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Feasibility study on the use of polyallyldiglycol-carbonate cell dishes in TUNEL assay for alpha particle radiobiological experiments

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Abstract

In the present work, we have studied the feasibility of a method based on polyallyldiglycol-carbonate (PADC) films to investigate the effects of alpha particles on HeLa cervix cancer cells. Thin PADC films with thickness of about 20 μm were prepared from commercially available CR-39 films by chemical etching to fabricate custom-made petri dishes for cell culture, which could accurately record alpha particle hit positions. A special method involving "base tracks" for aligning the images of cell nuclei and alpha particle hits has been proposed, so that alpha particle transversals of cell nuclei can be visually counted. Radiobiological experiments were carried out to induce DNA damages, with the *T*dT-mediated d*U*TP *N*ick-*E*nd *L*abeling (TUNEL) fluorescence method employed to detect DNA strand breaks. The staining results were investigated by flow cytometer. The preliminary results showed that more strand breaks occurred in cells hit by alpha particles with lower energies. Moreover, large TUNEL positive signals were obtained even with small percentages of cells irradiated and TUNEL signals were also obtained from non-targeted cells. These provided evidence for the bystander effect.
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Keywords: PADC; CR-39; Solid-state nuclear track detector; SSNTD; Radiobiology; Alpha particle

1. Introduction

Ionizing radiation leads to production of reactive oxygen species in human cells, which can damage cellular macromolecules including DNA and produce single-strand breaks (SSBs) and double-strand breaks (DSBs) in the DNA [1]. It is generally believed that DNA strand breaks induced by ions with high-linear energy transfer (LET) are severe due to clustering of ionization in the DNA. The effects of ionizing radiation can occur in irradiated (or targeted) cells or in non-irradiated (or non-targeted) cells. The latter are known as bystander effects which occur when an irradiated cell communicates with non-irradiated cells via secreted factors and/or gap junction communication. These untargeted cells show responses which are characteristic of irradiated cells. DNA strand breaks can be detected using the *T*dT-mediated d*U*TP *N*ick-*E*nd *L*abeling (TUNEL) fluorescence method. Here, the fluorescein labels incorporated in nucleotide polymers are detected and quantified by fluorescence microscopy or flow cytometry.

In the present work, we study the feasibility of a method based on polyallyldiglycol-carbonate (PADC) films to investigate the effects of alpha particles on HeLa cervix cancer cells. To directly provide evidence for the dependence of DNA strand breaks on the LET or for the presence of bystander effects, accurate alpha particle hit positions on the cells are needed. We will first give details on the fabrication of thin PADC films as cell-culture substrates to record the positions where the alpha particles hit the cultured cells. A special method involving "base tracks" for aligning the images of cell nuclei and alpha particle hits will then be introduced. Finally, the application of

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TUNEL assay to evaluate the DNA damages in the cells will be described.

2. Methodology

2.1. Preparation and use of thin PADC films

PADC films (commercially available as CR-39 detectors) are one of the most commonly used solid-sate nuclear track detectors (SSNTDs). A recent review on SSNTDs has been given in [2] while a review of uses of SSNTDs in cellular radiation biology can be found in [3]. There are distinct advantages of using PADC films as cell-culture substrates in alpha particle radiobiological experiments [4–6] although other films such as cellulose nitrate films have also been studied for such purposes [7,8]. For example, PADC films are transparent, relatively biocompatible [9] and do not dissolve in alcohol during sterilization.

For alpha particle radiobiological experiments, it is easier to quantify the alpha energies incident on the cell nuclei if the alpha particles pass through the substrate to strike the cells because there is always a nutritive layer with variable thickness on top of the cells. However, the substrate should then be thin enough to allow passage of alpha particles with nominal energies (e.g. those from ^{241}Am source). According to the SRIM program [10], the range of 5 MeV alpha particles in PADC is 28.77 μm. However, the thinnest commercially available CR-39 SSNTDs are ~100 μm thick (e.g. from Pershore) and are thus not thin enough. In the present work, we prepared thin PADC films (< 20 μm) from commercially available CR-39 SSNTDs with a thickness of 100 μm (from Page Mouldings (Pershore) Limited, Worcestershire) by chemical etching in NaOH/ethanol [4]. After etching, the thickness of the film was measured using a micrometer (Mitutoyo, Japan) with an accuracy of ±1 μm. These thin PADC films were then glued by an epoxy (Araldite® Rapid, England) to the bottom of petri dishes (Orange Scientific) with a diameter of 5 cm, with a hole of 1 cm diameter drilled at the bottom, to form the PADC cell dishes as in Fig. 1.

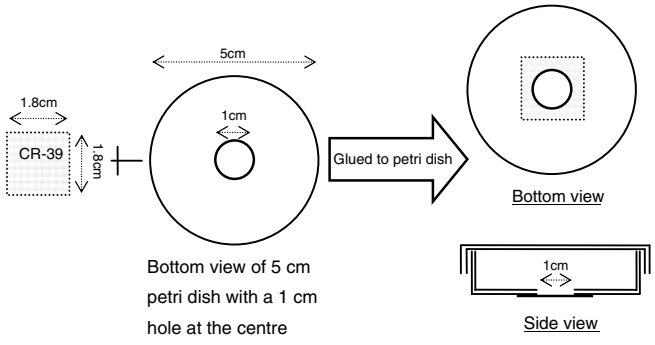

Fig. 1. Preparation of a PADC cell dish by gluing a thin PADC film onto the bottom of a petri dish with 5 cm diameter and with a 1 cm hole drilled at the center of the bottom.

2.2. Generation of base tracks on the PADC cell dishes

As mentioned in the introduction, accurate positions of alpha particle hits on the cells are needed. In this section, a special method involving generation of base tracks on the PADC films will be introduced for subsequent alignment of images of cell nuclei and alpha particle tracks. After gluing the thin PADC films onto the custom-made cell dishes, the bottom of PADC films were irradiated by 1 MeV alpha particles for 10 min to generate the base tracks.

The alpha particle source employed in the present study was a planar ^{241}Am source (main alpha energy = 5.49 MeV under vacuum). The final alpha energies incident on the film were controlled by the source to film distances in normal air. The relationship between the alpha energy and the air distance traveled by an alpha particle with initial energy of 5.49 MeV from ^{241}Am was obtained by measuring the energies for alpha particles passing through different distances in normal air using alpha spectroscopy systems (ORTEC Model 5030) with passivated implanted planar silicon (PIPS) detectors of areas of 300 mm^2. The parameters of the tracks formed on a PADC film by 1 MeV alpha particles (for generating base tracks) and 5 MeV alpha particles (for irradiating the cells) after chemical etching show distinct characteristics [11]. For example, 1 MeV alpha particle tracks have larger track openings and are shallower than 5 MeV alpha particle tracks [12]. In this way, the base tracks can be conveniently distinguished from the tracks formed by alpha particles employed to irradiate the cells.

After irradiated by the 1 MeV alpha particles, the bottom side of the cell dishes was etched by 14 N KOH solution at 37 °C (corresponding to a bulk etch rate of 0.64 ± 0.01 μm/h determined by the masking method [13,14]) for 3 h 40 min. Such a low-etching temperature was chosen to prevent the epoxy from being dissolved in the strong etchant at high-temperatures. Base tracks will be formed on the bottom of the PADC films upon chemical etching.

2.3. Cell cultivation, capture of cell images and alpha particle irradiation

After the base tracks are generated, the PADC cell dishes were sterilized by submerging them into 75% (v/v) ethyl alcohol for 2 h and then submerging in absolute alcohol. These cell dishes were then used for culturing National Institutes of Health HeLa cervix cancer cells obtained from the American Type Culture Collection. The cell line was maintained as exponentially growing monolayers at low-passage numbers in Dulbecco's modified eagle medium (D-MEM) supplemented with 10% fetal borine serum, 1% (v/v) penicillin/streptomycin. The cells were cultured at 37 °C in humidified atmosphere containing 5% CO_2. Subcultivation was performed every 3–4 d. Penicillin/streptomycin was produced by Gibco (Karlsrube, Germany). The cells were trypsinized for 4 min with 0.5/0.2% (v/v) trypsin/EDTA (ethylenediamine-tetra-acetic acid; Gibco),

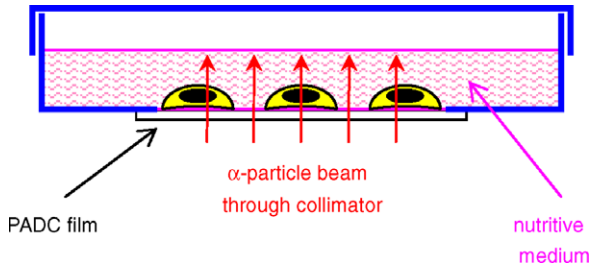

Fig. 2. The irradiation of the cell monolayer through the custom made PADC cell dish.

adjusted to a concentration of about 3×10^5 cells ml^{-1} and plated out on the PADC cell dishes.

Before alpha particle irradiation, images of the cells on the surface of the PADC films in the area planned for irradiation were captured. A volume of 1 ml of nutritive medium was retained in the cell dishes to prevent the cells from drying up. The images captured would include those of the 1 MeV base tracks spread over the whole area together with those of the HeLa cells. After capturing the images, the PADC cell dishes were irradiated from the bottom with 5 MeV alpha particles under normal incidence through a collimator for about 1 h from the same source as depicted in Fig. 2. During irradiation, some of the energy of the alpha particles will be absorbed while passing through the PADC film. The absorbed energy depended on the thickness of the PADC film and the residual energy could be calculated from the outputs of the SRIM program [10]. In order to investigate the response of the cells to different alpha particle energies, PADC films with various thicknesses were used in the experiments that would be subjected to irradiation of 5 MeV alpha particles.

2.4. TUNEL assay

After alpha particle irradiation, adherent cells on the PADC cell dishes were trypsinized for 4 min with 0.5/0.2% (v/v) trypsin/EDTA (Gibco) and these cells were collected from the cell dishes immediately. The collected cell suspensions would be transferred into a 1.5 ml microcentrifuge tube and washed by phosphate buffered saline (PBS) once. Before the cells are fixed, 20 μl of the cell suspensions were collected for counting the total cell number by using a hemocytometer (Tiefe Depth Profondeur, Marienfeld, Germany). Cell viability was assessed by staining with 0.2% Trypan blue (Sigma) which only enter across the membranes of dead/non-viable cells.

The washed cell suspension was then fixed by adding freshly prepared fixation solution (2% paraformaldehyde (Sigma) in PBS) and resuspended well with the centrifuged cells. The incubation conditions were 1 h at 15–25 °C. The cells were then washed once with PBS and resuspended in 1 ml permeabilisation solution (0.1% Triton X-100 (Sigma) in 0.1% sodium citrate (Gibco)) for 10 min on ice (2–8 °C). After permeabilisation, all the cells were washed twice with PBS.

A positive control sample was then prepared by adding DNase I (grade I) to digest the DNA for 15 min at 15–25 °C. After that, the cells in all samples were resuspended in 50 μl/tube TUNEL reaction mixture made of 90% (v/v) label solution (Roche Diagnostics GmbH, Germany) and 10% (v/v) TUNEL enzyme (Roche Diagnostics). The cell suspension was then incubated for 1 h at 37 °C in a humidified atmosphere in dark for reaction to take place. The two negative controls were unirradiated samples in a TUNEL buffer without terminal transferase and those in TUNEL reaction mixture, respectively. To study the effect of light from the microscope on the cells during image capture before irradiation, the samples of cells that had been exposed to light for the same time interval of image capture were also stained with the TUNEL reaction mixture. The samples were then washed twice in PBS.

Finally, the cells were transferred in a specialized tube to a final volume of 500 μl in PBS. The samples were analyzed by a flow cytometer (Becton DICKINSON, FACSCalibur cytometer) by counting 10 000 cells for each sample to detect TUNEL signals that involve an excitation wavelength in the range of 450–500 nm (e.g. 488 nm) and detection in the range of 515–565 nm (green).

2.5. Final track formation

After the cells were trypsinized from the surfaces of the PADC cell dishes, the bottom sides of the cell dishes were further etched for 6 h by a 14 N KOH solution at 37 °C to get sufficiently large tracks for identification of alpha particle hit positions. At this stage, there were no cells in the PADC cell dishes so a long etching time would not be a problem.

3. Results and discussion

3.1. Identification of alpha particle hit positions

After the second-time etching, two types of alpha particle tracks appeared on the bottom side of a PADC film,

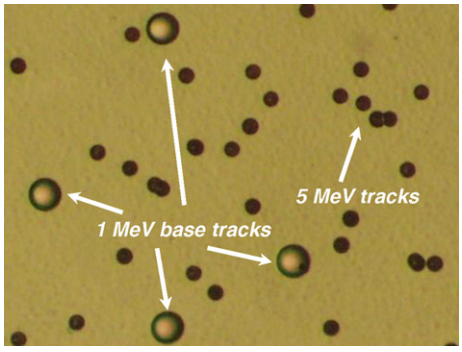

Fig. 3. The images (with a magnification of 200×) of base tracks formed from 1 MeV alpha particles that have been etched for a cumulative period of 9 h 40 min, together with the images of tracks from 5 MeV alpha particles that have been etched for 6 h in 14 N KOH solution at 37 °C.

namely, (1) 1 MeV alpha particle tracks which had been etched for a cumulative period of 9 h 40 min and (2) 5 MeV alpha particle tracks which had been etched for 6 h as shown in Fig. 3. Finally, the images of the cells together with the base tracks would be superimposed with the images of both 1 MeV base tracks and 5 MeV tracks captured after the final etching.

An image of base tracks together with HeLa cells is shown in Fig. 4(a). However, the cell nuclei cannot be seen clearly in this view as the focus is on the alpha particle tracks developed on the bottom of the PADC film. Therefore, another image should be captured at the same position but with the focus on the cells, which is shown in Fig. 4(b). By superimposing these two images using a photo editing software, both the cell nuclei and the tracks can be seen clearly.

In the next step, the images mentioned above were further superimposed with the image containing both the 1 MeV base tracks and 5 MeV tracks captured after the final etching as described in Section 2.5. An example is shown in Fig. 5. Here, there are in fact three image layers, namely, (1) the layer with base tracks, (2) the layer with clear images of cell nuclei and (3) the layer with enlarged base tracks and 5 MeV alpha particle tracks. As demonstrated in Fig. 5, the positions of alpha particle hits by 5 MeV alpha particles on the cell nuclei could be determined by aligning the enlarged base tracks with the original base tracks.

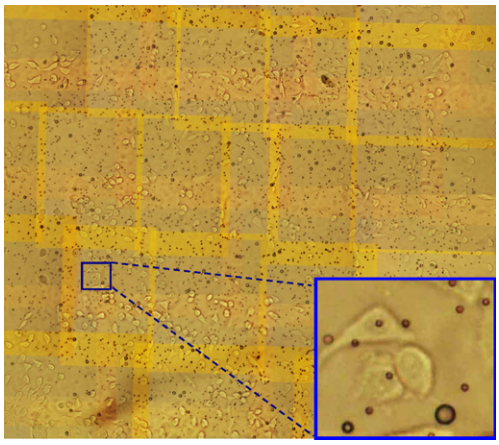

Fig. 6. An overall image of the area of cells irradiated by 5 MeV alpha particles, together with base tracks from 1 MeV alpha particles.

Fig. 6 gives a combined image of the area of cells hit by 5 MeV alpha particles with the enlarged base tracks. In this way, the number of alpha particle hits on the cell nuclei can

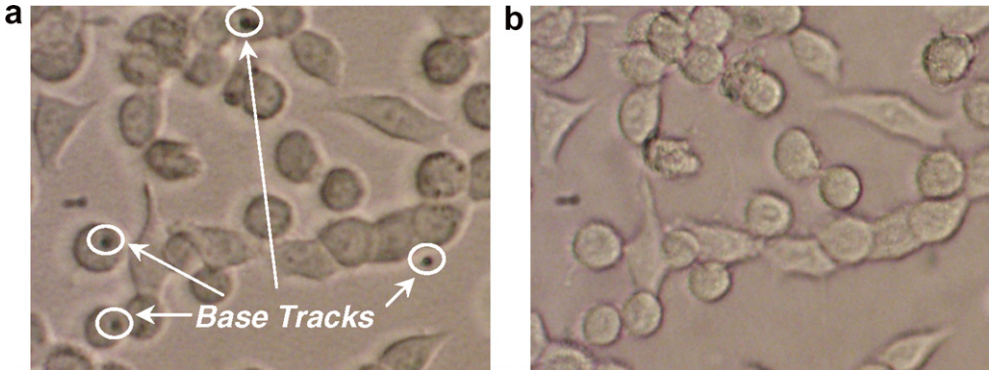

Fig. 4. The images of base tracks from 1 MeV alpha particles with HeLa cells on the surface of the PADC film with a magnification of 200×: (a) with focus on the tracks and (b) with focus on the cells.

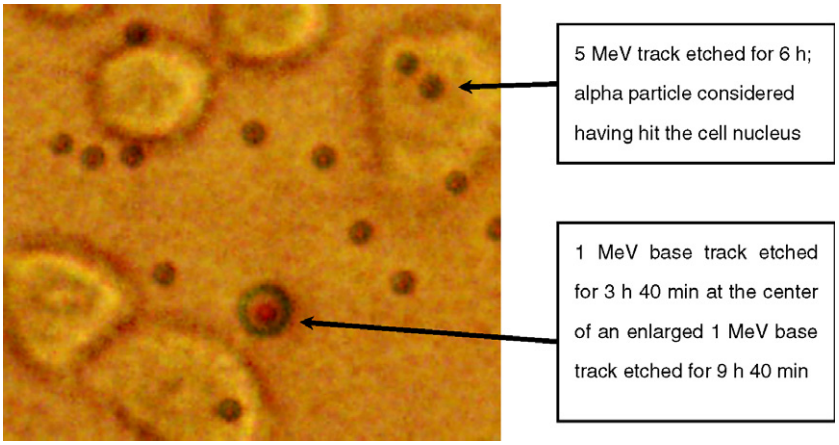

Fig. 5. The image of cells clearly showing the cell nuclei, together with the base tracks for alignment and the tracks from 5 MeV alpha particles.

Table 1
The results of TUNEL assay of the cells irradiated by alpha particles with different residual energies, with the number of alpha particle hits on cell nuclei also shown

Residual energy on cells (MeV)	% of TUNEL positive (considered with negative control)	Total cell number on the cell dishes	Number of alpha particle hits on cell nuclei	Number of cell nuclei hit by alpha particles	Number of alpha particle hits per cell nucleus
0.15	13.94	743 750	1527	827	1.85
0.15	17.43	1 106 250	906	495	1.83
0.15	19.72	1 181 250	868	682	1.27
0.39	16.77	1 150 000	2104	1276	1.65
0.39	14.65	1 093 750	2206	1429	1.54
0.39	8.11	1 625 000	1639	1115	1.47
0.96	4.23	1 287 500	1609	966	1.67
0.96	6.48	1 881 250	1412	1002	1.41
0.96	10.61	1 487 500	1535	1015	1.51
1.23	15.65	1 212 500	382	254	1.50
1.23	12.06	1 031 250	917	755	1.21
1.23	28.28	1 318 750	1171	752	1.56
1.47	3.91	1 137 500	931	634	1.47
1.47	4.05	2 043 750	564	415	1.36
1.47	5.16	1 568 750	1432	1023	1.40
1.70	4.15	1 193 750	804	592	1.36
1.70	3.57	1 700 000	1336	986	1.35
1.70	5.03	1 550 000	993	692	1.43
2.35	7.80	1 500 000	1924	1061	1.81
2.35	4.65	3 718 750	1592	1062	1.50
2.35	7.37	3 437 500	1060	613	1.73
2.80	8.37	1 300 000	1278	879	1.45
2.80	0.00	1 531 250	2687	1864	1.44
2.80	0.00	1 712 500	1939	1413	1.37

Each residual energy has three sets of data.

be counted. The results are summarized in Table 1. The residual energies of the alpha particles hitting the cells are calculated from the alpha particle energy incident on the bottom of the PADC film together with the thickness of the PADC film through using the outputs from the SRIM program [10].

3.2. TUNEL assay

Fig. 7 shows the results from the flow cytometer, which measures the strengths of fluorescence signals from the samples. The blue area represents the signal from the negative control, in which unirradiated cells were stained with the TUNEL reaction mixture. This region is defined as M1, which corresponds to no TUNEL signals. In contrast, the region corresponding to the presence of TUNEL signal is defined as M2.

The blue peak represents the signals from the sample irradiated by 5 MeV alpha particles (with a residual energy of 1.23 MeV on the cells) for 1 h. This sample showed that (30.12–1.84)% = 28.28% of cells had TUNEL positive results after adjustment for the negative signal, i.e. the irradiated sample had 30.12% of signals in the M2 region and the negative control sample stained with the TUNEL reaction mixture had 1.84% of signals in the M2 region (peak shaded in purple). TUNEL positive means that there are DNA strand breaks in the cell sample. The pink peak

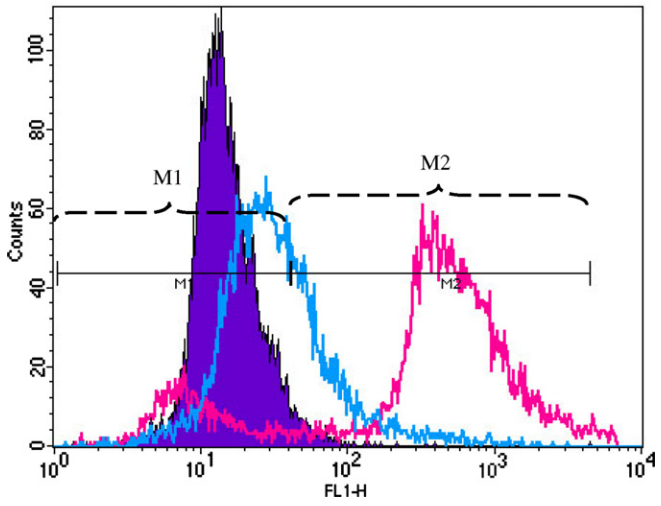

Fig. 7. The results from the flow cytometer that give the strength of fluorescence signals from the samples. The *x*-axis represents the strength of fluorescence signal while the *y*-axis represents the number of cells counted. Region M1 corresponds to fluorescence signals without TUNEL signals, while region M2 corresponds to fluorescence signals that has TUNEL signals. The purple shaded peak, blue peak and pink peak are obtained from a sample of negative control (1.84% TUNEL positive), a sample irradiated for 1 h by 5 MeV alpha particles which had a residual energy of 1.23 MeV on the cells (28.28% relative TUNEL positive) and a positive control (83.52% relative TUNEL positive) stained by TUNEL reactive mixture, respectively. (For interpretation of the figure in colour, the reader is referred to the web version of this article.)

represents the signals from the positive control sample with 83.52% TUNEL positive. The TUNEL results for the cells irradiated by alpha particles with different residual energies are shown in Table 1, with 3 sets of data for each residual energy.

The preliminary results here show that more strand breaks occur in cells hit by alpha particles with lower residual energies, as Fig. 8 shows an increase in the percentages of strand breaks with the residual energy of alpha particles striking the cells lower than about 1.5 MeV. The TUNEL positive signals are all lower than 10% for residual energies higher than 1.5 MeV. In contrast, the TUNEL positive signals are generally higher than 10% for residual energies lower than about 1.5 MeV. This is likely an effect from the LET as alpha particles with very high-energies have low-LET values. The dependence of the TUNEL positive signals on the alpha particle energy is also shown in Table 2. The TUNEL positive signal is also related to the average number of alpha particle hits on each cell nucleus and the percentage of the total hit cells as shown in Table 2.

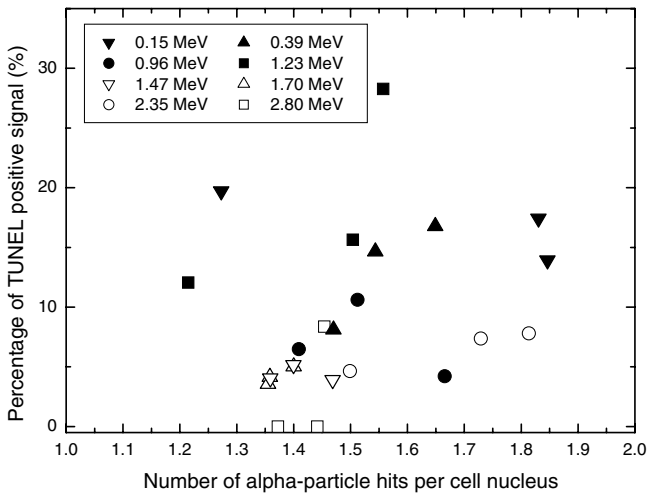

Fig. 8. The results showing the relationship between the TUNEL positive signal against the number of alpha particle hits per cell nucleus, with the residual energy as a parameter.

From Table 2, we notice that a TUNEL positive signal close to 30% occurs even with less than 0.1% of cells irradiated. This can be considered an evidence of the bystander effect. Previous studies have shown that bystander-induced effects would be maximized for low-dose cases, including a single alpha particle traversal [15]. From Table 2, we see that two samples with about 1.2 alpha particle hits per cell nucleus have TUNEL positive signals larger than 10%.

In order to further illustrate the effects on the non-irradiated cells, TUNEL assay was used to study whether there were TUNEL signals from the non-targeted cells. This was achieved by only trypsining the cells in the targeted area of irradiation and then collecting the trypsinized irradiated cells. After that, all the remaining non-targeted cells were then trypsinized and the steps of TUNEL assay were followed. Two sets of experiments were carried out: (1) Two samples with irradiated cells trypsinized and collected as mentioned above (two PADC films with thickness that result in a residual energy of 0.15 MeV were chosen) were

Table 2
The results of TUNEL assay of the cells irradiated by alpha particles with different residual energies, with the number of alpha particle hits on cell nuclei and percentage of total hit cells also shown

Residual energy on cells (MeV)	Percentage of TUNEL positive (%) (considered with negative control)	Number of alpha particle hits per cell nucleus	Percentage of hit cells (%)
0.15	13.94	1.85	0.11
0.15	17.43	1.83	0.04
0.15	19.72	1.27	0.06
0.39	16.77	1.65	0.11
0.39	14.65	1.54	0.13
0.39	8.11	1.47	0.07
0.96	4.23	1.67	0.08
0.96	6.48	1.41	0.05
0.96	10.61	1.51	0.07
1.23	15.65	1.50	0.02
1.23	12.06	1.21	0.07
1.23	28.28	1.56	0.06
1.47	3.91	1.47	0.06
1.47	4.05	1.36	0.02
1.47	5.16	1.40	0.07
1.70	4.15	1.36	0.05
1.70	3.57	1.35	0.06
1.70	5.03	1.43	0.04
2.35	7.80	1.81	0.07
2.35	4.65	1.50	0.03
2.35	7.37	1.73	0.02
2.80	8.37	1.45	0.07
2.80	0.00	1.44	0.12
2.80	0.00	1.37	0.08

Table 3
The results of TUNEL assay for the non-targeted cells for a residual energy of 0.15 MeV

Residual energy on cells (MeV)	% of TUNEL positive (considered with negative control)	Total cell number on the cell dishes	Number of alpha-particle hits on cell nuclei	Number of cell nuclei hit by alpha particles	Number of alpha particle hits per cell nucleus
0.15	22.60	1062500	1812	1249	1.45
0.15	11.04	556250	1012	612	1.65

Table 4
The results of TUNEL assay for both targeted and non-targeted cells for a residual energy of 1.23 MeV

	Residual energy on cells (MeV)	% of TUNEL positive considered with negative control)	Total cell number on the cell dishes	No. of alpha particle hits on cell nuclei	No. of cell nuclei hit by alpha particles	Number of alpha particle hits per cell nucleus
Irradiated and non-irradiated cells	1.23	13.88	787500	1299	719	1.81
Only non-irradiated cells	1.23	9.34	1093750	1679	1142	1.47

measured for TUNEL positive signals; (2) One sample followed the normal procedures of TUNEL assay as described in Section 2.4 while another one with irradiated cells trypsinized and collected as mentioned above (two PADC films with thickness that result in a residual energy of 1.23 MeV were chosen). The results for experiment sets (1) and (2) are shown in Tables 3 and 4, respectively.

Table 3 shows that there are TUNEL positive signals even when the irradiated cells are not present in the samples. Furthermore, despite the limited number of samples, Table 4 shows that the TUNEL positive signal from all cells, including irradiated and non-irradiated cells, is higher than that from untargeted cells only. These suggested the presence of the bystander effect.

Finally, the TUNEL results of the control samples used for illustrating the effect of the light source of the microscope and stained with the TUNEL reaction mixture are: (1) 0.50% TUNEL positive signal for unirradiated cells and (2) 0.66% TUNEL positive signal for unirradiated cells exposed to the light source of the microscope. The similarity of the results demonstrated that exposure to light from the microscope would not induce DNA damages in the cells and the TUNEL results above would not be affected. The average value for the negative control that has been exposed to the light source is $0.80 \pm 0.40\%$.

4. Conclusions

In the present work, we have demonstrated the feasibility to use cell dishes based on thin PADC films for TUNEL assay to detect DNA strand breaks. With a specially designed method involving generation of base tracks for aligning the images of cell nuclei and alpha particle hits, alpha particle transversals of cell nuclei can be visually counted. As a feasibility study, we have obtained preliminary results showing that more strand breaks occurred in HeLa cervix cancer cells hit by alpha particles with lower energies and showing the presence of the bystander effect.

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