. After sufficient colonies have formed, label and count the cell colonies as described in **steps 5 and 6** under Subheading 3.1 above.

3.3 Immunoblotting

1. Prepare and treat cells seeded in 6-well plates with nanoparticles and radiation as desired. At the appropriate time-point following irradiation (as determined by the protein target of interest), prepare protein lysates as described in the Bio-Rad General Protocol for Western Blotting [21]. Generally, one confluent well yields sufficient protein for loading multiple gel lanes; however, it is recommended to prepare at least three replicates of each treatment condition when running multiple gels.
2. Determine the overall protein concentration of each cell lysate using the Pierce BCA Protein Assay. Protein lysates should be stored at -80°C .
3. Preheat a hot plate to 100°C . Place the lysis buffer, Precision Plus protein standards, reducing agent, and protein lysate on ice.
4. Prepare all reagents. The running, washing, and transfer buffers can be prepared in advance. The sample buffer and blocking buffer should be prepared fresh each time.
5. Place 1–4 gels on electrode assemblies, adding a buffer dam for odd numbers of gels. Fill the center of each electrode assembly with running buffer to verify the gasket is not leaking. Finish filling the buffer tank to the appropriate line, based on the number of gels being run. Using running buffer, wash each loading well by pipetting up and down several times (*see* Notes 4 and 5).
6. Aliquot the protein lysate into microcentrifuge tubes and dilute to $\frac{1}{2}$ the final volume using with lysis buffer. Add $2\times$ sample buffer, so that 50 % of the final volume is sample buffer and 50 % is protein lysate in lysis buffer (*see* Note 6).
7. Heat the protein samples at 100°C for 5 min, and then centrifuge at 3000 rcf for 1 min, to collect the sample at the bottom of the tube.
8. Load each gel from left to right, placing 5–10 μL of Precision Plus Protein standard into the first well. Place the cover on the buffer tank and set the power supply to 150 V, 75 mA. Run the gel for desired amount of time to separate the

⁴Make sure to remove tape from bottom of gel before placing in the gel box. Make sure when placing electrode assemblies into the buffer tank, the correct electrodes are on the correct side.

⁵When selecting or casting a gel, the size of the loading wells should be selected based on volume of protein to be loaded and the number of samples. Typically, as the well size increases, the number of available wells decreases.

⁶The amount of protein prepared for a given gel is dependent on the concentration of the target protein and is generally determined experimentally when optimizing the use of each antibody.

protein band(s) of interest, generally 45–60 min, and then turn off power supply (*see* Note 7).

9. To prepare for gel transfer, soak two transfer stacks per gel in transfer solution. Soak PVDF or nitrocellulose transfer membranes in 95 % ethanol for 3 min, followed by 3 min in transfer solution. In the meantime, open each gel, and place them into the transfer solution. For an even transfer, it is recommended to remove the tops of gels using a knife to create a straight top edge. If multiple gels are used with different protein concentrations, make note of which one is placed where for later reference when adding primary antibody.
10. Place a transfer stack into the transfer cassette, followed by the transfer membrane, then the gel, and finally another transfer stack. Make sure there are no air bubbles beneath each layer as it is placed down. Complete the assembly of the transfer cassette and place it into the transfer system. Set the power supply to 25 V, 1.0 A for 30 min. These time and power settings may vary based on the transfer method used.
11. When the transfer is finished, remove membranes from transfer cassette and place each into its own labeled plastic box. Add 15 mL of blocking buffer to each container and shake on an orbital shaker for 60 min to block non-specific binding (*see* Notes 8 and 9).
12. Remove the blocking buffer and add the primary antibody. If you plan to run multiple antibodies per membrane, cut the membrane and separate the pieces into new containers. Primary antibodies are diluted in blocking buffer as recommended by manufacturer. Place container in the fridge or on a refrigerated shaker overnight.
13. The next day, place the membranes on a shaker at room temperature for 2 h. Remove primary antibody, and wash four times with 10–15 mL TBST at 10-min intervals. Shake continuously (*see* Note 10).
14. Remove the TBST and replace with 15 mL of secondary antibody diluted in blocking buffer as per manufacturer's instructions. Make sure to use a secondary antibody that that is designed to bind the Fc domain of the primary antibody. For chemiluminescent detection, also add 1 μ L of Strep Tactin HRP conjugate. Shake at room temperature for 1 h.
15. Remove the secondary antibody and wash four times with 10–15 mL TBST at 10-min intervals. Shake continuously.
16. Freshly prepare the chemiluminescent developer solution by combining Peroxide solution and Luminal Solution (1:1) from the Western ECL Substrate Kit.

⁷For proteins of low molecular weight, a shorter running time is needed. If left too long proteins, will run off the bottom of the gel. For proteins of high molecular weight, or when using one membrane to sample multiple proteins, a longer running time is generally needed to separate the bands of interest.

⁸In addition to labeling the protein loading concentration, it is helpful to record the primary antibody, antibody dilution, and the secondary antibody to be used.

⁹This blocking step can also be performed using non-fat dry milk instead of BSA.

¹⁰The primary antibody dilution can be saved and reused several times if stored at -20°C .

Prepare just enough to cover each membrane evenly. Remove one membrane at a time from the TBST, pat dry with a lint-free wipe, and place into developer solution for 5 min while shaking gently. After developing, pat the membrane dry, and cover in cling wrap, removing as many bubbles as possible. Image using a chemiluminescent imager (*see* Note 11).

17. Blots can be quantified using ImageJ software. More information can be found in Sect. 30.13 of the online ImageJ User Guide [22].

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¹¹For imaging, choose a standard exposure to start and then increase or decrease the exposure time based on the initial image. Take several images to verify that you have a good blot.

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Fig. 1. The proposed mechanism by which metallic nanoparticles enhance radiation sensitivity in cells. Auger electrons released from the nanoparticle following X-ray radiation result in hydrolysis of water molecules within the cell, producing free radicals that interact with DNA to cause large numbers of single-stranded DNA breaks and eventual cell death. Adapted from [10]

Fig. 2. Colonies of Capan-1 pancreatic cancer cells after 14 days of cell culture. (a) Cells were plated at a density of 2000, 1500, and 1000 cells/well (*left to right*) in 6-well plates 4 h after irradiation. (b) Colony counting following different doses of radiation yield a dose-dependent survival curve

Table 1

Selected biomarkers for assessing X-ray radiation enhancement

Protein	Function	References
BAX	Pro-apoptotic regulator responsible for cell commitment to apoptosis	[16,17]
BRCA1	DNA repair protein activated by double-stranded DNA breaks	[18]
BRCA2	DNA repair protein activated by single-stranded DNA breaks	[18]
Caspase-3	Protease activated during cell apoptosis	[19]
DNA polymerase theta	DNA repair protein activated by double-stranded DNA breaks	[18]
γ H2AX	DNA damage marker activated by double-stranded DNA breaks	[18, 19]
KRas	Cell cycle regulator that can induce cell cycle arrest, apoptosis, or replicative senescence	[20]
Parp-1	DNA repair protein activated by single-stranded DNA breaks	[18, 19]
P53	Tumor suppressor protein activated by DNA damage	[17]
Rad51	DNA repair protein activated by double-stranded DNA breaks	[18]