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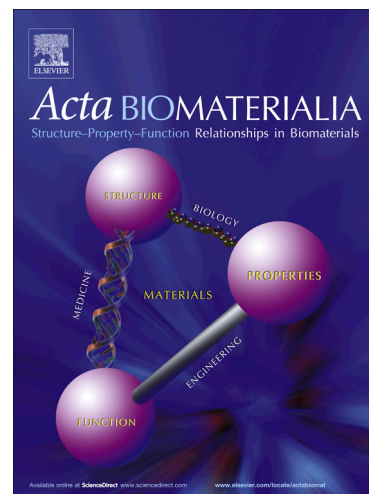
Design of Spherically Structured 3D *In vitro* Tumor Models -Advances and Prospects

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Abstract

Three-dimensional multicellular tumor models are receiving an ever-growing focus as preclinical drug-screening platforms due to their potential to recapitulate major physiological features of human tumors *in vitro*. In line with this momentum, the technologies for assembly of 3D microtumors are rapidly evolving towards a comprehensive inclusion of tumor microenvironment elements. Customized spherically structured platforms, including microparticles and microcapsules, provide a robust and scalable technology to imprint unique biomolecular tumor microenvironment hallmarks into 3D *in vitro* models. Herein, a comprehensive overview of novel advances on the integration of tumor-ECM components and biomechanical cues into 3D *in vitro* models assembled in spherical shaped platforms is provided. Future improvements regarding spatiotemporal/mechanical adaptability, and degradability, during microtumors *in vitro* 3D culture are also critically discussed considering the realistic potential of these platforms to mimic the dynamic tumor microenvironment. From a global perspective, the production of 3D multicellular spheroids with tumor ECM components included in spherical models will unlock their potential to be used in high-throughput screening of therapeutic compounds. It is envisioned, in a near future, that a combination of spherically structured 3D microtumor models with other advanced microfluidic technologies will properly recapitulate the flow dynamics of human tumors *in vitro*.

Abbreviations: Three-dimensional Multicellular Tumor Spheroids (3D-MCTS), Tumor Extracellular Matrix (Tumor-ECM), Three-Dimensional (3D), Tumor Microenvironment (TME), Mesenchymal Stem Cells (MSCs), Poly-L-Lysine (PLL), Human Adipose-derived Stem Cells (hADSCs), Alkaline Phosphatase (ALP), Matrix Metalloproteinases (MMPs), Epithelial-to-Mesenchymal-Transition (EMT), Green Fluorescent Protein (GFP), Epidermal Growth Factor (EGF), Basic Fibroblast Growth Factor (bFGF), Platelet-Derived Growth Factor (PDGF), Hyaluronic Acid (HyA), Stromal Cell-Derived Factor A (SDF-1α), Vascular Endothelial Growth Factor (VEGF), Bone Morphogenetic Protein 2 (BMP-2), Human Bone-Marrow Derived Mesenchymal Stem Cells (hBM-MSCs), Decellularized Matrices (dECMs), Poly(ethylene glycol) (PEG), Polylactic Acid (PLA), Poly(Glycolic Acid) (PGA), Poly(Lactic-co-Glycolic Acid) (PLGA), Poly-ε-Caprolactone (PCL), Polyvinyl Alcohol (PVA), Polystyrene (PS), Cancer-Associated Fibroblasts (CAFs), Prostate Cancer Stem-Like Cells (PCa-CSCs), Human Umbilical Vein Endothelial Cells (HUVECs), Poly(ethylene glycol) Diacrylate (PEGDA), Hematoxylin-Eosin (H&E), Red Fluorescent Protein (RFP).

1. Introduction

2D flat cell cultures of cancer cells remain the most commonly used *in vitro* platforms for screening anti-cancer therapeutics, despite their recognized inability for mimicking three-dimensional (3D) cellular organization and tumor proliferation kinetics [1–3]. Moreover, these *in vitro* models lack the ability to correctly mimic tumor stromal heterogeneity and tumor-ECM components. Adding to these limitations, nutrients, oxygen, and pH gradients are not recapitulated, resulting in a recognized inability to realistically mimic *in vivo* tumors [3]. Overcoming the shortcomings of conventional 2D cultures through the engineering of more robust *in vitro* models capable of simulating *in vivo* solid tumors, could improve the efficacy of anti-cancer drug discovery and biological performance screening [1]. The development of such models could contribute to reduce the number of false-positive results obtained during preclinical validation of novel compounds and improve the *in vitro/in vivo* correlation.

In this context, three-dimensional (3D) *in vitro* models have been gaining increasing momentum in the field of drug-screening and cancer research, due to their improved ability for recapitulating the complexity of the tumor microenvironment (TME) [4,5]. 3D culture models are capable of recapitulating tumors cellular heterogeneity, cell-cell interactions, and spatial architecture. In fact, the reproduction of such characteristics in 3D promotes the establishment of nutrient, oxygen, and signaling factor gradients, as well as the establishment of unique gene expression patterns similar to those observed in *in vivo* solid tumors [6].

From the currently available *in vitro* tumor models, 3D multicellular tumor spheroid models (3D-MCTS) remain one of the most commonly explored [7,8]. Their relative ease of assembly, reproducibility, and the ability to capture cellular heterogeneity (e.g., co-cultures of cancer-stromal cells), renders them suitable tumor surrogates for preclinical validation of novel therapeutic compounds [9]. Up to date, various 3D-MCTS *in vitro* models have been used to modulate the cellular components present in the TME of different tumors including those of breast [10], colon [11], pancreas [12] and lung [13]. However, most of these models still lack a complete representation of tumor-specific and disease stage-specific ECM. This is a critical component which is recognized to extensively influence cancer evolution through key biochemical and biomechanical cues [14,15].

3

To overcome these limitations, various studies have attempted to include ECM mimetics in the form of spherically structured scaffolds, namely microparticles or microcapsules. These technologies have been extensively used in the field of tissue engineering and stem cell research [16–18], and offer further opportunities to mimic the complexity of the TME *in vitro*. In fact, this approach opens the possibility to study biochemical and tumor-ECM dependent mechanical cues through the inclusion of modular matrix-mimetic scaffolds [19]. The inclusion of tumor-ECM components in a 3D spherical geometry allows researchers to control various key parameters, such as: (i) pH, oxygen, and nutrient perfusion gradients, (ii) cell-cell interactions, (iii) morphology, and (iv) tumor models overall size. Importantly, previous studies have associated variations in 3D tumor microtissues size and morphology with variability in phenotype, gene expression profile, as well as with the degree of response to anti-cancer or anti-stromal therapeutics [20,21]. The integration of spherically structured scaffolds morphology into tumor modeling is herein demonstrated to not only allow an increased control over produced spheroids biophysical properties, but also to increase models' reproducibility in terms of shape and size, both of which are major aspects that must be considered in drug screening assays. Furthermore, implementation of spherical designs facilitates incorporation of tumor-ECM components in well-established methodologies for spheroids analysis [22]. This unlocks the opportunity to model cell-ECM interactions and to evaluate the influence of ECM components inclusion in the response to anti-cancer therapeutics in a high-throughput compatible mode.

On this focus, this review showcases and critically discusses the recent advances in the field of complex 3D *in vitro* tumor models' assembly via spherical scaffolds. We begin by summarizing current scaffold-free and scaffold-based 3D microtumor production technologies and present up-to-date examples on the use of microparticles, microspheres, and microcapsules to assembly advanced 3D-MCTS. A critical perspective regarding future developments on new models that fully recapitulate *in vitro* the cellular and acellular components of the TME is also provided.

2. *In vitro* 3D Models Production

Ideally, 3D tumor models should be able to recapitulate the cell-cell and cell-tumor ECM crosstalk established during tumor progression. This communication is well recognized to contribute to the establishment of either pro-tumoral or anti-tumoral

4

microenvironments [23,24], depending on the type of cells and ECM properties. Such dichotomy is well portrayed by the communications that can be established between immune cells and mesenchymal stem cells (MSCs) present in the TME. In fact, MSCs-immune cells communications can lead both to immunosuppression and promotion of angiogenesis, or to the increased recognition of cancer cells by infiltrating immune cells. Such crosstalk is precisely mediated by the release of anti- and pro-inflammatory cytokines and growth factors, which can exert different effects depending on their amount and combinations [25,26]. Therefore, to recapitulate human tumors, 3D models must mimic this dichotomy, as well as ECM mechanical properties, cells spatial arrangement, and spatiotemporal biochemical composition of the TME. *In vitro* 3D microtumors should also be produced to dynamically recapitulate specific disease stages (e.g., from proliferation to metastasis) under highly reproducible conditions, so as to assure a direct correlation between *in vitro* and *in vivo* data [27].

3D multicellular tumor models production methodologies can be divided into three major categories: (i) scaffold-based models which take advantage of diverse natural, or synthetic materials that aim to mimic *in vivo* tumor-ECM [28–30]; (ii) scaffold-free models, which take advantage of cells suspension or hanging-drop techniques for assembly of 3D-MCTS [31–34]; and (iii) combinatorial hybrid technologies such as microencapsulation or microparticle-based approaches, which seek to combine the cell-cell aggregation obtained in scaffold-free based methods with the ECM representing capacities of 3D scaffold-based platforms. The following sections will discuss the differences and common advantages/disadvantages of currently employed technologies for the establishment of 3D-MCTS. In section 3, a focus is provided on spherically structured assembly of 3D *in vitro* models as these technologies have potential to emulate the different components of *in vivo* tumors TME.

2.1. Scaffold-free 3D Models Production

Scaffold-free methods are based on the implementation of cell cultures under non-adherent conditions (Figure 1). The main aim of this strategy is to promote the production of 3D spherical (spheroids) or more loosely aggregated microtissues (cellular aggregates) [35–37]. Despite scaffold-free models nomenclature remains non-regulated to date, in general terms it can be classified into three distinct categories: (i) multicellular tumor spheroid models (3D-MCTS), initially implemented in the 1970s by Sutherland and co-

5

workers [38], (ii) organotypic multicellular spheroids (e.g., fragments of tumor tissue cultured in non-adherent conditions), and (iii) tissue-derived tumor spheres obtained by partial disruption of tumor tissues through mechanical and/or enzymatical dissociation [39]. These microtissues are easy to assemble, to culture *in vitro*, and have been extensively used in the field of 3D *in vitro* disease modeling for drug screening and more fundamental biology studies.

Static-based, scaffold-free methods such as forced-floating or hanging-drop techniques allow the assembly of highly reproducible 3D-MCTS in terms of size and morphology [40,41]. These techniques employ super-hydrophobic surfaces, or for example, poly(2-hydroxyethyl methacrylate) [42], or agarose coated multiwell plates [31,43,44] (Figure 1), to prevent adhesion to culture plate surfaces and promote instead cell-cell interactions that lead to intercellular adhesion and aggregation [32]. These 3D-MCTS can be formed either by monotypic or heterotypic co-cultures (e.g., comprising cancer and stromal cells) [45,46]. Over time, the newly formed 3D-MCTS start to secrete their own ECM, which increases their density and diminishes their size, further approaching these models to compact solid tumors similar to those obtained *in vivo* [47].

Dynamic, stirring-based technologies can be grouped into two classes: (i) stirring tank bioreactors in which the culture media is internally impelled (e.g., by spinning blades), and (ii) rotational (microgravity) bioreactors [48] (Figure 1). Such methods take advantage of mechanical forces that maintain cells in continuous suspension during culture [35].
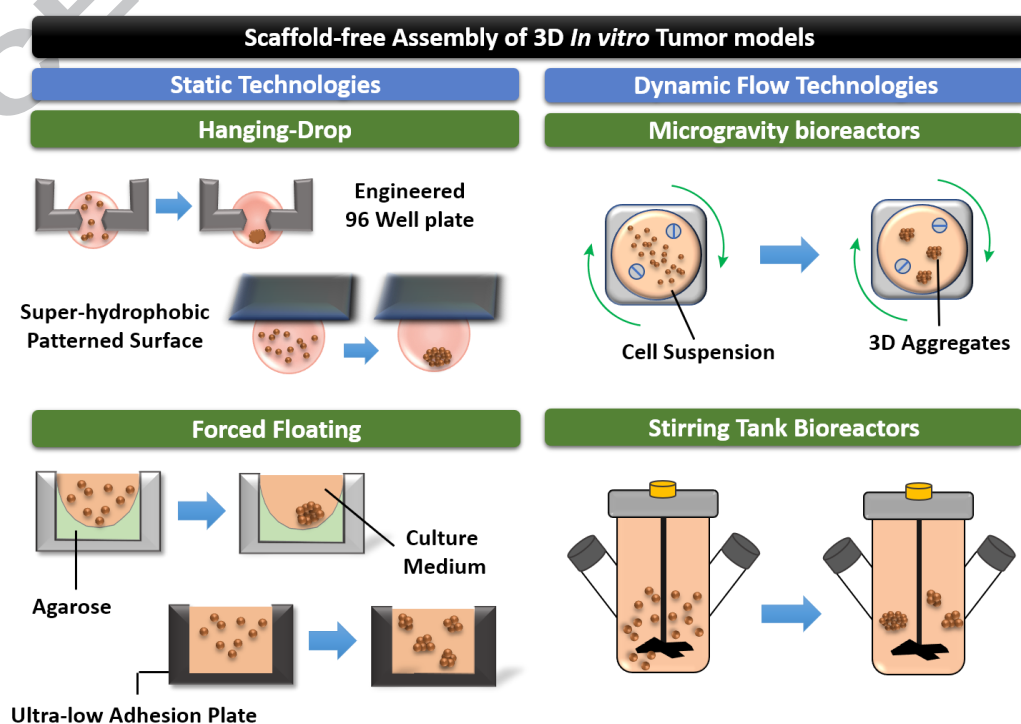

6

Figure 1. Schematics of scaffold-free technologies used for 3D *in vitro* tumor spheroids assembly. Static technologies are useful to obtain 3D microtissue with highly reproducible sizes but are unable to mimic the mechanical forces that cells/cellular 3D aggregates experience when cultured under dynamic flow conditions. Image layout adapted from Picollet-D'hahan and co-workers [49], with permission from Elsevier.

By comparison to stirred tank bioreactors, rotational (microgravity) bioreactors have the advantage that the internal flow is generated by rotation of the container and not through blade mechanisms, thus imparting a lower shear-stress to the cultivated cells [50].

In comparison to static methods, stirring-based methodologies are able to effectively produce high amounts of 3D-MCTS, having the advantage of allowing easy culture medium exchange and modifications to cell culture conditions *in situ* [51]. However, regarding 3D aggregates reproducibility, stirring-based techniques lack a suitable level of control over 3D spheroids morphology and size, an important parameter that is generally present in static-based methodologies [52]. In fact, 3D spheroids and cellular aggregates obtained in bioreactors frequently exhibit variable shapes and density, leading to inconsistent responses to anti-cancer therapeutics [27,41]. Since cellular concentration is dictated for the entire batch, different internal dynamic flows can also result in aggregates with different sizes [53].

The combination of stirring and forced adhesion methods could overcome such limitations. For example, one could combine hanging-drop technique to obtain highly uniform 3D spheroids, and then translocate them into bioreactors or microfluidic platforms for evaluating fluid dynamics influence [54]. Overall, the main advantages of scaffold-free combination with dynamic culture methods are: (i) the ease of manufacturing multiple and reproducible 3D microtissues per batch; (ii) the ability to maintain prolonged culture times; (iii) the ability to modify the culture media and growth conditions (e.g., media perfusion flow rate, nutrient starvation); and (iv) the ability examine 3D models evolution in time either by single direct analysis in situ [55], or by placing them in hanging-drop super-hydrophobic, on-chip arrays [33,56]. In a study performed by Oliveira and co-workers [57], such super-hydrophobic surfaces were employed to produce osteosarcoma spheroid arrays for anti-cancer drug-screening. Superhydrophobic surfaces patterned with wettable spots were successfully applied for osteosarcoma cells (Saos-2) 3D spheroids formation, and allowed the establishment of dynamic platforms capable of simulating

7

chemotherapeutics clearance through means of dynamic media renewal [57]. In addition, these platforms were also recently explored by Oliveira and co-workers [58], for stem cell-based tissue engineering. In this study, human adipose-derived stem cells (hADSCs) 3D spheroids were established under either direct or indirect co-culture with 2D cell layers. The cells were cultured in the flat microarray surface where a droplet of cells that formed the 3D spheroids was also formed. Co-culture of 3D hADSCs with 2D layers of HUVECs or Saos-2, resulted respectively in a significant decrease and slight increase in alkaline phosphatase (ALP) expression, an enzyme associated with hADSCs osteogenic differentiation, Therefore, such high-content imaging compatible, spheroid forming arrays, could be used in the future to study diverse stromal cells and 3D tumor spheroids direct or indirect interactions [58].

2.2. Scaffold-Based 3D Models Production

Tumor evolution *in vivo* is intimately correlated with the interactions between cells of the TME and their supporting ECM which provides both structural and signaling functions [59]. In a general perspective, the ECM is comprised mainly by fibronectin, collagen (types I-V), elastin, entactin, fibrillin, fibulin, vitronectin, laminin, as well as other glycoproteins and proteoglycans such as hyaluronic acid [60]. The exact composition of the ECM surrounding the primary tumor site can vary according to the type of tissue. Hence, the design of 3D *in vitro* models must take into account tissue, and patient, ECM specificity, since this variability can lead to different response rates to candidate anti-cancer therapeutics [27]. Furthermore, during tumorigenesis, alterations in matrix composition and structure occur over time due to cancer and stromal cell-mediated ECM deposition or degradation (mainly through matrix metalloproteinases (MMP) enzymatic digestion (e.g., MMP-9, MMP-2). This matrix rearrangement often involves collagen deposition and matrix stiffening, a phenomenon that is associated to an increased metastatic potential [61]. Moreover, various reports indicate that increased hyaluronan deposition and degradation occurs in several cancers. Such increases angiogenesis, metastasis and possibly drug-resistance [62–64].

It is this dynamic nature of the interactions established between all the cells in the tumor microenvironment and the surrounding ECM that scaffold-based tumor models aim to fully recapitulate. Scaffold-based 3D *in vitro* tumor models take advantage of natural, synthetic or hybrid biomaterials to culture cells in a TME-like milieu [19,35]. Each of

8

these classes presents its own advantages and disadvantages in mimicking *in vivo* tumors on an *in vitro* setting (Table 2). While naturally derived ECM mimetics have relatively low batch-to-batch reproducibility, scaffolds formulated with synthetic materials although more reproducible may require functionalization with bioactive molecules or inclusion of naturally-derived bioactive cues (e.g., peptides, adhesion proteins, etc), thus originating hybrid scaffolds. Despite the increased chemical modifications required in synthetic or hybrid scaffolds, the introduced biofunctionalizations can provide the opportunity to manipulate ECM mesh alignment, elasticity and swelling behavior, either by using static, or reversible precision chemistry reactions [68]. Hence, key events such as matrix stiffening can be simulated and precisely controlled [69,70].

Table 1. Advantages and disadvantages of different origin materials used for the production of scaffold-based 3D *in vitro* tumor models.

Class	Origin	Examples	Ref.	Advantages	Disadvantages
Natural	Mammalian	Collagen	[66,71]	▪ Contain *in vivo* similar domains (e.g., laminin, elastin, fibronectin) ▪ Cellular adhesive properties ▪ Recapitulate cells-ECM interactions present *in vivo* ▪ Enzymatically degradable	▪ Exact composition is unknown ▪ Batch-to-batch variability ▪ Limited level of control over matrix stiffness along time
		Matrigel™	[72,73]		
		Hyaluronan	[74,75]		
		Gelatin	[76,77]		
		Decellularized Matrix	[78,79]		
	Non-mammalian	Alginate	[80,81]	▪ Cell adhesion properties ▪ High biocompatibility ▪ Affordable	▪ May require further modification to simulate *in vivo* tissues ECM components ▪ Fabrication methods can be cytotoxic
		Chitosan	[82,83]		
		Silk-fibroin	[84,85]		
Synthetic		Polyethylene glycol (PEG)	[86]	▪ Good structural definition and chemically defined ▪ Highly tunable mechanical properties	▪ Lack ECM-mimicking domains ▪ Require further modification to increase bioadhesion and biocompatibility ▪ Degradation can result in
		Polylactic acid (PLA)	[87,88]		
		Poly-ε-caprolactone (PCL)	[89,90]		

9

	Poly (lactic-co-glycolic acid) PLGA	[87,88]		acidic by-products	
Hybrid	Alginate-RGD	[91,92]	• Combine the ease of chemical modification and the presence of ECM-like domains	• High-costs	
	PEG-RGD	[93,94]		• Representation of few ECM components	
	PEG-fibrinogen	[95,96]			

These ECM mimetic scaffolds can be manufactured into diverse structures such as: (i) fibrillar porous meshes, (ii) porous and non-porous microstructures (including microparticles or microcapsules), and (iii) micro-patterned surfaces, via 3D bioprinting technologies [28,35,97–101]. The production methodologies of scaffold-based *in vitro* models have been extensively reviewed elsewhere [19,102–104]. The different materials that are used for the manufacture of these scaffolds are selected by their specific characteristics, such as the rate of biodegradation, biocompatibility, elasticity, ease of manipulation and similarity to tumor-specific ECM. In this context, the following sections will review the most commonly used materials for these models, starting with natural-derived scaffolds and moving to synthetic and innovative combinatorial hybrid approaches used for correct ECM recapitulation.

2.2.1. Natural materials-based Scaffolds

From natural material-based scaffolds, the most commonly used hydrogel type for *in vitro* production of 3D-MCTS [28] is Matrigel[TM], a hydrogel matrix comprised by basement-membrane extracts obtained from Engelbreth–Holm–Swarm mouse tumors [73]. The assembly of this hydrogel often requires laborious preparation, involving cooling of all the materials and thawing in ice to prevent premature polymerization, since the material is liquid at 4º C and jellifies above 10ºC [105]. When crosslinked, Matrigel[TM] forms a randomly weaved mesh of fibers which withholds a large amount of fluid. Several types of Matrigel[TM] formulations with various concentrations can be obtained commercially, such as those produced by Corning Life Sciences[®], BD Biosciences[®], and Trevigen[®] [106]. This scaffold has been used to establish different types of 3D organoids (Figure 2A) and 3D spheroid (Figure 2B) models of various cancers, including those recognized by a highly aggressive progression such as pancreatic cancer [107].
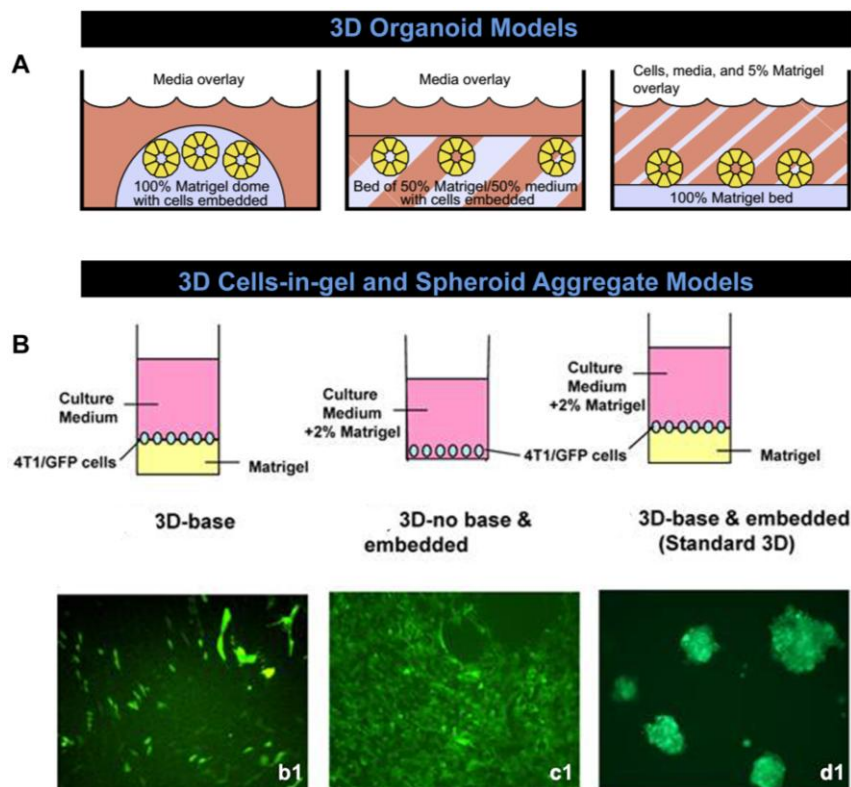
10

Figure 2. Different types of 3D *in vitro* models that can be assembled by using Matrigel[TM]. (A) Cross section schematics of different types of assemblies to establish 3D pancreatic tumor organoids. (B) Cross section schematics of different types of assemblies to establish 3D cell cultures, namely cells in Matrigel[TM] and spheroids. (b1, c1, d1) Fluorescence microscopy micrographs of green fluorescent protein (GFP) expressing 4T1 breast cancer cells after 5 days of culture. (b1) Pre-coated 96 well plates with a 3D base comprised of Matrigel[TM], where 4T1-GFP cells are cultured in normal cell culture medium. (c1) 4T1-GFP cells cultured in cell culture medium containing 2% Matrigel[TM]. (d1) Culture of 4T1-GFP cells in gel bed and gel containing medium, clear cellular aggregates are observable. Reproduced from Baker and co-workers [107], and from Li and co-workers [108], with permission from Elsevier and Ivyspring International Publisher, respectively.

Due to its *in vivo* origin, Matrigel[TM]-based scaffolds introduce ECM-specific signaling molecules and binding domains, such as laminin, collagen, elastin, entactin, fibronectin, fibrinogen and different growth factors (e.g., Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), Platelet-derived growth factor (PDGF)), among others [109]. Such bioactive components provide for example integrin and MMP binding sites, both of them imperative in tissue organization and cancer metastasis [60]. As a result of their origin, natural-based scaffolds exhibit similar structural interactions to those found in humans providing a suitable *in vivo*-like matrix where different cell lines can proliferate and acquire a stem-like phenotype [109,110]. However, due to the animal origin of Matrigel[TM], this material also exhibits significant batch-to-batch variability [109], which results in low data reproducibility [27]. Furthermore, the complex and variable protein composition reduces the possibility to mimic tissue-specific ECM environments [27]. For

11

example, Matrigel[TM] does not contain proper ratios of collagen type I or hyaluronan as those found in the matrix of *in vivo* tumors [105]. Moreover, it is important to emphasize that dynamic control over matrix stiffness in a dynamic mode is not possible [111]. However, it is possible to obtain Matrigel[TM] formulations with different protein concentrations and thus manufacture gels with tunable elastic moduli. Such is an important aspect in 3D *in vitro* tumor models' establishment, especially since matrix stiffness has been directly correlated with cells migration processes [112].

Other examples of natural based scaffolds commonly used for the assembly of 3D tumor models include collagen, hyaluronic acid, alginate, chitosan and silk fibroin hydrogels, as well as decellularized ECM. Most of them present, tunable mechanical properties, high biocompatibility and cell adhesive features [88]. The advantages and disadvantages of the use of these materials for assembly of 3D tumor models are summarized in Table 1. These materials have recently received increased focus as scaffolds for assembly of 3D tumor models in several reports [66,85,117–125,88,102–104,113–116].

Collagen-based 3D models can be assembled either through physical or chemical\enzymatic crosslinking [126], leading to the establishment of a fibrillary scaffold. Collagen is the common constituent of the tumor microenvironment, being increasingly deposited during tumor progression for example in breast, lung, and colon cancers [127,128]. These natural 3D cell culture platforms contain key cellular adhesion domains and trigger signaling events capable of stimulating *in vivo* like morphology and gene expression, as demonstrated by Cheng and co-workers [66]. In this study, breast cancer cells (MCF-7) seeded on collagen porous scaffolds expressed high levels of pro-angiogenic factors (vascular endothelial growth factor (VEGF), bFGF and IL-8), matrix metalloproteinases (MMP-2, MMP-9). Additionally, cancer stem cells (CSC)-like populations (CD44^{+}/CD24$^{-/low}$) exhibiting increased epithelial-mesenchymal transition (EMT) markers expression (e.g., CT4A, SOX2, SOX4, JAG1) were detected [66]. Moreover, through the variation of protein content, or addition of synthetic cross-linking agents scaffolds stiffness, elasticity and fiber alignment of collagen gels and scaffolds can be varied to simulate matrix stiffening that occurs during disease progression [86]. As such, collagen hydrogels have been extensively used by various researchers to establish *in vitro* models for evaluation of EMT and cellular migration. For example, collagen has been

used to study breast cancer cell invasion through variations of fiber alignment and scaffold stiffness [71]. A study by Fraley and co-workers [117], analyzed the effect of distinct collagen gels crosslinking level, pore size and varying fibrillar alignment combinations on cancer cells mobility and ability to migrate in complex ECM mimetic matrices [117]. The main results demonstrated that according to fiber alignment the protrusion rate of cancer cells and their respective orientation was greatly influenced. Furthermore, the levels of matrix metalloproteinase activity and their respective inhibition were highly dependent on matrix structure and collagen density, mimicking the complexity of *in vivo* tumor-associated matrix. This capacity to modulate cancer progression and invasion highlights the necessity of standardizing ECM mimetic microstructure characterization and composition. However, given the origin and composition of collagen, these *in vitro* scaffolds are prone to batch-to-batch variations, similar to those obtained with Matrigel[TM].

Other natural origin material that has been extensively investigated for assembly of 3D *in vitro* tumor models is hyaluronic acid (HyA). HyA is a major glycosaminoglycan found ubiquitously in normal and malignant tissues ECM, being comprised of D-glucuronic acid and D-N-acetylglucosamine residues [129]. HyA has been closely associated with cancer progression [130,131], and the presence of elevated quantities of HyA in the tumor stroma has been associated with poor patient outcome [132]. Given HyA importance in the TME a diverse array of studies has employed HyA-based scaffolds to establish 3D *in vitro* tumor models. Some of these studies take advantage of HyA unique chemical versatility that allows tailoring of its mechanical properties via precision chemical modification. One recent study that explores this possibility is that reported by Shen and co-workers [116], which developed a 3D scaffold based on acrylated hyaluronic acid hydrogels containing MMP-1 and MMP-2 sensitive peptides and bioadhesive RGD-based domains for the culture of fibrosarcoma cells (HT-1080). Using varying concentrations of the MMP sensitive peptides as a bridging moiety between acrylated hyaluronic acid chains, hydrogels with diverse degrees of crosslinking and different mechanical properties were formulated (Figure 3A). The use of an MMP-responsive HyA hydrogel with different crosslinking degrees allowed to study fibrosarcoma cells pro-angiogenic potential in different conditions that mimicked the native TME. An analysis of HT-1080 cells angiogenic sprouting potential demonstrated that in atmospheric conditions angiogenic induction only took place in soft and mediumly crosslinked HyA gels.

13

Whereas, the establishment of hypoxic conditions lead to sprouting even in highly dense HyA hydrogel networks (Figure 3F).
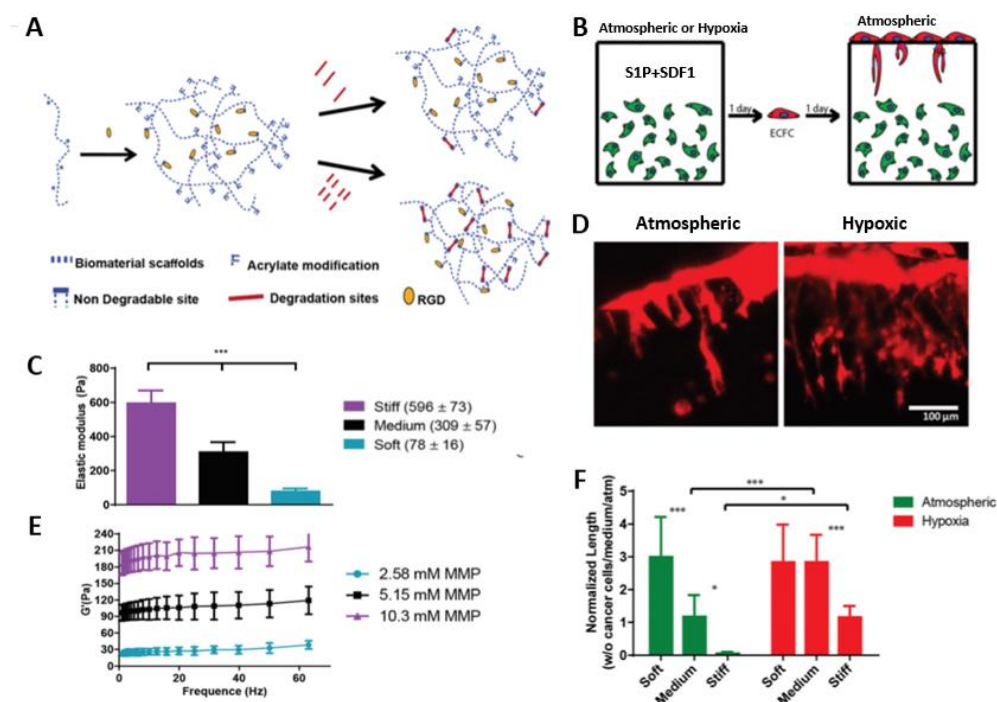

Figure 3. Establishment of 3D *in vitro* tumor models based on Hyaluronic acid hydrogels. (A) Schematic representation of Acrylated HyA hydrogels demonstrating crosslinking through MMP sensitive peptide bridging and functionalization with RGD-containing peptides. (C, E) The concentration of MMP sensitive crosslinkers allowed the control over gels viscoelasticity. Fibrosarcoma cells were cultured in acrylated HyA gel with varying stiffness, and under atmospheric or hypoxic conditions. (B) Schematic representation of sprouting assay. (D) Fluorescence microscopy of lectin stained (red) endothelial cells infiltrating the hydrogel containing fibrosarcoma cells supplemented with Stromal cell-derived factor α (SDF-1α). (F) While under non-hypoxic conditions no penetration occurred in stiff hydrogels, in 1% oxygen sprouting was observable. Reproduced from Shen and co-workers [106], with permission from The Royal Society of Chemistry.

Alginate, chitosan, and silk fibroin are examples of biopolymers derived of non-mammalian origins that have also been used for establishing 3D *in vitro* tumor models. These materials present low immunogenicity and suitable biocompatibility for *in vitro* cell culture [88]. Alginate is derived from brown algae and formed by repeating units of α-L-guluronic acid and β-D-mannuronic acid. Variations in the relative concentration of the two monomeric units produce changes in alginate physical and chemical properties, and the formation of gels with higher or lower water holding capacity and tunable porosity [133]. Alginate has been extensively used for cell encapsulation due to its capacity to rapidly crosslink under physiological conditions, easily forming a matrix that allows for medium and metabolite exchange [134,135]. However, it is important to mention that alginate possesses no cell adhesion properties, being often chemically modified with peptide-moieties or combined with other types of bioactive polymers [136].

14

Chitosan is another biopolymer that has been extensively used to form porous scaffolds for cell culture and tissue engineering due to its biodegradable, non-immunogenic and cationic nature which also promotes a facile functionalization with anionic molecules such as anionic glycosaminoglycans (e.g., HyA, heparan sulfate, heparin, chondroitin sulfate, keratan) [137]. The overall cationic charge of chitosan is attributed to its polymeric backbone which is comprised of glucosamine and N-acetylglucosamine units [137]. Chitosan has been conjugated with other types of biopolymers (e.g., alginate, HyA) [138,139] or macromolecules (e.g., bioadhesion domains, signaling cytokines such as VEGF or bone morphogenetic protein 2 (BMP-2)) to form biofunctional 3D scaffolds for tumor *in vitro* modeling [140,141].

Combinations of both alginate and chitosan to produce 3D *in vitro* disease models are one of the most common. For example, in 2013, Kievit and co-workers [118], formulated a highly porous polyelectrolyte chitosan-alginate (CA) composite scaffold that was used to establish a 3D glioblastoma model to study cancer cells (U-87) migration. The manufactured CA scaffold presented well-defined and aligned-fibers with small diameters ranging from 200 nm to 1.1 μm. This promoted glioblastoma cells 3D migration and evidenced that this may be a cost-effective testing platform to screen for anti-metastatic therapies. Furthermore, later in 2016, Kievit and co-workers employed a similar scaffold to study glioblastoma cells interactions in diverse ECM mimetic or non-mimetic scenarios [119]. In this study, different CA scaffolds, namely those coated with poly-ε-caprolactone (PCL) (lacking bio-adhesion domains), or coated with HyA (allowing interaction with cell receptors – CD44/HyA interaction) were investigated (Figure 4G). The produced scaffolds where then used for glioblastoma 3D cell culture in mono or co-culture conditions, using astrocytes and endothelial cells to better mimic glioblastoma TME and its complex cell populations (Figure 4D, E, F) [119]. The obtained results showed that cells cultured in scaffolds coated with ECM mimetic HyA had an increased expression of stemness markers (CD44 and CD133) (Figure 4G, H).
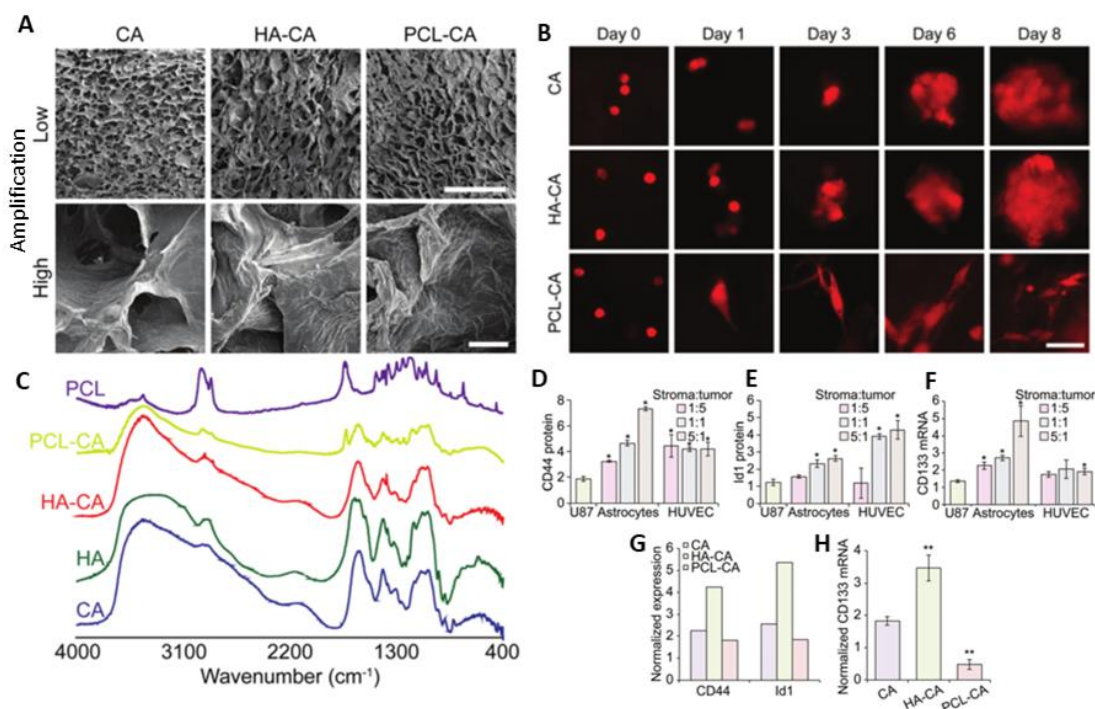
Figure 4. Alginate-chitosan scaffolds formulated for establishment of glioblastoma 3D tumor models. (A) SEM images of different alginate-chitosan scaffolds namely: uncoated (CA), Hyaluronic acid (HyA) coated scaffolds, polycaprolactone (PCL) coated scaffolds. Scale bars in SEM images correspond to 500 μm in low magnification, and 20 μm in high magnification. (C) Fourier-transform infrared spectroscopy (FTIR) characterization demonstrating the presence of the distinct coatings. (B) U-87 MG cells expressing RFP and cultured in the diverse platforms exhibited highly different morphologies derived from their ability to effectively interact with its surrounding hydrogel matrix. Moreover, in U-87 cells mono or co-cultures the level of CSC markers (G, H) increased significantly in HyA containing scaffolds. These results demonstrate both the ability of matrix-cell interactions and cell-cell communication (D, E, F) to influence tumor progression and establishment of CSCs-like phenotypes which resulted in increased expression stemness markers CD44 and CD133 as well as of Id1 a transcriptional regulator associated with apoptosis, angiogenesis, and neoplastic transformation. Adapted from Kievit and co-workers [119], with permission from The Royal Society of Chemistry.

Silk fibroin, has also been extensively used for the development of 3D *in vitro* models, particularly for the study of cancer-to-bone metastasis [120,121], due to its resistance to protease degradation, as well as mechanical and cell adhesion properties (e.g., resultant from the naturally occurring RGD sequences of the fibroin protein). Adding to this silk fibroin can also be conjugated with different biomolecular cues (e.g., BMP-2, EGFR, SDF-1) [142,143]. To date, these scaffolds have been used for establishing *in vitro* 3D models of primary tumors including those of prostate [85], and osteosarcoma [122].

Decellularized matrices (dECMs) such as those obtained from malignant/healthy tissues decellularization, or by through *in vitro* ECM production by 2D cultured cells [124] have been gaining increasing attention given their remarkable ability to provide native ECM components. However, the difficulty in producing such matrices in high-throughput compatible platforms, combined with the lack of structural and architectural control still

16

hinders decellularized matrices widespread use as scaffolds for 3D *in vitro* tumor models assembly. Hence, recent studies have been mainly focused on the production and characterization of highly reproducibly cell-derived dECMs [124], or in the production of disease-specific decellularized matrices [125]. Using this concept, Rijal and co-workers described the development of a 3D tissue matrix scaffold, obtained from decellularized breast tissues, that was processed either into hydrogel or porous scaffolds which recapitulate native tissue architecture and resilience [125]. The obtained dECMs allowed the culture of breast cancer cells in microenvironments similar to those found in mammary tissue. Moreover, by comparing cellular proliferation of MM231 breast cancer cells in diverse decellularized platforms and matrix extracts, the authors demonstrated the important role of disease-specific ECM. In this study, the results indicate that MM231 cells grown in decellularized tumor matrices exhibit the highest proliferation in comparison to MCF-10A breast fibroblasts. Interestingly, a pro-tumoral phenotype of T47D and BT474 breast cancer cells tumoroids was obtained when anti-cancer drug-screening assays were performed in dECMs in comparison with other tested scaffolds (Collagen, Laminin rich ECM, PLGA) [125].

2.2.2. Synthetic Materials based Scaffolds

Alternatives to naturally derived scaffolds include synthetic, polymer-based, scaffolds that can be precisely manufactured to include ECM-mimicking cues and tweaked biophysical properties [103]. Several synthetic polymers exhibiting bioactive, biocompatible and biodegradable properties have been synthesized and reported in the literature in the recent decades, namely poly(ethylene glycol) (PEG), polylactic acid (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA) and poly-ε-caprolactone (PCL) [5,133]. Overall, batch-to-batch variability and the lack of a precise control over scaffolds mechanical properties associated with natural scaffolds is eliminated when using well-defined, synthetic polymer-based scaffolds [144]. Moreover, when functionalized with bioactive molecules they serve both as bioinstructive and structural anchors until *de novo* matrix deposition by cancer and stromal cells occurs [28].

However, cells cultured in purely synthetic platforms can proliferate devoid of tumor-like gene expression patterns, presenting inconsistent tumorigenicity, metastatic potential or drug-resistant phenotypes, when compared to those of *in vivo* tumors [28,145].

17

Expectedly, such issues affect the production of robust tumor-mimicking 3D *in vitro* models. To overcome such drawbacks synthetic materials are often combined with other polymers such as polyvinyl alcohol (PVA), or with naturally derived biopolymers such as chitosan, hyaluronic acid or polydopamine, in order to attain more *in vivo*-like conditions [87,146,147].

2.2.3. Hybrid Scaffold Based Models

The development of hybrid-based scaffolds for the assembly of 3D models is based on the incorporation of natural bioactive molecules (e.g., growth factors), and bioadhesive moieties (e.g., peptides), into the highly tunable/controllable matrix of synthetic scaffolds [88,133]. Synthetic materials can act as a 'white-canvas' and be conjugated with natural polymers such as fibrin [148], HyA [74], or specific bioactive molecules (e.g., BMP-2, RGD peptides). PEG and its derivatives (PEG-diacrylate) have been the most widely used synthetic polymers for conjugation with bioactive molecules and to ultimately form 3D biofunctional hybrid hydrogel scaffolds for cancer *in vitro* modeling. As demonstrated by Weiss and co-workers, PEG polymeric backbone was successfully functionalized with a peptide containing RGD domains [149], which resulted in increased cellular adhesive properties. In addition, MMP or plasmin-sensitive sequences have also been chemically coupled into PEG [114,144]. The inclusion of these bioactive moieties increased cellular interactions (e.g., cell-ECM and cell-cell), and mimicked tumor-ECM specific degradability [150]. One example of this strategy is the study performed by Roudsari and co-workers [151], in which PEG-based hydrogels, containing both MMP sensitive (GGGPQGIWGQGK), and cell adhesion (RGD) peptides, were used to co-culture lung adenocarcinoma cells (3445Q) with endothelial and pericyte vascular cells. These proteolytically-degradable models promoted tubule-like network formation guided by interactions with cancer cells and provided a suitable platform to study tumor neo-vascularization.

Compared to conventional natural or synthetic scaffold-based approaches, advanced hybrid scaffold-based 3D *in vitro* models represent more robust platforms in which several aspects of tumor progression can be recapitulated. In fact, hybrid scaffolds could allow to evaluate the role of specific ECM components in events such as metastasis [152] or cancer cells proliferation [153]. However, the manufacture of hybrid scaffold-based models

18

generally requires laborious production procedures, an important aspect that limits their use in high-throughput screening platforms [27]. Combination of hybrid scaffold-based methodologies with scaffold-free based techniques has provided novel and interesting platforms that can be used to modulate, with relative ease, several aspects of tumor progression, such as ECM deposition, metastasis, genetic drift and angiogenesis.

Recapitulating the diverse facets of tumor progression in multifactorial hybrid-based approaches, allows more predictive models to be obtained. A recent study by Hirt and co-workers, demonstrated the combination of bioreactor-based methodologies to develop a drug-screening model of colon cancer with HT-29 cells cultured in porous scaffolds under perfusion flow [154]. The model showed a high correlation with tumor xenografts regarding the testing of a cytotoxic compound (5-Fluorouracil), and a clinically effective compound (BCL-2 inhibitor ABT-199), with 2D cultures evidencing antagonistic responses [154].

Other promising models have combined microfluidic platforms with collagen matrix hydrogels [155]. Microfluidic systems entail the use of micrometer-sized channels that open the possibility to produce 3D microtumor models under flow perfusion conditions [156]. Ultimately, scaffold-microfluidic combinations allow for a precise control of cancer cells growth by dynamically controlling cell culture media composition and manipulating drugs mass transfer via modification of liquid flow rate [157]. These characteristics make microfluidic systems ideal to perform angiogenesis, migration, or flow perfusion studies in the context of tumor perfusion and tumor invasion, EMT, cells dissemination and metastasis [28,158,159].

From the abovementioned materials to assemble 3D tumor models for drug discovery, several have been translated into commercially available platforms in recent years. As summarized in table 2, these 3D *in vitro* cell culture tools take advantage of both scaffold-based and scaffold-free strategies to assemble 3D microtumors in TME mimicking environments and in a monotypic or heterotypic co-culture mode. Moreover, some of the most advanced platforms also allow to model nutrient flow dynamics and are amenable to screen anti-cancer candidates in high-throughput and high-content imaging settings (Table 2).

Table 2. Summary of commercially available technologies for establishment of 3D *in vitro* tumor models.

Technology	Product Name	Description	Services	Ref
Scaffold-Free	Corning™ Ultra-Low Attachment Multiple Well Plates	Forced Floating 3D spheroids formation via culture in round bottom, ultra-low attachment (ULA) multi-well plates	-	[160]
	GravityPLUS™	Hanging-drop based culture platform for 3D spheroids assembly and that uses a patented plate design which allows fast and user-friendly recovery of cultured microtumors	Insphero® offers an on-demand 3D spheroids development service using scaffold-free platforms	[161]
	Nexcelom3D™	Ultra-low attachment (ULA) multi-well plates with flat or round bottom.	-	[162]
	Nunclon Sphera Surface™	Ultra-low attachment (ULA) multi-well plates with round bottom	-	[163]
	OncoPanel™ 3D	A drug profiling platform comprised of more than 100 types of cell line-based 3D spheroid models suitable for drug-screening and validation. The technology used for 3D spheroids assembly is not disclosed	Provides a service of drug profiling, regarding penetration and anti-proliferative screening in 3D spheroid models for more than 18 different tissue types	[164]
	Synthecon® Rotary Cell Culture Systems (RCCS)	Rotary platforms based in NASA microgravity bioreactors, ideal for 3D spheroids culture under low-shear stress conditions	-	[20]
Scaffold-Based	3D Insert™	Scaffolds with well-defined porous structures comprised either by PCL, polystyrene (PS), or PLGA and suitable for 3D microtissues assembly	-	[165,166]
	Advanced Biomatrix® Matrices and ECM Select® kits	ECM Select® Array Kit Ultra-36 is an array of ECM-mimetic scaffolds based mainly in natural derived ECM constituents (e.g., silk fibroin, collagen types I, II, III and IV, hyaluronic acid, or adhesion proteins – vitronectin, fibronectin, laminin, etc) in which 3D tumor models can be established	Provides an ECM platform in which cells can proliferate and be analyzed. This technology is useful for screening optimal matrix composition and mechanical properties that allow cells to grow in an environment that mimics *in vivo* conditions	[167]
	AlgiMatrix®	Alginate-based scaffold with a highly porous structure suitable for 3D cell culture and microtissues formation	-	[168]
	Alvetex®	Highly porous PS scaffold suitable for 3D cell culture and microtissues formation	-	[169]
	Cellusponge	Disc shaped Collagen type I or Galactose-based scaffold that allow cells to be cultured in easy to use 3D environments	-	[170]

20

Cultrex®	Murine basement membrane extract obtained from Engelbreth-Holm-Swarm tumors, available in reduced growth factor or concentrated growth factor form. This gel allows 3D cell culture in a bioactive environment	-	[171]
Cytodex™	A group of crosslinked dextran matrix - based particles, which can be used for 3D cells expansion	-	[91,172]
Geltrex®	Soluble form of reduced growth factor basement membrane extract purified from murine Engelbreth-Holm-Swarm tumor	-	[173]
HydroMatrix™ Peptide Hydrogel	A self-assembled scaffold, based on synthetic peptide nanofibers. It offers precise control of 3D matrix architecture. Suitable for 3D cell culture and spheroids assembly	-	[174]
HyStem® Hydrogels	A diverse set of thiol-modified scaffolds that can be comprised of Hyaluronan (Glycosil®) or Hyaluran and heparin (Heprasil®). Offers the possibly of being combined with Thiol-reactive PEGDA crosslinkers (Extralink®) or Thiol-modified collagen (Gelin-S®)	-	[175]
Matrigel™	Engelbreth-Holm-Swarm sarcoma solubilized basement membrane extract. Available in both concentrated and reduced growth factor forms	-	[176]
MaxGel™	Human extracellular matrix extract derived from human basement membrane. Suitable for 3D cell culture and invasion assays	-	[177,178]
Qgel® Vials and Qgel® High-throughput kits	An extensive panel of specialized, PEG-based ECM mimetics with well characterized mechanical properties and chemical composition, specifically tailored for tissue-specific cell lineages or primary cultures. The ECM-like matrices can be provided in modified 96-well plates and/or high-throughput compatible kits, suitable for drug-screening assays	Qgel® provides a specific artificial matrix tailoring service with the objective of finding or designing scaffolds that better recapitulate tumor/tissue specific ECM	[179]
SeedEZ™ and GradientEZ™	Glass fiber-based disc or flower shaped bioinert scaffolds mainly used to study the influence of compound or growth factor gradients in 3D cultured cells	-	[180]
SpongeCol®	Type I collagen-based scaffold with cross-linked structure for increased mechanical strength and durability. Suitable for 3D cell culture and microtissue formation in a well-defined biodegradable micro-porous structure	-	[181]
TrueGel3D™ Hydrogels	Diverse array of scaffolds based in either PEG, PVA or dextran matrices, designed for 3D cell culture in tailored conditions i.e., fast ('FAST-PVA') or slowly ('SLO-Dextran') gelling gels, pH responsive gels, or gels tailored by the	-	[182]

21

		addition of specific cell adhesion domains		
Hybrid – Bioreactor-based Platforms	3D Perfusion Bioreactor	Combination of bioreactor technology with the 3D Biotek® PCL disc inserts for the formation of perfused microtissues	-	[160]
	3DKUBE™	3D cell scaffold-culture chambers which allows the establishment of independent scaffold-based cell cultures under perfusion	-	[161]
Hybrid – Microfluidic-based Platforms	Ibidi™ μ-Slide III 3D Perfusion,	A set of microfluidic devices capable of working in static or fluid perfusion conditions, in which cells included in 3D scaffold-based models (e.g., Matrigel or other gel-based system) can be cultured. These platforms are suitable for simulating perfusion conditions, allowing for example drug administration under flow, chemotaxis and migration studies to be performed. The tumor models in chips/slides can be analyzed in real-time by microscopy-based analysis	-	[183,184]
	TissUse™ Organ-on-a-Chip	Organ-on-a-chip microfluidic devices that can accurately mimic physiological flow in microchannels. These can work in either free-circulation or closed-loop setup, allowing communication between reservoirs that can contain 3D scaffold-based models of tumor and healthy tissues developed by the user	Provides a specific service of chip design and tailored healthy tissue organoid integration, oriented for drug screening	[185]
	MIMETAS™ Organplate Models	High-throughput compatible, organ-on-a-chip platforms that allows insertion of scaffold-based 3D models (e.g., gel-based), into close-loop microfluidic platforms. These platforms allow direct contact between scaffold containing sections and fluid containing channels by employing a patented phase guide system	MIMETAS™ offers services of OrganPlate® model design for drug development, efficacy screening and toxicity studies in its facilities	[186–188]
	SynTumor™ 3D Cancer Models	Microfluidic devices engineered with tortuous channels with the aim of mimicking tumor-associated erratic microvasculature and transport across the vessel walls. These channels open into a central reservoir that can contain scaffold-based 3D tumor models	Provides real-time screening of tailor made tumor models. Services include target validation, compound screening, biomarkers analysis, adsorption, distribution, metabolism, toxicity and studies regarding mechanisms of action	[189]

*3D - Three-Dimensional; PCL - Poly-ε-caprolactone; PEG - Polyethylene Glycol; PEGDA - Poly Ethylene Glycol Diacrylate; PGA - Poly Glycolic Acid; PLGA - Poly Lactic-co-Glycolic Acid; PS – Polystyrene; PVA - Poly Vinyl Alcohol

Although a significant number of commercial platforms has been developed, to date the majority of scaffold-based models, particularly those based on hydrogels, fail to achieve a precise control over 3D microtumors spherical morphology in a sense similar to

22

that obtained in 3D tumor spheroids assembled by scaffold-free approaches. However, despite the fact that 3D spheroids present reproducible morphological features and tunable size, the absence of pre-existing tumor ECM components also remains a significant drawback [19,190]. The following section will describe advanced technologies based on spherically structured 3D platforms that aim to bridge the gap between the lack of gel-grown microtumors reproducible morphological control and the absence or pre-existing ECM components in 3D scaffold-free assembled spheroids.

3. Spherically structured 3D *In vitro* Tumor Models

Considering the specificities of the drug-screening process, namely the necessity of high-throughput, ease of analysis, reliability and predictable potential of the preclinical validation models [39,191], an ideal approach could require the combination of scaffold-based models to represent ECM biochemical and mechanical complexity, along with the ease of analysis obtained from simple scaffold-free spherical models. Following the example of stem cells research in tissue engineering [17], such a combination could be achieved for example through the inclusion of microparticles containing specific ECM mimetic components. These combinations would allow the inclusion of TME specific matrix and cellular components into a spherical scaffold (Figure 5), thus leading to the formation of composite multicellular spheroids compatible with current analysis methodologies. An alternative methodology to the inclusion of microparticles would be the encapsulation of cancer cells inside hydrogel microcapsules [16]. Microencapsulation techniques have shown the capacity to confine cells and promote a reproducible spheroid growth, while providing ECM-like components that would otherwise be lacking [16,192,193]. Several spherical *in vitro* tumor mimicking cancer models have been developed so far, providing innovative platforms for the study of tumor biology and drug-screening assays (Figure 5). Herein, microencapsulation and microparticle scaffold will be critically reviewed in light of recent reports.
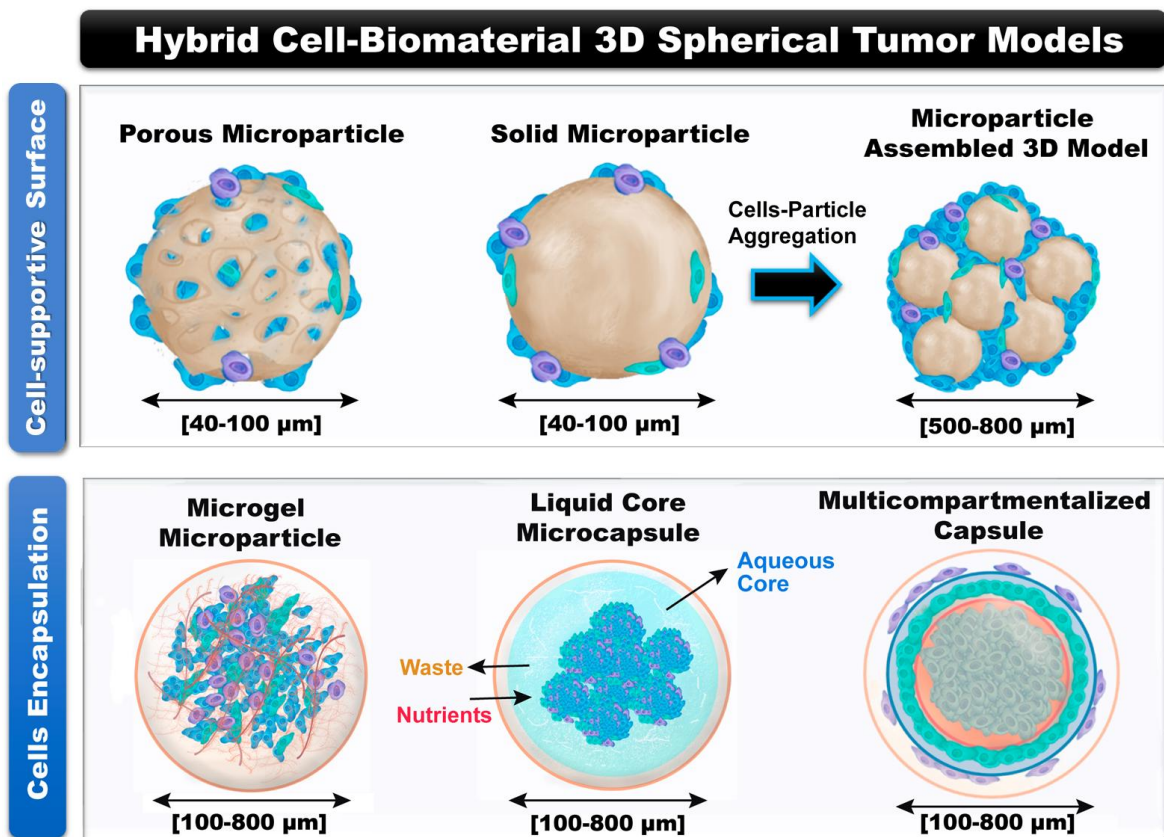
Figure 5. Schematic representation of the diverse technologies used for assembly of spherically structured 3D *in vitro* tumor models.

3.1. Microparticles for 3D Tumor Models Assembly

As previously stated, 3D-MCTS are seen has golden standard *in* vitro models for performing drug-screening tests [194], due to their ability to correctly recapitulate several features of the tumor microenvironment, such as: (i) cell-cell interactions; (ii) matrix deposition; (iii) cell-ECM interactions; (iv) internal structure organization (responsible for hypoxia and consequently necrotic core formation) and (v) drug-resistance, stemming both from the acquisition of a resistant phenotype by continuous low drug dose stimulation, and drugs diffusional limitations in dense tumor masses [22]. These models provide a platform that can be easily assembled and facilitates high-throughput studies [195], in comparison with more complex scaffold or microfluidic-based models. However, as previously discussed in section 2.1, conventional spheroid-based models have inherent limitations that could be overcome by including biofunctional microparticles.

Microparticles have been extensively applied in the field of tissue engineering mainly in four areas of application: (i) delivery of incorporated or surface-attached molecular cues or tethered protein into tissues or cell aggregates; (ii) reporting changes in

24

culture conditions; (iii) serving as scaffolds for cell attachment and providing necessary cues for cell differentiation or phenotype stimulation, and (iv) introduction or preservation of local targeted heterogeneity or homogeneity [17,196]. To date, most studies have explored of microparticles mainly for structural support of for providing molecular cues, with limited works exploring microparticles potential in the context of tumor modeling. Studies involving microparticles-based scaffolds for the production of *in vitro* tumor models [87,95,134,197–200], employing either non-modified synthetic polymers, or hybrid scaffolds such as the previously discussed PEG-Fibrinogen model, later developed by Pradhan and co-workers into the format of microspheres that allowed the assembly of spherical cancer models [95].

As mentioned, in comparison to scaffold based models, 3D-MCTS main limitation is the lack of a pre-existing ECM-like supporting structure. As a result, contrarily to what happens *in vivo*¸ the ECM will not be able to guide or influence cancer cells and stromal cells from the onset, ultimately failing to provide the necessary initial cues for characteristic cancer phenotypes to arise [201,202]. Consequently, the acquisition of phenotypes that do not resemble those found *in vivo* can be observed in certain culture settings. For instance, Brancato and co-workers [203], reported that for spheroids assembled either for cancer-associated fibroblasts (CAF) or normal fibroblasts, different cell metabolism, cell growth, matrix deposition rates and mechanical properties were observed when culturing cells with or without the support of porous gelatin microparticles [203]. Through the use of such microparticles, the authors were able to better replicate the functional and metabolic differences found *in vivo* between healthy and neoplastic tissues containing CAFs [203]. This study exemplifies that the introduction of microparticles into spheroid-based tumor models allows to surpass such limitations.

Emerging reports have described the use of microparticle-based scaffolds as a means of introducing previously lacking ECM components into 3D-MCTS, promoting stem-like or multidrug resistance profiles [197,199,203]. The production methodologies for these microparticles mainly involve the application of modified double emulsion methods and sieving, with the combination of both techniques allowing a high yield of microparticles in the desired size ranges [95,197,198,200,203]. A significant body of knowledge on the methodologies to prepare polymer and hydrogel microparticles has been accumulated in the field of drug delivery systems in the past decades [204], and could be

transposed to support spherical cancer models production. One can envisage that simple compact particles to the surface of which cells can adhere, or porous micro- or macroparticle formulations (with enhanced surface area for cell attachment and adding additional volume for cell colonization) (Figure 5), can provide a structure capable of recapitulating aspects of the TME. Sahoo and co-workers [87], produced porous microparticles based scaffolds which allowed cells to interact with a semi-rigid or rigid ECM-like structure. The obtained PLGA/PLA microparticles exhibited diameters ranging from ~100-260 μm and consequently significant surface area for cell attachment. After a period of 5 days, microparticles were completely covered in cell layers, that ultimately formed a spherical aggregate in which compact cell-cell adhesion characteristics found in 3D scaffold-free techniques were reproduced. This approach allows the establishment of 3D-MCTS when used in combination forced-floating, hanging drop or stirring-based methodologies [87].

In the context of particle porosity, the work of Kang and co-workers [200], established a cryopreservable tumor model of MCF-7 using PLGA microspheres with an average particle diameter of 393±5 μm, an exterior pore size ranging from 10-70 μm with intertwined porosity (Figure 6). The particles were used for cancer cells culture in stirred suspension bioreactors, achieved an elevated growth rate (2.8-fold cell expansion over seven days), increased resistance to doxorubicin when compared to 2D counterparts, as well as maintained viability and metabolic profiles after the process of cryopreservation. Moreover, the model exhibited increased effectiveness in establishing tumors on athymic female mice, with MCF-7 cells cultured on microspheres presenting a 4-fold increase in tumor formation [200].
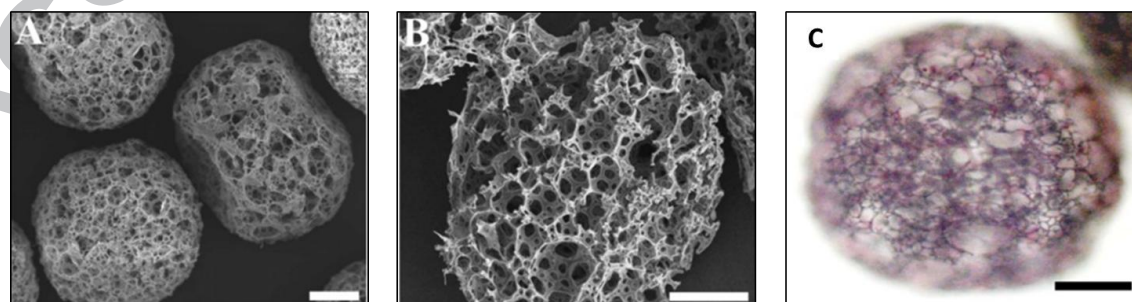

Figure 6. (A, B) PLGA microspheres produced as a cryopreservable model SEM micrographs, presenting pores with suitable size for cells impregnation in the scaffold. (C) H&E staining of MCF-7 cells cultured on PLGA microspheres inside a spinner flask, image acquired at 5 days of culture. White and Black bars represent 100 μm. Adapted from Bae and co-workers [200], with permission from Elsevier.

26

So far, most studies involving tumor modeling produced polymeric microparticles as supporting scaffolds for assembling breast cancer cell spheroids, but mostly restricted to the MCF-7 lineage [95,197,198,200,203,205]. Several studies made use of Microparticles scaffolds as a mean of culturing breast cancer cells for measuring the cytotoxic effect of diverse pharmacological compounds such as doxorubicin, cisplatin, gemcitabine, paclitaxel, and tamoxifen [197,198,200]. In this context, Horning and co-workers [198], used a combination of PLA and chitosan to create microparticles with diameters of 160-182 μm for culturing MCF-7 cells and evaluating its cytotoxicity profile against doxorubicin, paclitaxel, and tamoxifen [198]. The authors performed a comparison analysis between 2D and 3D models and observed that drug internalization was significantly delayed in the 3D model. (Figure 7). In fact, while in 3D models containing microparticles doxorubicin only reached the spheroid core region after 8h of incubation, in 2D models such observations were visible within the first hour.
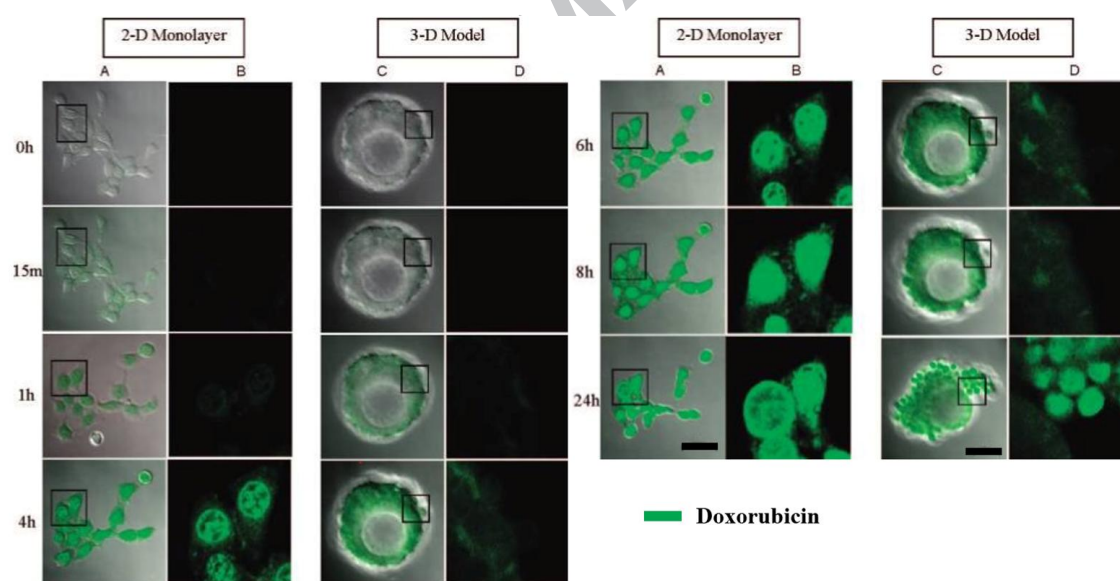

Figure 7. Confocal images of doxorubicin penetration over several time periods (15 min, 1h, 4h, 6h, 8h and 24h) in both 2-D monolayer cultures and 3-D of MCF-7 breast cancer cells, following incubation with 2.500 ng/ml of doxorubicin (in green). B and D columns of each section are enlarged areas of the images present in to their left. Analysis of doxorubicin penetration demonstrated slower penetration in MCF-7 spheroids when compared to 2D monolayers, taking almost 24 h to achieve the same level of doxorubicin inclusion in the 3D structures. Adapted from Horning and co-workers [198], with permission of American Chemical Society (ACS).

The ability to recapitulate *in vivo* arrangements and expression patterns may pave the future for screening novel therapeutics targeting specific TME hallmarks. Another study by Brancato and co-workers improved on the previous porous gelatin microparticle-based model of stroma through the addition of MCF-7 cancer cells. This breast cancer co-

culture model containing porous gelatin Microparticles, was used to test a targeted nanoparticle drug delivery system [197]. A comparative analysis confirmed elevated expression of MMP-2 and other metalloproteinases in the 3D model versus 2D cultures, hence better mimicking *in vivo* overexpression by breast cancer cells in the TME. Interestingly, this enzymatic overexpression was effectively exploited via an enzyme-responsive targeted delivery system, comprised by PLGA-PEG nanoparticles and a tumor targeting pro-drug activated by MMP-2 degradation. The obtained results evidenced increased specificity of targeting system in microparticles-based models, with the efficacy of the nanoparticles being confirmed through increased cytotoxicity in the 3D model (Figure 8) [197].
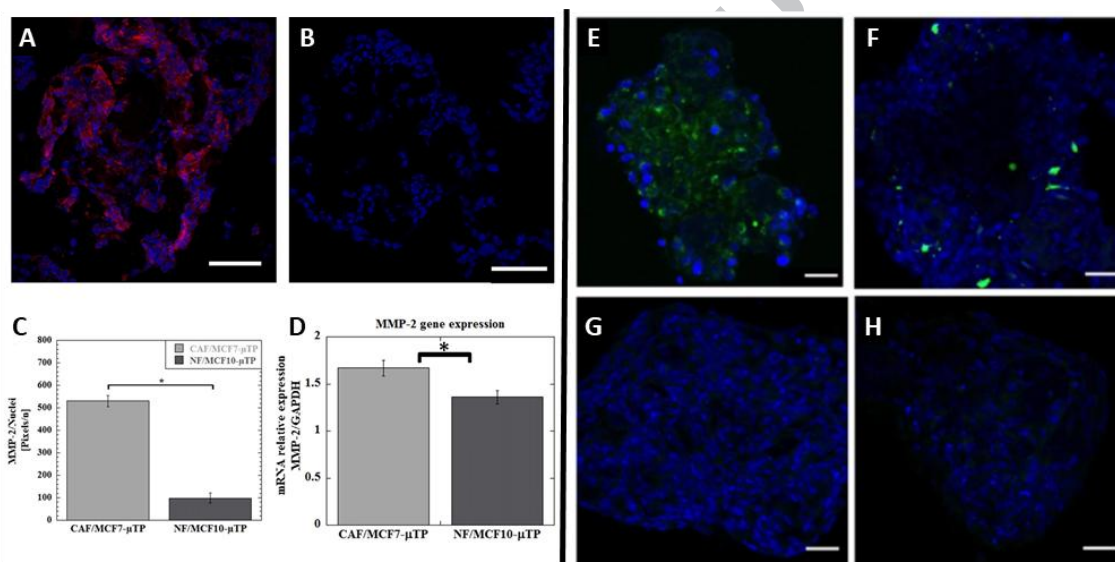

Figure 8. Fluorescence microscopy (A, B, C) and RT-PCR (D) analysis of MMP-2 expression in both tumor mimicking (CAF/MCF-7) and normal breast tissue 3D spheroids (MCF-10A) containing porous gelatin microparticles (cell nuclei in blue, MMP2 protein in red; scale bar 75 μm). (F, G, J, K) Fluorescence microscopy analysis of doxorubicin penetration (green) (scale bar 50 μm); in tumor mimicking spheroids and healthy spheroids with (F, J) or without (G, K) MMP sensitive targeting nanocarrier particles. CAF/MCF-7 spheroids exhibited higher (C – left side) doxorubicin penetration especially when treated with MMP-2 sensitive doxorubicin carrying nanoparticles. (G, H) Alternatively, in normal tissue spheroids, no doxorubicin release was observed. Results demonstrate gelatin microparticles potential for mimicking *in vivo* overexpression of metalloproteinases. Adapted from Brancato and co-workers [197], with permission from Elsevier.

Despite extensive implementation of microparticle-based scaffolds in tissue engineering applications, there is still a tremendous untapped potential for exploring these assemblies in the field of *in vitro* tumor modeling. In fact, most models containing microparticles merely focus their utilization as cell culture vehicles or for culture in bioreactors. Production of finely tuned microparticle structures using advanced 3D printing and micropatterning technologies [206], or through the use of flow-focusing microfluidic devices [207], could allow to study the role of specific signaling cues in *in vitro* expanded

malignant cells. These novel approaches may shed light upon biomolecules specific roles and enhance our capacity to modulate the TME *in vitro*.

3.2. Microencapsulated 3D Models

Encapsulation of cancer cells or spheroids is a promising strategy for tumor modeling that has received considerable attention in recent years [5]. Microencapsulation can serve as a mean of representing spatially defined ECM mimicking scaffolds. This strategy allows cancer and stromal cells to grow (in mono or co-cultures), and establish both cell-cell and cell-ECM interactions in a confined, yet, not fully isolated environment. Furthermore, the encapsulation of cancer cells, particularly in spherical-shaped, size-controlled microcapsules with semi-permeable membranes, allows bidirectional diffusion of nutrients, oxygen, therapeutic compounds and low/medium molecular weight signaling molecules (e.g., growth factors and cytokines). In addition, microencapsulation can be employed to prevent the penetration of high molecular weight objects such as antibodies and immune cells [208], having been originally used as a tool for cell transplantation and immune isolation. In the field of tumor modeling, microencapsulation has been employed in a diverse set of ways that will be discussed in the following examples [16].

The ability to restrain direct cellular contact makes microencapsulated 3D-MCTS as an ideal model to study the diverse paracrine interactions occurring in the TME between the different key cellular populations such as immune cells and mesenchymal stem cells [209]. This capacity of microencapsulated models was exploited by Yeung and co-workers [210], to study non-direct communication between neuroblastoma and bone-marrow derived mesenchymal stem cells. By using a collagen microsphere system, the authors demonstrated mesenchymal stem cells ability to promote neuroblastoma growth [210]. Such combinations of diverse cell populations in indirect contact can also be achieved in a hierarchically structured way, as demonstrated by the multilayered models produced by Fang and co-workers [192]. These authors manufactured hierarchically-assembled microencapsulated tumor models of prostate cancer cells (PKD1), and prostate cancer TME associated stroma cells (WPMY-1) in an alginate hydrogel. By separating the diverse cellular populations into different particle sub-layers, the authors achieved an ideal model in which to study the paracrine interactions established between both cell populations (Figure 9).
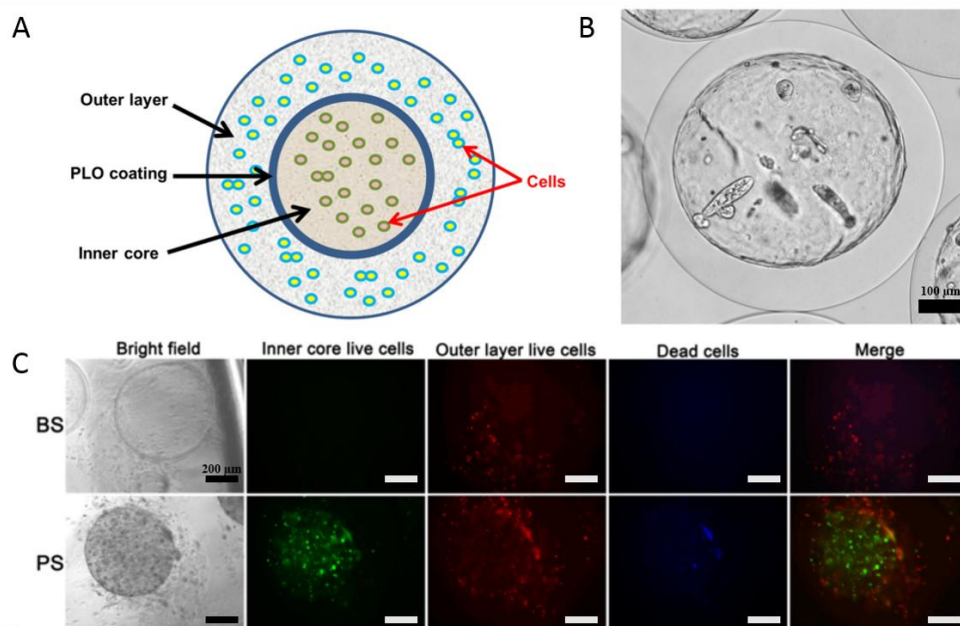
29

Figure 9. Formulation of multi-layer spherical tumor models for compartmentalized 3D co-culture. (A) Schematic representation of the double-layered 3D models containing diverse cellular populations, and their respective analysis through (B) optical contrast light microscopy of stromal cells growth in the inner core with an empty outer-layer (7 days), and (C) live dead analysis of cells cultured in the double-layered model show that cells remain viable for over 30 days. Adapted from Fang and co-workers [192], reproduced under Creative Commons License.

In a recent study by Lu and co-workers [211], compartmentalized hydrogel microparticles containing ECM-mimetic scaffolds were produced through the combination of multi-fluidic electrospray of hydrogel particles with ionic/thermal gelation mechanisms (Figure 10). The ability to obtain compartmentalized platforms is important for several fields of tissue engineering and 3D *in vitro* disease models, a point which the authors demonstrated by utilizing the novel system to perform 3D cultures of small intestinal organoids, as well as tumor and hepatic microtissues. By using this platform, the authors were able to produce size-controlled microcapsules at a high rate (10 000 particles min^{-1}), containing direct or indirect co-cultures of MDA-MB-231 breast cancer cells, MCF-10A normal mammary epithelial cells, and normal human lung fibroblasts (Figure 10Q, R). In culture, cells could communicate either by direct contact or via paracrine interactions, according to the compartmentalization settings. Furthermore, given the ability to control microgels size from 95 to 725 μm, the authors easily obtained encapsulated cultures with 600 μm of diameter and capable of recapitulating the hypoxic conditions seen in *in vivo* avascular solid tumors. The capacity of recapitulating direct and indirect interactions, the ability to represent both physical (hypoxic gradients) and biochemical (ECM composition) properties combined with the ability to sequentially retrieve cultured cells, makes this an

30

ideal platform for further studies on gene expression, cell-cell signaling, and drug screening.
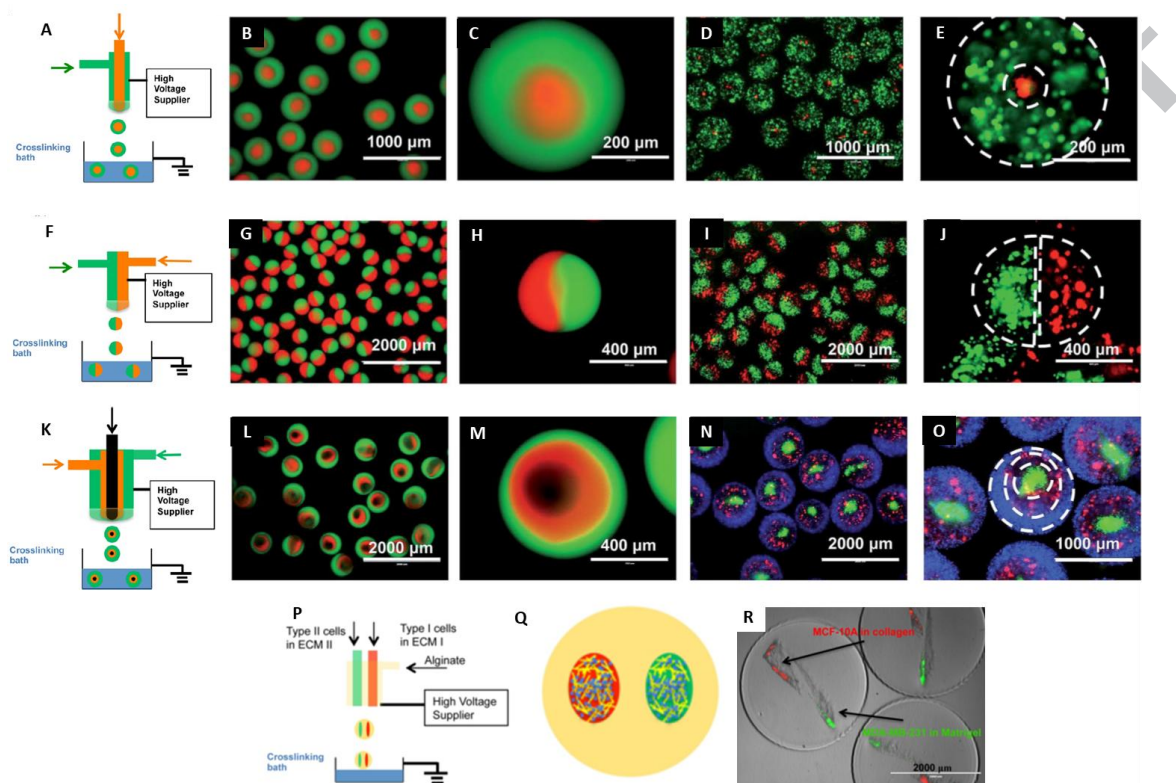

Figure 10. (A, F, K, P) Schematic representation of the diverse multi-fluidic geometries used for production of hydrogel microparticles containing (P, Q) or not (A, F, K) two distinct ECM cores (P, R). Fluorescence Microscopy images of double-layer (B, C, G, H) and triple-layer (L, M,) microparticles containing fluorescently labeled alginate (either green or red). (D-E, I-J, N-O) Fluorescence microscopy images of co-cultured cells (green cells: MDA-MB-231 expressing GFP; red cells: normal human lung fibroblasts expressing RFP; blue cells: MCF-10A stained with Hoechst. (Q, R) These microparticles allowed indirect co-culture of diverse cell populations in distinct ECM-like environments, such as co-culture of MCF-10A cells in collagen (red), and MDA-MB-231 cells in Matrigel™ (green). Adapted from Lu and co-workers [211], with permission from Royal Society of Chemistry (RSC).

Layer-based microencapsulation can also be for the proliferation of key tumor sub-populations as demonstrated by Rao and co-workers [212], for prostate cancer stem-like cells (PCa-CSCs). By manufacturing liquid core microcapsules with an alginate hydrogel shell, the authors were able to accelerate PCa-CSCs production from 10 days to 2 days. When compared to growth in ultra-low attachment plates, microencapsulated prostate spheroid models presented a higher degree of stem cell surface receptor markers and higher pluripotency, which combined with the rate of production, renders this platform suitable for the production of PCA-CSCs *in vitro* [212].

On a different perspective, microencapsulation can also provide a platform for enhanced recovery of both cells and cell-secreted factors [213,214]. A study by Cui and co-workers [215], demonstrated the feasibility of easy cell recovery through thermal-

31

dissociation of microcapsules incorporating HeLa cells which were readily recovered as aggregates under specific temperature conditions. Another study, by Huang and co-workers [193], reported a microcapsule model formed by gelation of a newly discovered peptide for encapsulation of MCF-7 cells. The methodology employed for microcapsule formation allowed cells to be encapsulated at physiological pH and temperature, in minimum essential medium, decreasing cytotoxic effects sometimes associated with microencapsulation processes [16,216]. Through shear stress, caused by pipetting, the gel was easily converted back to its liquid form allowing recovery of breast cancer cells [193]. Furthermore, cytotoxicity assays with cisplatin revealed that the models were suitable for drug-screening assays, by allowing free penetration of the drug. Such ability to isolate specific cells further increases the capacity of studying genetic and phenotypic alterations in specific sub-sets of the cultured cells. In a recent study by Yang and co-workers [217], spherical alginate-based microcapsules were used to cultivate low passage human mucoepidermoid cells, and also to isolate angiogenesis-related molecules released from these cancer cells. The analysis of 3D cultures phenotypes and genotypes revealed a higher expression of pro-angiogenic genes and hypoxia associated factors in comparison to those obtained in standard 2D cultures [217].

Microencapsulation can also be exploited for the assembly of hierarchic 3D tumor models. In a recent report, Agarwal and co-workers [218], microencapsulated breast cancer cells (MCF-7) in collagen I and alginate core-shell semi-permeable microcapsules (~400 μm) that served units for the bottom-up assembly of a 3D microtumor. This hierarchical model was able to promote *de novo* vasculature establishment and organization when encased under perfusion in a collagen I hydrogel containing human umbilical vein endothelial cells (HUVECs) and hADSCs (Figure 11B). This merged structure was then placed inside a microfluidic chip, being subjected to physiologic perfusion conditions. By introducing such physical and biochemical cues, through paracrine communication with encapsulated cancer cells, the authors effectively mimicked HUVECs vasculogenic morphogenesis. Furthermore, *in vitro* analysis revealed highly increased resistance to doxorubicin when compared to avascular and 2D models containing the same cellular ratios (4.7 and 139.5 times respectively) (Figure 11D).
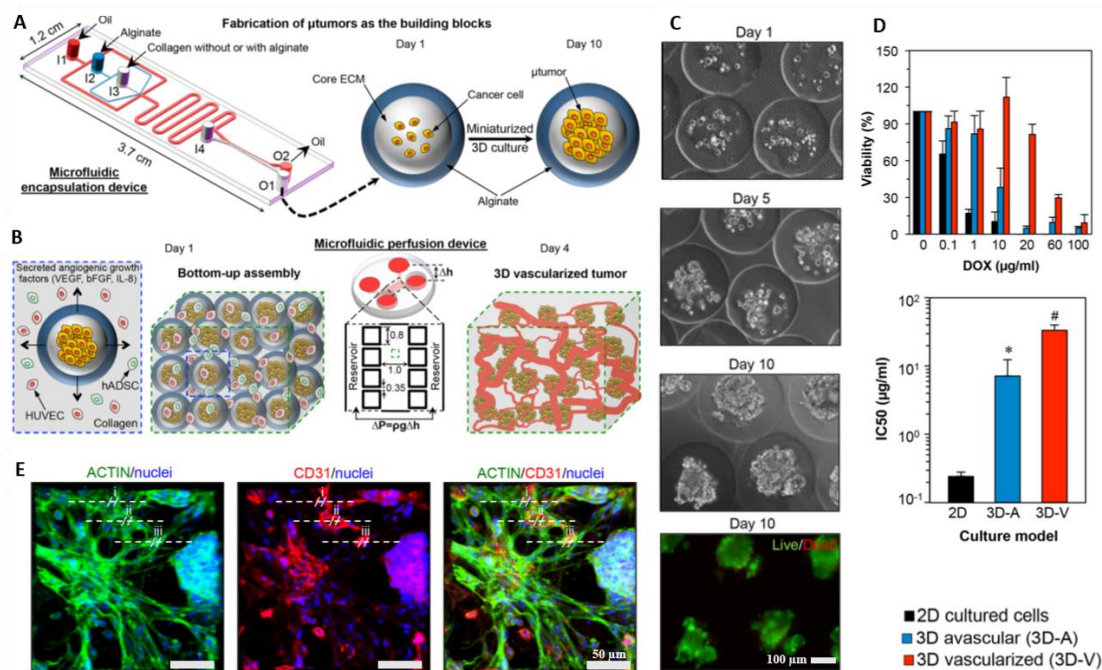
Figure 11. (A) Schematic representation hierarchically structured, vascularized 3D breast cancer models. Cells were encapsulated in Alginate-Collagen microcapsules (~200 µm) by using a microfluidic chip, (C) being cultured for up to 10 days with no loss of viability due to the radius of ~200 µm. (B) These microtumors were then assembled in a bottom-up approach into a vascularized tissue by encasing in a collagen I hydrogel in a perfusion microchip, containing HUVECs and adipose derived stem cells. (E) The combination of biological and physical interactions leads to *de novo* formation of functional vasculature mimicking the processes of angiogenesis seen *in vivo*. (D) This capacity noticeably contributed to a significant increase in models' resistance Doxorubicin. Adapted from Agarwal and co-workers, [218], with permission from the American Chemical Society (ACS).

Similarly, to microparticles, microcapsules can also serve as a technology for the inclusion of tumor ECM components. Several studies demonstrated that for neuroblastoma [210], lung [219] and breast [29,208,210,220] cancer microencapsulated spheroids establish cell-cell signaling interactions similar to those observed *in vivo*. Moreover, internal ECM matrix components deposition occurs inside the microcapsules, leading to increased resistance when compared to conventional 2D models. Such makes these 3D microencapsulated models possibly suitable for drug-screening assays and research in tumor drug resistance. Interestingly, microencapsulated 3D-MCTS models can mimic for example both solid tumor density [29], cell-matrix interactions, and the mechanical and physical pressures resultant from uncontrolled expansion of tumor masses, which can promote cancer metastasis and lead to profile alterations in cancer cells [216]. In fact, as demonstrated by Guzman and co-workers [221], depending on the elasticity of the chosen microcapsule, these can allow the study of the invasive processes carried out for example by invasive breast cancer [221] and other epithelial tumors [222], recreating the breaching of the involving basement membrane layer that surrounds the primary tumor site.

33

Furthermore, as elegantly demonstrated by Alessandri and co-workers [216], microcapsules can be used to study the buildup of intra-tumoral pressure, decurrent from the increasing of tumor mass generating increasing pressure on adjacent tissues and conversely compressing the tumor [223].

Microcapsule-encompassed spheroids are assembled through several methodologies, the most common of which being generation of liquid-core structures by employing microfluidic devices and hydrogel reticulation methods [29,208,216,224–226]. Frequently assembled microcapsules present diameters in the order of a few 100 μm to 500 μm, an exception being the study produced by Pradhan and co-workers [205]. The authors assembled poly (ethylene glycol) diacrylate (PEGDA) milibeads through the usage of a single droplet emulsion technique in which the PEGDA droplets were crosslinked in oil solutions through a dual-photoinitiator system. The authors consistently created monodisperse milibeads with geometric diameters that ranged from 1671.24 ± 34.91 μm to 3089.07 ± 55.58 μm (Figure 12) for encapsulating MCF-7 cells [205]. Moreover, the developed model achieved good cell-cell and cell-matrix adhesion, proliferation and establishment of extensive necrotic chore regions at day 5 of culture, accompanied by proliferative outer rims, akin to those characteristic to *in vivo* tumors (Figure 12G) [205].
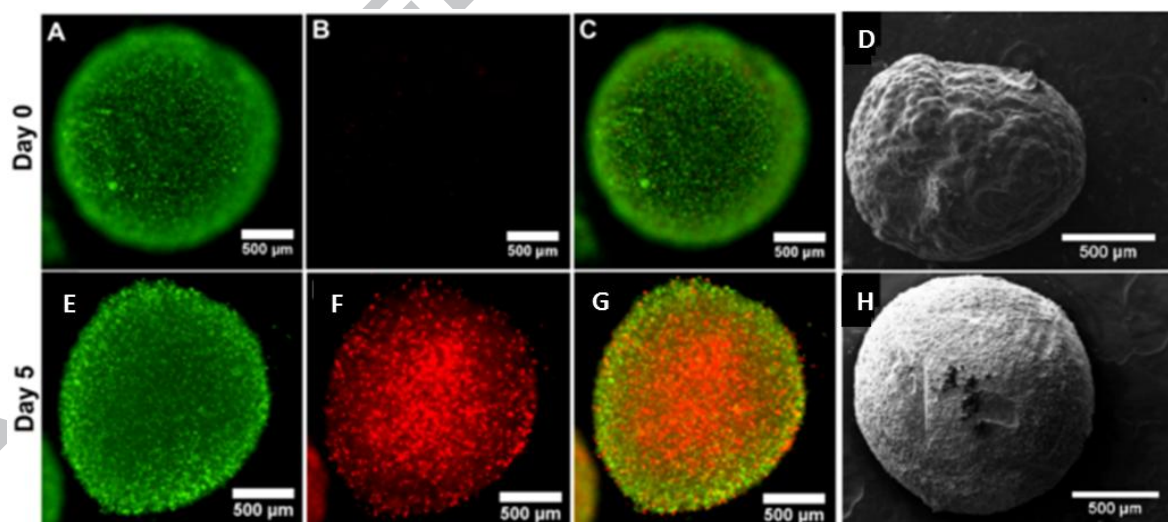
Figure 12. Tumor mili-sized particles capable of recapitulating tumor conditions lead to the establishment of necrotic cores around 5 days of culture. Live cells are stained with a green fluorescent marker, while dead ones appear in red. Difference between day 0 (A, B, C) and 5 days of culture (E, F, G). Ultrastructure of tumor mili-sized beads without (H) and with (D) encapsulated tumor cells, as observed through SEM. Adapted from Pradhan and co-workers [205], with permission from Langmuir.

Microfluidic-based approaches use flow-focusing, T-junction chips or more complex channel designs [16,194] to generate droplets of cell suspensions mixed with pre-selected biocompatible polymers or hydrogels. In these strategies, encapsulation into

spherical structures is achieved by exposure to a crosslinking agent, such as calcium bath solutions or UV light, which triggers gelation and produces microcapsules containing the desired cells. Encapsulated cells assemble over time to form matrix-encapsulated spheroids capable of establishing both cell-cell and cell-matrix interactions [227]. An excellent example of such application is the formerly mentioned study by Alessandri and co-workers [216]. In this study, a model of colon carcinoma based on murine CT26 colon cancer cell line was assembled through a simple and highly reproducible method, based on a microfluidic co-extrusion chip assembled by co-centering three glass capillaries extruding sequentially cell solution, calcium free solution and alginate solution into a calcium bath (Figure 13) [216]. The authors were able to assemble highly elastic spherical microcapsules that acted as quantitative mechanical sensors to measure the internal pressure resultant from the expanding tumor cells. Moreover, the researchers found that peripheral cells inserted in the encapsulated model readily escaped the spheroid environment, while the spheroid invasive profile was not present in non-confinement 3D models (Figure 13I) [216].
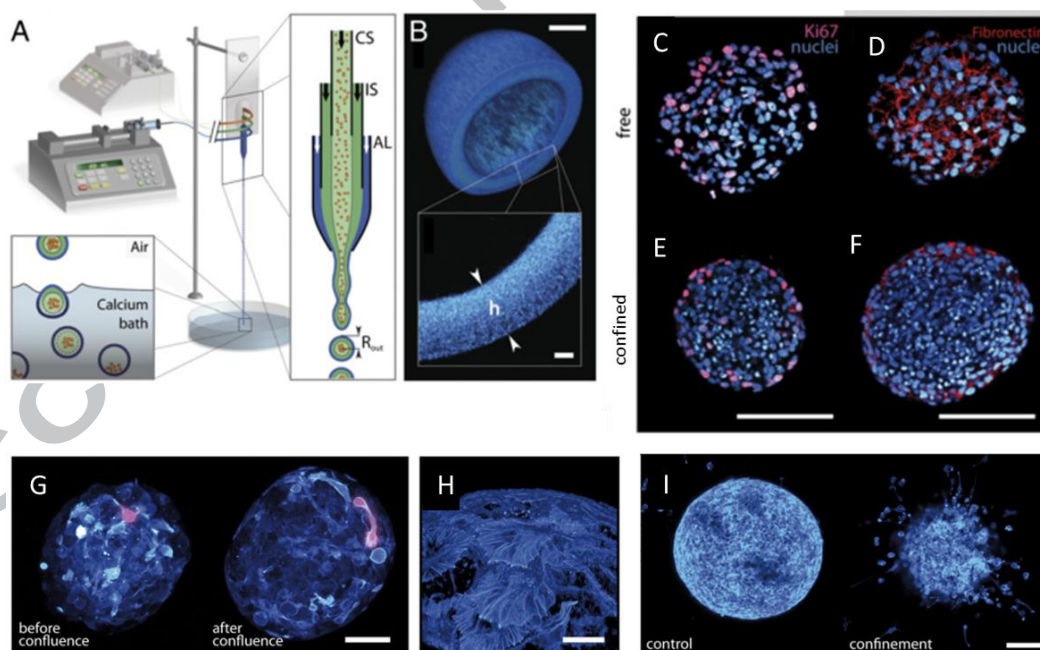

Figure 13. Production of spherical alginate microcapsules for development of 3D tumor models. (A, B) Schematic of the process used to produce alginate microcapsules, and confocal micrographs after staining with dextran (B). CT26 cultured in alginate matrix (E, F) and in free-spheroid form (C, D). (H) 3D models were cryosectioned and analyzed through immunolabeling DAPI (blue), Ki-67 (magenta), and fibronectin (red). Magnified confocal microscopy image of the surface of a fixed spheroid after staining with phalloidin-Alexa 488 (Hot LUT, cyan) (H). After reaching confluence both confined and control spheroids (G) were inserted into a collagen-based scaffold to access invasion capacity; (I) After 48 h cultured cells in confined models started to invade collagen matrix while freely formed spheroids retained their spherical shape. Scale bars: B=50 μm; C, D, E, and F=100 μm; G=50 μm; H=10 μm; I=100 μm. Adapted from Alessandri and co-workers [216], with permission from Proceedings of the National Academy of Sciences of the United States of America (PNAS).

Additional approaches to microcapsule production consist in the utilization of coaxial electrospray-based encapsulation, or alternatively, aerosol-based microencapsulation [226]. Leong and co-workers [226], demonstrated the feasibility of microencapsulating keratocytes (HaCaT) and cancer cells of oral squamous cell carcinoma (ORL-48) inside alginate microcapsules polymerized in a calcium bath. Produced microtissues were capable of self-arranging into spheroids inside the alginate microcapsules, remaining viable until after 16 days of culture. Other commonly employed techniques for microcapsule production involve simple procedures such as emulsion technique-based microencapsulation, or syringe pump extrusion and micromolding [16]. The work developed by Lee and co-workers [225], is an excellent example of the latter technique. In this study, the authors used diffusion-mediated encapsulation, performed in PDMS-micromolds where hepatocarcinoma spheroids were previously assembled. Such spheroids were subjected to posterior deposition of an encapsulating alginate hydrogel through nano-porous membranes, which allow a control over crosslinking agents deposition rates [225]. Lastly, 3D bio-printing has also been used by Xu and co-workers [228], to produce high-throughput automated encapsulation of ovarian cancer cells and fibroblast co-culture droplets in Matrigel™. This approach allows the study of co-culture interactions in diverse settings due to high control over initial cell density and spatial arrangement of the patterned structure of the model, paving the way for the development of more complex and precise spherical scaffold-based tumor models to be generated.

Identically microparticles, microcapsules can be used as a means of incorporating specific ECM mimetic components allowing the establishment of *in vivo* like interactions between internalized cells and tumor ECM as shown by Xu and co-workers [219]. In this study, the authors encapsulated A549 cells in a gelatin and glycosaminoglycan matrix modified with VEGF, bFGF, and a laminin peptide to improve cell adhesion, in an attempt to substitute the commonly used Matrigel™ and establishing an improved xenograft model using enriched 3D encapsulated lung cancer cells *in vivo*. The results showed that the functionalized gelatin membrane was comparable to Matrigel™-based models, but allowed a complete control over initial matrix composition [219].

3.3 Design parameters for fabrication of Spherically Structured 3D Tumor Models

Establishing reproducible and easy to analyze spherically structured *in vitro* 3D tumor models containing bioinstructive tumor-ECM moieties requires the manipulation of key parameters including: (i) the inclusion of multiple cellular components of the tumor stroma (e.g., fibroblasts, mesenchymal stem cells, adipocytes, etc) and their cell-cell ratios; (ii) type of culture medium; (iii) scaffolds biodegradability; (iv) selection and degree of functionalization of bioactive ECM-mimetics; (v) 3D spheroids size, as this parameter influences the formation of the characteristic necrotic core of solid tumors; and (vi) microparticles/microcapsules porosity due to its role in nutrients and cells diffusion through the scaffolds. Moreover, other parameters such as tumor ECM mechanical and viscoelastic properties must be controlled to closely mimic those of the native diseased tissues. This one of the most important parameters since as demonstrated by Alessandri and co-workers [216], the elasticity of the matrix in which cells adhere can influence the establishment of a pro-metastatic phenotype, simulating the pressure exerted by surrounding tissues over the tumor mass [222]. Careful consideration must also be given to the manufacturing processes required for the fabrication of spherical 3D models based on microparticles or microcapsules, since the crosslinking processes might require cells exposure into deleterious, non-physiological conditions such as acidic/basic pH [216], organic solvents, or exposure to high-intensity UV light during photo-crosslinking reactions, all of these resulting in loss of cellular viability[16].

4. Conclusions and Future Perspective

The need for expediting drug research both at the preclinical validation level and discovery of novel targets is crucial for the management of currently incurable diseases such as cancer. Research regarding the development of novel 3D *in vitro* models is increasingly contributing to this goal by providing innovative platforms capable of efficiently, predictively, and robustly mimicking *in vitro* the complex *in vivo* reality of the TME in what regards its cellular and ECM components. Among the vast array of 3D cell culture methodologies that have been developed to date for *in vitro* tumor modeling, 3D spheroid-based models are the most promising regarding the production of high-throughput usable and affordable tumor mimetics. Microencapsulation and microparticle-

based production technologies are capable of recreating complex cell-cell, mechanical and physiological characteristics that recapitulate *in vivo* solid tumors, at both the cellular and ECM level. These are highly valuable characteristics since standard 3D spheroid models lack correct ECM representation and confinement of soluble mediators (e.g., growth factors, cytokines) in controlled environments such as those found in human tumors niche.

Overall, there is a tremendous potential for improving 3D spheroid-based drug screening platforms by combining the knowledge acquired in scaffold-based methodologies with microencapsulation or microparticle inclusion techniques to form spherical microtumor constructs compatible with already implemented analysis methodologies (e.g., high-content imaging) [16,17]. Such complex hybrid spherical approaches to 3D tumor modeling, combined with the implementation of co-culture models have the potential to mimic a plethora of features that extend beyond the capacity of conventional 3D-MCTS. The inclusion of populations such as immune-cells (e.g., macrophages, dendritic cells, T-cells etc), adipocytes, mesenchymal stem cells, endothelial cells and tumor stroma associated fibroblasts (CAFs) is paramount importance for a full TME recapitulation. We envision that making efforts towards the inclusion of multiple cells in spherically structured compartmentalized-like capsular models could allow the study of paracrine signaling and provide platforms for discovery of innovative immune-oncological therapeutics. Moreover, microencapsulation provides the means of studying in detail both direct and indirect cell-cell and cell-matrix interactions found in the TME. A deeper knowledge about such crosstalk and events will open the opportunity to develop more advanced therapies that for example inhibit the process of tissue invasion and metastasis. Given that the vast majority of cancer-related deaths is associated with metastasis [229], from a therapeutic perspective, metastasis inhibition will open a new window of opportunity to significantly increase patient survival rates past 5 years.

In a future perspective, the nature of 3D spheroid models and the unique features provided by microencapsulation and microparticle technologies could also be combined with dynamic bioreactor-based culturing technologies to provide an added layer of *in vivo*-like conditions under dynamic flow conditions. Moreover, further improvements to hybrid spherical 3D tumor models are expected upon their combination with advanced organ-on-a-chip platforms and through the implementation of methodologies already used in the field of tissue engineering such as those related to cells microencapsulation cells-

microparticle adhesion, as well as the formation of healthy tissue surrogate constructs. These healthy tissue constructs could be used to study the metastasis process from 3D tumor models in multi-compartment organ-on-a-chip platforms. This is envisioned to contribute to a faster discovery of more effective anti-cancer and anti-metastatic compounds or compound combinations.

Acknowledgements

Conflict of interest

The authors disclose that they have no conflict of interest.

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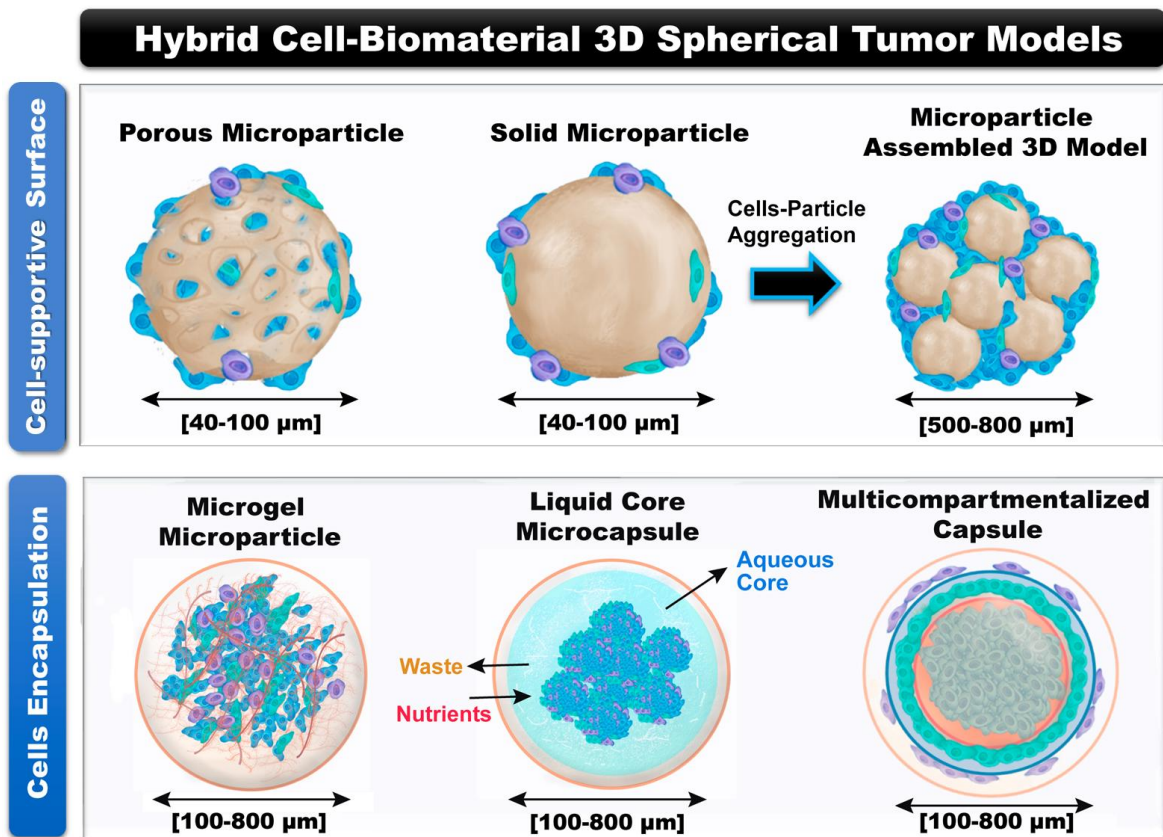
Statement of Significance: The ability to correctly mimic the complexity of the tumor microenvironment *in vitro* is a key aspect for the development of evermore realistic *in vitro* models for drug-screening and fundamental cancer biology studies. In this regard, conventional spheroid-based 3D tumor models, combined with spherically structured biomaterials, opens the opportunity to precisely recapitulate complex cell-extracellular matrix interactions and tumor compartmentalization. This review provides an in-depth focus on current developments regarding spherically structured scaffolds engineered into *in vitro* 3D tumor models, and discusses future advances toward all-encompassing platforms that may provide an improved *in vitro/in vivo* correlation in a foreseeable future.

Table 2. Advantages and disadvantages of different origin materials used for the production of scaffold-based 3D *in vitro* tumor models.

Class	Origin	Examples	Ref.	Advantages	Disadvantages
Natural	Mammalian	Collagen	[66,71]	▪ Contain *in vivo* similar domains (e.g., laminin, elastin, fibronectin) ▪ Cellular adhesive properties ▪ Recapitulate cells-ECM interactions present *in vivo* ▪ Enzymatically degradable	▪ Exact composition is unknown ▪ Batch-to-batch variability ▪ Limited level of control over matrix stiffness along time
		Matrigel™	[72,73]		
		Hyaluronan	[74,75]		
		Gelatin	[76,77]		
		Decellularized Matrix	[78,79]		
	Non-mammalian	Alginate	[80,81]	▪ Cell adhesion properties ▪ High biocompatibility ▪ Affordable	▪ May require further modification to simulate *in vivo* tissues ECM components ▪ Fabrication methods can be cytotoxic
		Chitosan	[82,83]		
		Silk-fibroin	[84,85]		
Synthetic		Polyethylene glycol (PEG)	[86]	▪ Good structural definition and chemically defined ▪ Highly tunable mechanical properties	▪ Lack ECM-mimicking domains ▪ Require further modification to increase bioadhesion and biocompatibility ▪ Degradation can result in acidic by-products
		Polylactic acid (PLA)	[87,88]		
		Poly-ε-caprolactone (PCL)	[89,90]		
		Poly (lactic-co-glycolic acid) PLGA	[87,88]		
Hybrid		Alginate-RGD	[91,92]	▪ Combine the ease of chemical modification and the presence of ECM-like domains	▪ High-costs ▪ Representation of few ECM components
		PEG-RGD	[93,94]		
		PEG-fibrinogen	[95,96]		

Table 2. Summary of commercially available technologies for establishment of 3D *in vitro* tumor models.

Technology	Product Name	Description	Services	Ref
Scaffold-Free	Corning™ Ultra-Low Attachment Multiple Well Plates	Forced Floating 3D spheroids formation via culture in round bottom, ultra-low attachment (ULA) multi-well plates	-	[160]
	GravityPLUS™	Hanging-drop based culture platform for 3D spheroids assembly and that uses a patented plate design which allows fast and user-friendly recovery of cultured microtumors	Insphero® offers an on-demand 3D spheroids development service using scaffold-free platforms	[161]
	Nexcelom3D™	Ultra-low attachment (ULA) multi-well plates with flat or round bottom.	-	[162]
	Nunclon Sphera Surface™	Ultra-low attachment (ULA) multi-well plates with round bottom	-	[163]
	OncoPanel™ 3D	A drug profiling platform comprised of more than 100 types of cell line-based 3D spheroid models suitable for drug-screening and validation. The technology used for 3D spheroids assembly is not disclosed	Provides a service of drug profiling, regarding penetration and anti-proliferative screening in 3D spheroid models for more than 18 different tissue types	[164]
	Synthecon® Rotary Cell Culture Systems (RCCS)	Rotary platforms based in NASA microgravity bioreactors, ideal for 3D spheroids culture under low-shear stress conditions	-	[20]
Scaffold-Based	3D Insert™	Scaffolds with well-defined porous structures comprised either by PCL, polystyrene (PS), or PLGA and suitable for 3D microtissues assembly	-	[165,166]
	Advanced Biomatrix® Matrices and ECM Select® kits	ECM Select® Array Kit Ultra-36 is an array of ECM-mimetic scaffolds based mainly in natural derived ECM constituents (e.g., silk fibroin, collagen types I, II, III and IV, hyaluronic acid, or adhesion proteins – vitronectin, fibronectin, laminin, etc) in which 3D tumor models can be established	Provides an ECM platform in which cells can proliferate and be analyzed. This technology is useful for screening optimal matrix composition and mechanical properties that allow cells to grow in an environment that mimics *in vivo* conditions	[167]
	AlgiMatrix®	Alginate-based scaffold with a highly porous structure suitable for 3D cell culture and microtissues formation	-	[168]
	Alvetex®	Highly porous PS scaffold suitable for 3D cell culture and microtissues formation	-	[169]
	Cellusponge	Disc shaped Collagen type I or Galactose-based scaffold that allow cells to be cultured in easy to use 3D	-	[170]

56

		environments	
	Cultrex®	Murine basement membrane extract obtained from Engelbreth-Holm-Swarm tumors, available in reduced growth factor or concentrated growth factor form. This gel allows 3D cell culture in a bioactive environment	-	[171]
	Cytodex™	A group of crosslinked dextran matrix - based particles, which can be used for 3D cells expansion	-	[91,172]
	Geltrex®	Soluble form of reduced growth factor basement membrane extract purified from murine Engelbreth-Holm-Swarm tumor	-	[173]
	HydroMatrix™ Peptide Hydrogel	A self-assembled scaffold, based on synthetic peptide nanofibers. It offers precise control of 3D matrix architecture. Suitable for 3D cell culture and spheroids assembly	-	[174]
	HyStem® Hydrogels	A diverse set of thiol-modified scaffolds that can be comprised of Hyaluronan (Glycosil®) or Hyaluran and heparin (Heprasil®). Offers the possibly of being combined with Thiol-reactive PEGDA crosslinkers (Extralink®) or Thiol-modified collagen (Gelin-S®)	-	[175]
	Matrigel™	Engelbreth-Holm Swarm sarcoma solubilized basement membrane extract. Available in both concentrated and reduced growth factor forms	-	[176]
	MaxGel™	Human extracellular matrix extract derived from human basement membrane. Suitable for 3D cell culture and invasion assays	-	[177,178]
	Qgel® Vials and Qgel® High-throughput kits	An extensive panel of specialized, PEG-based ECM mimetics with well characterized mechanical properties and chemical composition, specifically tailored for tissue-specific cell lineages or primary cultures. The ECM-like matrices can be provided in modified 96-well plates and/or high-throughput compatible kits, suitable for drug-screening assays	Qgel® provides a specific artificial matrix tailoring service with the objective of finding or designing scaffolds that better recapitulate tumor/tissue specific ECM	[179]
	SeedEZ™ and GradientEZ™	Glass fiber-based disc or flower shaped bioinert scaffolds mainly used to study the influence of compound or growth factor gradients in 3D cultured cells	-	[180]
	SpongeCol®	Type I collagen-based scaffold with cross-linked structure for increased mechanical strength and durability. Suitable for 3D cell culture and microtissue formation in a well-defined biodegradable micro-porous structure	-	[181]
	TrueGel3D™ Hydrogels	Diverse array of scaffolds based in either PEG, PVA or dextran matrices, designed for 3D cell culture in tailored conditions i.e., fast ('FAST-PVA') or slowly ('SLO-Dextran') gelling gels, pH	-	[182]

		responsive gels, or gels tailored by the addition of specific cell adhesion domains		
Hybrid – Bioreactor-based Platforms	3D Perfusion Bioreactor	Combination of bioreactor technology with the 3D Biotek® PCL disc inserts for the formation of perfused microtissues	-	[160]
	3DKUBE™	3D cell scaffold-culture chambers which allows the establishment of independent scaffold-based cell cultures under perfusion	-	[161]
Hybrid – Microfluidic-based Platforms	Ibidi™ μ-Slide III 3D Perfusion,	A set of microfluidic devices capable of working in static or fluid perfusion conditions, in which cells included in 3D scaffold-based models (e.g., Matrigel or other gel-based system) can be cultured. These platforms are suitable for simulating perfusion conditions, allowing for example drug administration under flow, chemotaxis and migration studies to be performed. The tumor models in chips/slides can be analyzed in real-time by microscopy-based analysis	-	[183,184]
	TissUse™ Organ-on-a-Chip	Organ-on-a-chip microfluidic devices that can accurately mimic physiological flow in microchannels. These can work in either free-circulation or closed-loop setup, allowing communication between reservoirs that can contain 3D scaffold-based models of tumor and healthy tissues developed by the user	Provides a specific service of chip design and tailored healthy tissue organoid integration, oriented for drug screening	[185]
	MIMETAS™ Organplate Models	High-throughput compatible, organ-on-a-chip platforms that allows insertion of scaffold-based 3D models (e.g., gel-based), into close-loop microfluidic platforms. These platforms allow direct contact between scaffold containing sections and fluid containing channels by employing a patented phase guide system	MIMETAS™ offers services of OrganPlate® model design for drug development, efficacy screening and toxicity studies in its facilities	[186–188]
	SynTumor™ 3D Cancer Models	Microfluidic devices engineered with tortuous channels with the aim of mimicking tumor-associated erratic microvasculature and transport across the vessel walls. These channels open into a central reservoir that can contain scaffold-based 3D tumor models	Provides real-time screening of tailor made tumor models. Services include target validation, compound screening, biomarkers analysis, adsorption, distribution, metabolism, toxicity and studies regarding mechanisms of action	[189]

*3D - Three-Dimensional; PCL - Poly-ε-caprolactone; PEG - Polyethylene Glycol; PEGDA - Poly Ethylene Glycol Diacrylate; PGA - Poly Glycolic Acid; PLGA - Poly Lactic-co-Glycolic Acid; PS – Polystyrene; PVA - Poly Vinyl Alcohol