A new potential radiosensitizer: ammonium persulfate modified WCNTs

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ABSTRACT. Radiotherapy plays a very important role in cancer treatment. Radiosensitizers have been widely used to enhance the radiosensitivity of cancer cells at given radiations. Here we fabricate multi-walled carbon nanotubes with ammonium persulfate, and get very short samples with 30-50 nanometer length. Cell viability assay show that *f*-WCNTs induce cell death significantly. We hypothesize that free radicals originated from hydroxyl and carbonyl groups on the surface of *f*-WCNTs lead cell damage.

As is well known, radiotherapy plays a very important role in cancer treatment, and the cure effect strictly relied on the intrinsic radiosensitivity of target cancer cells. Besides of very fewer sensitive examples, such as atxia telangiectasia [1] cells, most malignancy cells are of moderate radiosensitivity, even some of them are radioresistant [2]. In order to achieve better treatment effect, radiosensitizers [2-8] are frequently used in vivo or in vitro performances. Radiosensitizer introduce a higher radiotherapy effect mainly through capturing electrons in target volume under irradiations to prevent them from recombination with radiation-injured samples [3].

In recent years, ability of carbon nanotubes [9-13] to transport across cell membranes has received significant interest. Since the carbon nanotubes appear to be non toxic for the cell lines, they can be considered as a new tool to deliver poorly penetrating drug, peptides, peptidomimetics, protein or small organic probe molecules into cancerous cells [9-14], though, very little sight is thrower on carbon nanotubes itself in radiobiology. Due to its very unique structure characteristic and subsequent physical and chemical properties, simple carbon nanotubes would display unanticipated radiobiological function.

In present study, we describe preparation of multi-walled carbon nanotubes modified with ammonium persulfate. The modified carbon nanotubes are not only able to readily enter cervix cancer cells (HeLa cells), but also to effectively kill the cancerous cells under low dose of gamma radiations. Furthermore, both *p*-WCATs and *f*-MWCNTs themselves appear non-toxic for HeLa cells. Upon increasing concentration of *f*-MWCNTs in growth medium when exposed to radiation, there is a significant decrease of the cell survivals, exhibiting dose-dependent toxicity.

The Stable aqueous suspensions of purified, shortened, and functionalized carboxylic acid nanotubes is obtained by oxidation and polishing [15-17] of laser-ablated raw multi-walled carbon nanotubes (purchased from Shenzhen Nanotech Port Co. Ltd.). In order to eliminate metal catalysts, the carbon nanotubes was afterward dispersed in 6 M HCl under ultrasonic agitation, washed with sodium hydroxide solution and deionized water to neutrality and dried. The purified MWCNTs is suspended in a 3:1 mixture of concentrated H_2SO_4/HNO_3 in 500 mL flask and sonicated in a water bath for 24 h at 35–40 °C. Centrifugation (7000 rpm, 5 min) removed larger unreacted impurities from the resultant suspension to afford a stable suspension of MWCNTs. The cut nanotubes is recovered by filtration with polytetrafluoroethylene membrane with a pore size of 0.22 μm and rinsed with deionized water. Subsequently, they are then further polished by suspension in a 4:1 mixture of concentrated H_2SO_4/30% aqueous H_2O_2 and stirring at 70 °C for 30 min. After filtering and washing again, the resulting MWCNTs can be relatively dispersed in water, this resulting material was regarded as *p*-WCNTs. Then, 50mg *p*-WCNTs are added into 10ml deionized water, sonicated for 10 minutes. Thereafter, ammonium persulfate is added into upper solution with the terminated concentration of 0.5M, and stirring for 48 hours at 50 °C. The rinse and filtration process is repeated as described above, at the end, we get *f*-WCNTs.

All of the tested materials are conducted in sterile PBS, and kept the cells from contaminant. Hela cells purchased from ATCC (America) are cultured in DMEM (Invitrogen-Gibco), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells are incubated in a humidified atmosphere of 5% CO_2–95% air at 37 °C in a 75 cm^2 flask, and supplied with fresh medium every three days.

Incubation of cells is done by adding PBS of the *p*-MWCNTs, *f*–MWCNTs into the culture medium

(concentration ranges from 0 to 50 μ g/mL in the culture medium), and the incubation duration is always 4 h. After incubation, the cells are washed with PBS and resuspended in fresh culture medium.

All confocal images are taken immediately after the incubation and washing steps except for the radiation experiment and cell viability assay. The cell suspension (20 μL) is dropped onto a glass-bottomed dish and image by a Zeiss LSM 510 confocal microscopy.

Cells are randomly divided into three groups: two are adding *p*-WCNTs, *f*-WCNTs into cell culture medium, respectively; and another group is regarded as control with no other materials added into. Cells are irradiated with ^{60}Co gamma rays with dosage range from 0 to 6 Grays (Dose rate was 1Gray per minute).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) is used to determine cell survival in a quantitative colorimetric assay. Various dehydrogenase enzymes in active mitochondria, forming a blue-colored insoluble product, cleave its tetrazolium ring formazan. The HeLa cells are incubated with MTT (5 mg/mL) added to the culture medium for 4 h at 37 °C. The medium is then aspirated and the formazan product is dissolved in dimethyl sulfoxide and quantified spectrophotometrically at 490 nm with control of 650 nm. The results are expressed as a percentage of control culture viability.

To examine the dispersion state of the MWCNTs in water solutions, one drop of the water solution with MWCNTs (1 mg/mL) is dropped on a silicon-oxide substrate for SEM analysis, the image reveal mostly short (about 100 nm–1 μm) *p*-MWCNTs and 30-100nm *f*-WCNTs with diameters in the range of 20–60 nm corresponding to mostly isolated individual MWCNTs (Fig. 1a, 1b). No significant amount of particles is observed on the substrate, suggesting good purity of short MWCNTs in water solution. In pure water, the suspension of the black MWCNTs is stable for extending periods of time and does not agglomerate, which is likely relative to amount of shortened MWCNTs, which is not contradictory with

that polishing makes carbon nanotubes flocculate more in the reported literature [15-16]. In physiological buffer solutions containing ~0.2 M salt, the suspension of MWCNTs is less stable and start to aggregate after 2 h. We use Zetasizer 3000HS (Malvern Instruments Ltd, UK) achieve the zeta potential of *p*- and *f*-WCNTs,which is 42mv and 54mv, respectively. This indicates the more negative electronical groups existed on the surface of *f*-WCNTs than that of *p*-WCNTs (Fig. 1c, 1d). Furthermore, IR analysis showe that both carbonyl and hydroxyl peak number of *f*-WCNTs are higher than that of *p*-WCNTs (Fig. 1e, 1f). These groups will change into free radicals in aqueous atmosphere when exposed to ion radiation, and consequently induce cell damage [18].

To visualize the interaction of *p*-MWCNTs, and *f*–MWCNTs with cells, the HeLa cells are incubated with these nanomaterials (50 μ g/mL) for 4 h at 37 °C. After the cells are carefully washed with PBS and digested by steapsin, and a fresh culture medium is added. Subsequently, the cells are observed directly in glass-bottomed dishes under confocal microscope. Fig. 2a, 2b show the cells with dark cytoplasm and apparent nuclei free of MWCNTs, indicative of intracellular and not extracellular localization of the MWCNTs.

To further verify the celluar uptake of both *p*-MWCNTs and *f*–MWCNTs, negatively charged single-stranded DNA (ssDNA) labeled with 6-carboxy fluorescein is bound to the sidewall of MWCNTs via hydrophobic interaction. The dispersive complexes of the MWCNTs/ssDNA are dialyzed for 2 h with constant stirring in PBS to eliminate free ssDNA. The confocal images indicate that the stable complexes of MWCNTs/ssDNA in PBS reveal green fluorescence, which confirms the ssDNA can be strongly absorbed on sidewall of MWCNTs. We then study the interactions of these resulting complexes with the HeLa cells. Fig. 2c show that these complexes appear to uniformly accumulate in the cytoplasm in the HeLa cells after internalization, and not adhering to the cells extracellularly, which further

confirm the intracellular uptake of the complexes. As negative-control experiment, the cells are incubated with a solution that contained only fluorescently labeled ssDNA. No fluorescence of the cells is detected, which mean that the MWCNTs can traverse the cell membranes and transport adsorbed ssDNA into the cells.

Having discovered the ability of *p*-MWCNTs and *f*–MWCNTs to enter Hela cells, we further seek to examine their potential toxicity due to the delivery of these nanomaterials into cells. Toward this end, the *p*- and *f*-MWCNTs are used as the toxic control assay, and the cell survival is conducted by the observed MTT experiments. The cells are separately incubated with pure DMEM culture medium (contain 10% BSA), *p*-MWCNTs and *f*–MWCNTs for 4 h, and rinsed with sterile PBS. Subsequently, these cells are transferred into 96 bores board. After 48 h incubation, the MTT is added into the each bore in the board. After 5 h, the clear solution into each bore is obtained by centrifugation, and respectively transferred into the other 96 bores board. As show in Fig. 3, along with increasing the concentration of *p*- and *f*–MWCNTs in the incubation solution (concentration ranges from 0 to 50 μg/mL in the culture medium) survival rate of the HeLa cells is stable at about 95%, which is similar with that of control group, no significant changes of cell survival are observed. These results indicate that p- and *f*-MWCNTs themselves ar little toxic to HeLa cells after the cells are incubated even in highly concentrated solutions.

The *p*- and *f*-MWCNTs are conducted under high pressure and high temperature, and that all of the tested materials are conducted in sterile PBS, and kept the cells from contaminant. Thus, the observed endotoxin is nearly nonexistent when these cells are exposed to the *p*- and *f*-MWCNTs in our experiment. Since these materials are immediately dispersed by sonication within a few seconds, the cell survivals are not likely influenced by the residual transition metal contaminants into the MWCNTs.

When absent of radiations, *p*- and *f*-MWCNTs have been verified no toxicity to HeLa cells, regardless of its concentration. After irradiation, cell viability is observed decreased along with the increase of radiation dosage and material concentration. For *f*-WCNTs group, there is a sharp decrease, compared with *p*-WCNTs and control group; and the cell survival of *p*-WCNTs group is low than that of control group. Cell viability is similar with our previous report [19], under 6 Gy gamma radiation, there is a 40 percent cell survive with no WCNTs addition, and the cell survival curves are linear-quadratic, which is consistent with the classical low linear energy transfer radiation model. After further 72 hours incubation in fresh DMEM culture medium, there is an obvious repair effect in control group (see Fig.4d), and this repair effect is not observed in p- and *f*-WCNTs groups (see Fig.4e, f), it indicates that the WCNTs induces radiation damage is lethal attack to cells.

The ammonium persulfate oxidized and polished areas of the MWCNTs contain large amount of negatively charged carbonyl, hydroxyl groups along the sidewalls, such groups are of feasibility to be radioanalyzed to become hydroxylic and other free radicals under radiation. These free radicals are of the direct killing ability to cells, possibly including cell membrane, plasma, nuclei and other substructures. The unoxidized areas of the MWCNTs may still afford regions of appreciable hydrophobicity, and the DNA molecules can be strongly adsorbed on surface of MWCNTs presumably via hydrophobic interaction with sidewalls of the carbon nanotubes [13-14]. The MWCNTs can nonspecifically associate with hydrophobic regions of the cell surface and internalize by endocytosis [20-21]. Therefore, when the ssDNA was complexed to the MWCNTs they readily entered the cancerous cells.

In conclusion, the ammonium persulfate functionalized MWCNTs are soluble, and can enter cancer cells, exhibiting dose-dependent cytotoxicity under radiation. Thus, because of the unique

biocompatibility, and bioabsorption of the MWCNTs, it provids the basis for new classes of materials for enhancing the cancer cell radiosensitivity, though the targeting nature of WCNTs is not discussed in present study. This indicates that WCNTs is of great importance in developing a new kind of radiosensitizer.

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FIGURE CAPTIONS:

Figure 1. Figure caption. Physical and chemical properties of *p*-WCNTs and *f*-WCNTs, (a) SEM image of *p*-WCNTs, (b) SEM image of *f*-WCNTs, (c) Zeta potential of *p*-WCNTs, (e) Zeta potential of *f*-WCNTs, (e) IR spectrum diagram of *p*-WCNTs and *f*-WCNTs

Figure 2. Figure caption. Confocal images of HeLa cells after incubation in solution of *p*-MWCNTs and *f*–MWCNTs: (a) after incubation in *p*-MWCNTs, (b) after incubation in *f*–MWCNTs, (c) after incubation in the MWCNTs/ssDNA complexes at 37 °C.

Figure 3. Figure caption. Confocal images of HeLa cells after incubation in solution of *p*-MWCNTs and *f*–MWCNTs: (a) after incubation in pure DMEM culture medium with 10% fetal bovine serum, (b) after incubation in *p*–MWCNTs, (c) after incubation in *f*–MWCNTs, (d) cell viability showed by OD value. $P<0.05$

Figure 4. Figure caption. MTT analysis of HeLa cell survival ratio after gamma radiation. (a) 4Gy radiation; (b) 4Gy+25μg *p*-WCNTs; (c) 4Gy+25μg *f*-WCNTs; (d) 4Gy irradiation and 72 hours repair; (e) 4Gy+25μg *p*-WCNTs and 72 hours repair; (f) 4Gy+25μg *f*-WCNTs and 72 hours repair; (g) Cell violability curves. $p<0.05$

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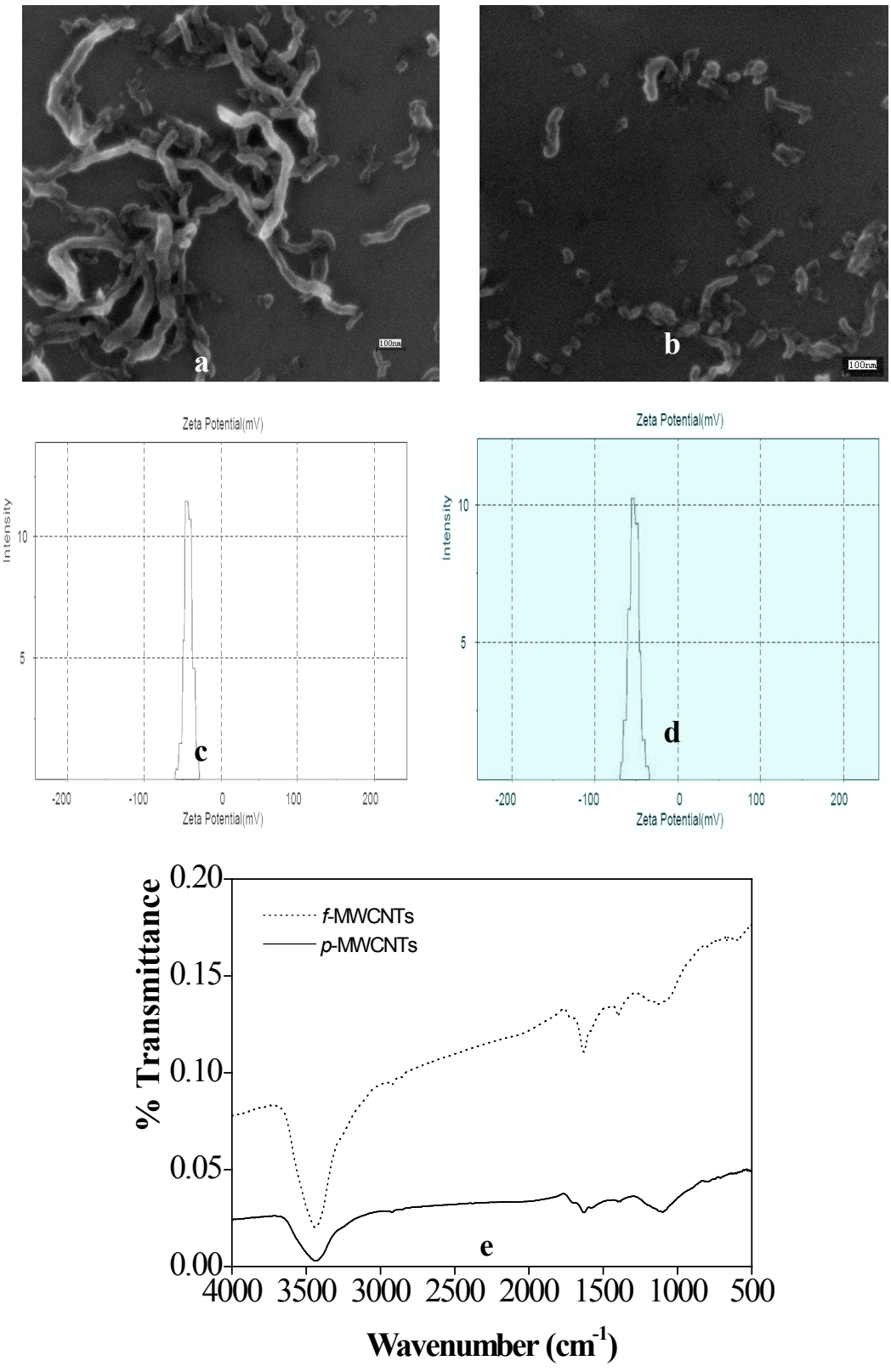
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b
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Intensity
10
5
c
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-100
0
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200
Zeta Potential(mV)
Zeta Potential(mV)
Intensity
10
5
d
-200
-100
0
100
200
Zeta Potential(mV)
% Transmittance
0.20
0.15
0.10
0.05
0.00
f-MWCNTs
p-MWCNTs
e
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

Figure 1

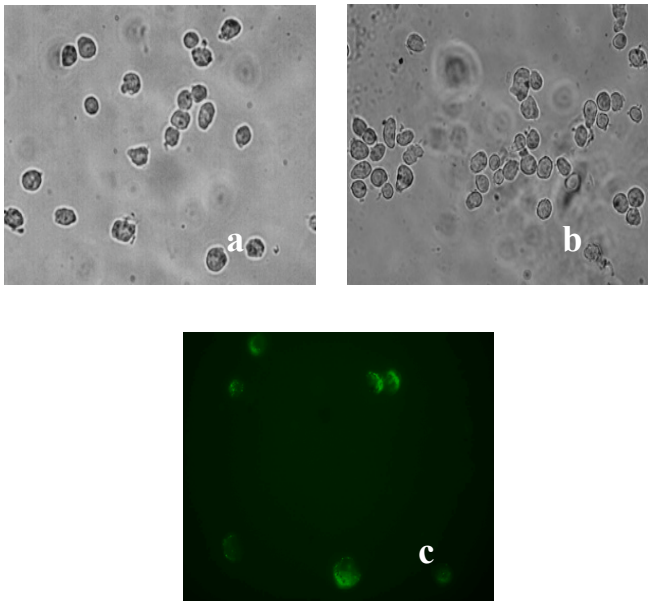
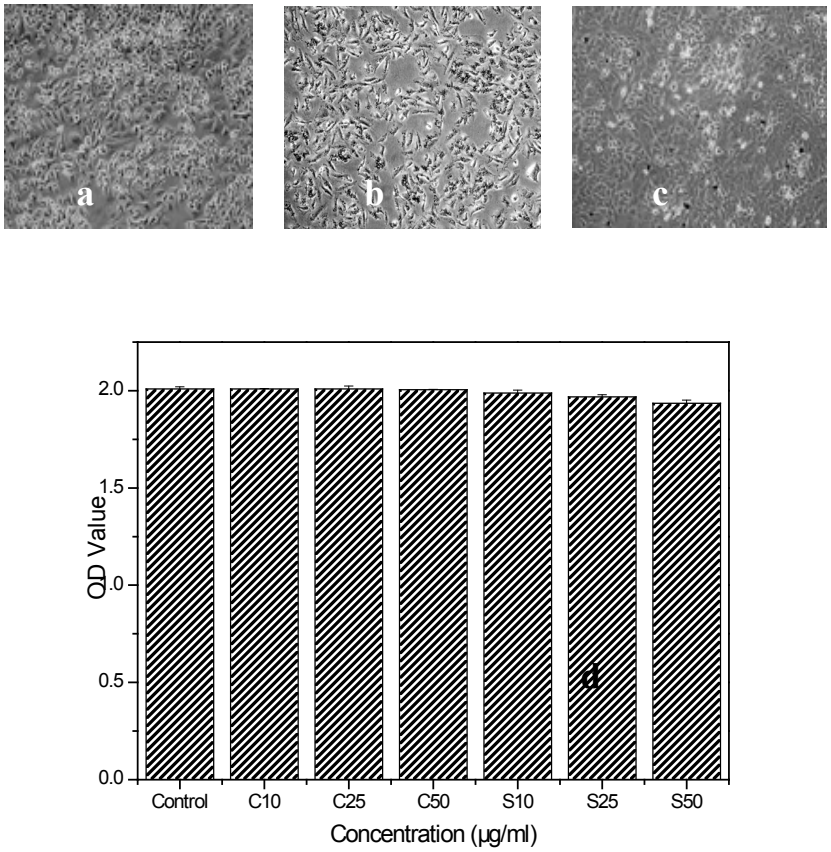


Figure 2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C25
C50
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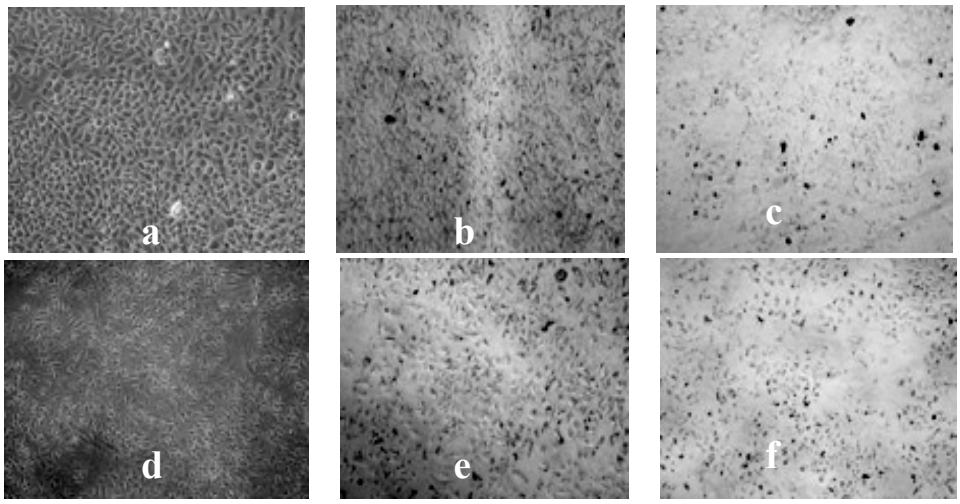
S25
S50
Concentration (μg/ml)

Figure 3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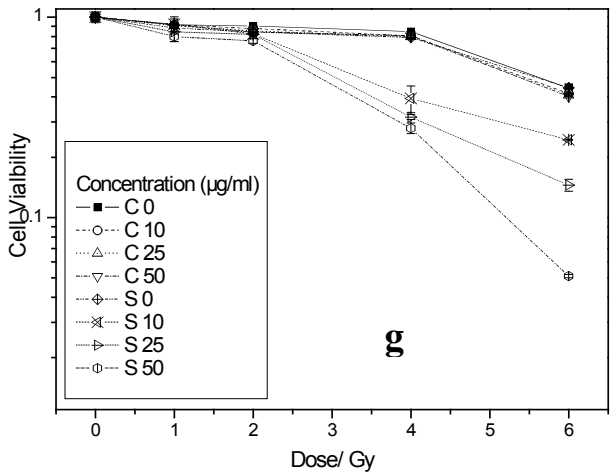
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Cell Vialbility
Concentration (μg/ml)
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C 10
C 25
C 50
S 0
S 10
S 25
S 50
g
0
1
2
3
4
5
6
Dose/ Gy

Figure 4