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NOTE

Flowthrough of ^{239}PU and ^{55}FE during RNA extraction

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Abstract

Analysis of gene expression has become an important tool in understanding low-dose effect mechanisms of ionizing radiation at the cellular level. Metal binding to nucleic acids needs to be considered when interpreting these results, as some radioactive metals, particularly actinides, may produce free radicals and cause oxidative stress damage via chemical means at rates much higher than free radical formation related to their radiological properties. Bacteria exposed *in situ* to low dose rates of plutonium-239 (^{239}Pu) and iron-55 (^{55}Fe) were previously analysed for gene expression. The work herein was motivated by an interest in more precisely identifying the distribution of radionuclides in these bacteria as well as the practical need to ensure appropriate transport and handling of the associated ribonucleic acid (RNA) extractions. RNA extractions were performed on bacteria growth media with and without bacteria cells (i.e. with and without RNA) at several different concentrations of ^{239}Pu and ^{55}Fe to inform the level of specificity of the extraction membrane as well as provide insight into internal (uptake) vs external (sorption) accumulation of these radionuclides in bacteria cells. Results of the study suggest that ^{239}Pu and ^{55}Fe detected in RNA extraction samples during long term cell studies is the result of binding to RNA prior to the time of extraction, as opposed to flow through or binding after cell lysis, and it highlights the practical importance of nucleic acid sample characterization to radiation protection more generally.

1. Introduction

1.1. Ionizing radiation effects

The genome of a cell, made of deoxyribonucleic acid (DNA), contains the full set of genetic information for living organisms, with sequences that control everything from morphology to function [1]. If ionizing radiation interacts with DNA, any resultant damage that is not successfully repaired by the cell may cause cell death or produce a non-fatal mutation [2, 3]. In humans, excessive cell death associated with high dose-rate exposures to ionizing radiation results in tissue reactions which may occur acutely or many years after exposure, while non-fatal mutations, under the right conditions, may result in carcinogenesis [4–6]. Studies of effects in individual cells cannot be used directly to develop a dose-response relationship for carcinogenesis in humans, that belongs in the realm of epidemiology, e.g. [7], but cellular studies are important in understanding fundamental mechanisms, particularly for low doses and low dose rates [8].

Radiobiological studies indicate that a combination of events may better explain low dose radiation response, especially when there is damage to proteins, the cell membrane, and mitochondria [9]. One popular field for examining cellular response to damage is transcriptomics, a branch of biological study that examines response through gene expression [1]. An impacted cell may differentially express (e.g. 'turn off' or 'turn on') genes coded on its genome in response to damage or stress to open repair pathways, protect the

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cell, send extra-cellular messages, etc. Gene expression includes the transcription of ribonucleic acid (RNA) from DNA, and differentially expressed genes are quantified by analysis of RNA produced in populations of cells through sequencing or amplification of previously identified genes of interest [1].

1.2. Fe and Pu binding to nucleic acids

In general, DNA has a strong negative charge and consists of a cyclic sugar (deoxyribose), purine (adenine and guanine) and pyrimidine (cytosine and thymine) bases, and a phosphate group. DNA is usually found in a double strand, where hydrogen bonds between the purine and pyrimidine groups connect the two molecules. RNA is found in a single strand form and is otherwise similar to DNA except that the cyclic sugar is ribose and uracil is used instead of thymine [10]. The potential for redox-active species of Fe to bind to DNA in cells has been studied because of Fe's ability to accelerate free radical reactions that cause DNA damage. Iron is known to bind to multiple sites on DNA molecules under physiologically relevant chemical conditions [11]. Both Fe(II) and Fe(III) bind to DNA at sites located on the phosphate backbone as well as the guanine base, where the strongest affinity is between Fe(III) and phosphate groups [12]. Potential binding sites for Fe on DNA also apply to RNA molecules. Additionally, binding sites on RNA for Mg related to RNA folding can also be used by Fe [13, 14]. Plutonium(IV) often follows the biochemistry associated with Fe(III), so it is expected that many binding sites available to Fe will also be available to Pu when uptake occurs in cells [15, 16].

Low-dose exposures to high energy, low-LET radiation can be achieved through external irradiation with radiation generating devices or sources, such that only radiological interactions occur without the introduction of direct chemical stressors. Studying the response of cells to low doses of alpha emissions or low-energy electrons, however, requires the introduction of radiological materials directly to the cell culture environment because of the short path length of these types of radiations. DNA and RNA are likely to bond with metal cations, with evidence in the literature of bonding to radiologically relevant metals besides Fe and Pu, such as Y, Ce, Sm, Sr, Cs, U, and Pb [10]. As we strive to better understand low-dose effect mechanisms of ionizing radiation, metal binding to DNA and RNA cannot be ignored, especially for actinides like U and Pu which may produce free radicals and cause oxidative stress damage via chemical means at rates much higher than free radical formation related to their radiological properties [17, 18].

1.3. Relevance for radiological materials handling

The work presented herein was spurred by the practical need to radiologically characterize RNA extraction samples from bacteria cultures exposed to ^{55}Fe and ^{239}Pu, because they would be removed from a radiologically-controlled laboratory and brought to a shared, non-radiological laboratory facility quality control and sequencing processes for transcriptomic analysis. To ensure high quality data, DNA and RNA extraction processes are designed to be highly selective for DNA or RNA so that the extraction product contains few impurities, though physical efficiency values are not generally available due to the proprietary nature of these extraction processes. Because of this selectivity, we expected little to no detectable radiological content in our extraction samples. But an initial semi-quantitative analysis suggested otherwise. Thus, the investigation was expanded and RNA extraction samples generated in our laboratory were appropriately characterized so that we could develop proper transport and handling procedures.

After considering the affinity of DNA and RNA for metal cations, we postulated that the radiological content of our RNA extraction samples could be used to gain insight into internal (uptake) vs external (sorption) accumulation of ^{55}Fe and ^{239}Pu in our bacteria cultures. The use of the RNA extraction radiological data for this purpose, however, depended on demonstrating evidence that the ^{55}Fe and ^{239}Pu found in our samples was bound to RNA when the cell cultures were being incubated and studied. It was determined that there were two other potential routes for how the ^{55}Fe and ^{239}Pu could end up in the RNA extraction samples:

(a) Process flowthrough: because of the previously mentioned lack of physical efficiency data for impurity removal, it was possible that the ^{55}Fe and ^{239}Pu may end up in the RNA extraction samples as impurities directly from the growth media.
(b) Binding with RNA during the extraction process: we also considered the possibility that metals present in the growth media or released from the cell surface when the cells were lysed as part of the extraction process could quickly bind to RNA at that point.

To determine if these two pathways better explained the quantities of ^{239}Pu and ^{55}Fe in our RNA extraction samples, we designed an experiment that considered process flowthrough by completing RNA extractions on solutions containing radioactive material spikes in growth media with no cells. We also

considered metal binding with RNA during the extraction process by using non-contaminated cell culture samples and spiking them with ^{239}Pu immediately before the cell lysing step.

2. Materials and methods

The methods applied to this study sought, primarily, to mimic previous studies in our laboratory with where *Pseudomonas putida* and *E. coli* were exposed to ^{239}Pu and ^{55}Fe in their growth media [19, 20]. As such, sample volumes and radionuclide concentrations were selected to be relevant to our studies.

2.1. RNA extraction process overview

For clarity, the RNA extraction process is graphically described in in figure 1. The RNA extraction process begins with a 100–400 μl aliquot collected from a liquid culture of bacteria, where the volume of which is based on the optical density (OD) of the culture (OD600). Solutions used to lyse the cells (which releases RNA from the cells) and preserve the RNA are added. The solution is run through a proprietary exchange column that is centrifuged. During this step, RNA and other compounds bind to the exchange column. The exchange column is then washed with various solutions to remove non-RNA content before a final wash with the goal of producing an aqueous RNA sample with few impurities. Non-RNA content is either released in the effluent of the process or remains on the exchange column and is disposed of all RNA extraction simulations were performed using a Qiagen RNeasy kit. Extractions were performed according to manufacturer instructions.

2.2. Plutonium solution preparation

Plutonium solutions were prepared from two working solutions of ^{239}Pu in 0.01 M HCl, one solution where ^{239}Pu was complexed with citrate in a ratio of 1000:1 and another without citrate complexation. Based on oxidation state analysis of the stock solution, the oxidation state was assumed to be primarily Pu(V) (∼75%) with some Pu(IV) and Pu(VI) prior to the addition of citrate. Based on oxidation state measurements of growth media spiked immediately prior to inoculation, the oxidation state of Pu in treatment groups where citrate was added was found to be primarily Pu(IV) (75%–80%) with the remainder mostly comprised of Pu(V) [20]. The solution of ^{239}Pu complexed with citrate was diluted with 0.01 M HCl to produce a working solution with an activity concentration of 1.37×10^{1} kBq ml^{-1}. Using this working solution, four additional solutions were produced via serial dilution (with 0.01 M HCl) with activity concentrations of 1.37 kBq ml^{-1}, 1.37×10^{-1} kBq ml^{-1}, 1.37×10^{-2} kBq ml^{-1}, and 1.37×10^{-3} kBq ml^{-1}. The dilution process was also completed using the ^{239}Pu solution that was not complexed with citrate. There were 10 total ^{239}Pu solutions used as ^{239}Pu -spiked process blanks: 5 complexed with citrate and 5 without. The activity of solutions used for each experiment conducted is summarized in table 1.

2.3. Iron-55 solution preparation

Process blanks for ^{55}Fe were made from a stock solution of $^{55}FeCl_3$. An aliquot of the stock solution was diluted to a concentration of 1.81×10^{4} kBq ml^{-1} in approximately 0.01 M HCl. Four additional working solutions were prepared as process blanks via serial dilution (with 0.01 M HCl) with concentrations of 1.81×10^{3} kBq ml^{-1}, 1.81×10^{2} kBq ml^{-1}, 1.81×10^{1} kBq ml^{-1}, and 1.81 kBq ml^{-1}. The process resulted in 5 total ^{55}Fe blanks with different concentrations that were used (table 1).

2.4. Sample preparation for ^{239}Pu and ^{55}Fe blanks

The RNA extraction process was first completed using solution blanks to examine flow-through of ^{239}Pu and ^{55}Fe from the extraction process without the presence of cells. Activity concentration for both ^{239}Pu and ^{55}Fe solutions were based on laboratory experiments conducted in our laboratory with different microorganisms [19, 20]. The middle concentration of the five concentrations used for each radionuclide was chosen to reflect the approximate activity concentration of bacteria solutions used in previous experiments (1.37×10^{1} kBq ml^{-1} for ^{239}Pu solutions and 1.81×10^{2} kBq ml^{-1} for $^{55}FeCl_3$), such that our experiment included solutions 2 orders of magnitude greater and 2 orders of magnitude fewer than the bacteria cultures studied in our laboratory. The activity concentration of each solution used was verified by liquid scintillation counting (LSC) analysis of an aliquot of each solution.

The RNA extraction process was completed according to manufacturer's instructions starting with the transfer of 100 μl of ^{239}Pu or ^{55}Fe solution to a 2 ml microcentrifuge tube. The process is outlined in figure 1(b). Solutions containing ^{239}Pu, ^{239}Pu complexed with citrate, or $^{55}FeCl_3$ were added to the ion exchange column in step 2 of the RNA extraction process (step 1 of figure 1 was not completed for process blanks, as there were no cells used). The process was completed in triplicate for each concentration used. The concentrations used are summarized in table 1. Throughout the RNA extraction process, ∼2 ml of liquid

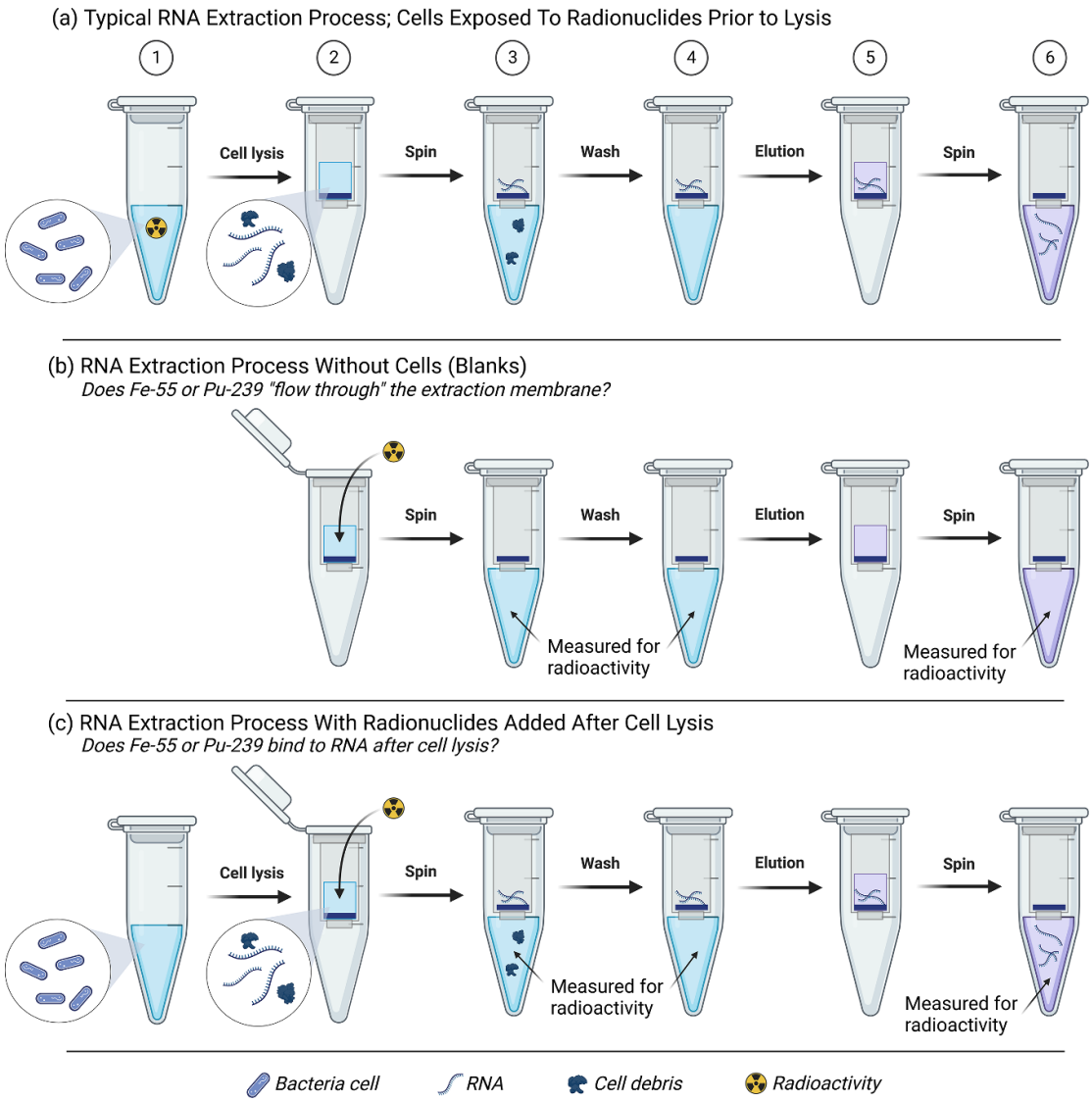

Figure 1. (a) Illustration of the general RNA extraction process as compared to the processes completed for this work; (b) testing for potential process flow through by performing an 'RNA extraction' without cells; (c) testing for potential binding with RNA during the extraction process by adding the radionuclide after cell lysis. Created with BioRender.com.

effluent waste was produced (steps 3 and 4, figure 1). The waste effluent from each extraction column was collected in a 20 ml LSC vial for the purpose of activity/mass balance. The final liquid effluent from the RNA extraction process, which under normal circumstances would contain the RNA sample to be used for bioinformation analysis, was collected in a separate 20 ml LSC vial (step 6, figure 1).

2.5. Plutonium and P. putida sample preparation

To address the potential for radionuclides to bind to RNA as a result of the chemical changes inherent to the RNA extraction process, several control bacteria samples were spiked with ^{239}Pu prior to RNA extraction. The process used for the ^{239}Pu and *P. putida* samples is outlined in figure 1(c). Aliquots (150 μl) were collected from bacteria cultures grown with no radiological contaminants and prepared for RNA extraction. After the cell lysing agent was added, the samples were spiked with 10 μl of the previously prepared Pu solutions with activity concentrations (table 1) of 1.37 kBq ml^{-1}, 1.37 $\times$ 10^{-1} kBq ml^{-1}, and 1.37 $\times$ 10^{-2} kBq ml^{-1} of ^{239}Pu complexed with citrate and 1.37 kBq ml^{-1}, 1.37 $\times$ 10^{-1} kBq ml^{-1}, and 1.37 $\times$ 10^{-2} kBq ml^{-1} of ^{239}Pu without citrate complexation creating six different exposure scenarios (step 2, figure 1). As completed in the process blank investigations, the waste effluent was collected for LSC analysis (steps 3 and 4, figure 1), in addition to the RNA extraction product (step 6, figure 1). The mass of samples was collected for mass balance and quality analysis.

Table 1. Summary of the radiological solution activity concentrations used.

Experiment	Activity concentrations (kBq ml^{-1})				
RNA extraction without cells					
Process blank ^{239}Pu	1.37×10^{1}	1.37	1.37×10^{-1}	1.37×10^{-2}	1.37×10^{-3}
Process blank ^{239}Pu with citrate	1.37×10^{1}	1.37	1.37×10^{-1}	1.37×10^{-2}	1.37×10^{-3}
Process blank $^{55}FeCl_3$	1.81×10^{4}	1.81×10^{3}	1.81×10^{2}	1.81×10^{1}	1.81
RNA extraction with radionuclide spike after cell lysis					
P. putida and ^{239}Pu		1.37	1.37×10^{-1}	1.37×10^{-2}	
P. putida and ^{239}Pu with citrate		1.37	1.37×10^{-1}	1.37×10^{-2}	

2.6. LSC

All samples were prepared for LSC with 10 ml of Optiphase HiSafe 3 (Perkin Elmer) liquid scintillation cocktail. Plutonium-239 samples were quantified based on activity from alpha emissions assuming 100% efficiency. Iron-55 samples were quantified based on activity from beta emissions, assuming an efficiency of 42%. All samples were corrected for variation in volume based on their mass.

2.7. Sample terminology

Two samples were produced for each RNA extraction performed. The first sample, collected in steps 3 and 4 of the process as illustrated in figure 1, is referred to as an effluent sample. The second sample, collected in step 6 of the process as illustrated in figure 1, is referred to an RNA extraction sample. While most of the samples considered in this work were blanks, processed exclusively to examine the flow-through of radionuclides, and thus contain little to no RNA, this terminology will be used for all samples as they were intended to represent the product of the RNA extraction process.

3. Results and discussion

While the radiological content of RNA samples was initially analysed for the purpose of developing appropriate protocols for the handling of radiological materials, the discovery of ^{239}Pu and ^{55}Fe in RNA samples lead to our decision to collect RNA extraction samples specifically for the purpose of radiological characterization during experiments. In our work, we use the radiological content of RNA samples as evidence to differentiate uptake of ^{239}Pu and ^{55}Fe into the cells, versus sorption to the outside of the cell membrane. To use the RNA extractions as evidence of uptake, however, we needed to rule out that the presence of ^{239}Pu and ^{55}Fe was not the result of something related to the extraction process. By examining process blanks, with no cellular content including RNA, we were able to demonstrate that the presence of ^{239}Pu and ^{55}Fe in our RNA extractions was not the result of process flow through.

There was no detectable ^{239}Pu in any RNA extraction sample analysed when ^{239}Pu was complexed with citrate. When process blanks for ^{239}Pu without citrate complexation were analysed, Pu was detected in only one RNA extraction sample. The sample with detectable ^{239}Pu was from the largest sample-blank concentration, 1.37×10^{1} kBq ml^{-1}, and had an activity of 0.87 ± 0.04 Bq. The activity of the RNA extraction sample represents 0.064% of the total activity used in this sample. The results of this part of the experiment demonstrate that the presence of ^{239}Pu in RNA extraction samples is not the result of process flow-through.

By studying the radiological content of the extraction process effluent samples, we were also able to observe the movement of the radionuclides considered through the extraction process and better understand where radiological waste may be produced. Plutonium-239 was detected in effluent samples from sample blanks containing ^{239}Pu complexed with citrate from the three largest concentrations (1.37×10^{1} kBq ml^{-1}, 1.37 kBq ml^{-1}, and 1.37×10^{-1} kBq ml^{-1}), with no ^{239}Pu detected in effluent samples from the 1.37×10^{-2} kBq ml^{-1} and 1.37×10^{-3} kBq ml^{-1} blanks. The activity of ^{239}Pu found in these samples varied between blank concentrations by about a factor of 10, which is consistent with the serial dilution method used to create the working solutions. Note that 1 of the 3 effluent replicates for the 1.37×10^{1} kBq ml^{-1} was excluded from the data set as the sample was spilled during processing. The Pu activity of the effluent samples for blanks containing Pu complexed with citrate are provided in figure 2.

When considering the total activity contained in each sample analysed, the effluent blanks left about half of the ^{239}Pu unaccounted for in samples where ^{239}Pu was complexed with citrate for the three highest concentrations used. We assume that the exchange column used in this process has some affinity for Pu and the remaining activity was disposed of with the exchange columns. The percent of Pu activity that was assumed to remain on the exchange column at the end of the RNA extraction process is provided in figure 3.

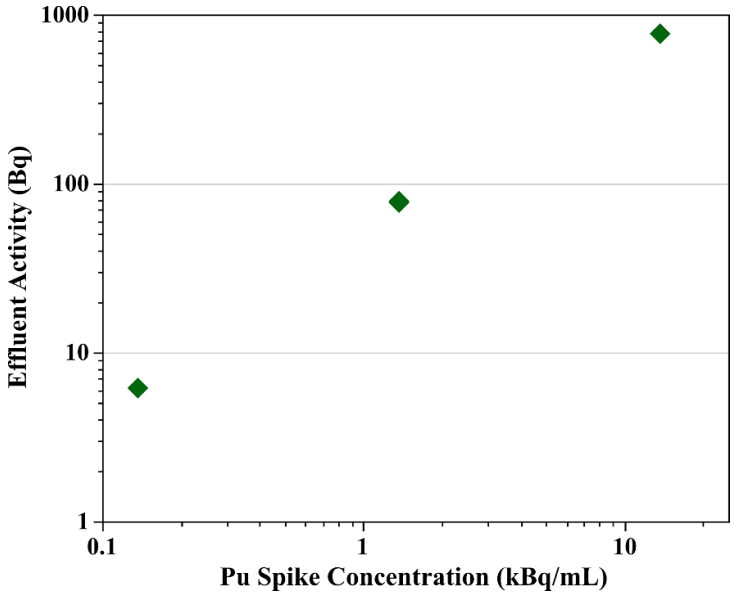

Figure 2. Activity of effluent samples for RNA extraction blanks using Pu complexed with citrate.

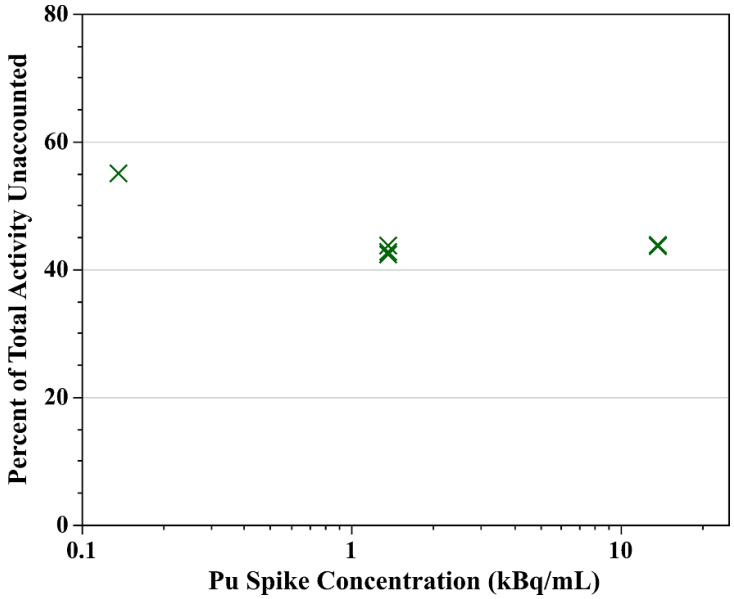

Figure 3. Percent of total sample activity that was not accounted in the RNA extraction or effluent samples.

When effluent samples from process blanks containing ^{239}Pu that was not complexed with citrate were analysed, ^{239}Pu was detected only in effluent samples from the blanks containing 1.37×10^{1} kBq ml^{-1} ^{239}Pu. The effluent samples from the 1.37×10^{1} kBq ml^{-1} ^{239}Pu without citrate group also contained a lower concentration of ^{239}Pu when compared effluent samples from the same ^{239}Pu concentration complexed with citrate. In all three replicates, 99.5% of the activity was unaccounted for and assumed to have remained on the exchange column when the 1.37×10^{1} kBq ml^{-1} blanks were processed, with 100% remaining on the exchange column for all other concentrations. The effluent data for ^{239}Pu both with and without citrate complexation suggests that the affinity of the exchange column for ^{239}Pu is greater for Pu(V) than Pu(IV).

Iron-55 was detected in RNA extraction samples for all replicates of blanks with the largest concentration of ^{55}Fe, 1.81×10^{4} kBq ml^{-1}, as well in one sample from the 1.81×10^{3} kBq ml^{-1} set of blanks, and one sample from the smallest concentration, 1.81 kBq ml^{-1}. All other RNA extraction samples did not contain detectable quantities of ^{55}Fe. The results are provided in figure 4.

The detected ^{55}Fe in the RNA extraction sample from a 1.81 kBq ml^{-1} blank is inconsistent with two replicate data points and also inconsistent with triplicate data from two higher concentrations of ^{55}Fe blanks.

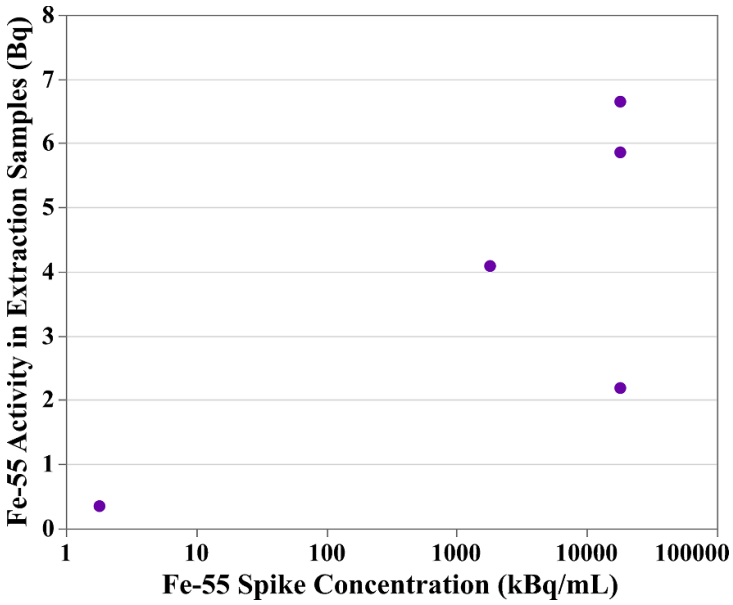

Figure 4. Iron-55 content of RNA extraction samples for RNA extraction completed with ^{55}Fe process blanks.

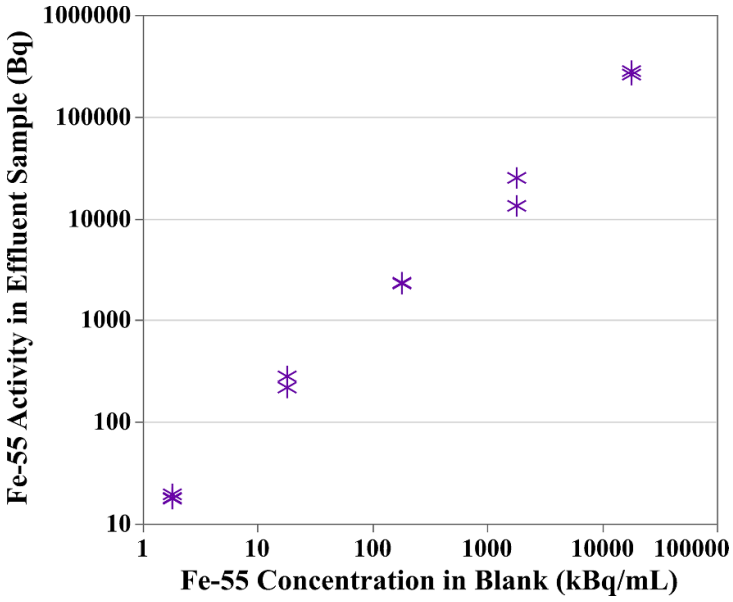

Figure 5. Concentration of ^{55}Fe in effluent samples.

Furthermore, the activity in the 1.81 kBq ml^{-1} extraction sample that was reported is 1.92% of the total activity in the sample analysed. In other RNA extraction samples where ^{55}Fe was detected, the extraction sample activity represented between 0.00121% and 0.0226% of the total activity in the samples analysed. The results have been presented in their complete form for the purpose of transparency, however it is most likely that the RNA extraction result related to the 1.81 kBq ml^{-1} sample is the result of cross contamination during the RNA extraction process because of its lack of consistency with all other data, which was collected in triplicate. The content of ^{55}Fe in RNA extraction samples also demonstrate that ^{55}Fe content in our previous RNA extraction studies was not the result of process flow through [20].

Iron-55 was detected in all effluent samples, where the ^{55}Fe activity in effluent samples increased by a factor of about 10 for each concentration of ^{55}Fe used as a process blank. The ^{55}Fe activity in effluent samples for process blanks is provided in figure 5.

As observed with the ^{239}Pu blank effluent samples, the total quantity of activity from the original sample is not accounted for by the RNA extraction and effluent samples, so it is assumed the remainder of activity remained on the extraction column. The amount of ^{55}Fe that was assumed to remain on the extraction

column more closely resembles the activity balance results from ^{239}Pu without citrate complexation, where the percent of ^{55}Fe that was unaccounted for ranged from 97.1% to 99.3% across all samples. As stable Fe would be likely be found in trace quantities in normal cell culture studies, and the RNA extraction process strives to produce an extraction contain few impurities, the extraction column is likely designed to have an affinity for Fe.

In addition to process flow through, we also prepared an experiment to demonstrate that ^{239}Pu did not bind to RNA after the cells were lysed, during the RNA extraction process. RNA extraction samples collected from *P. putida* samples spiked after lysing cells with 1.37 kBq ml^{-1}, 1.37 $\times$ 10^{-1} kBq ml^{-1}, and 1.37 $\times$ 10^{-2} kBq ml^{-1} ^{239}Pu complexed with citrate and the same concentrations of ^{239}Pu without citrate complexation did not contain detectable quantities of ^{239}Pu. Only one effluent sample, effluent from the *P. putida* sample spiked with the greatest quantity of Pu complexed with citrate, the 1.37 kBq ml^{-1} spike, contained detectable ^{239}Pu. The results of this analysis indicate that ^{239}Pu found in RNA extraction samples is not the result of ^{239}Pu binding to RNA after the cell is lysed.

4. Conclusions

Plutonium-239 was only found in one set of RNA extraction replicates, the set containing over 100 times the quantity of ^{239}Pu without citrate complexation that would be expected in cell studies conducted as a part of our work. Iron-55 was only consistently found in extraction samples with 100 times the quantity of ^{55}Fe used in our cell cultures, and in one replicate with 10 times more ^{55}Fe. The results of this work show that process flow through is not a significant pathway for ^{239}Pu or ^{55}Fe in RNA extraction samples as observed in our previous work. By analyzing the radiological content of effluent samples, the study also demonstrated that the extraction columns used have an affinity for both Pu and Fe. The study supports the assumption that ^{239}Pu detected in RNA extraction samples during long term cell studies is the result of ^{239}Pu that was bound to RNA prior to the time of extraction. While similar studies were not completed with ^{55}Fe due to availability at the time of experimentation, the results for ^{239}Pu also suggest that ^{55}Fe was also bound to RNA prior to the time of extraction, as ^{239}Pu uptake is primarily the result of using Fe pathways. Because Pu has similar biochemistry to Fe(III), it is expected that similar behaviour would occur with both metals, consistent with our other observations on ^{55}Fe and ^{239}Pu behaviour [12].

Validating a model for radiation protection for doses less than about 150 mGy remains a priority in the field of radiological science, and the use of -omics technologies to understand low dose effects, especially transcriptomics and proteomics, will likely increase in the coming years. A 2022 Consensus Study of the National Academy of Sciences reaffirms the prioritization of a low dose research program in the United States of America for radiological doses below 100 mGy and dose rates of 5 mGy h^{-1} or less. Specifically, the report calls for leveraging modern biological research techniques for the study of low dose and low dose-rate mechanisms of effect and integrating this data with epidemiological studies to examine risk assessment models [21]. The relevance of our observations regarding the radiological content of RNA samples is two-fold. The first area of relevance of this work is the practical concern of handling radiologically contaminated samples. Biological equipment utilized for transcriptomic research, especially equipment used for RNA sequencing, is costly and requires specialized training to use. Sequencing equipment is often located in centralized facilities at laboratories and universities, and, in some cases, sequencing is completed off-site. Researchers preparing RNA or DNA extraction samples from cells exposed to radiological contaminants, especially metals, should take care to characterize samples so that appropriate procedures for transport and handling are developed in accordance with federal, state, and facility regulations. Appropriate characterization will also help prevent contamination of specialized equipment.

This work also highlights the importance of considering how effects may be impacted when radiological metals are bound to DNA and RNA. Radiologically characterizing the content of DNA and RNA samples may provide quantitative data needed to differentiate between chemical and radiological response in low-dose research efforts. Separating chemical and radiological effects will not only be useful when attempting to identify unique responses to radiation exposure, it may also help identify responses related to specific radiological contaminants. Finally, radiological content found in DNA and RNA samples may also be useful in quantifying uptake rates which could have implications for dosimetry, as advanced biological assessment techniques should be matched with fine-tuned dose estimates if dose-effect relationships are developed.

Data availability statement

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

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