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Experimental setup for studying the effects of alpha particles on zebrafish embryos

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Abstract

In the present work, we have studied the feasibility to use an experimental setup based on polyallyldiglycol-carbonate (PADC) films to study effects of alpha particles on dechorionated zebrafish embryos. Thin PADC films with a thickness of 16 μm were prepared from commercially available CR-39 films by chemical etching and used as support substrates for holding zebrafish embryos for alpha-particle irradiation. These films recorded alpha-particle hit positions, quantified the number and energy of alpha particles actually incident on the embryo cells, and thus enabled the calculation of the dose absorbed by the embryo cells. Irradiation was made at 4 h post fertilization (hpf) with absorbed doses up to 2.3 mGy. Images of the embryos at 48 hpf were examined for identification of morphologic abnormalities. The preliminary results showed that absorbed doses corresponding to the abnormally developed embryos ranged from 0.41 to 2.3 mGy, which was equivalent to 0.21–1.2 mGy in human.

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1. Introduction

It has been common to study DNA damage responses in vertebrates using cell cultures. However, such experiments cannot be used to study dynamic *in vivo* processes such as temporally and spatially regulated patterns of gene expression [1]. In recent years, the zebrafish, *Danio rerio*, a small vertebrate from Southeast Asia, has become a preferred model for studying human disease, including carcinogenesis. The most important advantage is that the human and zebrafish genomes share considerable homology, including conservation of most DNA repair-related genes [2]. Rapid embryonic development is another advantage in that major organ systems become evident within 48 h postfertilization (hpf).

Recently, a number of research works using the zebrafish embryo as an *in vivo* model to study the DNA damage response to ionizing radiation have emerged. For example, Bladen et al. [3] studied the DNA damage response and Ku80 mRNA function in the zebrafish embryos irradiated with ^{137}Cs gamma rays. McAleer et al. [4] evaluated the effects of 250 kVp X-rays in combination with a known radioprotector (free radical scavenger Amifostine) or radiosensitizing agent (tyrosine kinase inhibitor AG1478) with a view to validate zebrafish embryos as a screen for radiation modifiers. McAleer et al. [5] also used zebrafish embryos to study radiosensitizing effects of flavopiridol in normal tissues exposed to ^{137}Cs gamma rays or 250 kVp X-rays. Daroczi et al. [6] evaluated the radioprotective effect of the nanoparticle DF-1, which was a fullerene with antioxidant properties, in zebrafish embryos exposed to

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^{137}Cs gamma rays. Geiger et al. [1] studied the effects of ^{137}Cs gamma rays and concurrent treatment with Amifostine on the development of the zebrafish embryos.

Despite the success of using the zebrafish embryos to study the DNA damage response to ionizing radiation in these studies, only energetic photons (X-rays and gamma rays) were used. Studies using alpha particles will be of interest because alpha particles are also an ionizing radiation, and with high linear energy transfer (LET). Furthermore, alpha particles are emitted from radon and its progeny, which are ubiquitous in our natural environment, and constitute the largest natural radiation dose to human and can induce lung cancers [7].

However, in order to study the DNA damage response to alpha particles using the zebrafish embryos, we have to solve two major problems. The first one concerns the absorption of alpha-particle energies in (a) the holder for the zebrafish embryos, (b) the fluid in which the zebrafish embryos are immersed, and (c) the chorions of the zebrafish embryos. An alpha particle will lose its energy to the surrounding material as it travels. The amount of energy loss can be determined using the publicly available computer program called Stopping and Range of Ions in Matter (SRIM) [8]. By using the SRIM output results, it is noticed that significant portions of the alpha-particle energies can be absorbed in these three media. The second problem concerns the quantification of alpha-particle dose absorbed by the zebrafish embryo cells, which relies on the number of alpha particles actually incident on the embryo cells and the average energy of these alpha particles. In the present work, we study the feasibility of an experimental setup based on polyallyldiglycol-carbonate (PADC) films to tackle these two problems in order to study the effects of alpha particles on zebrafish embryos.

2. Methodology

2.1. PADC-film based holders for zebrafish embryos

To quantify the alpha-particle dose received by the zebrafish embryo cells, alpha-particle hit positions are needed. In the present work, we propose to use PADC films as support substrates to record the positions where the alpha particles hit the embryos as shown in Fig. 1. PADC films (commercially available as CR-39 films) are one of the most commonly used solid-state nuclear track detectors (SSNTDs). A recent review on SSNTDs has been given in [9] while a review of uses of SSNTDs in cellular radiation biology can be found in [10]. There are distinct advantages of using PADC films as substrates in alpha-particle radiobiological experiments [11–14] although other films such as cellulose nitrate films have also been studied for such purposes [15,16]. For example, PADC films are transparent and relatively biocompatible [17].

For alpha-particle irradiation of zebrafish embryos, it is only feasible to quantify the alpha energies incident on the embryos if the alpha particles pass through the substrate to strike the embryo cells, because there is always a fluid layer with variable thickness above the embryos. However, the PADC 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 [8], the range of 5 MeV alpha particles in PADC is 28.77 μm. However, the thinnest commercially available CR-39 films 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 films with a thickness of 100 μm (from Page Mouldings (Pershore) Limited, Worcestershire) by chemical etching in NaOH/ethanol [11]. After etching, the thickness of the film was measured using a micrometer (Mitutoyo, Japan) with an accuracy of ±1 μm. For simplicity, we unify the thickness of these thin PADC films as 16 μm in the present work. Most of the background counts in PADC films was due to defects present on the surface, and would thus be effectively eliminated on such heavy etching [18].

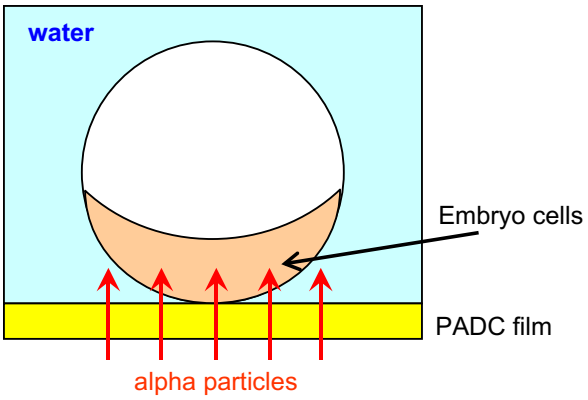

Fig. 1. Schematic diagram showing alpha-particle irradiation of a zebrafish embryo, with the embryo cells resting on the bottom, and assuming that the alpha particles strike the PADC film vertically.

These thin PADC films were then glued by an epoxy (Araldite® Rapid, England) to the bottom of a custom-made holder made of acrylic resin with 8 × 6 holes drilled at the bottom. The holes had a diameter of 2 mm, and the holes were separated at 8 mm. A photo of the custom-made PADC-film based holder is shown in Fig. 2.

2.2. Embryo dechorionation

The chorions of zebrafish embryos will absorb a significant fraction of the alpha-particle energies, so in the present experiments, the chorions were removed before alpha-particle irradiation. The embryos were first dried by sucking all the water away using a dropper. A volume of 2.4 ml E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.1% methylene blue) and 100 μl enzyme called pronase were mixed and were added to the embryos. The embryos were checked from time to time through a microscope to see whether the enzymes had sufficiently digested the chorions. When the chorions were being

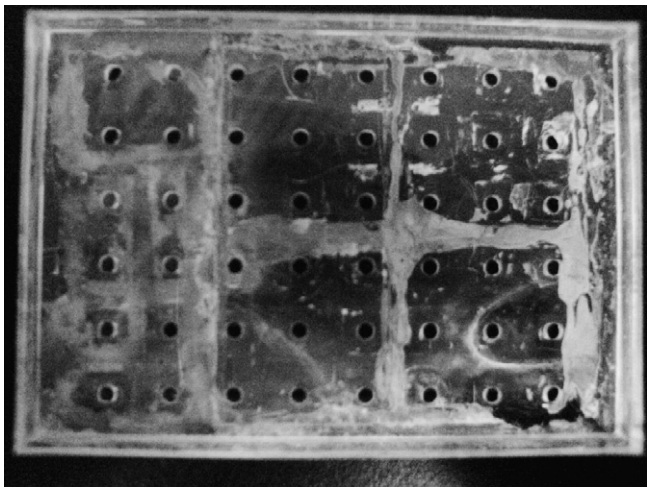

Fig. 2. A custom-made PADC-film based holder for zebrafish embryos.

detached, the embryos were transferred to a beaker of water and washed. The embryos were then transferred to a petri-dish containing E3 solution using a dropper. A dropper was used to create an agitating water current to facilitate the liberation of the embryos. Around 100 dechorionated embryos were transferred to a petri-dish and were placed into the incubator and allowed to develop for 4 h post fertilization (4 hpf). This early stage yielded radiosensitive results and thus made the currently proposed method a sensitive one.

2.3. Alpha-particle irradiation of zebrafish embryos

Around 40 dechorionated embryos were transferred into the custom-made PADC-film based holder shown in Fig. 2, with 4 embryos in one hole. A viscous solution (1.5% methyl cellulose) was added into the holes to minimize the movement of the embryos. At 4 hpf, half of the embryos were irradiated for 4 min with alpha particles from a planar ^{241}Am source (with an alpha-particle energy of 5.49 MeV under vacuum and an activity of 0.1151 μCi) from the side of the PADC film. The other half of the embryos without irradiation was taken as control samples. Images of the embryos were captured immediately after the alpha-particle irradiation, which would be employed to superimpose with the images of the alpha-particle tracks revealed after chemical etching (discussed in Section 2.4 below) to obtain the alpha-particle hit positions on the embryo cells.

2.4. Number of alpha particles incident on embryo cells

After irradiating the embryos, all the embryos were labeled and separately transferred into individual containers and allowed to develop into 48 hpf. The PADC films were etched in 6.25 N NaOH at 70 °C for 3 h. Images of the PADC films with visible alpha-particle tracks were captured with a digital camera attached to a microscope with a magnification of 200×. A total of four images covering different areas of a PADC film were taken and combined to reconstruct an overall image of the PADC film at the bottom of the hole as shown in Fig. 3. The previous captured image of the embryos (described in Section 2.3) was then superimposed onto the current image using Adobe® Photoshop®. An example is shown in Fig. 4.

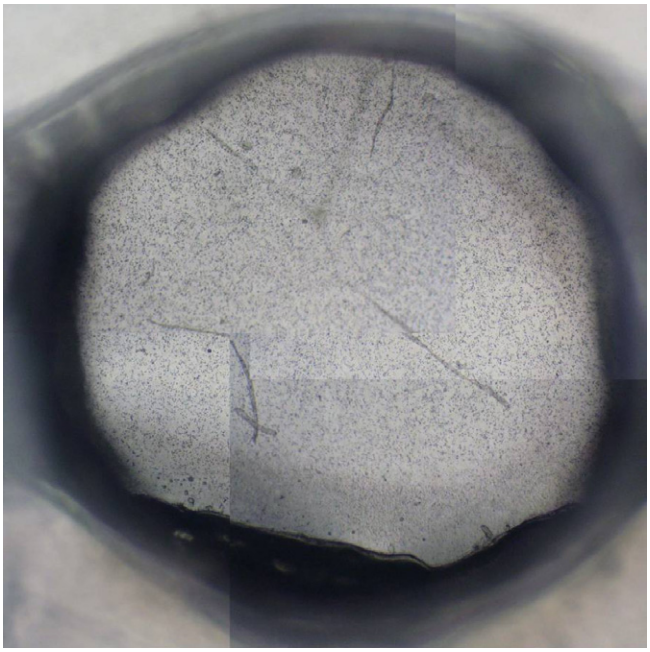

Fig. 3. An overall image of a PADC film at the bottom of the hole with visible alpha-particle tracks, reconstructed from four images covering different areas.

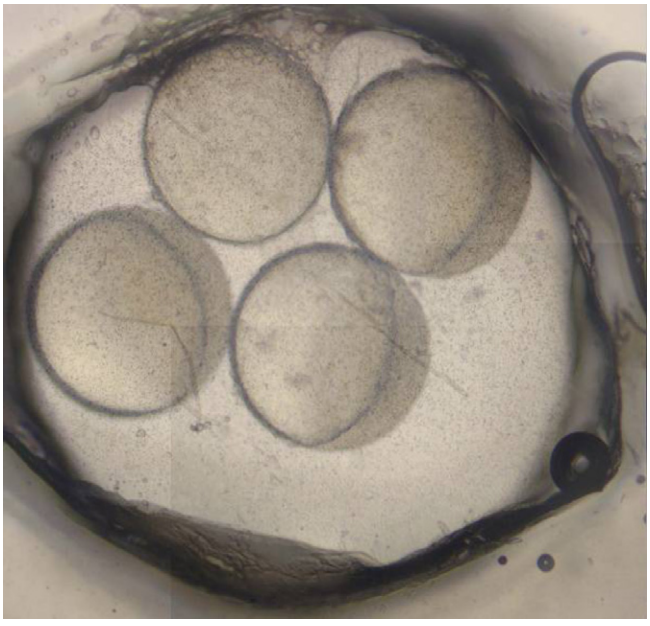

Fig. 4. Superposition of (a) the image of the PADC film at the bottom of the hole with visible alpha-particle tracks shown in Fig. 3 with (b) the image of embryos.

However, not all the alpha particles traversing the PADC film could reach the embryo because some would lose all their energies while passing through the water column. By using the outputs from the SRIM program [8], the residual energy of the 5.49 MeV alpha particles (from the source) after traversing the 16 μm PADC film was 3.49 MeV, and alpha particles with this residual energy could not further travel more than 21.6 μm in water. From this distance, we can determine the effective area containing the alpha-particle tracks which corresponded to the alpha particles that could finally reach the embryos, and the number N_α of these tracks should be counted. For most cases, the embryos could be adequately approximated by spheres. If the radius is R, the radius r_{max} of the circular effective area can be obtained by

$$r_{max} = \sqrt{R^2 - (R - 21.6)^2} \quad (1)$$

as shown in Fig. 5. In exceptional cases, the embryo can become ellipsoidal so the effective area should be an ellipse defined by a major axis and a minor axis.

After setting up the criteria of the effective area, the area was drawn on the superimposed image of the embryo cells and the alpha-particle tracks (as the one shown in Fig. 4) using the freely available image analyzing software called ImageJ (available at http://rsb.info.nih.gov/ij/). The zebrafish embryo was first outlined by a circle (or ellipse in exceptional cases) and the radius (or major and minor axes in exceptional cases) was calculated in pixels. The effective area was then plotted using the "enlarge" function in ImageJ by using negative values. This step ensured the same center for the circle representing the effective area and that containing the embryo.

In general, however, it was difficult to rotate the embryos so that the embryo cells were exactly resting on the bottom. Instead, these embryos were more likely tilted as shown in Fig. 6. In such cases, a part of the effective area may be void of embryo cells, and only the number of alpha-particle tracks (N_α) in the intersection between the effective area and the embryo cells should be counted.

Fig. 7 shows an example of the effective area drawn on a superimposed image of the embryo cells and the alpha-particle tracks using ImageJ. Here, the effective area fell entirely in the area of the embryo cells, so all the tracks within the effective area should be counted to determine the radiation dose.

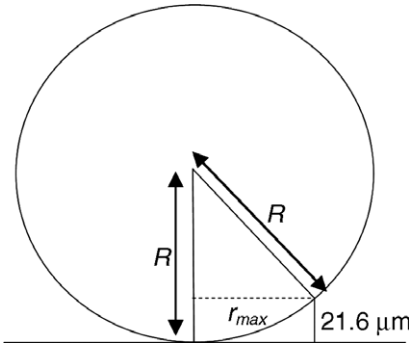

Fig. 5. Determination of the radius r_{max} of the circular effective area in the general case where the embryo is a sphere with a radius R.

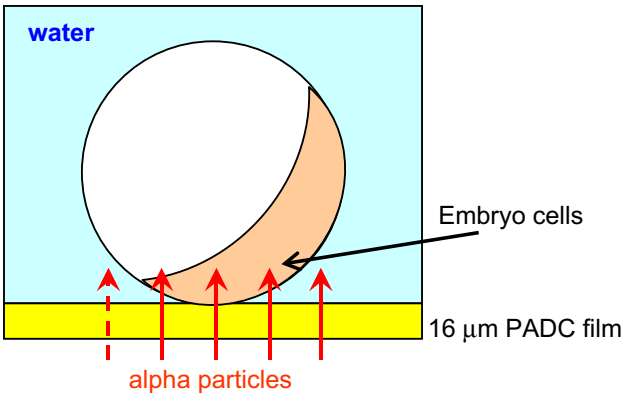

Fig. 6. Schematic diagram showing alpha-particle irradiation of a tilted zebrafish embryo. The alpha particle represented by the dashed arrow does not hit embryo cells and should therefore be discarded when calculating the radiation dose.

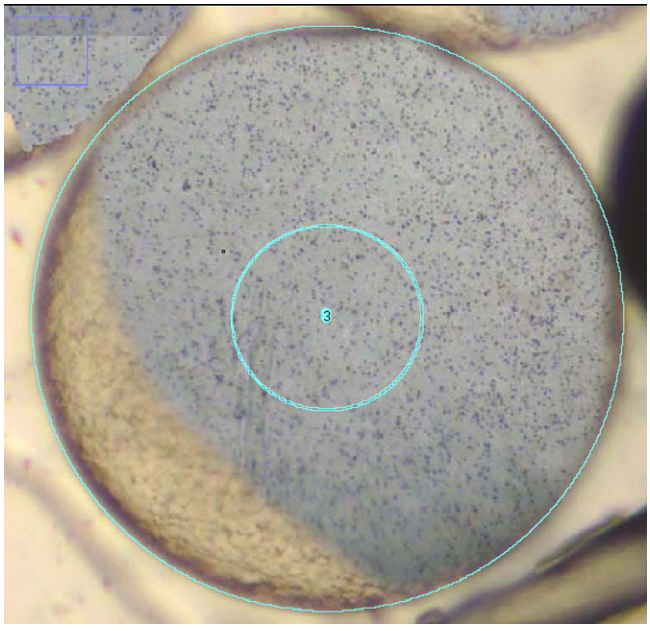

Fig. 7. Outlines of the zebrafish embryo (outer circle) and the effective area (inner circle). The central dot represents the common center of the two circles.

2.5. Alpha-particle radiation dose absorbed by embryo cells

Absorbed dose is the amount of energy absorbed by a unit mass of the material, which is the embryo cells in the present case. The number and energy of alpha particles incident on the embryo cells are required to compute the dose of alpha particles absorbed by the embryo cells.

As can be inferred from Figs. 1 and 6, the energy of the alpha particles reaching the embryo cells varied according

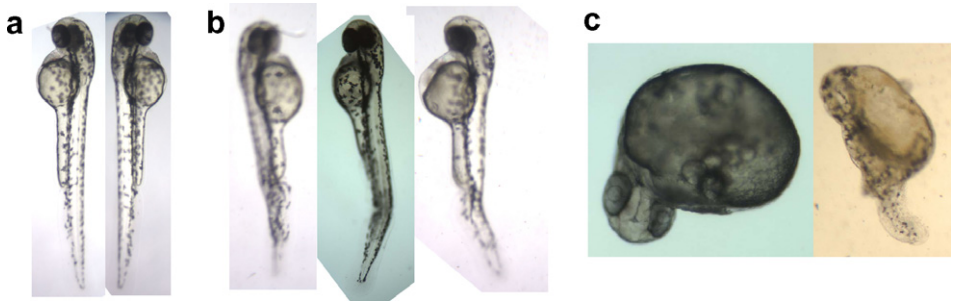

Fig. 8. Appearance of control embryos and morphologic abnormalities. (a) Control embryos; (b) abnormal embryos with malformed tails; (c) abnormal embryos with drastic malformations.

to the height of the water column they traveled. By definition, the alpha particles corresponding to the tracks on the rim of the effective area would just lose all their energies to the water column before reaching the embryo cells. To enable the determination of the alpha-particle radiation dose absorbed by embryo cells, it is therefore necessary to first determine the average energy of the alpha particles reaching the embryo cells, considering that the residual energy of the alpha particles is 3.49 MeV after passing through the PADC film. The average energy $\langle E_\alpha \rangle$ is given by

$$\langle E_\alpha \rangle = \left(\int_0^{r_{\max}} E \cdot 2\pi r \mathrm{d}r \right) \Big/ (\pi r_{\max}^2), \tag{2}$$

where E is the residual energy of an alpha particle after passing through a water column at a radial distance r from the contact point between the embryo and the PADC film, and E is determined from the outputs from the SRIM program [8]. By definition, $E = 0$ MeV for $r = r_{\max}$ which is the radius of the effective area, while $E = 3.49$ MeV for $r = 0$. Since $\langle E_\alpha \rangle$ depends on $r_{\max}$ which in turn depends on R (see Eq. (1)), $\langle E_\alpha \rangle$ has to be determined for individual embryos. The alpha-particle radiation dose absorbed by embryo cells is then given by $\langle E_\alpha \rangle \times N_\alpha / M$, where M is the mass of the embryo cells. The volume V of the embryo cells was calculated using the radius of the circular interface between the embryo cells and the yoke within the embryo, together with the radius R of the embryo, both being obtained through the microscopic images. By assuming a density ρ of 1000 kg m^{-3} for the embryo cells, the mass can be simply obtained as $M = V \times \rho$.

3. Results and discussion

Images of the embryos at 48 hpf were captured for comparisons with the control samples and for identification of morphologic abnormalities. Three sets of experiments were carried out on three separate days, with 20, 14 and 20 irradiated embryos and 17, 15 and 20 control embryos, respectively. Among the 54 irradiated embryos, 5 morphologic abnormalities were identified (2, 1 and 2 cases from the three sets), while no morphologic abnormalities were found in all the 52 control embryos. The rates of morphologic abnormalities were thus statistically significant ($p = 0.011$) for irradiated and control embryos. The images of the 5 abnormal embryos and typical images of control embryos are shown in Fig. 8. Abnormal developments ranged from malformations in the tails to morphologic abnormalities in the whole body.

The counted track numbers corresponding to these five morphologically abnormal embryos were 58 ± 8, 64 ± 8, 355 ± 19, 155 ± 13, 268 ± 17, and the absorbed doses for five embryos were 0.45 ± 0.06, 0.41 ± 0.05, 2.3 ± 0.1, 0.58 ± 0.05 and 1.1 ± 0.1 mGy (with the highest absorbed dose for all embryos as 2.3 mGy).

These values are about four orders of magnitude smaller than those employed in the literature to produce morphologic abnormalities in the zebrafish embryos. For example, Geiger et al. [1] found severe morphologic abnormalities in the embryos that survived 10-Gy irradiation of ^{137}Cs gamma rays at 4 hpf. McAleer et al. [4] found adverse effects on the morphologic development of the embryos after exposure to 4–6 Gy of 250 kVp X-rays at 4 hpf. The dose range of 0.41–2.3 mGy is equivalent to 0.21–1.2 mGy in human, which has a 2-fold larger genome [3]. However, considering the small sample size being examined, further studies may be needed to confirm the findings.

4. Conclusions

In the present work, we have demonstrated the feasibility to use an experimental setup based on PADC films to study effects of alpha particles on dechorionated zebrafish embryos. Thin PADC films with a thickness of 16 μm were fabricated and used as substrates for holding zebrafish embryos for alpha-particle irradiation. These films enabled quantification of the number and energy of alpha particles actually incident on the embryo cells and thus the calculation of the absorbed dose. Irradiation was made at 4 hpf with absorbed doses up to 2.3 mGy. Images of the embryos at 48 hpf were examined for identification of morphologic abnormalities.

Among the 54 irradiated embryos, five abnormal developments were identified while no abnormal case was

found in all the 52 control embryos. The absorbed doses corresponding to the abnormally developed embryos ranged from 0.41 to 2.3 mGy, which is equivalent to 0.21–1.2 mGy in human.

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