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Cell viability assessment using the Alamar blue assay: A comparison of 2D and 3D cell culture models

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Abstract

Comparisons of 2D and 3D cell culture models in literature have indicated differences in cellular morphology and metabolism, commonly attributed the better representation of *in vivo* conditions of the latter cell culture environment. Thus, interest in the use of 3D collagen gels for *in vitro* analysis has been growing. Although comparative studies to date have indicated an enhanced resistance of cells on collagen matrices against different toxicants, in the present study it is demonstrated that non-adapted protocols can lead to misinterpretation of results obtained from classical colorimetric dye-based cytotoxic assays. Using the well established Alamar Blue assay, the study demonstrates how the transfer from 2D substrates to 3D collagen matrices can affect the uptake of the resazurin itself, affecting the outcome of the assay. Using flow cytometry, it is demonstrated that the cell viability is unaffected when cells are grown on collagen matrices, thus the difference seen in the fluorescence is a result of a dilution of the resazurin dye in the collagen matrix, and an increased uptake rate due to the larger cell surface exposed to the surrounding environment, facilitating more effective diffusion through the cellular membrane. The results are supported by a rate equation based simulation, verifying that differing uptake kinetics can result in apparently different cell viability. Finally, this work highlights the feasibility to apply classical dye-based assays on collagen based 3D cell culture models. However, the diffusion and bioavailability of test substances in 3D matrices used in *in vitro* toxicological assays must be considered and adaption of the protocols is necessary for direct comparison with the traditional 2D models. Moreover, the observations made based on the resazurin dye can be applied to drugs or nanoparticles which freely diffuse through the collagen matrices, thus affecting the effective concentration exposed to the cells.

Keywords: Alamar blue assay, 3-D Cell culture, collagen gels, extracellular matrix, cell viability, flow cytometry,

1. Introduction

Significant efforts have been devoted towards the development of more realistic *in vitro* cell culture models, better mirroring *in vivo* conditions. For example, it is well accepted that, in an environment mirroring the *in vivo* conditions encountered by the cells, the observations made are more representative of the cancerous cell phenotype compared to those found on conventionally used 2D cultures (Breslin and O'Driscoll, 2012; Elliott and Yuan, 2011; Kimlin et al., 2011). Moreover, providing a microenvironment with adequate adhesion and proliferation has been reported to allow a more accurate investigation of cellular homeostasis, differentiation and migration (Kim, 2005; Kim et al., 2004). The use of collagen gels and more complex multi-component systems such as Matrigel has become increasingly popular, as they provide the cells with a matrix which more accurately represents the extra cellular matrix (ECM) (Petersen et al., 1992; Prestwich, 2008; Weaver et al., 1995). The impact of such matrices on the cell phenotype and metabolism has been already documented in the literature. For example, it has been shown that antibodies against B1-integrins exhibit different behaviour when tested in 2D compared to 3D models (Wang et al., 1998); induced doxorubicin-resistance by the extra cellular matrix in human osteosarcoma and HT1080 cells has been demonstrated (Fourre et al., 2008; Harisi et al., 2007); reduced radiation induced toxicity when cells are grown in a 3D environment has also been reported (Sowa et al., 2010). The use of collagen matrices as 3-D cell culture matrices has greatly increased in the last few years and numerous other publications can be found in the literature describing modification of the

cell phenotype, metabolism or composition when grown in a 3D matrix, compared to 2-D environments (Lupanova et al., 2010; Wu et al., 2009).

Although 3D matrices are increasingly routinely employed for cellular analysis, the impact of the ECM like microenvironment on the cells is still under investigation. The degree of modification of the cellular behaviour and metabolism remains unclear and the comparison between conventional 2D substrates and 3D models is still ongoing. However, the use of a 3D cell culture environment could lead to difficulties in adapting established cytological protocols as well as in the interpretation of the results. Although already intensively used in cancer research, collagen gels have not been adapted to other fields such as cyto- or nano- toxicology. Moreover, although efforts have been devoted to explaining the difference of cell behavior when grown on 3D substrates such as collagen gels, no consideration has been given to the differing cell geometry and more specifically the cell surface exposed to the microenvironment.

In the present study, the Alamar blue (AB) *in vitro* cytotoxicity assay has been used as a model to demonstrate the link between the cell substrate used and the cell geometry, ultimately also influencing the outcome of the colorimetric assay employed, independent of the cell viability or proliferation. The results are supported by a rate equation based simulation, verifying that differing uptake kinetics can result in apparently different cell viability (Supplementary Information).

2. Materials and Methods

2.1. HeLa Cell line

HeLa cells, immortalized human cervical cells, were obtained from the ATTC (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 1% L-glutamine (200 mM) and 10% foetal bovine serum and maintained in a humidified atmosphere containing 5% CO₂ at 37°C. HeLa cells were cultured until they reached approximately 80% confluency before preparing the plates for the cytotoxicity assay. Cells were seeded at a density of 2×10^4 cells per well in 6-well-plates (Nunc Lab-Tek®). Half the plates were prepared with collagen gels at a concentration of 2.5 mg/mL, and the other half were directly grown on the plastic base of the plate as controls. The experiments were performed in triplicate and readings of the cell viability using the AB assay were performed after 24h. Moreover, it is important to point out that the whole experiment (in triplicate) has been repeated twice within three weeks interval in order to confirm the reproducibility of the observations made.

2.2. Collagen gels

Solutions of collagen I from rat tail tendons (Gibco) were used for preparation of the collagen gels. The 5mg/mL solution was mixed with sterile 10X phosphate buffered saline (PBS), sterile distilled water (dH₂O) and 1M NaOH. The appropriate relative quantities of these components is determined by the final concentration (2.5 mg/mL) and volume needed. All the steps were carried out on ice to slow the gelation process. After mixing, 500 µL of the solution were either placed in 6-wells-plates for the AB cytotoxicity assay or 1 mL of the solution was placed in a 25 flask for the flow

cytometric analysis, before incubation at 37 °C degrees in a 95% humidity incubator until a solid gel was formed (about 30 minutes).

2.3. Alamar blue assay

The Alamar Blue® assay is designed to quantitatively measure the proliferation of human and animal cell lines, bacteria and fungi (Kuda et al., 2003; Mosmann, 1983; O'Brien et al., 2000; Pettit et al., 2005; S.Al-Nasiry et al., 2007). Over the past 50 years, the AB assay has been widely used in studies of cell viability and cytotoxicity for biological and environmental applications (Rampersad, 2012; Vega-Avila and Pugsley, 2011; White et al., 1996). The bioassay can also be used to establish the relative cytotoxicity of agents within various chemical classes (Bopp and Lettieri, 2008; Borra et al., 2009; Mikus and Steverding, 2000; Miret et al., 2006). Using the REDOX indicator resazurin (oxidised form), it is possible to spectrophotometrically measure the cellular proliferation. Resazurin is blue and non-fluorescent, whereas resorufin (reduced form) is red and highly fluorescent. Thus, measuring the changes in the fluorescence of the dye in the intracellular environment, modifications in the number of metabolic active cells can be detected. Tetrazolium salts can deliver similar information regarding cell growth, but present incompatibility problems, the most limiting being the high toxicity of the DMSO or HCl/isopropanol required for reading the results (Mosmann, 1983). Thus, the AB assay is generally preferred for kinetic studies. The oxidation-reduction potential of resazurin is +380 mV at pH 7.0, 25 °C, which means it can be reduced by NADPH ($E_o = 320$ mV), FADH ($E_o = 220$ mV), FMNH ($E_o = 210$ mV), NADH ($E_o = 320$ mV), as well

as cytochromes ($E_0 = 290 \text{ mV}$ to $+80 \text{ mV}$), all part of the cellular respiration metabolic reactions. However, other enzymes such as the diaphorases (dihydrolipoamine dehydrogenase (Matsumoto et al., 1990)), NAD(P)H:quinoneoxidoreductase (Belinsky and Jaiswal, 1993) and flavin reductase (Chikuba et al., 1994) located in the cytoplasm and the mitochondria can also reduce Resazurin. Therefore, AB reduction is the result of multiple metabolic reactions and does not necessarily specifically indicate a mitochondrial dysfunction, but remains a suitable indicator of the cellular health and viability (Ahmed et al., 1994).

The AB assay was carried out according to manufacturer's instructions. Briefly, control medium was removed; the cells were rinsed with PBS and 2 mL of an AB solution (5% [v/v] solution of AB dye) prepared in fresh medium (without FBS or supplements) were added to each well. Following 3 hours incubation, AB fluorescence was quantified at the respective excitation and emission wavelength of 540 and 595nm using a Tecan Genios microplate reader. The results were averaged over 3 different independent experiments ($n=3$, each conducted with one week interval) with 3 replicates per experiment (3 x 6 well plates), each replicate being prepared from different T75 flasks in order to take into account the biological variability. Finally, for each plate the reading was also done in triplicate (values obtained from 3 different wells averaged) in order include the technical variability due to the efficiency of AB assay, sensitivity of the plate reader or simply related to the sample preparation. For each experiment, wells containing only the AB solution without cells were also prepared and incubated for 3h. The fluorescence measured in those was used as a background and subtracted. However, although this protocol is routinely used in the literature, some variations in the AB solution

concentration and exposure time have been made purposely throughout this study. These modifications will be clearly highlighted and discussed at the appropriate points in the manuscript, for clarity. Again, each experiment was conducted in triplicates (3 x 6 well plates) which have been prepared from different flasks in order to have independent replicate based on different cell populations.

2.4 Flow cytometry assays

2.4.1. Apoptosis assay

This study was performed using a Partec CyFlow® Space Flow Cytometer (Partec UK Limited, Germany). The default Partec FloMax® flow cytometry software has been used for the analysis of the samples, but the Beckman Coulter Summit software and the FCSExpress Research Edition have been used for the reanalysis of the samples. The QC control of the instrument was performed using Spherotech 6 and 8 peak beads. HeLa cells (1×10^6 cells//flask) were seeded in T-25 flasks and incubated in a 5% CO₂ incubator at 37°C for 24 hours. These conditions were selected to avoid cells becoming over confluent, which might lead to misinterpretation of the apoptosis assay due to cellular death caused by the stress of the culture conditions and medium depletion causing cell starvation. The experiments have been conducted in triplicate. However, in order to keep replicates as independent from each other as possible, 3 T75 flasks were initially seeded and from each one of them two T25 were prepared for flow cytometry, with only one containing a collagen gel as described above. Following incubation, the cells were washed twice with pre-warmed PBS and were collected by trypsinization, after which the trypsin was removed by centrifugation. The cells were then washed twice with pre-

warmed PBS and stained with the YO-PRO1/Propidium iodide (PI) dyes (Life Technologies), whereby 1 μ L of YO-PRO1 dye (100 μ M) and 1 μ L of PI (1mg/mL) were used to stain 1×10^6 cell/ml. After staining of a cell population, apoptotic cells show a green fluorescence, whereas necrotic cells show green and red fluorescence. After incubation on ice for 30 min, the cells were analysed by flow cytometry within 30 minutes, using 488 nm excitation and reading the fluorescence of YoPro with a IBP 527/30G filter and PI with a IBP 682 B filter. Unstained and single control samples have been used in order to set up the instrument and gates in order to visualize 3 groups: live cells, apoptotic cells and necrotic cells. Samples with over-confluent cell populations leading to medium depletion which is ultimately associated with a high cellular death have been used as biological controls. 15,000 single cells per sample have been analysed.

3. Results

3.1. Cell viability monitoring using the AB assay

Conventional *in vitro* cytotoxicity assays such as AB, Neutral Red and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) have been developed for rapid screening of cellular proliferation, viability, metabolic activity, lysosomal and mitochondrial activity (Davoren et al., 2007; Herzog et al., 2007; Mukherjee et al., 2010; Naha et al., 2010). AB is widely used for cytotoxicity assay and has been selected for the low induced cytotoxicity from the redox indicator giving more freedom in the concentration of reazurin used.

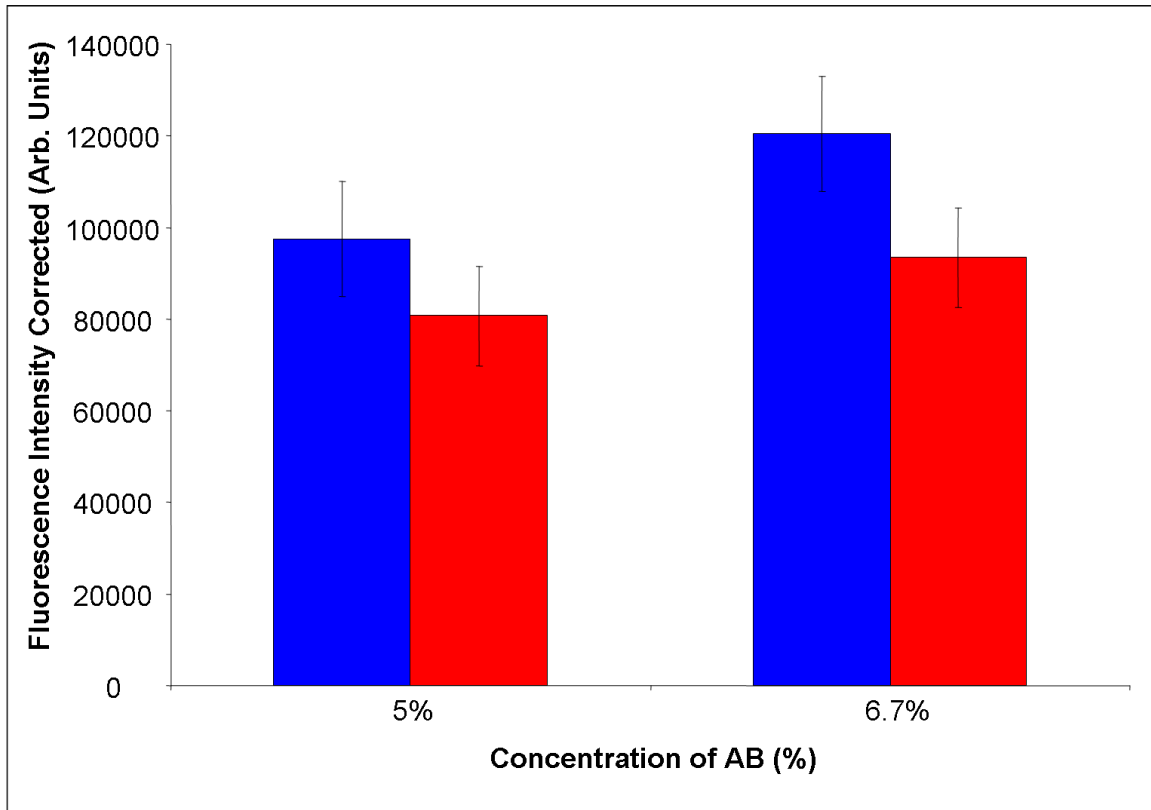


Figure 1: Comparison of Alamar blue assay results obtained after compensation of the AB solution concentration for cells grown on collagen gels (blue) and plastic (red) for 24hrs using both 5% and 6.7% AB solutions. The experiments have been repeated twice in triplicates and the error bars indicate the overall variability observed

The viabilities of the HeLa cells grown on a 2D plastic surface and a 3D collagen matrix have been evaluated using the AB assay according to the standard protocol described in Section 2.3. Figure 1 displays the AB fluorescence measured after 24h for cells cultured on both plastic substrates and collagen gels. The Y axis is the fluorescence intensity after subtraction of background signal of the AB with no cells but incubated for 3 hr.

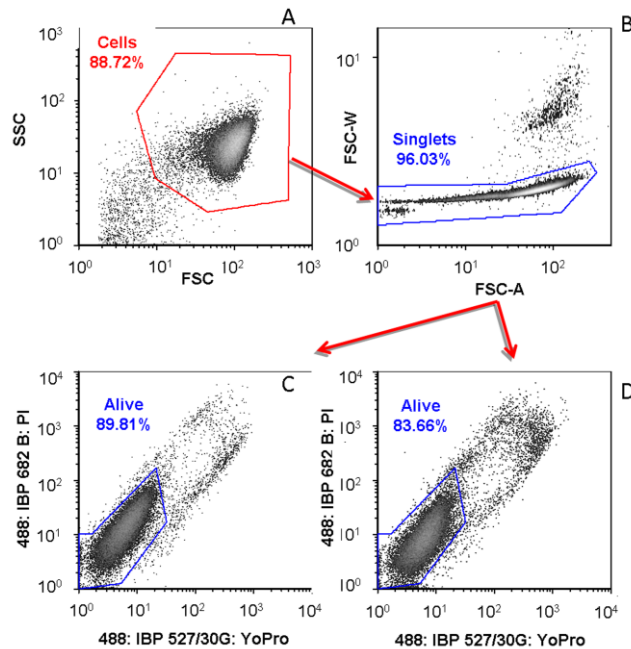
After 24h incubation, the cells grown on collagen gels exhibit significantly higher (~21%) fluorescence intensity than those grown on the 2-D substrate ($p < 0.001$). The statistical variations represented by the error bars show small variance in the fluorescence read at 24h. The difference in the fluorescence measured indicates a higher degree of

reduction of the AB dye by the cells grown on collagen gels. In absence of exogenous agents with toxic properties for the cells, the difference in the readings indicates an increased viability compared to the cells seeded directly on plastic. Therefore, in order to validate this observation, further approaches have been used.

3.2 Flow cytometry

As collagen gels are purported to better represent *in vivo* conditions, a possible cause for the strong increase of the cell viability obtained using the AB assay is a better biocompatibility of the cells with the 3D matrix. In order to validate the observations made using the AB assay, additional assays were needed to give more information regarding the cellular behaviour when grown on plastic compared to the collagen gels. Flow cytometry is a powerful tool for the analysis of large cell population based on fluorescent dyes. Moreover, the main advantage for this study was the possibility to label the cells while in suspension rather than still attached and thus the labeling process should be independent of the growth substrate.

For the purpose of this work, the apoptosis assay has been selected to visualize the amount of live / dead cells in the suspension to get an indication of the possible higher toxicity/better biocompatibility of the substrates.



*Figure 2: Flow cytometry apoptosis assay using the Yo-pro/PI dyes. **A:** Debris have been excluded by gating them out based on the scattered characteristics (FSC vs SSC), gate 1, **B:** Aggregated events have been excluded with gate 2 in FSC-Area vs FSC-Height; **C and D:** Cells characterized as alive respectively from plastic (2C) and collagen gels substrates (2D). The experiments have been repeated in triplicate from 3 different T25 flasks.*

The results of the apoptosis assay performed after 24h incubation are presented in figure 2. The plot presents the fluorescence measured for both the YOPRO1 (figure 2C and D - X axis) and the PI (figure 2C and D - Y axis) dyes. The distribution of the data indicates the proportion of live versus dead cells present in the suspensions harvested from the collagen gels (figure 2C) and plastic (figure 2D). Apoptotic and necrotic cells incorporate different levels of the two fluorescent dyes, as well as scatter changes as can be observed in the scatter bivariate histogram. In the present study, and for comparison with the AB assay, only the viable cells are of interest. As shown in figure 2, in the case of the cells grown both on collagen and plastic, the cluster is mainly located in the bottom left area

(with no uptake of any of the dyes), indicating a high proportion of live cells. After performing the experiments in triplicate from 3 different sets of T25 flasks, the percentage of live cells has been evaluated to be 84.6% (+/- 5.2%) for those harvested from the collagen gels whereas for the plastic substrate it was found to be 87% (+/- 2.9%). Therefore, neither of the substrates used has a statistically significant toxicity that could possibly affect the outcome of the AB assay after 24h incubation, and notably, according to the standard deviation calculate from the triplicates, there is no significant difference between the cell populations grown on 2D substrates and 3D matrices.

3.3 Effect of the collagen gels on the AB solution concentration

The observations made using flow cytometry clearly demonstrate that the apparent difference in the cell viability indicated by the AB assay is not linked to differences in cell viability. Thus, the question remains as to how to understand and interpret the origin of the differences in the fluorescence readings of the AB assay.

The collagen matrices are prepared from a solution of collagen initially at 5 mg/mL which is diluted for the needs of the experiments to 2.5 mg/mL. All the reagents used are water based and, after gelation and formation of the collagen fibers, the matrices are similar to wet sponges. Thus, the first concern is about any reduction of the effective concentration of the AB solution, as presented to the cells. Wells free of cells and only containing the collagen gels covered with 2 mL of AB solution (5%) were prepared and incubated for 1h, 2h and 3h. After each time point, the AB was pipetted out of the wells and the absorbance at 570 nm was measured. The results are expressed as a percentage of the absorbance obtained from the AB solution placed in the well without any collagen.

After 1h, the absorbance is equal to 76% of the control, after 2h 74% and after 3h 76%. This demonstrates how quickly the dye from the AB solution can diffuse through the collagen gels and therefore how the concentration of the AB solution used can be affected by dilution of the dye solution by the water contained in the matrix. As the collagen gel is mostly water, it is important to take into account the volume of collagen used to form the matrix into the calculation of the solution used for the assay.

The effect of the AB solution concentration on the assay can be easily visualised. Based on the absorbance measurements, it can be estimated that the effective concentration of the nominally 5% AB solution is reduced by ~25% in the collagen gel, resulting in the cells being exposed to only 3.75% AB solution. To compensate the dilution of the dyes into the collagen gels, the assays have been run in parallel using 6.7% AB solutions. Figure 1 presents the results obtained after 24h for both AB solutions tested on cells grown directly on plastic and collagen gels. According to the observations made on the dilution of the dyes in the collagen gels, the results obtained from the cells grown on plastic with a 5% AB solution should be compared to those obtained from the cells grown on collagen gels exposed to a solution at 6.7%. Under these conditions, the difference in the cell viability found is even greater, with a fluorescence intensity 49% more intense after 24h incubation.

3.4 Understanding the Alamar blue assay

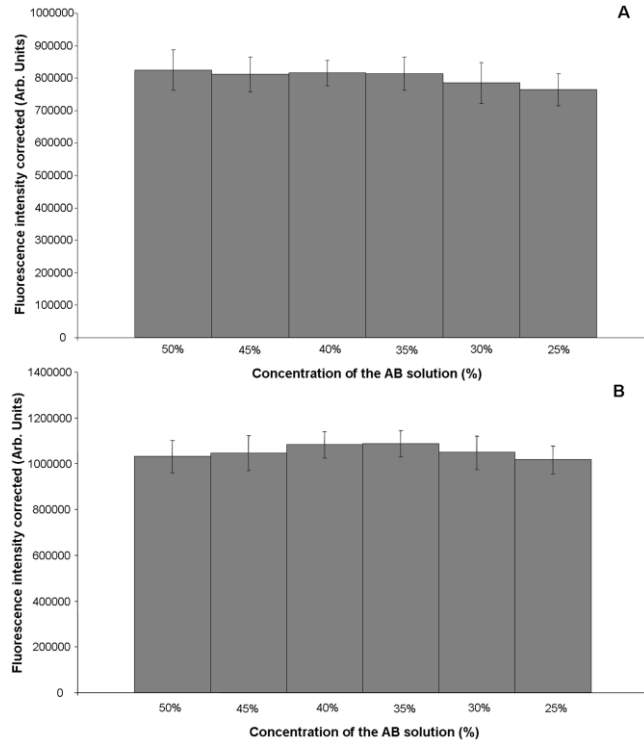


Figure 3: Alamar blue performed on cells grown on collagen gels with high concentration of AB solution ranging from 25% to 50%. Cells have been exposed to the AB solution for 3h (top) and 5h (bottom). The error bars indicate the variability observed over 3 different replicates.

The cell viability as measured using flow cytometry highlighted that there is no increased cell death on the plastic substrates compared to the collagen gels, which rules out potential toxicity due to the environment of the cells. Thus, two hypotheses could explain the difference in the fluorescence measured using the AB assay. Firstly, an increased metabolic activity of the cells when grown on the collagen gels leading to an increased conversion rate of the resazurin to its fluorescent form. Secondly and more simply, different rates of uptake of the AB dye by the cells in the two culture environments. The organization of the collagen matrix can be compared to a network formed by the polymerized fibers. In this work, the cells have been seeded onto the collagen gels, but, as discussed above, the dyes from the AB solution freely diffuse through the gels. Uptake

of the dye by the cells occurs by diffusion through the cytoplasmic membrane. Whereas the cells seeded on plastic have only one side exposed to the surrounding medium, when seeded on the collagen matrix, the underside of the cells also have direct contact with the AB solution and thus present a significantly larger surface (membrane) area through which the dye can diffuse. A higher uptake rate of the AB solution by the cells would result in a higher fluorescence reading, indicative of a higher metabolic activity or larger cell population.

Ideally, the measurement of the actual amount of AB dye present in the cells would be the best approach to investigate differing uptake rates by the cells. However, the dye diffuses passively through the membrane of the cells and is continuously reduced in the cytosol. A measurement of the absorbance would register the unreacted, resazurin (oxidized) form of the dye, whereas fluorescence registers the reacted, resorufin (reduced) form of the dye.

In order to estimate the effects of different uptake rates of the dye by the two cell culture models, the protocol for the AB assay has been modified to operate in the regime of saturated uptake, in which different uptake rates do not affect the quantity of dye uptaken. One of the advantages of the AB assay is the low cytotoxicity of the dye at low concentrations which allows the kinetics of the cellular viability over a prolonged time to be monitored. However, for the purpose of this study, the concentration of the AB solution was increased up to 50% in order to visualize a point of saturated dye uptake/conversion, as indicated by the fluorescence intensity reaching a maximum. Figure 3A presents the results obtained for cells grown on collagen exposed to 25%, 30%, 35%, 40%, 45% and 50% AB solution for 3h. Although, for concentrations up to

35%, the fluorescence slightly increases, it appears that higher concentrations do not induce a statistically significant increase of the fluorescence. This indicates either saturation of the dye uptake or of the reduction rate of the resazurin into resorufin.

For varying AB concentrations, measurement of the fluorescence was made every 45 minutes for 7 different time points. After 5h exposure to the AB solution (figure 3B), the fluorescence still exhibits a maximum intensity at 35% AB solution, but for higher concentrations, a slight decrease can be seen, likely related to the toxicity of the AB solution at high concentrations. Similar observations can be made for the cells grown on plastic substrates (data not shown). A 35% AB concentration was therefore selected in order to avoid interferences from the cytotoxicity of the solution on the cells which could make the interpretation of the results more difficult. Figure 4 shows the fluorescence measured for incubation periods from 3h to 7h15 min (7 time points). Although a small difference can be seen, with a slightly higher fluorescence for the cells grown on collagen, it is not comparable to the 21 % difference observed for the standard assay protocol (3hr, 5% AB). For more precise visualisation, figure 5 displays the difference in the fluorescence intensities, but also this difference expressed as a percentage of the total fluorescence measured. The fluorescence is between 1% and 5% higher for the cells cultured on collagen gels, which is considerably less than the 21% difference found when working with 5% AB solution. This highlights that, using a higher concentration, the system has reached saturation. The fact that the fluorescence exhibits similar intensities also indicates that the cells have reached their maximum reduction rate, which, importantly, is the same for cells grown on both collagen and plastic. Therefore, the metabolic activity of the cells as determined by the AB assay is the same for both cell

cultures. This is confirmed in figure 4 as the gap between the two curves remains constant over time, indicating identical reduction rates of the resazurin into resorufin in the two cell culture models. If the metabolic activity was higher for cells grown on collagen, the difference would increase over time. However, the fluorescence difference between the two substrates remains similar with a maximum difference of about 5% after 4h 15 min. These observations demonstrate that the metabolic activity of the cells in the two cultures is comparable and therefore the difference in the fluorescence observed under the standard assay conditions is not related to higher cell viability or proliferation but to a higher uptake of the dye by the cells. The larger surface area exposed to the medium from the cells grown on 3D collagen gels increases the passive diffusion into the cytosol inducing the slightly higher reading obtained which reflects the delay between the 2 models until reaching the saturation point.

The impact of the differences in the effective cell surface areas in 2D vs 3D cell culture models can be further illustrated using a rate equation model as described in the Supplementary Information. The model clearly demonstrates how the apparent differences in cell viability as indicated by the AB assay can be affected by differing uptake rates of the dye, as a result of different effective cell surface areas (see supplementary materials). Notably, such a numerical approach could potentially be employed as a guide to adapting 2D protocols to 3D cultures.

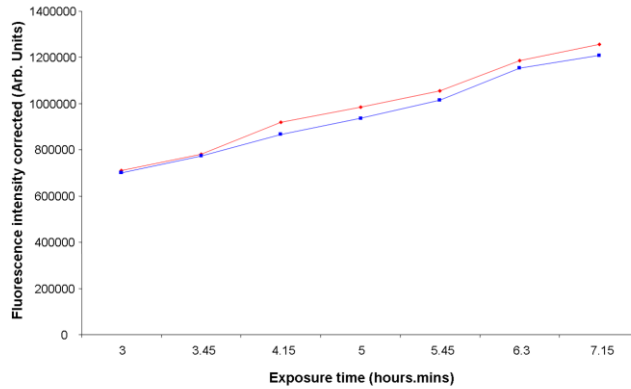


Figure 4: Kinetic of the fluorescence obtained using an AB solution at 35% for cells grown on plastic (blue) and collagen gels (red) from 3h exposure up to 7h15mins. Reading s have been performed every 45 mins. The values presented are mean fluorescence calculated from experiments conducted in triplicate.

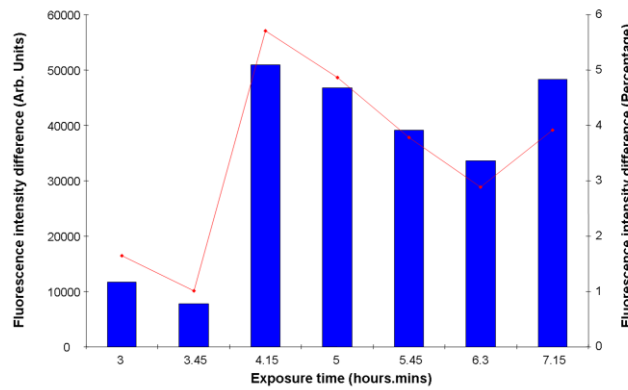


Figure 5: Representation of the difference of the fluorescence obtained for cell grown on plastic and collagen gels when performing the AB assay using a 35% solution for each time point. The histogram represents the difference in the fluorescence intensity while the curve is the difference expressed in percentage. The results presented have been obtained from experiments conducted in triplicate and averaged.

4. Discussion

According to the observations made throughout this study, there is no evidence that the transfer from the 2D plastic to the 3D collagen gels has any influence on the cell health and viability. Clearly, however, the effects of diffusion of the active dyes through the matrices and their subsequent bioavailability to the cells can lead to misinterpretation of

the results obtained. The concern addressed in the present study relates to the relevancy of the change of cell behavior, proliferation or resistance against active agents documented in the literature. The AB assay has been used as a model to highlight the importance of the cell culture model geometry on the outcome of cytotoxic investigation. While the cell surface exposed to the surrounding environment will have an impact of the uptake rates, it is essential to take into consideration the added volume added to the cell environment associated with the use of 3D protein based substrates. Importantly, the observations made regarding the resazurin can be applied to other models. Thus, both the dilution of the solution by the gel and the different uptake routes will also apply to all solutions/dispersions *in vitro* such as toxicants, chemotherapeutic agents and nanoparticles. The 3D matrix acts as a sponge which results in a dilution factor of the medium and thus of any active agent, drug or nanoparticle in suspension. However, this effect is often neglected in *in vitro* studies investigating the cytotoxicity of drugs or toxicants on cells (Godugu et al., 2013; Lee et al., 2008; Millerot-Serrurot et al., 2010). Although the collagen gels may appear to have a significant impact on the cell behavior and metabolism, when comparing IC₅₀'s or EC₅₀'s calculated from 2D and 3D models, neglect of the dilution effect in 3D matrices makes the observations questionable. Apparent systematic variations in viability can be a result of a reduced effective concentration of the agent or assay used in 3D matrices compared to 2D cultures, resulting in a dilution factor which could account for the difference in the IC₅₀ calculated (Godugu et al., 2013; Lee et al., 2008; Millerot-Serrurot et al., 2010) and/or increased cell resistance to toxic/chemotherapeutic agents⁴⁵.

Conclusion

The use of 3D collagen gels as growth substrates has been reported to affect the cell phenotype and behavior, exhibited as different degrees of resistance to toxicants or modified levels of metabolic activity. In the literature, many studies aim to understand and explain how the use of 3D matrices can induce considerable changes in the results obtained when testing toxicants, chemotherapeutic agents or nanoparticles compared to the well known 2D surfaces. Although the interaction of the cells with their surrounding environment can trigger different signaling pathways in the cells, modifying their behavior, the present study demonstrates that adaption or comparison of the results obtained from 2D models with 3D matrix cell culture systems requires consideration of the geometry and morphology of the cell/substrate interface. Therefore, the diffusion and bioavailability of test substances in 3D matrices used in *in vitro* toxicological assays must be considered and adaption of the protocols is necessary for direct comparison with the traditional 2D models. The example of the well established AB cytotoxicity assay highlights that, what can be first interpreted as an increase of the cell viability, is in fact a result of a reduction of the effective concentration of the assay by dilution in the collagen matrix, and a difference in the cell area exposed to the surrounding environment of the cells, resulting in a higher rate of dye uptake by the cells. Thus, in order to clearly understand how the 3D cell culture model affects the cell resistance and survival, the protocols used for 2D models need to be carefully improved and adapted to allow direct comparison of the results obtained.

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Cell viability assessment using the Alamar blue assay: A comparison of 2D and 3D cell culture models

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Supplementary Information

S.1 Mathematical model describing the relation between uptake rate and cell surface area

The system can be simply modeled using a rate equation approach, by which the differences between low dose and high dose responses, as well as the impact of AB solution dilution by the collagen matrix and the greater uptake rate of cells in 3D gels compared to 2D substrates may be more easily visualized. The diffusive uptake and release of the AB dye by the cell is described by

$$\frac{dN_{\text{int}}}{dt} = k_{12} \cdot A \cdot B \cdot D - k_{21} \cdot A \cdot N_{\text{int}}(t) \quad \text{Equation 1}$$

where N_{int} is the amount of dye internalized by the cells, k_{12} is the rate of internalization, D is the % dose of the dye, and k_{21} is the rate of reverse diffusion from the cells. A is a factor which normalizes for the effective exposure of the cells, depending on the

substrate, and B normalizes for the reduction of the effective concentration of the dye solution in the 3D gels.

The saturated uptake of the dye is simulated by introducing a concentration of receptors (Black and Leff, 1983), N_{recp} , which are occupied according to

$$\frac{dN_{\text{recp}}}{dt} = k_A \cdot N_{\text{recp}}(t) \cdot N_{\text{int}}(t) \quad \text{Equation 2}$$

where k_A is a receptor/dye binding rate. The fluorescence reading, I_{AB} , from the AB can then be represented by

$$\frac{dI_{\text{AB}}}{dt} = k_A \cdot N_{\text{recp}}(t) \cdot N_{\text{int}}(t) - k_{\text{tox}} \cdot N_{\text{int}}(t) \quad \text{Equation 3}$$

The second term allows for a reduction of AB fluorescence due to dye toxicity.

Using such a model, the complete time evolution and concentration dependence of the AB fluorescence can be simulated. To simulate the differences between the 3D and 2D cell culture environments, the effective area parameter A, is reduced from 1 for 3D to 0.5 for 2D, mimicking a 50% reduction of the effective area of the cells exposed to the AB solution. In the 3D environment, the effective concentration of the AB solution is reduced by 25%, and so a value of $B = 0.75$ is used. For the purpose of demonstrating the appropriateness of the model for this study, Figure S.1 shows, for example, the dose dependent %fluorescence predicted for 3hrs and 5 hrs. For collagen gels, values of $A=1$ and $B=0.75$ were employed. The figure qualitatively reproduces the behavior observed in figure 5, whereby an increasing %fluorescence is observed in the range 25-50% concentration of AB solution after 3hrs, whereas a maximum in %fluorescence is observed at a concentration of ~30% after 5hrs.

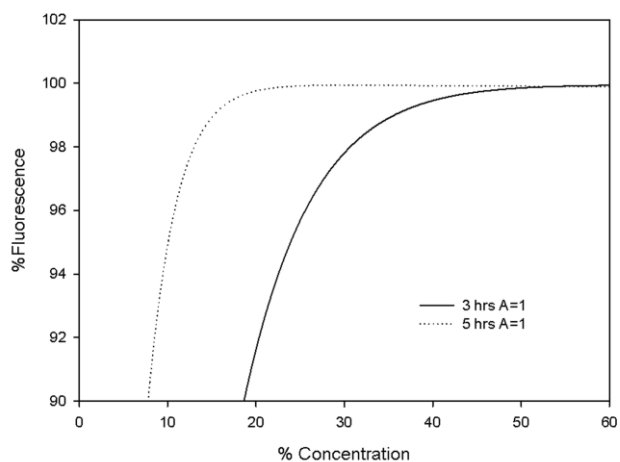


Figure S.1: Comparison of the simulated AB fluorescence after 3 and 5 hrs as a function of %Concentration of AB solution for the 3D gels ($A = 1$, $B = 0.75$)

Using the same parameters, the simulated AB fluorescence from the 3D matrices ($A = 1$, $B = 0.75$) after 3hrs can be compared with that of the 2D substrates ($A = 0.5$, $B = 1$), as shown in Figure S.2. It can be seen that, although there is a significant difference of ~25% between the viability values for the two cell culture substrates at an AB concentration of 5%, this has reduced to ~5% at an AB concentration of 35%. Although it cannot be considered a precise description of the system, the numerical simulation does indicate that the observed differences in AB fluorescence can be accounted for simply by the differences in the cell surface area which is accessible by the dye for diffusion.

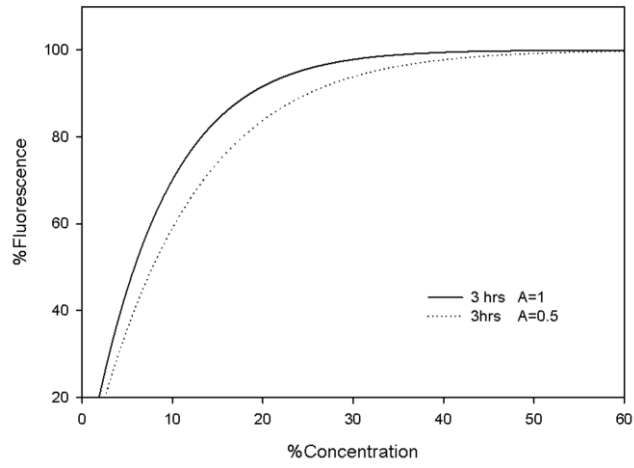


Figure S.2: Comparison of the simulated AB fluorescence after 5 hrs as a function of %Concentration of AB solution for the 3D gels ($A = 1, B = 0.75$) and the 2D substrates ($A = 0.5, B = 1$)