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The cost-efficient techniques to evaluate the age-based structural heterogeneity and morphological analysis of multicellular tumor spheroids

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Abstract: Tumor tissues being resistant to penetration of drug molecules causing hindrance to cancer therapy. Multicellular tumor spheroids (MCTSs) are used as an *in-vitro* tumor model and its detailed exploration is the need of the day. MCTSs were generated by liquid overlay technique, their penta-physical characteristics including diameter, cell number, volume per cell, viability status and estimated shell of viable and core of dead cells, were determined via confocal microscopy and haemocytometry. The growth of spheroids was linear over the first week but declined in the 2nd week. Compaction of spheroids occurs from day 3 to day 7, with the mature spheroids having a lower amount of extra cellular space compared to intracellular volume. Age-oriented growth of MCTSs provides a rationale to predict less rapid penetration as spheroids get older and could be correlated with *in-vivo* tumors to predict pharmaceutical and therapeutic intervention.

Keywords: Interconversion of spheroid parameters; spheroid composition; compaction of spheroids; disaggregation of spheroids; confocal microscopy; haemocytometry.

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INTRODUCTION

For better investigation of cancer progression, effective drug delivery to the site of action and to bridge the gap between *in-vitro* cancer cell suspension with clinical findings, the multi cellular tumor spheroids (MCTSs) are the most appropriate intermediate *in-vitro* experimental model to corelate *in-vitro* and *in-vivo* research studies (Han *et al*., 2021; Mitrakas *et al*., 2023; Olofsson *et al*., 2021; Xie *et al*., 2023). *In vivo* or *in vitro* exposure to carcinogens may transform normal animal cells into cancer cells (Stiles *et al*., 1976). MCTSs imitate in-vitro micro metastasis and the avascular stage of real tumor development, presenting a good situation to study tumor biology and to assess the effects of various therapeutic approaches (Guirado *et al*., 2003; Sant & Johnston, 2017). The three-dimensional multicellular tumor spheroids (3-D MCTSs) acquire similarities with *in-vivo* tumor tissues with respect to structural characterization. MCTSs have been used as an in vitro tumor tissue model to mimic *in vivo* tumor studies (Kim, 2005; Kunz-Schughart, 1999; Mitrakas *et al*., 2023). Therefore, the selection of cell lines, proficiency in cell culture and spheroid generation are the core prerequisites for tumor tissue research. Penetration of drug molecules or cargo-drug conjugates varies from cell line to cell line (Hällbrink *et al*., 2001; Mann & Frankel, 1991; Tunnemann *et al*., 2006) and selection of a particular cell line is essential according to the aims of biomedical research projects. Skills in cell culture and spheroid generation enhance insight into research outcomes. It has been reported that researchers must characterize and optimize the growth conditions for spheroid cells, which are selected for investigation (Sutherland & Durand, 1984). HT-29 (a human colon adenocarcinoma cell line) possesses a good experimental system for the study of factors concerned with the differentiation of epithelial cells. The cytoskeleton adjusts accordingly as the cell changes its shape and environment or when it divides (Cohen *et al*., 1999). While working with cancer cells, principles have been described, such as facilities in the lab, disposal methods, accessories and cell lines to work within the laboratory efficiently (Cree, 2011).

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MCTSs serves as morphological and biochemical in-vivo tumors model for cancer research (KUNZ-Schughart *et al*., 1998). It exhibits similarities with tumor tissues in terms of composition, microenvironment, cellular behaviors, oxygen and pH gradients as well as expression of anoxic, quiescent, hypoxic and necrotic cells subpopulations. Old-age spheroids with size greater than 500µm, hypoxic and necrotic core expressed along with a shell of viable cells (Casciari *et al*., 1992; Kostarelos *et al*., 2005). Hypoxic cells, being resistant to penetration of molecules, is a target for cancer therapy (Wilson & Hay, 2011).

Numerous methods developed for multicellular tumor generation have been developed including Pellet Culture, Rotating Wall Vessel, Micro fluidics, Magnetic Levitation, Hanging Drop, Spinner Culture and Liquid Overlay (Mitrakas *et al*., 2023; Ryu *et al*., 2019). The capabilities for spheroid formation of different cell lines focuses the need for standardization of spheroid generation protocols for better comparison (Froehlich *et al*., 2016). Moreover, among MCTS formation methods, appropriate method should be chosen carefully based on the desired application. The current trend in spheroids exploitation is treatment with live and dead stains and hypoxia probes for the quantification of sub-populations using confocal laser scanning microscope (CLSM). But, inability of the performance of confocal microscopy to detect specimen depth is one of obstacles for accurate measurement (Diaspro *et al*., 2002). Various characterization techniques have been conducted to explore spheroids for its suitability to exploit it for cancer and biomedical research including drug delivery strategies (Han *et al*., 2021; Hulo *et al*., 2024; Maitra Roy *et al*., 2023; Olofsson *et al*., 2021; Tartagni *et al*., 2023).

Spheroidal age has been considered as a critical parameter which impacts drug diffusivity in 3D-MCTSs models (Eilenberger *et al*., 2019; Ur Rahman *et al*., 2020). Development of such a technique that explores characteristic features of spheroids, such as the correlation of cell number with the age of spheroids, viability status of cells, extracellular volume per cell and distinct cellular layer in spheroids, could be a research-oriented therapeutic approach in the field of cancer research. To study spheroid composition and growth characteristics, we addressed spheroid geometry and explored many interesting observations/ features for the characterization of spheroids. Changes in these features along with the age of spheroids can be correlated with the age of *in-vivo* tumors, which can be used as a silent tool for cancer/tumor research.

MATERIALS AND METHODS

Materials

Accessories for cell culture and spheroid generation

T-25 cm^2 and T-75 cm^2 cell culture flasks, 96-well plates, 15 ml centrifuge tubes were purchased from Corning (USA), Gilson pipettes of 20µl, 200µl, 500µl and 1000µl capacity (France made), multipipette (Swiss made), 20ml Universal tubes, 10ml pipettes (Barloworld Scientific Ltd, UK), optical microscope (Olympus Optical Co Ltd, Japan) and Neubauer haemocytometer chamber.

Cell culture media and reagents

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle's medium (DMEM) without phenol red, fetal bovine serum (FBS), L-glutamine, trypsin/ EDTA were purchased from Invitrogen (UK), penicillin/streptomycin (P/S), phosphate-buffered saline (PBS) tablets, trypan blue solution (0.4% w/v), agarose powder and Accutase reagent were purchased from Sigma (UK), and Tat-FITC was purchased from Cambridge Bioscience (UK). The HT-29 cell line was kindly provided by a laboratory colleague in the drug delivery group at Manchester Pharmacy School, The University of Manchester (UK).

Monolayer cell culture in two-dimension (2-D)

The HT-29 cell line was grown in T-75cm^2 cell culture flasks and kept in an incubator maintained at 37°C in a humid atmosphere and 5% CO_2. The culture medium was changed on alternate days. When the cell confluence reached approximately 70%, cells were detached from the flasks using either trypsin/EDTA or Accutase.

Generation of 3-D multicellular tumor spheroids

Spheroids were generated by the liquid overlay technique (Carlsson *et al*., 1983). According to this technique, approximately 200µl of cell suspension containing 2000 cells/well (HT-29 cell line) was transferred to each well of a 96-well agarose gel-coated plate. The cell suspension was composed of cell culture media (DMEM containing 4.5 g/l glucose) supplemented with 10% FBS (fetal bovine serum) and 1% (v/v) penicillin/streptomycin (P/S) and L-glutamine. The seeded 96-well plates were kept in an incubator maintained at 37°C, a humidified atmosphere and 5% CO_2 for 3 days (72 hrs.).

After a three-day incubation period, all wells of 96-well plates were checked for the formation and shape of spheroids. Observations regarding the shape and size of spheroids were recorded. A sample of spheroids has been depicted in

(a). Only those spheroids were selected for experimental observations that were of spherical shape and of the same size through observation by optical microscopy (Gheytanchi *et al*., 2021) after three days of spheroid generation and retained the same characteristics until the day of observation/experiment.

Disaggregation of spheroids

Five spheroids per day were selected for each disaggregating treatment, either through accutase or trypsin/EDTA. Each spheroid was disaggregated by Accutase or 0.5x trypsin/EDTA (T/E). Then, 100 µl of either disaggregating reagent was added to each spheroid

for disaggregation. Then, a sufficient volume of PBS (2.9 ml) was added to make up the cell suspension up to 3 ml of each spheroid and pipetted well to form a uniform suspension. Then, 20µl from these suspensions was mounted on a hemocytometer and the number of cells in each spheroid was counted.

For interconversion of spheroid parameters, the diameter and volume of a single cell is mandatory. Therefore, the HT-29 single-cell diameter was measured, which was approx. 15µm. Then, the volume of a single cell was calculated from the diameter, which was approximately equal to 1800µm^3. The diameter of spheroids was measured through confocal microscopy, while the total cell number and viability status of each spheroid were determined through haemocytometry. Afterwards, through interconversion of spheroid parameters, the diameter, volume per cell, number of cells in dead and viable cell regions of each spheroid and radii (radii of spheroids, shell of live and core of dead cell regions) were calculated.

Calculating the distinct parameters after spheroids disaggregation

After disaggregation of spheroids, the viability test of each spheroid was performed by staining with the trypan blue solution (0.4%). Dead cells were stained with the trypan blue solution, while viable cells remained unstained. Then, the numbers of viable and dead cells were counted separately through a hemocytometer.

To estimate the number of cells in a spheroid from its diameter, spheroids were washed with phosphate-buffered saline (PBS) and transferred from a 96-well culture plate to an 8-well plate along with one drop of PBS. Then, one side of the spheroid was mechanically dissociated with the tip of the pipette inside the 8-well plate, as shown in fig. 1(b). Then, we imaged the spheroids with confocal microscopy in DIC (differential interference contrast) mode. The mean diameter of these disaggregated cells is essential for the interconversion of spheroid parameters. These parameters include the number of cells in the spheroid, diameter of the spheroid, volume of the spheroid, cellular layer in a spheroid and estimation of dead cell and viable cell regions.

For this purpose, the diameter of 20 disaggregated cells, as mentioned in the encircled area of fig. 1(b), was measured through confocal software and the mean diameter was calculated. The calculated mean diameter (d) of a single cell was approx: 15 µm. The volume of a single cell (v) was also calculated by the formula $V=\frac{3}{4}\pi R^3$ (where r is radius of a single cell), which was approximately equal to 1800um^3

(i) The conversion of spheroid parameters was calculated by using mathematical formulas from diameter to spheroid volume and from volume to number of cells (without haemocytometry):

a) Radius was calculated from the diameter of spheroid R=D/2 where 'R' is the radius of spheroid and 'D' is its diameter.
b) To calculate volume of a spheroid, $V=\frac{3}{4}\pi R^3$ where 'V' is the volume of spheroid and 'R' is its radius.
c) To calculate number of cells from volume of spheroid, N=V/v Where 'N' is number of cells, 'V' is volume of spheroid and 'v' is the volume of single cell.

(ii) Conversion from number of cells to spheroid diameter (using haemocytometry):

a) Volume of spheroid is calculated from the number of cells by the formula: V=vxN
b) Where 'V' is volume of spheroid, while 'v' is single cell volume, and 'N' is number of cells in spheroid
c) To estimate the diameter of spheroid from its volume $D = (\frac{6V}{\pi})^{1/3}$

Where 'D' is diameter of spheroid and 'V' is its volume. Similar equations were applied to estimate the number of cells, volume and diameter in dead cell and viable cell regions.

The diameter and volume of a single cell is essential for the interconversion of spheroid parameters. Therefore, it was calculated after disaggregation of cells from a spheroid. It should be noted that the HT-29 cell monolayer, when attached to the glass surface, shows a higher diameter (18 µm) than the diameter obtained from disaggregated spheroid cells (15 µm). The reason for this variation in diameter is that HT-29 cells attached to the glass surface may attain a flat shape, while cells after disaggregation from spheroids are rounded in shape. Therefore, the diameter of freshly disaggregated cells was considered for calculation.

The approximate layers of cells in spheroids, dead cells and viable cell regions were estimated by the formula:
Layers of cells = Thickness of spheroid or region / single cell diameter

Interconversion of spheroids parameters

For interconversion of spheroid parameters, the diameter and volume of a single cell is mandatory. Therefore, the HT-29 single-cell diameter was measured, which was approx. 15 µm. Then, the volume of a single cell was calculated from the diameter, which was approximately equal to 1800um^3. The diameter of spheroids was measured through confocal microscopy, while the total cell number and viability status of each spheroid were determined through haemocytometry. Afterwards, through interconversion of spheroid parameters, the diameter, volume per cell, number of cells in dead and viable cell regions of each spheroid and radii (radii of

spheroids, shell of live and core of dead cell regions) were calculated.

Ethical approval

Not required because this study was not conducted on human or animal subjects.

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 5. Student t-test or analysis of variance (ANOVA) test were applied. $P < 0.05$ was considered as statistically significant.

RESULTS

Spheroid's geometry

From fig. 2, in the larger spheroids (spheroids number 1 and 2), the actual cell count is less than calculated based on volume per cell. Larger spheroids develop a central necrotic region and a hypoxic region (Minchinton & Tannock, 2006). Therefore, well-established hypoxic and necrotic regions might exist in more mature spheroids, day 7 and onward. The larger spheroids could also indicate that spheroids become unstable and necrotic after exceeding a diameter of 800 microns.

For the two smaller spheroids (spheroids 3 and 4), the volume per cell is very close to what we estimated as the intracellular volume (estimated from images of single disaggregated cells). This suggests that the spheroids (by this age and provided they do not get too big and necrotic) consist of very tightly packed cells with a small (~10% or less) extra cellular volume fraction like many tissues. When comparing spheroids with tumor tissue, it should be noted that the cell packing is similar, but the synthesis of extra cellular matrix (ECM) proteins might not be similar. It has been reported that in vivo ECM is mostly produced from host stromal cells and that in spheroids, it is produced from tumor cells. This difference causes variation in the gene-producing constituents of the ECM (Kim, 2005). The outermost shell of viable cells is very thin in old-age spheroids with only a few cells thickness. As trypan blue interacts with serum protein (Gao & Zhao, 2003), it may overestimate the dead cells and hence underestimate the viable fraction of cells when using trypan blue exclusion techniques.

Diameter and cell number versus age of spheroids

Spheroid diameter (determined by confocal microscopy of intact spheroids) has not yet faced the subsequent disaggregation treatment, so these measurements can be taken as primary controls for subsequent measurements. The diameter of spheroids versus age is depicted in fig. 3-a. The growth curve shows that the tumor mass does not grow at an exponential or even linear rate. Growth appeared linear over the first week after seeding, but after day 9, the growth rate appeared to decrease for both treatments (accutase as well as trypsin/EDTA) and form a plateau, and there appeared to be greater variability in spheroid size.

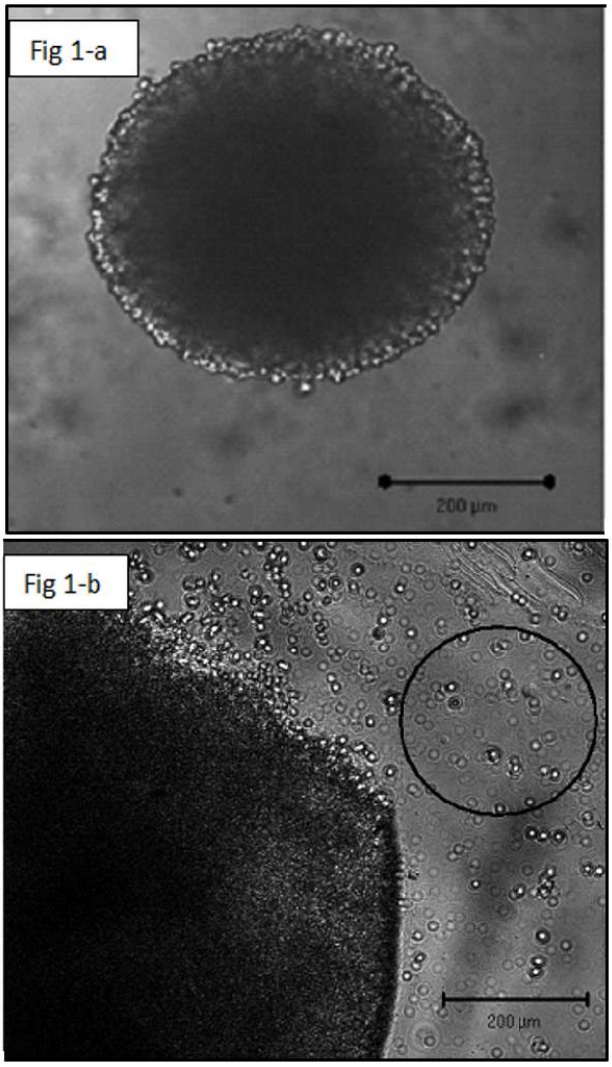

Fig. 1: (a) DIC image of 5-day-old spheroids obtained through confocal microscopy, (b) Mechanical disaggregation of a spheroid. The microscope objective setting was 10x/0.3. Scale bar represents 200 µm.

Cell number measurements were supposed to be affected by the spheroid disaggregation method used. Therefore, the data were split according to the enzymatic treatment (accutase *versus* trypsin/EDTA). The analyses show that the effect with respect to spheroid age is highly significant (ANOVA, $p<0.01$), but the comparison of disaggregation methods was statistically not significant (ANOVA, $p>0.05$). The cell number of spheroids versus age is shown in fig. 3-b. The growth of cells (in terms of cell number) appeared linear over the first week after seeding, but the rate (cell number per day) appeared to decline after 9 days. It is also evident that the Accutase disaggregation method appears to yield higher cell counts, particularly in the more mature spheroids. The possible

reason may be that dead or dying cells seem to be more sensitive to trypsin/EDTA than accutase treatment. Therefore, more cells were obtained by accutase treatment than trypsin/EDTA treatment.

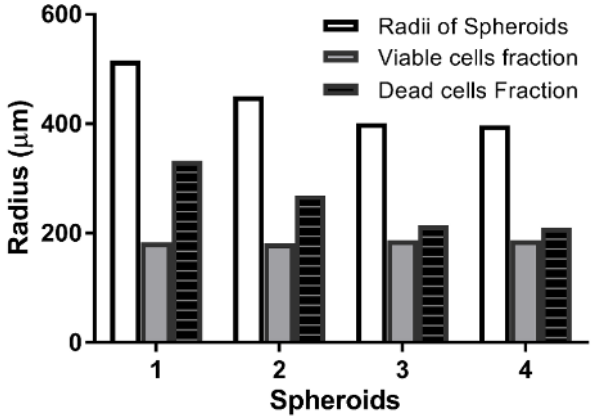

Fig. 2: Spheroid geometry based on haemocytometry. Four spheroids were taken for observations; numeric 1-4 represents the respective spheroids.

Volume per cell versus age of Spheroids

After diameter measurement through confocal microscopy, the spheroids were subjected to disaggregation through Accutase reagent or trypsin/ EDTA. The volume per cell obtained is shown in fig. 4. The difference in volume/cell obtained with Accutase reagent versus trypsin/ EDTA was statistically non-significant ($p>0.05$). The volume per cell via accutase treatment is more realistic and has comparative similarity with theoretical calculations in terms of volume per cell, most particularly on the 7th day of spheroids age (spheroid compaction stage). Therefore, we prefer accutase treatment to trypsin/EDTA treatment as a spheroid disaggregating reagent. Observations by confocal microscopy led to the calculation that the diameter of a single HT-29 cell was 15µm, which corresponds to a volume of 1,800µm^3 if the cell is assumed to be a sphere. Within the spheroid, there will be an additional extra cellular volume associated with each cell. One approach is to combine spheroid volume and estimated cell number to explore the volume per cell within the spheroid, excluding the trypsin-treated group for reasons discussed previously.

Cells viability versus age of spheroids

The viability measurement was performed by trypan blue staining. The cell viability decreased with the age of the spheroids, as shown in fig. 5. Variations in the viable fraction as a function of spheroid age and disaggregation method were noted. However, as noted previously, the effect of the disaggregation method was not statistically significant ($P>0.05$).

Thickness of distinct cell regions versus age of spheroids

Cellular layers of distinct cell regions, such as radii of spheroids, core of dead cell regions or thickness (shell) of live cell regions, were estimated and are shown in fig. 6. The presented data could be applied to a simple model of spheroid structure, in which a core of nonviable cells is covered with a shell of viable cells.

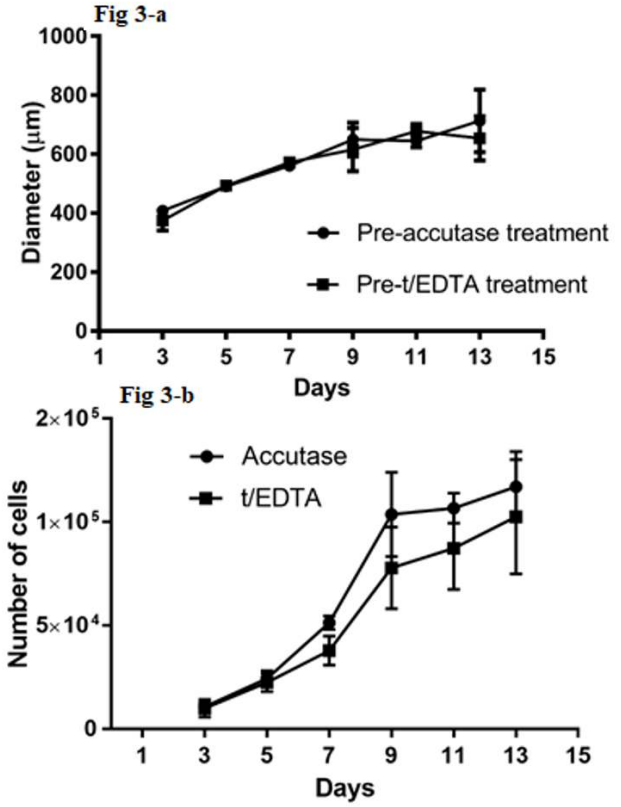

Fig. 3: (a) Diameter *versus* age of spheroids, (b) Cell number *versus* age of spheroids. Each value is the mean ± SD, n = 5.

Cellular layers of distinct cell regions versus age of spheroids

The analysis suggests that the outer layer of viable cells grows during the first week, as shown in fig. 7, but by day 7, it stabilizes at a maximum value of approximately 80 µm (~5 cell diameters). On the other hand, the nonviable core continues to grow during the second week, albeit at a slower rate.

DISCUSSION

Different cancer cell-lines have variability to efficiently generate spheroids. Some cell-lines form compact spheroids, others form aggregates, while some cell-lines are unable to generate spheroids. Generation of spheroids also dependent on seeding density and the method to generate spheroids (Han *et al*., 2021). Spheroid generation from HT-29 colon cancer cell-line has been reported as robust and reproducible (Gheytanchi *et al*., 2021), and therefore, HT-29 cell-line was selected for current study. The ratio of live and dead cells as well as the diameter of the necrotic region in a spheroid is an important issue for

the delivery and penetration of molecules. Necrosis might be due to insufficient in- and out-flow of oxygen, nutrients and other negative positive regulators (Durand & Sutherland, 1973; Gheytanchi *et al*., 2021; Kunz-Schughart, 1999). Furthermore, it has also explored that age of spheroid directly influences the drug response as a result of changes in the internal micro-structure of spheroid (Eilenberger *et al*., 2019). Penetration in all live cells is the most efficient and advantageous, but there is no simple method to estimate the ratio of live and dead cells and to estimate the necrotic region. Therefore, we proposed that this method could cover the gap and could estimate the various parameters of spheroids through simple techniques. This is a simple method for estimating the diameter, number of cells, volume per cell, thickness of distinct regions and cellular layers in spheroids.

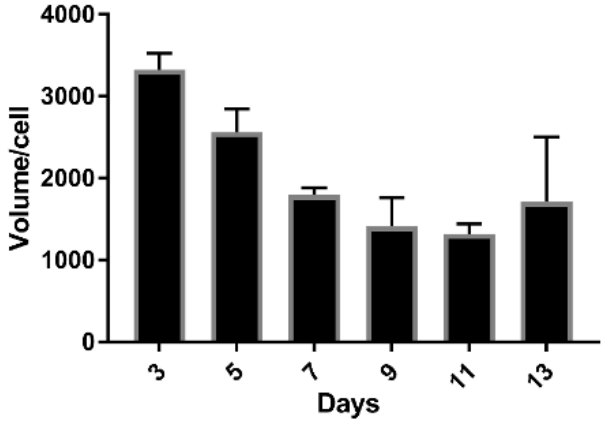

Fig. 4: Volume per cell versus age of spheroids. Each value is the mean ± SD, n = 5.

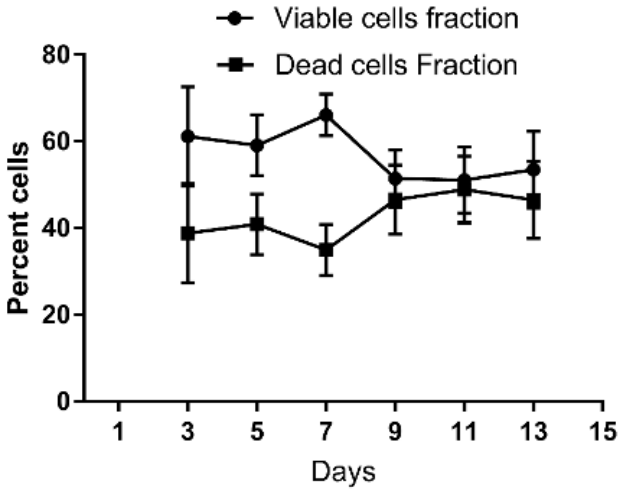

Fig. 5: Dead and viable cell fractions versus age of spheroids. Each value is the mean ± SD, n = 10.

The possible reason for spheroid diameter variability may be due to an increase in spheroid age and the accumulation and concentration of dead cells increases in the inner region. This results in an increase in waste products and their flow across the spheroid, which might affect the growth rate. It has been reported that spheroids from different cell-lines grow differently and the Gompertz model has frequently applied to describe the growth kinetics of spheroids (Gheytanchi *et al*., 2021). It is best reflected by the results in this study, where the MCTSs growth/cell number *versus* culture time (days) representing three distinct phases: (a) the initial exponential phase, (b) linear phase, and (c) plateau as shown in fig. 3-b. The cell number along the age of spheroids indicates that accutase treatment leads to a greater degree of disaggregation into single cells and hence a higher and more accurate estimate of the total number of cells in the spheroid. The haemocytometry results from the accutase group were more reliable than trypsin/EDTA treatment because we obtained a greater number of cells in mature spheroids with former than latter. Therefore, in subsequent experimental procedures, accutase treatment was used exclusively.

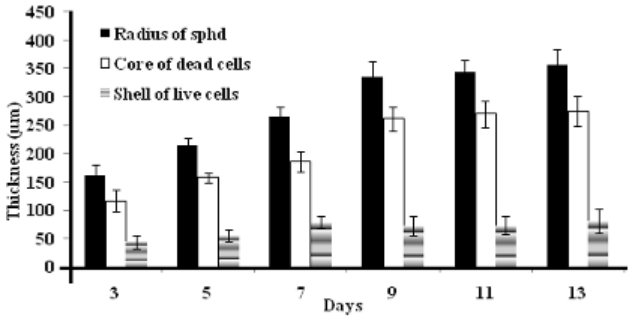

Fig. 6: Thickness of distinct regions along with the age of spheroids. Each value is mean ±SD, n = 10.

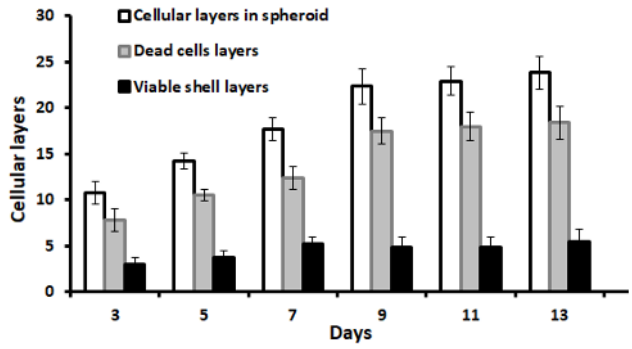

Fig. 7: Cellular layers of distinct regions along the age of spheroids. Each value is mean ±SD, n = 10

The volume per cell analysis appears to show dependence on spheroid age. This observation could be explained based on the three-stage process of spheroid growth, comprising an initial loose form followed by a packing/compaction process during spheroid formation, followed by the development of a hollow necrotic core in the larger more mature spheroids. Here, it should be noted that we assumed that the volume of dead and necrotic cells equals that of live cells. In actual cases, the volume of dead cells may be quite lower than that of live cells. Moreover, necrotic region estimation is also based on the volume difference of the actual cell volume and the

calculated cell volume. Therefore, taking into consideration the effect of dead cell volume and necrotic region volume, the volume per cell along the age of spheroids may increase and lead to the development of a necrotic core in larger more mature spheroids. There was initially a loose packing of cells, but as time progressed, the volume per cell approached 1500 $\mu m^{3,}$ meaning that cells were tightly packed and extracellular volume was negligible in the live cell region, as evident from day 7 onward. Additionally, there was possibly development of a well-established necrotic region from day 7 onward, which justified that the age of spheroids could play a role in the penetration of molecules. Therefore, it addresses the objectives that spheroid geometry/distinct cellular distribution changes with the passage of time and could alter penetration of drug/fluorophore (s) in spheroids.

As far as the viability of cells along the age of spheroids is concerned, the effect of spheroid age shows that there is a small but significant reduction in the viable cell fraction after the first week of growth. This reduction in the viable cell fraction might be due to the accumulation of dead cells and degraded products that affect the growth of viable cells. On the other hand, the fraction of dead cells increased at day 9 and then stabilized.

Regarding the thickness of distinct regions in spheroids with age, it has been reported that larger spheroids develop a central necrotic region and a hypoxic region (Minchinton & Tannock, 2006). Therefore, well-established hypoxic and necrotic regions might exist in more mature spheroids, day 7 and onward. Trypan blue also interacts with proteins (Gao & Zhao, 2003); therefore, the dead cell count might be overestimated. At an early age (day 3 particularly & day 5), cell–cell contact in spheroids may be loose, leading to compaction. Moreover, at these early age spheroids, distinct cells may be mixed with each other, and the hypoxic region may not be well established. On day 7 and onward, a well-established hypoxic region and necrotic region might take place. To compare our finding with the existence literature, it has been reported as a result of spheroidal-analysis that spheroid size and shape both are important factors in determining the drug penetration response (Hou *et al*., 2018; Schmitz *et al*., 2017; Zanoni *et al*., 2016). The results of the model illustrated above, which represent the estimated radii of spheroids, estimated radii (core) of the dead cell region and estimated thickness (shell) of the live cell region which could contribute to the diffusion process of drugs along the age of spheroids. The cellular layers suggest that necrotic and hypoxic regions in larger spheroids (Minchinton & Tannock, 2006) significantly increase with the age of spheroids, while the viable cell layer decrease is prominent, which could lead to poor penetration of drugs in old-age spheroids. Moreover, age of spheroids can be corelated with the age of *in-vivo* tumor to improve clinical findings.

CONCLUSION

The penta-physical measurements of MCTSs through current technique are cost effective, simple and the growing spheroids could be corelated with the stage of *in-vivo* tumors. In the tested spheroids, it has been concluded that from day 7 to 11, the diameter of spheroids ranged from 550-650 µm. At this interval, the spheroids could be in a compact form and will possess three distinct regions: A well-developed proliferating region (composed of growing viable cells), oxygen-deficient or hypoxic (stagnant viable cells), and necrotic regions (dead cells and degraded products). It is proposed to be the appropriate duration to use spheroids as tumor tissue models for experimental/ anticancer drug delivery purposes. Cell-lines grow differently; hence each kind of cell-line should be characterized for shape, size, and heterogeneity accordingly. The results and analysis of spheroid heterogeneity revealed that morphological changes occur as they get older and meet the objectives that spheroid dynamics changes with the passage of time, and it could alter penetration of drug/fluorophore(s) in spheroids.

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