



Fabrication of pseudo three-dimensional PADC cell culture substrates for dosimetric studies

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ABSTRACT

Pseudo three-dimensional (3D) polyallyldiglycol carbonate (PADC) cell culture substrates were fabricated by creating micrometer-size pores on thin PADC films by alpha-particle irradiation and subsequent chemical etching. Our results showed comparatively fewer 53BP1 foci in alpha-particle irradiated as well as bystander HeLa cells cultured on pseudo 3D PADC substrates than in those cultured on two-dimensional substrates.

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1. Introduction

Polyallyldiglycol carbonate (PADC) films are commercially available as CR-39 detectors and are common solid-state nuclear track detectors (Nikezic and Yu, 2004). Thin PADC films ($\sim 20 \mu\text{m}$) were fabricated from commercially available thicker films (Chan et al. 2007a) and employed as “PADC substrates” in radiobiological experiments (Chan et al. 2007b, 2008; Law and Yu, 2009; Wong et al. 2009; Yum et al. 2007, 2009, 2010; Choi et al. 2010). Gaillard et al. (2005, 2009) also studied 2D cell cultures on thin PADC films ($\sim 10 \mu\text{m}$) fabricated by UV polymerization. PADC substrates record alpha-particle hit positions and energy (Nikezic and Yu, 2003, 2008).

Two-dimensional (2D) cell monolayers cultured on flat substrates do not recapitulate the suitable level of *in vivo* cellular responses (Lee et al. 2009). Ng et al. (2010) fabricated thick ($\sim 1000 \mu\text{m}$) three-dimensional (3D) substrates by creating micrometer-size pores on PADC films by alpha-particle irradiation and subsequent chemical etching, and observed that cells grown in the pores formed multiple directions and layers, and better mimicked *in vivo* conditions.

In the present study, we fabricated thin pseudo 3D PADC substrates, and studied the effects of alpha-particle irradiation on HeLa cells cultured on these substrates, including the radiation-induced bystander effect (RIBE). RIBE was revealed by Nagasawa and Little (1992), and refers to the phenomena where irradiated

cells affect cells nearby through released stress signal factor(s) or affect cells that have received the medium conditioned by the irradiated cells. RIBE under 3D conditions were also studied (e.g. Gerashchenko and Howell, 2004; Belyakov et al. 2005). We employed fluorescent detection of foci formation using p53 binding protein 1 (53BP1) as a marker of DNA damages (Tartier et al. 2007; Han et al. 2010a,b) to quantify the radiation effects including RIBE. 53BP1 is required for phosphorylation of numerous ataxia-telangiectasia-mutated substrates during the double-strand break (DSB) response (Abraham, 2002; Mochan et al. 2004). 53BP1 was shown to relocalize into foci shortly after irradiation, with the number of foci closely paralleling the number of DSBs (Schultz et al. 2000).

2. Methodology

2.1. Cell culture and cell thickness measurement

Handling of HeLa cells was described in detail by Ng et al. (2010). The cells were trypsinized, adjusted to 3.5×10^5 cells in a 35 mm dish and plated out on the raw $2 \times 2 \text{ cm}^2$ (1 mm thick) PADC substrates for 1 d. The HeLa cell thickness was measured using a digital holographic microscope (Lyncée tec-DHM T1000).

2.2. Fabrication of 2D and 3D substrates

We prepared thin PADC films with a size of $1.5 \times 1.5 \text{ cm}^2$ from 100 μm thick CR-39 detectors (Page Mouldings) by chemical

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etching in NaOH/ethanol (Chan et al. 2007a). Pseudo 3D substrates were fabricated by irradiation of thin PADC films with 5 MeV alpha particles, which were then etched in 6.25 N aqueous NaOH at 70 °C for 30 h, and then further etched for 5 min in 1 N NaOH/ethanol at 40 °C (Ho et al. 2003; Li et al. 2006). The final bulk thickness of the substrate (where there were no pores) was 35 μm , while the minimum thickness (measured from the bottom of the pores) was 17 μm , which were determined from their lateral images (Dörschel et al. 1997). Flat 2D PADC substrates with a thickness of 17 μm were also prepared by etching with 1 N NaOH/ethanol at 40 °C.

2.3. Experimental setup

The cells were trypsinized, adjusted to 1.2×10^5 cell/ml cells for a total of 120 μl medium and plated out on PADC substrates for 1 d. The cells were then irradiated as shown in Fig. 1 for 20 min with 5 MeV alpha particles having traveled a distance of 5 mm in air from an ^{241}Am alpha-particle source (main alpha-particle energy of 5.48 MeV) with incident angles between 70.8° and 90° (normal incidence) controlled by a collimator.

2.4. Flow cytometric analysis

2D/3D PADC films (with thickness of 1 mm and area $2 \times 2 \text{ cm}^2$) were sterilized by submerging into 75% (v/v) ethyl alcohol for 1 h and then immersed in MilliQ water for 1 h before cell culture. Concentrations were adjusted to 5×10^6 cells before they were plate out in a 95 mm dish (five 2D and 3D films for each dish) for 1 d, with a total of three dishes. Cells attached on 2D/3D substrates were rinsed three times with PBS before being removed by trypsin-EDTA solution for 5 min, which were then collected by washing with PBS. After centrifugation and supernatant decant, the cell pellet was resuspended in PBS, and these procedures were repeated one more time. The resuspended cells were stored overnight in 70% ethanol at -20 °C. After that, the cells were centrifuged, and the collected cell pellet was resuspended, and these procedures were repeated one more time before the cells were stained with propidium iodide (PI) for 15 min. The cell cycle distribution was then determined by flow cytometry.

2.5. Detection of 53BP1 foci and hit positions

Immunochemical staining of cells was performed largely following the procedures as described by Aten et al. (2004) with

some modifications. Optical and fluorescent images of cells were captured by a fluorescent microscope (Nikon ECLIPSE 80i). The cells on PADC substrates were then removed by running water before the substrates were etched in a 14 N KOH solution at 37 °C for 3 h to reveal visible tracks corresponding to alpha particles striking the substrates. The epoxy remained intact at this etching temperature (Chan et al. 2008). Optical images of these tracks were captured, which were then superimposed onto images of cells described above, from which the positions of alpha-particle hits on cells could be determined. Due to the short irradiation time, very few cells were hit by more than one alpha particle.

3. Results and discussion

The thickness of live HeLa cells seeded on raw PADC films were measured using a digital holographic microscope as $3.77 \pm 0.87 \mu\text{m}$. In the present work, the incident angles of alpha particles on the cultured cells ranged from 70.8 to 90°. For a 2D film, a 5 MeV alpha-particle traveled 17 μm in the film, had its energy reduced to $2.54 \pm 0.09 \text{ MeV}$ before hitting a cell cultured on the film, and ultimately deposited an energy of $0.579 \pm 0.016 \text{ MeV}$ in the cell. For a 3D PADC film, a 5 MeV alpha particle had its energy reduced to an average of $1.71 \pm 0.19 \text{ MeV}$ before hitting a cell cultured on the film, and ultimately deposited an energy of $0.723 \pm 0.009 \text{ MeV}$ in the cell.

Through staining of trypsinized cells by Hoechst 33342 for 4 min, the radius and volume of the cell nucleus were calculated as $5.98 \pm 0.39 \mu\text{m}$ and $895 \pm 176 \mu\text{m}^3$, respectively. On the other hand, the HeLa cell volume was found as $2600 \mu\text{m}^3$ by Zhao et al. (2008). With these volumes, the average absorbed doses in the cell nucleus or the whole cell for a single alpha-particle traversal were: 0.104 ± 0.003 and $0.0356 \pm 0.0099 \text{ Gy}$, respectively, for the cells cultured on a 2D PADC film; and 0.129 ± 0.002 and $0.0445 \pm 0.0006 \text{ Gy}$, respectively, for the cells cultured on a 3D PADC film.

The fluorescent images of the cell nuclei and the 53BP1 foci together with the epoxy were first captured and combined as shown in Fig. 2(a). Epoxy was auto-fluorescent. After etching the substrate in KOH solution, the transmitted bright field images of the etched tracks together with the epoxy were captured and combined as shown in Fig. 2(b). The transmitted bright field images and the fluorescent images were then superimposed by aligning the positions of epoxy. The space resolution was estimated to be better than 1 μm . The superimposed images gave information on

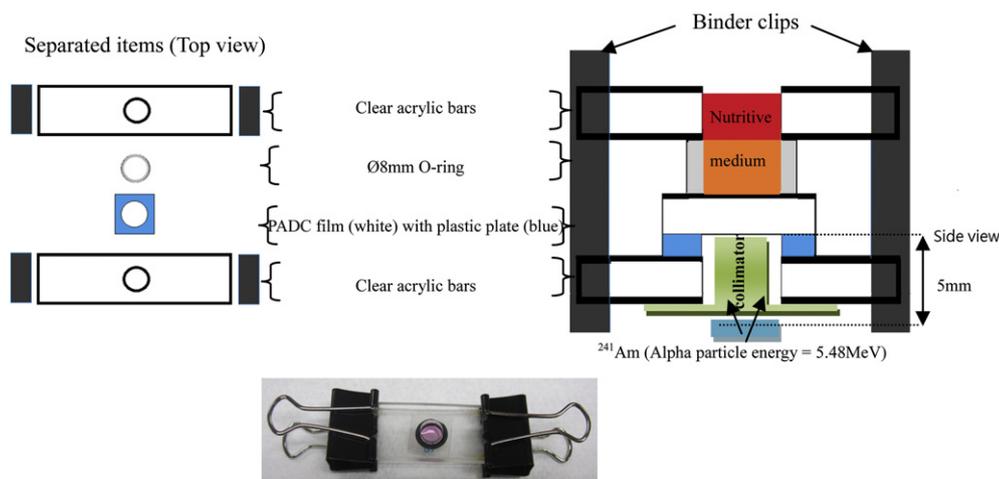


Fig. 1. Cell culture setup for PADC substrates. A $1.5 \times 1.5 \text{ cm}^2$ PADC film was fixed on a plastic plate (with a 7 mm hole at the center), and sandwiched by O-rings and clear acrylic bars (with dimension of 5.5 cm (L) \times 2 cm (W) \times 2 cm (H), and with a 7 mm hole drilled at the center) to prevent leakage of nutritive medium. The two ends of the bars were fixed by binder clips. Bottom: photo showing the setup with cell culture medium added.

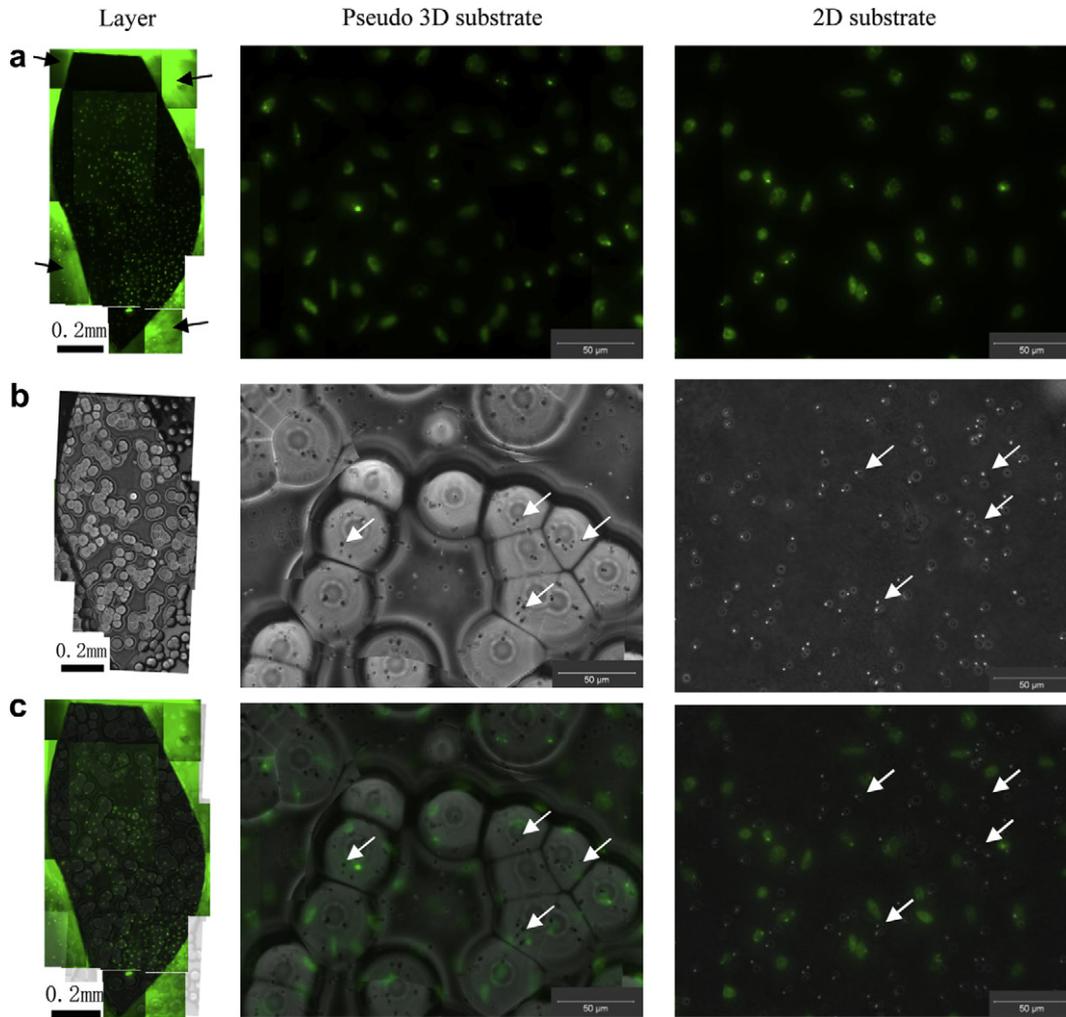


Fig. 2. (a) Fluorescence images of 53BP1 foci, with arrows showing the epoxy; (b) optical images of tracks (alpha particles passing through films), with arrows showing the positions of alpha-particle tracks after etching in 14 N KOH; (c) superimposed images of those in (a) and (b) to determine the positions of alpha-particle hits on cell nuclei, with arrows showing the positions of alpha-particle tracks after etching in 14 N KOH.

whether the nuclei were crossed by alpha particles. Fig. 2(c) shows the superposition of images in (a) and (b).

Fig. 3 shows the number of 53BP1 foci on controls (unirradiated cells), irradiated cells (one alpha-particle hit only) and bystander cells (unirradiated cells partnered with the irradiated cells) cultured on pseudo 3D and 2D substrates. We had the following observations.

3.1. Comparison between irradiated cells and control samples

- (a) irradiated cells cultured on 2D substrates had marginally fewer and more numbers, respectively, for 0 focus and 1–3 foci when compared with control samples ($p < 0.08, n = 3$);
- (b) irradiated cells cultured on pseudo 3D substrates did not have significant difference in the numbers for 0 focus and 1–3 foci when compared with control samples.

3.2. Comparison between bystander cells and control samples

- (a) bystander cells cultured on 2D substrates had significantly more and fewer numbers, respectively, for 0 focus and 1–3 foci when compared with control samples ($p < 0.05, n = 3$);

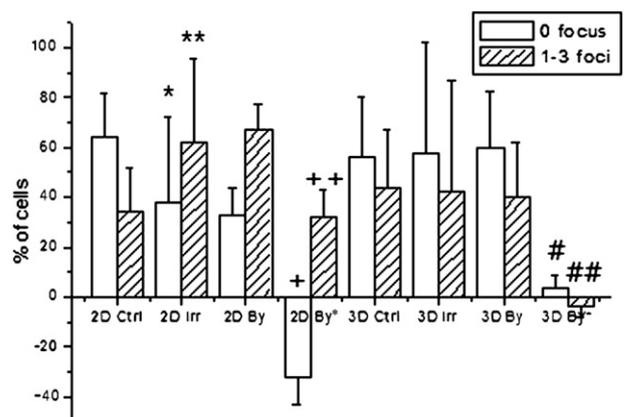


Fig. 3. Number of 53BP1 foci on control (Ctrl), irradiated (Irr) and bystander cells (By) cultured on 2D and pseudo 3D substrates. (“By*” = “By” – “Ctrl”). Error bars represent one standard deviations. For both 0 focus and (1–3) foci, results for “2D Ctrl” and “2D Irr” are marginally different (* and ** represent $p < 0.08, n = 3$); results for “2D Ctrl” and “2D By*” are significantly different (+ and ++ represent $p < 0.05, n = 3$); results for “2D By*” and “3D By*” are significantly different (# and ## represent $p < 0.05, n = 3$).

(b) bystander cells cultured on pseudo 3D substrates did not have significant difference in the numbers for 0 focus and 1–3 foci when compared with control samples.

3.3. Comparison between cells cultured on pseudo 3D substrates and 2D substrates

(a) irradiated cells cultured on 2D substrates did not have significant difference in the numbers for 0 focus and 1–3 foci when compared with irradiated cells cultured on pseudo 3D substrates, no matter whether the numbers had been corrected for the numbers in their controls;

(b) bystander cells cultured on 2D substrates had significantly more and fewer numbers, respectively, for 0 focus and 1–3 foci when compared with bystander cells cultured on pseudo 3D substrates, no matter whether the numbers had been corrected for the numbers in their controls ($p < 0.05$, $n = 3$).

Both irradiated and bystander cells cultured on 3D substrates had smaller response to radiation in terms of the number of 53BP1 foci, and thus might be more resistant to radiation damages and/or have speedier DNA repair. Possible reasons included differences in cell cycle distribution, cell-shape mediated changes in (repair-related) gene expression, and alterations in chromatin packaging which affected DNA repair (Feder-Mengus et al. 2008; Gorlach et al. 1994; Olive and Durand, 1994, and Sowa et al. 2010).

Burdak-Rothkamm et al. (2007) indicated that cells in the S phase might be more vulnerable to bystander effects. Luo and Yang (2004) found a lower proportion of S phase in 3D compared with 2D hybridoma cell culture. The distribution of G0/G1, G2/M and S phases of HeLa cells seeded on our 2D and 3D substrates were found as 61 ± 3 , 12 ± 2 and $28 \pm 2\%$ as well as 62 ± 2 , 12 ± 2 and $27 \pm 2\%$, respectively. These results showed no changes in the cell-phase distribution of cells cultured on 2D and 3D substrates, in contrast to that obtained by Luo and Yang (2004).

Since the absorbed energies and thus the absorbed doses are (slightly) higher for cells cultured on 3D substrates, one would not have expected fewer foci for them if the 3D substrates are not providing a microenvironment different from that being provided by the 2D substrates. Kashino et al. (2010) found an increase in 53BP1 foci per cell when the X-ray dose was increased from 1 to 2 Gy. However, fewer foci were actually observed, which supported that 3D substrates were providing a different microenvironment.

There are many differences in the biological functions between tumor cells in 2D and 3D cultures, such as metabolic profile modification and increase in lactic acid production (Fischer et al. 2007), decrease in proliferation capacity (Ghosh et al. 2005), decrease of HSP70 expression (Dangles-Marie et al. 2003), change in polarity and shape of cells (Yamada and Cukierman, 2007), gene expression profiles modification (Ghosh et al. 2005), sensitivity to IFN, drugs, irradiation or apoptosis induced by death receptor ligation (Santini et al. 2004). As such, different response of cells cultured on 2D and 3D substrates to alpha-particle irradiation in terms of the number of 53BP1 foci was not unexpected.

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