Multicellular Tumor Spheroids in Nanomedicine Research: A Perspective

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Multicellular tumor spheroids are largely exploited in cancer research since they are more predictive than bi-dimensional cell cultures. Nanomedicine would benefit from the integration of this three-dimensional *in vitro* model in screening protocols. In this brief work, we discuss some of the issues that cancer nanomedicine will need to consider in the switch from bi-dimensional to three-dimensional multicellular tumor spheroid models.

PROLOGUE

Researchers in the drug delivery field routinely take advantage of *in vitro* cell culture models to assess the performance of nanotechnology-based medicines, with the aim of optimizing them prior to *in vivo* evaluations in animal models and predicting their performance in patients. This is of vital importance during the development of nanoparticles (NPs) or any other nanotechnology-based drug delivery system intended to target solid cancers. Today, most of the early-stage development of cancer nanomedicine products is done using cell culture monolayers, also termed bi-dimensional (2D) models. 2D cell cultures have been an essential tool in early-stage drug delivery investigations but they tend not to be useful for prediction, because they fail to replicate the tumor macrostructure, the tumor-stroma interaction, and the heterogeneity of the tumor microenvironment (1, 2). The three-dimensional (3D) architecture of human solid tumors provides optimal conditions for cellular organization, proliferation, and differentiation (3).

The need for more reliable and predictive *in vitro* models that respect the principle of the 3Rs (Replacing, Reducing, and Refining the use of *in vivo* experimentation) has motivated researchers to develop advanced and more consistent *in vitro* models. In this context, 3D cell cultures are rapidly taking the place of conventional 2D models in biomedical research. However, despite a consistent body of evidence suggesting that 2D models largely fail to predict NP performance and/or drug efficacy and efficiency *in vivo*, 3D models have not been fully integrated into nanomedicine research and are not routinely employed in screening procedures.

Among the *in vitro* 3D cancer models under scrutiny, multicellular tumor spheroids (MCTSs) have attracted great attention and are now broadly exploited in the cancer research field (4). Indeed, over the past decades, MCTSs has been used as models to study tumor cell metabolism, tumor growth and progression in a more realistic 3D context. Being relatively simple to produce and to grow, MCTSs have been regarded as the first choice to replace 2D cell cultures in the development of cancer nanomedicines (5).

In this brief work, we will discuss the use of MCTSs in drug delivery research, with a focus on cancer nanomedicine. After an overview of MCTS general features and preparation methodologies, we will discuss some issues to consider when switching from 2D to this 3D model, and we give our perspective on the potential of MCTSs in nanomedicine. Comprehensive reviews on the subject may be found elsewhere (6–8).

MULTICELLULAR TUMOR SPHEROIDS

MCTSs are 3D aggregates of tumor cells, alone or in combination with other cell types, generally grown in absence of exogenous scaffolds (9). However, the use of scaffolds has been also contemplated. MCTSs generally have a spherical geometry, possess a 3D architecture and an extracellular matrix (ECM) in which the cells are dispersed (10). Spheroids with diameter larger than 400–500 μm have usually a structure consisting of an exterior layer of proliferating cells, an intermediated layer of quiescent cells and an internal necrotic core (**Figure 1A**) (5). As a result, the protein and gene expression profiles of cancer cells in spheroids are more similar to those of tumor cells *in vivo*, allowing more reliable evaluations of anticancer drug effectiveness when administered as a solution or loaded in nanoparticle-based carriers (11, 12). MCTSs may be maintained in culture for 2–4 weeks, thus allowing long-term experiments (13). In addition, to better mimic the tumor microenvironment (cell-cell interactions), hybrid cell spheroids, also known as mixed-cell spheroids, can be easily obtained by co-culturing cancer cells with fibroblasts, endothelial cells, immune cells, and other cell types relevant to the tumor environment (14–16).

Preparation Techniques

MCTSs may be produced by different protocols but the core principle, based on the anchorage-independent methodology, remains the same. By providing conditions in which cell-cell adhesive forces are greater than cell-substrate adhesive forces, cancer cells aggregate to form spheroids instead of adhering to a substrate (17). Hanging drop and liquid overlay are the most commonly employed methods to produce MCTSs (**Figures 1B,C**), but other methods, including agitation-induced systems, microencapsulation, and magnetic levitation, have been proposed (**Figures 1D–F**).

Hanging Drop

A drop of cell suspension is deposited on a dish lid and, upon inversion, cells are forced by gravity to accumulate at the free liquid–air interface and to form a single MCTS within the droplet (**Figure 1B**). This is the simplest and cheapest method to obtain MCTSs and co-cultures (mixed spheroids) since the use of dedicated materials, such as coated plates, might be avoided, but it requires MCTS transfer for further investigation. This method is very useful for generating uniform spheroids with a designated size and shape, even though long-term culture and media replacement remain a challenge (18). To overcome these limitations, companies have developed and commercialized different plates, such as Akura™ PLUS and Perfecta3D®, that simplify the culture procedures (19, 20).
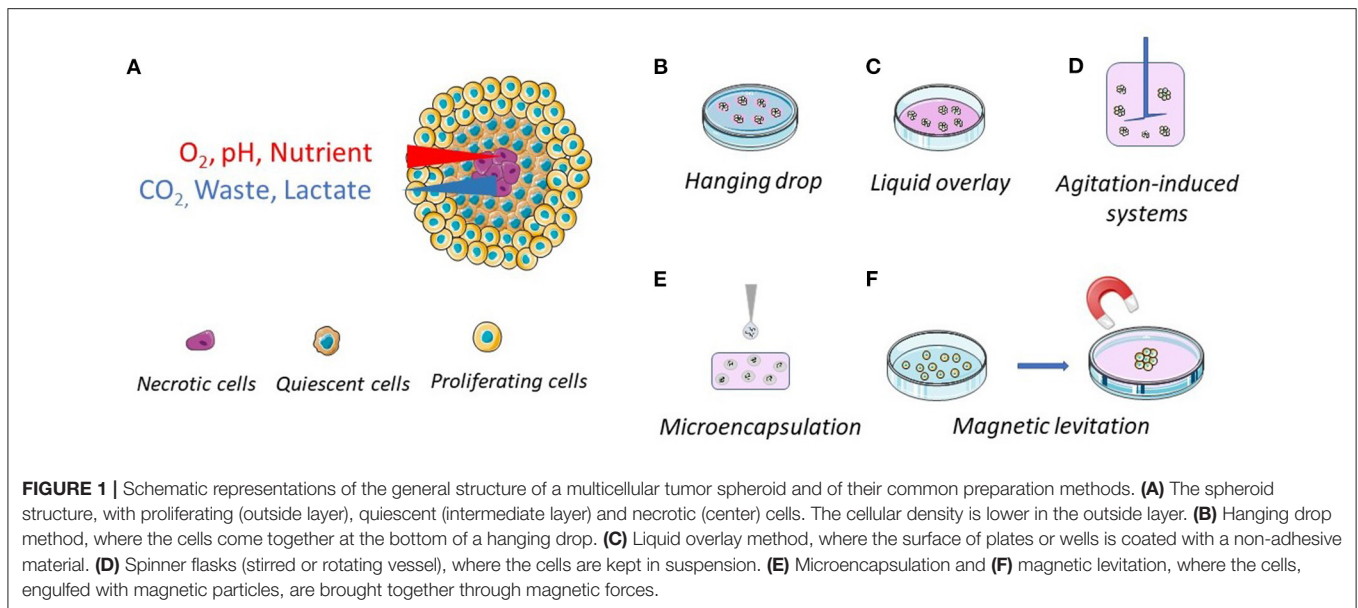
Liquid Overlay

MCTSs can be easily generated by the liquid overlay, reducing interaction between the cells and the culture surface and forcing cell aggregation (**Figure 1C**). Culture plates treated for low attachment or suspension culture are now widely available on the market. Otherwise, standard plates can be easily coated with non-adhesive substrates such as poly-hydroxyhethylmethacrylate (Poly-HEMA) (21), agar (22), or agarose (23). The major limit of this technique is the formation of MCTSs with a high variability in sizes and shapes. To overcome this limit, it is possible to apply U-bottom 96-wells plate where a single spheroid per well is obtained. Alternatively, researchers can use *ad hoc* developed plates with multiple microcavities that allow the formation of one aggregate per cavity, allowing the production of a considerable number of consistent size spheroids [examples are AggreWell™ Microwell Plates (24, 25), Corning® Elplasia® Plates (26) and Sphericalplate 5D (27)].

Agitation-Induced Systems

Non-adherent conditions can be obtained in rotating systems, including gyratory shakers, perfusion bioreactors and spinner flasks [e.g., NASA bioreactor (28)] (**Figure 1D**). In these systems, the cell suspension is maintained in motion, preventing cell interaction with the bioreactor surface. While agitation-induced systems enabled production of large pools of MCTSs, the obtained spheroids are heterogeneous in size, shape and number of cell populations. For this reason, spheroid selection is required if the spheroid size needs to be controlled (29). Even though spheroid generation via bioreactors requires expensive instruments and high quantity of culture medium, bioreactors still provide greater advantages, mainly at the industrial level due to their scalability (29).

Microencapsulation

Taking advantage of the knowledge gained in pancreatic islet encapsulation (30, 31), cancer cell microencapsulation in alginate-poly-l-lysine-alginate beads has been proposed as a method to produce MCTSs (**Figure 1E**) (32). This method has the advantage to be scalable and to allow a precise control of the spheroid size and shape. However, microcapsules with a core-shell structure, seem to be more suitable for cell growing and MCTS production. Indeed, this structure makes it possible to use different materials with diverse chemical and physical characteristics for the core and the shell. For instance, Sakai et al. developed core-shell microcapsules by first embedding the tumor cells in gelatine beads and then surrounding them by calcium alginate membranes. The gelatine core liquefies upon incubation at 37°C allowing the formation of MCTSs that can be freed from the calcium alginate membranes by incubation with sodium citrate or alginate lyase (33, 34). The core-shell particles make it possible to study cancer cell lines unable to form MCTS by the techniques described above. However, the alginate membrane generally reduces oxygen and nutrient supply, the contact between cells, and may introduce a bias. To address these issues, Alessandri et al. developed a simple microfluidics platform to produce permeable and elastic hollow microspheres. The

FIGURE 1 | Schematic representations of the general structure of a multicellular tumor spheroid and of their common preparation methods. **(A)** The spheroid structure, with proliferating (outside layer), quiescent (intermediate layer) and necrotic (center) cells. The cellular density is lower in the outside layer. **(B)** Hanging drop method, where the cells come together at the bottom of a hanging drop. **(C)** Liquid overlay method, where the surface of plates or wells is coated with a non-adhesive material. **(D)** Spinner flasks (stirred or rotating vessel), where the cells are kept in suspension. **(E)** Microencapsulation and **(F)** magnetic levitation, where the cells, engulfed with magnetic particles, are brought together through magnetic forces.

permeability of the gel allows easy diffusion of nutrients into the capsule and cell proliferation in a scaffold free environment (35).

Magnetic Levitation

Magnetic levitation was first developed by Souza et al. in 2010 (36). This system involves mixing the cells with magnetic particles and exposing them to a magnetic force during the culturing process (**Figure 1F**). It utilizes negative magnetophoresis, which mimics a weightless state. The magnetic force exerted causes the cells combined with magnetic particles to stay levitated or floating against gravity. This condition causes a geometry change of cell mass and stimulates cellular contact, leading to cell aggregation. In addition, this system can also facilitate multi-cellular co-culturing with different cell types (37–39). However, this technique presents a few drawbacks: beads are expensive and can be toxic to cells at a high concentration, and a limited number of aggregates can be produced (40).

MCTSs IN NANOMEDICINE RESEARCH

Switching From 2D to 3D *in vitro* Models

The use of MCTSs is a great opportunity in cancer nanomedicine research since it can provide more reliable information than that obtained using 2D cell cultures. However, the differences between a spheroid and a solid tumor are still enormous and the results obtained should be interpreted with caution. In fact, the size of a MCTS is at least one order of magnitude smaller than the tumor mass and the effects observed *in vitro* could hardly be reproduced *in vivo*. However, the differences in size might be mitigated somehow by the fact that, *in vivo*, NPs reach the tumor through the systemic circulation by blood vessels/capillaries, taking advantage of the EPR effect (41). The distance between vessels and the tumor necrotic area *in vivo* has been reported to be 50–250 μm, compatible with the distance

between the proliferating cell layer and the necrotic core in MCTSs (**Figure 1A**) (8).

When switching from 2D to 3D models, the data established in 2D models on NP penetration, drug release and anticancer effect will be difficult to compare with that obtained in the 3D models. For instance, the IC_{50} of an established anticancer drug can increase 1 log or more when moving from 2D to 3D, enhancing the concentrations necessary to have the effect (42). This can be even worst in the case of drug loaded NPs where NPs do not have to be taken up by a single cell layer, but must diffuse through multiple cell layers and the interstitial space.

Obviously, one must consider the drug or NP diffusion process from the medium to the spheroid. By considering a diffusion process that obeys Fick's laws (normal diffusion), the different surface-to-volume ratios of the two model systems must be taken into account. MCTSs have much lower surface-to-volume ratios than cell culture monolayers and only the peripheral cell layer is directly exposed to the drug/NP that will diffuse according to the concentration gradient. NPs are 1-2 orders of magnitude larger than a small molecule and are expected to have a lower diffusion coefficient, diffusing very slowly from the spheroid surface to the center, with the risk of remaining confined in the outer cell layers. Therefore, particle shape, size and aspect ratio are features that affect the rate of NP diffusion through MCTSs, determining the deepness of penetration and the time needed to reach the center of the cell aggregate (**Figure 2**) (43). Also, the experiment timeframe in 2D and 3D models is generally different, as longer times are needed in 3D model experiments to record the effect expected from the treatment (42).

Obviously, NP penetration and distribution within MCTSs will never be just the result of normal diffusion. It is difficult to draw general considerations, given the different processes/mechanisms involved, such as NP cell uptake by
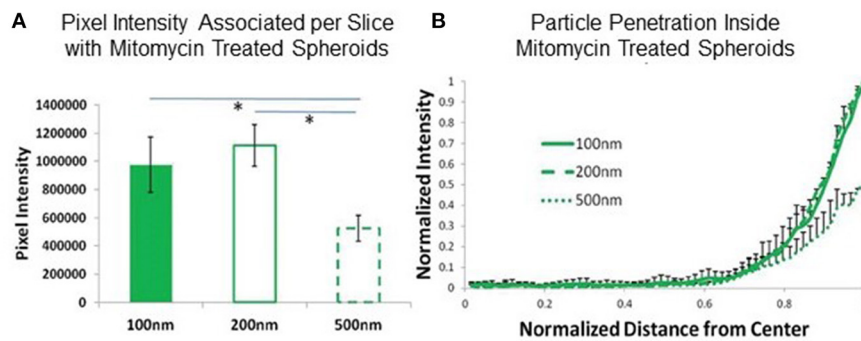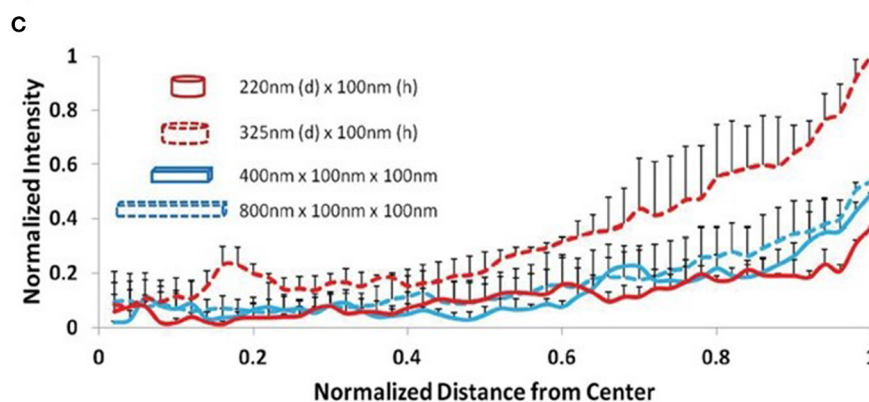
FIGURE 2 | Effect of NP size and shape on MCTS uptake and penetration. **(A,B)** Effect of NP size: uptake and penetration of spherical 100, 200, and 500 nm polystyrene particles in Mitomycin C-treated HEK 293 spheroids. **(A)** Graph showing comparison of normalized pixel intensity associated with spheroid per slice with different diameter polystyrene beads and **(B)** Normalized radial intensity distribution of polystyrene beads as a function of distance from the center of the spheroid. **(C)** Effect of NP shape: normalized radial intensity distribution of shape-specific nanoparticles as a function of distance from the center of the spheroid. Only positive error bars are shown, to allow proper visualization of data. Reproduced with permission from Agarwal et al. (43) (John Wiley and Sons License, license number 5306710208408).

endocytosis (pinocytosis and phagocytosis), NP intracellular trafficking, NP transcytosis, NP diffusion in the ECM.

By considering the not yet completely disclosed complexity of the two interacting systems (NP and MCTS), considerations on a case-by-case basis might be the best option. Indeed, "canonical" NP features, such as size, shape, surface chemistry/charge, surface decoration, are routinely measured and somehow controlled while others, such as the number of targeting moieties/nm^2 and the adsorption of biomolecules (biomolecular corona), are often neglected or underestimated.

On the other hand, MCTS characteristics, such as cell line, preparation method, mean size/size distribution, cellularity, ECM secreted, and the eventual presence of exogenous ECM (i.e., hydrogels), will play a crucial role in the NP/spheroid interaction and the effect observed (6, 7, 44). Also, MCTS comprehensive morphological and molecular characterization will be essential in order to give a correct interpretation of the results obtained and to assess the model reproducibility.

MCTSs and Medium/High-Throughput Screening

The success of MCTSs as a pre-clinical model in anticancer drug development will also depend on the possibility to use them in medium-throughput screening (MTS) or high-throughput screening (HTS) (45, 46). This possibility will also open great opportunities in nanomedicine research, a field pioneered by Prof. Langer about 20 years ago (47), which has never been really developed. MTS or HTS assays for NPs will have a tremendous benefit for nanomedicine, especially now that nanoparticle-based mRNA therapeutics have been well recognized in clinics (48, 49). Thus, the use of 3D culture technologies, MCTSs *in primis*, in cancer nanomedicine MTS/HTS is a compelling unmet need.

From the 3D model side, three significant technical challenges hamper the development of these technologies: liquid handling automation, culture optimization and assay variability, and automated imaging/visualization of the 3D structure.

The automation of liquid handling can be conducted in suspension cultures, through the use of ultra-low-attachment

microplates or the hanging drop technique (50). However, the application of automated liquid handling translates poorly when MCTSs are embedded in commercial thermogelling hydrogels. The use of thermogelling materials requires a highly controlled working environments and rapid processing due to their temperature-sensitive gelation conditions (51). Additionally, the batch-to-batch variability observed with some hydrogels considerably impacts the assay quality and reproducibility, crucial to ensure consistency when conducting MTS/HTS (52). Finally, MCTSs produced by co-culturing multiple cell types have much greater morphological complexity than simpler spheroids and 2D cultures, and multiparametric analysis will be required to investigate and quantify the cell response the treatment (53).

In the case of nanomedicine, an accurate analysis of nanoparticle uptake and/or localization would be of great value. However, their tridimensionality also poses a difficulty in computational image analysis and visualization: the complex topology and the thickness of MCTSs, as for other 3D models, make image analysis challenging, and are incompatible with most automated imaging systems due to low light penetration and absorption across the multi-layered structures (52).

Despite these challenging barriers, new culture platforms and imaging systems are being developed to overcome the technical issues that hinder the use of MCTSs in MTS/HTS. These developments include using synthetic hydrogels to generate more consistent 3D cell cultures, automated high-resolution imaging using light-sheet microscopy, and integrated computational platforms for data analysis and visualization of 3D structure (54–56).

However, from a nanomaterial perspective, the issue of producing and handling NP libraries remain unsolved. In fact, MTS/HTS can be relatively easily applicable to the material *per se* (polymers, lipids) but it become a struggle in the case of complex NPs designed and engineered for cancer drug delivery and targeting. Nanotechnology-based medicines under scrutiny today, given their surface functionalization with fragile biomacromolecules and their anticancer drug cargo, are much more difficult to handle than small molecule drugs.

CONCLUSIONS AND PERSPECTIVE

The application of MCTSs in cancer nanomedicine offers a great opportunity, since it is the first step to narrow the gap between pre-clinical and clinical research, making the process of nanotechnology-based medicine development more efficient and successful. More important, this will surely pave the way for the routine application of more advanced *in vitro* 3D models, such as organ-on-a-chip and organoids, in academia and industry. The use of MCTS in MTS/HTS is still challenging but recent technological advances might solve some of the issues, making this possible in the near future.

However, the switch from 2D to 3D *in vitro* models will not be painless, since researchers will be obliged to consider the higher complexity of the model during the design of the experiment, for example, adapting the experiment timeframe and the treatment concentrations as well as the assays needed to have the desired outcome. It will be necessary to go deeper in MCTS biological characterization (e.g., cell and molecular biology, biochemistry) to know and, if possible, control the experimental parameters that will affect the experimental results.

In addition, since most, if not all, *in vitro* data available from the last 40 years of nanotechnology-based drug delivery research were generated on classical 2D models, for which comparisons with 3D models will be extremely difficult, researchers will have to contemplate a sort of "re-evaluation" of NP technologies discarded because of their failure on cell culture monolayer assays.

In conclusion, MCTSs will become the standard *in vitro* model to assess the nanomedicines performance in adulthood cancers, and cell culture monolayers will be used to obtain complementary information, such as mechanistic understandings of NP uptake, binding affinity, and translocation into intracellular compartments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

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