Probing the relevance of 3D cancer models in nanomedicine research

Leong, David T.; Ng, Kee Woei

2014

Leong, D. T., & Ng, K. W. (2014). Probing the relevance of 3D cancer models in nanomedicine research. *Advanced drug delivery reviews*, in press.

<https://hdl.handle.net/10356/102502>

<https://doi.org/10.1016/j.addr.2014.06.007>

© 2014 Elsevier B.V. This is the author created version of a work that has been peer reviewed and accepted for publication by *Advanced Drug Delivery Reviews*, Elsevier B.V. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<http://dx.doi.org/10.1016/j.addr.2014.06.007>].

Downloaded on 26 Apr 2023 14:11:36 SGT

Core/shell
nanoparticle with
cancer drugs or
fluorophore

Nanorods &
nanotubes

Nanoparticles
decorated with
cell targeting
ligands

Supporting tissue matrix or synthetic scaffold

stroma

3D cancer cell spheroid or tumor explant culture
in which cancer cells are of different proliferation
capacity or at different stages of the cell cycle

Probing the Relevance of 3D Cancer Models in Nanomedicine Research

David T Leong ^{a*}, Kee Woei Ng ^{b*}

^a Department of Chemical & Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore 117585; email: cheltwd@nus.edu.sg

^b School of Materials Science and Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798; email: kwng@ntu.edu.sg

* Equal contribution and co-corresponding authors

Table of Contents

1. Overview 3

2. Potential roles of nanomedicine in managing cancer..... 5

 2.1 Drug carriers 6

 2.2 Therapeutics 7

 2.3 Homing devices 8

 2.4 Theranostics 9

3. 3D cancer models vs 2D cell culture models 10

 3.1 3D cell culture systems and materials 11

 3.2 Morphological differences leading to phenotype changes..... 12

 3.3 Remembering cell native states in 3D cultures 12

 3.4 Realistic diffusion in 3D culture 15

 3.5 Partially substituting animal use with 3D culture systems..... 15

 3.6 Cancer studies using 3D culture models 16

4. Developing nanomedicine strategies for managing cancer using 3D cultures..... 19

 4.1 Delivering the drug package in 2D vs 3D models 19

 4.2 Imaging uptake in 3D cultures 20

 4.3 Toxicological testing of nanomedicine in 3D 22

5. Conclusions and Future perspectives 24

6. Acknowledgment 26

References..... 27

Abstract

For decades, 2D cell culture format on plastic has been the main workhorse in cancer research. Though many important understanding of cancer cell biology were derived using this platform, it is not a fair representation of the *in vivo* scenario. In this review, both established and new 3D cell culture systems are discussed with specific references to anti-cancer drug and nanomedicine applications. 3D culture systems exploit more realistic spatial, biochemical and cellular heterogeneity parameters to bridge the experimental gap between *in vivo* and *in vitro* settings when studying the performance and efficacy of novel nanomedicine strategies to manage cancer. However, the complexities associated with 3D culture systems also necessitate greater technical expertise in handling and characterizing in order to arrive at meaningful experimental conclusions. Finally, we have also provided future perspectives where cutting edge 3D culture technologies may be combined with under-explored technologies to build better *in vitro* cancer platforms.

Keywords: Nanoparticles; Nanotherapeutics; Nanotoxicology; Nanotheranostics; Cell-nanoparticle interaction; Spheroid; 3D culture

1. Overview

Despite significant advancements in our understanding and development of clinically relevant therapies, cancer remains a major threat to human health. In our battle against cancer, *in vitro* cell culture remains a vital experimental model for both fundamental cancer biology studies as well as translational drug development testing. Conventionally, cell culture is performed on 2D synthetic plastic surfaces in a wide variety of formats. This offers simple, convenient and relatively reproducible means of probing biological processes in response to changes in the cellular environment, and has served scientists well since the late 19th century. However, it is obvious that “life isn’t flat” [1, 2]; most tumors are certainly not. 2D cell culture systems are not adequate in recapitulating the complex stimuli that the native 3D environment provides [2]. In conventional 2D culture systems, adherent cells typically express surface receptors that recognize cell attachment motifs that are adsorbed onto the culture surface. In their native 3D environment, cells regulate various functions through discrete interactions with the extracellular matrix (ECM) often not on a single planar surface that defines the physical, mechanical and biochemical characteristics of each tissue environment [3]. Not surprisingly, cellular interactions with their environment are highly complex and varied in diverse ECM makeup and configuration in different geometries, which in turn directly affect cell phenotype.

Inevitably, using the approach of 3D cell culture is fast gaining popularity. A search on PUBMED using the keywords “3D cell culture” presented an exponential increase in the number of publications on this topic (**Figure 1**). Even then, it has been estimated that less than 25% of all cell culture work is currently done in 3D, while only 30% of cell culture scientists would switch from 2D to 3D cell culture by 2015 [4]. It is therefore expected that a clear shift in cell culture paradigm towards 3D systems will happen over the coming years.

Consequently, 3D cell culture systems have also permeated into cancer research [5].

Pioneering work by Mina Bissell's group was central in providing the early experimental evidence that moving cell cultures into 3D systems made a difference. The group showed that when β 1-integrins on breast cancer cells were blocked in 3D spheroid cultures they regained their normal morphology and became non-cancerous [6]. This phenomenon was not observed in 2D cultures. In addition, protein secretion functions of mammary epithelial cells were enhanced only when they were organized into 3D acinus structures [7].

An exciting development in cancer therapy research is the introduction of nanomedicine. Nanomedicine is broadly defined as the use of nanotechnology in medicine [8]. Among the many manifestations of this definition the use of mobile engineered nanomaterials, generally termed nanoparticles, with respect to cancer research and treatment is one of the most intriguing. This is not only because of the versatility of these nanoparticles but also because of the multitude of nanoparticle parameters that could influence the complex biological outcomes through direct and indirect interactions with biological systems. These parameters include the chemical (composition of material and surface groups reactivities) and physical (shape, size, surface charge and surface area-to-volume ratio) characteristics of the nanoparticles, which directly influence the modes of their interaction with the surroundings and their state of aggregation in different media. This multitude of parameters coupled with the current know-how in synthesizing nanoparticles means that it is now possible to produce nanoparticles of almost infinite configurations [9, 10]. Promising demonstrations of using engineered nanoparticles as cancer therapeutics (nanotherapeutics) have been reported [8]. Nanoparticles in general, can act as drug carriers [11], delivery vehicles for receptor ligands

and agents to improve imaging and targeting [12-16]. They can also interfere with intricate cell biology to effect a desired outcome [17-25] and even directly as a “drug” [21, 26-30].

The marrying of 3D cell culture and nanomedicine will bring many exciting possibilities and open up new and promising frontiers to help advance our understanding of cancer and develop new cancer therapies. This review summarizes the significant developments in this field over recent years and takes a peek into what the 3D culture future holds.

2. Potential roles of nanomedicine in managing cancer

Nanomedicine refers to a wide range of applications of nanotechnology in medicine. These applications include drug delivery, therapeutics, imaging, diagnostic and active implants, among others. Comprehensive reviews of nanomedicine applications are now widely available [8, 31-35]. Mobile nanoparticles are particularly interesting in nanomedicine applications because they have the potential to reach parts of an organ or tissue that are hard to access using conventional approaches.

This suggests that nanoparticles can act as vehicles to transport specific agents to parts of the body, or even as the bioactive agents themselves to elicit a certain biological response. By designing nanoparticles with specific configurations of composition, size, shape, charge and surface functionalization, it may even be possible to achieve targeted delivery or homing of these particles to a specific location [12, 36-42]. Given this possibility, it was natural that nanoparticles began to be explored in cancer – for both research and therapy. Possible uses of nanoparticles in cancer management include photo-thermal and magneto-thermal probes, drug- and gene-delivery vehicles or radiation enhancers [11, 15, 43]. To date, applications of

nanomedicine in managing almost all types of human cancers have been explored. These, including brain [44], head and neck [43], prostate [45], skin [46], liver, pancreatic and gastrointestinal tract cancers [47] had all been expertly reviewed in the literature, and **only** broadly presented here.

2.1 Drug carriers

Cancer remains a major threat to human health today, with 1 in 3 people expected to develop cancer at some point in their lifetime [48]. Treatment of cancer has advanced significantly with the availability of many cancer drugs, mostly as chemotherapy agents, for various cancer types. Most of these are currently introduced into the body systemically by intravenous infusion. Consequently, in order to achieve desired concentrations within cancerous tumors, cancer drugs need to be administered at even higher concentrations, which in turn result in toxicity to the surrounding healthy tissues and increase the possibilities of side effects. With the discovery that cancer tumors have compromised lymphatic drainage and leaky vasculatures, a window of opportunity opened up for free circulating nanoparticles to accumulate in tumors, a phenomenon known as the enhanced permeability and retention (EPR) effect [49]. There is therefore a natural mechanism in place to allow drug-carrying nanoparticles to “home” into tumors and offload the drug. With the intercellular gaps within the endothelial layer of tumor blood vessels understood to be 400-600nm wide, at least in human colon adenocarcinoma [50], it appears that a wide range of nanoparticles or even submicron particles could do the job. Recently, it was also reported that some nanoparticles can induce endothelial cell leakiness (NanoEL) by themselves [51], suggesting that there could be a way to enhance the EPR effect through direct interaction between nanoparticles and the endothelium.

Nonetheless, it is likely that the EPR effect needs to be considered along with other mechanisms affecting the biodistribution of nanoparticles within an organism. Moreover, the EPR effect is only significant in matured tumors where a leaky vasculature exists. To achieve targeted delivery, the physicochemical parameters of the nanoparticles are clearly important factors. In addition, the ability of nanoparticles to evade clearance mechanisms is a major factor too. For instance, Glazer et al [52] showed that gold nanoparticles introduced systemically into rabbits implanted with liver Vx2 tumors not only accumulated at the tumors but also at the spleen and liver. In fact, after 24 hours the majority of gold nanoparticles were found in the liver. If this is indeed the general fate of nanoparticles introduced into the body systemically, then the advantage of more efficient cancer drug delivery to tumor sites based on the EPR effect could be lost.

2.2 Therapeutics

Advancements in nanotechnology have also given rise to significant concerns over the impact of nanomaterials on human health, fuelling the birth and rapid development of the field of Nanotoxicology [41, 53-55]. While nanotoxicology is a bane to our well-being, it may turn out to be a boon in our fight against cancer. Pristine nanoparticles may potentially be used directly as “drugs” to kill cancer cells. Not surprisingly, the potential of this approach has been demonstrated using a wide permutation of nanoparticle types and cancer cell lines. For example, Gonzalez et al [56] demonstrated that amorphous monodispersed silica nanoparticles resulted in genotoxic effects in the human lung carcinoma A549 cells with particle number and total surface area being the most important factors. Srivastava et al [57]

reported that multi-walled carbon nanotubes (MWCNTs) at concentrations of 10 and 50 mg/ml induced significant cytotoxicity, oxidative stress and apoptosis in the same A549 cells.

Since the liver tend to be the primary organ where nanoparticles accumulate due to the body's natural clearance mechanism [52], attempts were made to target liver cancer using nanoparticles. To this end, Paino et al [58] found that gold nanoparticles with diameters ranging from 7-20nm, at a concentration as low as 0.1 μ M and coated with either sodium citrate or polyamidoamine dendrimers, inflicted a greater extent of cytotoxicity and genotoxicity in human hepatocellular carcinoma cells (HepG2) compared with peripheral blood mononuclear cells. Other nanoparticles can also result in similar effects in HepG2 cells, such as anatase titanium dioxide (TiO₂) nanoparticles less than 25nm in diameter [59]. Further examples of other nanoparticle types inducing toxicity and genotoxicity in cancer cells are common in the literature, such as carbon nanotubes in human colon carcinoma cell line HT29 [60].

2.3 Homing devices

To achieve more efficient homing to cancer cells, functionalized nanoparticles have been developed by targeting specific moieties that are found on cancer cells [61]. Nanoliposomes carrying doxorubicin and conjugated with a peptide ligand for $\alpha_5\beta_1$ integrin were able to home more effectively to B16F10 melanoma cells to enhance intracellular uptake and induce cytotoxicity. These nanoliposomes also displayed stronger tumor inhibition and prolonged survival time in comparison with controls without the peptide ligand, in C57BL/6 mice bearing B16F10 tumors [62].

Epidermal growth factor (EGF) receptors are highly upregulated in cancers and are potential targets for cancer cell homing as well. At the same time, EGF receptor activation is also able to trigger nanoparticle endocytosis. Functionalizing nanoparticle surfaces with EGF receptor ligands is therefore a popular strategy. Tseng et al [63] fabricated gelatin nanoparticles decorated with biotinylated EGF as carriers of cisplatin and showed these to have improved specificity to A549 cells compared with non-EGF decorated controls. By aerosol delivery of the EGF-decorated nanoparticles into mice, lung tumor volume was also more effectively controlled. Surface functionalization approach can also help nanoparticles access hard to reach tissues, such as crossing the blood-brain barrier to reach brain tumors. For this purpose, nanoparticles can be decorated with a multitude of ligands such as EGF, integrins and transferrin receptors, strategies that are comprehensively reviewed elsewhere [44].

2.4 Theranostics

Theranostics is a new term coined for strategies that are used for both diagnosis and treatment simultaneously. The aim of this approach is to eliminate multiple step procedures and reduce delays in treatment so as to improve patient care [64, 65]. Given their versatility, engineered nanoparticles have been closely linked to the development of novel theranostics approaches, giving rise to yet another new spin in the nanotechnology revolution – nanotheranostics [14, 66-69]. Typically, multiple agents are packaged into nanoparticles, including ligands for cancer cell targeting, drugs for treatment and imaging modalities for detection or even real-time monitoring of the therapeutic agents. For example, in order to target melanoma cells, Vannucci et al [70] built nanoparticles using the heavy chain of human ferritin, and loaded these with α -melanocyte stimulating hormone for specific targeting, plus rhodamine for imaging. To stabilize and mask the ferritin to prevent binding with its native receptors, the

authors further coated the nanoparticles with polyethylene glycol molecules. These nanoparticles were specifically taken up by melanoma cells and demonstrated tumor targeting ability in melanoma-bearing mice. Other nanoparticle systems including metal oxides, magnetic nanoparticles, gold, carbon nanotubes and polymers have also been explored for theranostics applications in cancer [71-73]. While promising, nanotheranostics approaches present new technical challenges due to the need for multi-modal analysis for evaluating the efficacy of multi-decorated nanoparticles. Accuracy of imaging degradable, dye-conjugated nanoparticle systems is particularly difficult due to uncertainty of nanoparticle-dye binding profiles over time [74].

In summary, there is clearly a host of nanoparticle configurations and multiple approaches that have been demonstrated, or are being developed, for treating and understanding cancer. It is not the intention of this review to cover all of these possibilities but rather to give a glimpse of the possibilities. Although the developments described in this section are exciting and hold great promise for cancer management, the use of 3D culture systems for such development purposes is still in its infancy. Nonetheless current awareness of the relevance of 3D culture is high and acceptance of 3D experimental approaches as mainstream would likely happen in the foreseeable future. The rest of this review offers a closer look at the exciting new possibilities that 3D cultures can bring to realize the clinical potential of nanomedicine in cancer.

3. 3D cancer models vs 2D cell culture models

In every *in vitro* cell testing system, whether 2D or 3D, one of most important purposes is to recapitulate what is in the *in vivo* environment. The degree of mimicry at the chemical,

biological and physical level is targeted to bring *in vitro* system to be as close as possible to *in vivo* mimicry. Tumors like tissues in the body are very complex structures with a multitude of connectivity and hierarchy. To recapitulate that level of complexity on a 2D surface is preposterous [75]. While it is highly intuitive that 2D cell culture will not recapitulate the real *in vivo* settings as well as 3D cell culture, it is not too intuitive to actually know what is key. This section provides an overview of the different 3D cell culture systems currently available and describes the significance of using these in our attempts to mimic the *in vivo* environment with *in vitro* platforms for developing cancer management and research strategies with engineered nanoparticles.

3.1 3D cell culture systems and materials

Although 3D cell culture systems are not yet the mainstay of cancer research, a wide range of such systems are now available, and are summarized in **Table 1**. Choosing the right system to use for any particular scenario of testing nanoparticle interaction with cancer is not straightforward. The most realistic 3D model has to be the organ explant culture but this may not be the most practical to adopt due to limited availability and complexity in characterization. On the other hand, cell spheroids are the easiest and most convenient to prepare and are evidently popular in the literature [76] (**Figure 2**). Using various natural or synthetic biomaterials to create 3D culture systems allows greater flexibility in manipulating the cancer environment [5] (**Figure 3**). However the success of these systems requires specific material processing expertise. Clearly, there is unlikely to be a “one-size-fits-all” method for our purpose. In fact, it is probable that thorough *in vitro* testing of any nanoparticle system will have to be achieved by using a combination of different 3D culture systems. More details specific to 3D cell culture systems can be found elsewhere [77, 78].

3.2 Morphological differences leading to phenotype changes

When cells are plated on cell culture plastic, the focal adhesion forces a high contact surface with the cell culture surface and this leads to only a small fraction of the cell membrane to be actually in contact with the neighboring cells. When in such constrained conformation, membrane and transmembrane connections for paracrine communications will be severely altered. Even a simple change in cell shape can profoundly affect cellular function [79, 80]. The spatial orientation of these communication entities between cells are important and would be impossible to replicate in a 2D configuration. In contrast, when in 3D context, the intrinsic morphology of the cells are already more similar to that of the same cells in the original tissue architecture [81, 82]. Appropriate cellular and sub-tissue polarity could then be recapitulated. For instance, epithelial cells in 3D cell culture model can organize into apical and basolateral orientation which is critical in overall tissue organization and for luminal secretions [83, 84].

3.3 Remembering cell native states in 3D cultures

Within a tissue, several directions help cells to orientate themselves. For example, the lumen in the gut and gastrointestinal cells and also the mammary lumen help to tell cells which side is lumen and which side is basal. On a horizontal culture, even in a multi-layer format, gravity is always acting away from the lumen while in 3D, depending on the cell position, the lumen side could be aligned with or aligned opposite to gravity or any other angles between these two extremes which are closer to *in vivo* settings.

In 2D cultures, monolayer cells show stress fiber formation instead of cortical actin arrangement as in tissue native cells [85, 86]. This important cytoskeletal arrangement difference can profoundly affect the phenotype of the cell. This adhesiveness of cells on 2D cell substrate affects the migration ability of the cells [87]. Minimally the overall morphology of the cell is already very different due to the type of cytoskeletal structures formed when in 2D. In 3D, the eventual stable morphology of the cell is more similar to the original structure of the tissue [2, 88]. Appropriate cell and tissue polarity also dictate functionality. For instance, in epithelial cells, the cytoskeletal formation partially stems from the cell's perceived apical and basolateral orientation which again dictates overall tissue organization and secretion of products into the apical face [83, 84]. Even where there is apical and basolateral orientation, cells in 2D may still lack physiologically relevant tissue-like structures [2, 88] as there is general loss of tissue organization as isolated cells adapt to 2D monolayer culture [89]. For example, 2D monolayer cells are incapable to form the acinus structure found in liver [90].

The proper functioning of clusters of homogeneous or heterogeneous cells at a sub-tissue level clearly depends on the orientation of these cells in relation to one other. Simplistically, their spatial arrangement pre-determines their structure. These structures are not present in culture either because the cells have lost their spatial memory or it is impossible for the cells to adopt those structures due to missing essential building blocks. 3D culture system may reinstate their native tissue memory through allowing cells to self-assemble and to re-orientate their own native spatial arrangement.

Petri dish used for cell culture presents a stiffness that is mostly alien to the cells used to native substrate stiffness in tissues. Thus, 2D cells might not have the same characteristics as

cells in a real tissue [91-94]. Altered metabolism and gene expression frequently occur when isolated cells adapt to 2D cell culture model [95, 96]. Significant reduction of functional genes expression was observed when cells adapt to 2D cell culture [86]. In contrast, when in 3D cultures, cells are able to maintain some of the organ-specific activities, such as Cytochrome P450 (CYP) activity and albumin secretion for liver [86, 97], milk protein secretion for mammary epithelial acini [7].

3D cell culture can be used to form dense-tissue like cell clusters and to construct more complex cellular organization partly due to the independence from culture plastic substrates [98-100]. This independence forces the cells to form more realistic *in-vivo*-like interactions with one another resulting in a dense configuration. For example, primary hepatocytes cultured as spheroids could form bile canaliculi with multiple tight junctions [101]. The hepatocytes packed tightly with one another as contrasted to its otherwise 2D culture plastic configuration where the same hepatocytes would have packed loosely but tightly with the underlying substrate. Epithelial cells could still retain their original functional memory and could organize into spherical 3D structures with a lumen which resembles a lung alveolus , acinus of a gland and a kidney glomerulus [82, 102]. Besides that, primary stromal fibroblasts cultured in 2D condition lose its *in vivo* stromagenic characteristics momentarily [103]. For instance, hFOB1.19 cells even in high confluence monolayer cultures do not form bone specific matrix in the absence of osteogenic induction factors [104, 105]. Overall, the 3D models makes for a more realistic and functionally relevant model in testing the effects of drugs with the advantages of ease of assaying coupled with mimicking the animal studies. Multi-cell type culture model to mimic a real tissue with different cell types can be established by using 3D cell culture model [106, 107].

3.4 Realistic diffusion in 3D culture

2D cell culture model does not have a realistic concentration gradient for mass transfer as a real tissue. 2D cell model comprises of a monolayer of cells, which allows the materials to reach its intended target easily by diffusing through a short distance of cell membrane and cytoplasm often with no or little extracellular matrix on the top side of the cell layer. Whereas in 3D cell models, at least the densely packed cell clusters themselves and the appropriate extracellular matrix (ECM) layer simulates a more realistic mass transfer gradient [108-111]. ECM, which consisted of fibrous structural proteins, specialized proteins and proteoglycans [94], could affect mass transfer of certain compounds and establish a concentration gradient in the model [112].

3.5 Partially substituting animal use with 3D culture systems

In general, 3D cultures offer a relative ease in establishing a viable testing system and its associated assays when compared to *ex vivo* tissue or organ culture [110]. For example, real-time and fixed imaging of a 3D culture will be easier compared to visualizing a specimen group in the animal and let alone study the target tissue at the *in vivo* cellular level [113].

In addition, since the physiology, metabolism and cells in animals are different from humans, traditional *in vivo* animal tests often fail to predict the actual toxicity and drug efficacy to human beings [89, 114-116]. Furthermore, since pre-clinical trials forbid testing in humans, 3D models using human cells can potentially bridge this inter-species gap.

3.6 Cancer studies using 3D culture models

However, the current literature in cancer research still perpetuates to use 2D cultures as the common model to test hypotheses and drugs [5]. There are several studies that repeat some of the 2D experiments in 3D models and illustrated interesting findings. Most of these studies attempt to partially recapitulate major cancers.

Using spheroid 3D melanoma models, the tumor environment **can be** recapitulated. SBCL2, VM-115 and 451-LU cell lines, representing different stages of melanoma progression were first grown in hanging drops cultures to form spheroids and later mixed with skin fibroblasts and embedded into a collagen type I hydrogel to form an organotypic 3D culture. It was found that the cells in the 3D culture system is more susceptible to TRAIL-cisplatin but not to TRAIL-UVB as opposed to the 2D system using the same host of melanoma cell lines were susceptible to both treatment regimes [117]. This suggested that while small molecules like cisplatin could penetrate through the multi-layered construct while relatively low penetrating power radiation could not reach the tumor cells to achieve a synergistic effect with TRAIL.

In another study where a relatively large mouse melanoma B16-F10 spheroid of about 1cm in diameter was used as a model for GFP DNA plasmid transfection using electroporation techniques, it was found that even at that large dimensional scale, there were significant degree of GFP expressing cells showing successful transfection [118]. This model helps to recapitulate the tumor environment within and outside of the melanoma tumor and the findings suggested that one might still be able to deliver a specific DNA cargo for gene transfer therapy despite the sheer size of the tumor. It would be interesting if the spheroids

could be made in varying sizes to fine tune to the threshold size that would still be transfectable.

Oral squamous cell carcinoma (OSCC-3) cells seeded on a high porosity poly(lactide-co-glycolide) (PLG) scaffold could recapitulated some of the major characteristics of an *in vivo* tumor. There were clear demarcation of highly proliferative regions from necrotic and apoptotic regions in accordance with the nutrient environment. Tumor cells in this 3D construct interestingly grew at a similar rate as real tumors versus that grown in Matrigel which tended to be much faster [119-121]. Intra-tumoral oxygen tension in this PLG-OSCC-3 construct also mimics that of the real tumor, suggesting that angiogenesis formation within the tumor construct may likely follow that of the real tumor [120]. These are important design parameters in the push towards a realistic 3D tumor construct. When comparing cells grown in 2D culture, those grown in this PLG construct exhibited higher metastatic potential and were resistant to LY294002, a PI3K inhibitor used as an experimental drug. These findings further emphasized the need to retest some of the experimental drugs, that showed efficacy in 2D cultures, first in 3D cultures before progressing on to the animal studies and preclinical trials. Using surface feature imprinting techniques on a flat substrate surface, HT29 colon cells adopted a 3D aggregate culture instead of a monolayer of cells [122]. The adaption also recapitulates some of the features of a tumor. There was a hypoxic core with a corresponding upregulated expression of HIF-1, a master transcription factor for hypoxia induced genes. This hypoxic core drives tumor derived angiogenesis in an *in vivo* tumor, thus suggesting that cancer cells within the core of the construct can be used to test HIFs inhibitors [123].

In another study involving tumorigenic accessory cells like cancer associated fibroblasts, co-culture of spheroids of colorectal cancer cells encapsulated within cancer associated

fibroblasts in a collagen matrix started to show characteristic features of the cancer associated fibroblasts-tumor synergism [124]. Highly proliferative tumor cells within the spheroids started expressing phospho-Rb, similar to *in vivo* colon tumors [125]. As the spheroids continue to proliferate to around 450µm, a corona of proliferating cells starts to form over necrotic and apoptotic cells, again reminiscent of *in vivo* tumors [126, 127]. At the pathway level, being in this co-culture-spheroid system, there was synergistic upregulation of the Wnt pathway with canonical beta-catenin nuclear translocation. The Wnt pathway is a commonly activated cancer stem cell pathway in colorectal cancers [124, 128]. Also treatment with LY294002 reduced the growth of the spheroids in the co-culture system although it remains unclear whether the effect was due to growth inhibitory action on the cancer associated fibroblasts or the spheroid or both [124]. Nonetheless, this study showed minimally that the complexity and heterogeneity of *in vivo* tumors can be sufficiently replicated in their 3D-co-culture system where informative experiments can be reliably carried out.

The power of tissue engineered constructs may also be employed in the study of metastatic cancer. Through tissue engineering, the complex *in vivo* environment in 3D can be better recapitulated. This tissue engineered construct then becomes the more realistic testing ground for cancer cell behavior in the native environment. Huttmacher and co-workers tissue engineered human bone like constructs through a process of seeding human mesenchymal progenitor cells onto calcium triphosphate coated medical-grade polycaprolactone scaffolds. These constructs were first grown in static culture conditions and then transferred to a bioreactor and supplemented with pro-osteogenic differentiation media. The resulting bone-like constructs were subsequently implanted into NOD-SCID mice and allowed to mature *in vivo*. Metastatic prostate cancer cells (PC3) injected into circulation were found to be able to

home to those tissue engineered constructs and showed colonization in similar ways as the real human metastatic prostate cancer [129]. In similar ways, breast to bone metastasis could also be studied in humanized tissue engineered constructs [130-132]. To further push the limits of information that could be gleaned with tissue engineered bone constructs, the use of diseased bone cells in osteoporosis could now be included to study the complications arising from breast cancer metastasis in menopausal women.

4. Developing nanomedicine strategies for managing cancer using 3D cultures

The advantages and relevance of 3D cell culture models over conventional 2D ones are clear and well described above. These advantages are amplified in cancer models because of features unique to tumors which are not represented in 2D cultures. Research and development in nanomedicine approaches for cancer therapy has therefore benefitted from 3D cell culture models (**Figure 4**).

4.1 Delivering the drug package in 2D vs 3D models

For the purpose of drug delivery using nanocarriers, assessing the ability of the nanocarriers to navigate through a tumor stroma is essential. 3D cancer models offer a more realistic *in vitro* environment to do this. Mitra et al. [133] evaluated the efficacy of cancer drug-loaded chitosan-alginate nanoparticles in 3D cultures of Y79 retinoblastoma cells embedded in PLGA-gelatin microparticles. Notwithstanding potential discrepancies in the efficiency of drug delivery to cells within the 3D microparticles due to diffusion limitations, all three drugs tested – doxorubicin, carboplatin and etoposide recorded significant reductions in efficacy in the 3D cultures compared to conventional 2D cultures. A separate study using breast cancer

cells MDA-MB-231 showed the same difference in cancer drug efficacy between 2D monolayer cultures and 3D cultures in silk fibroin scaffolds [134]. For the purpose of targeting gliomas, which is challenging because of the need for delivery vehicles to cross the blood-brain barrier, Jiang et al [135] fabricated nanoparticles with poly(trimethylene carbonate) cores encapsulated with paclitaxel, and surfaced functionalized with integrin recognizing RGD peptides conjugated onto PEG chains. To test the efficacy of this system, U87MG glioma cells were cultured on agarose coated plates to produce 3D glioma spheroids and treated with the nanocarriers. Compared with conventional nanoparticles, the RGD-decorated nanocarriers showed the most efficient penetration and accumulation into the 3D glioma spheroids, along with significant microtubule stabilizing ability. These results corroborated with *in vivo* data, suggesting the relevance of using 3D glioma spheroids in such studies. Using a different approach, Cheng et al [136] made use of the tumorigenic migratory properties of human bone marrow derived mesenchymal stem cells to deliver nanoparticles for cancer targeting purposes. NeutrAvidin labeled polystyrene nanoparticles of 40 nm diameter were bound onto biotinylated plasma membrane to form nanoparticle patches. These cells were able to polarize towards liver tumor spheroids composed of mouse embryonic fibroblasts, human endothelial cells (HUVEC) and human liver cancer cells (Hep G2) cultured in 3D collagen I gels.

4.2 Imaging uptake in 3D cultures

One of the clear challenges of any bioimaging experiment in 3D is whether the signal could be captured from within the 3D construct. It has been shown that with confocal microscopy, uptake of fluorescent-labeled nanoparticles from within a cell spheroid can be detected (**Figure 2E**). Using X-ray microscopy, Chen et al. [137] demonstrated differences in the

uptake of bare and PEGylated gold nanoparticles by EMT-6 and HeLa cells cultured in 2D monolayers versus 3D spheroids. Differences were also observed in uptake among cells within the same cultures, which is likely due to cell cycle variations [138]. Similarly, England et al [139] used 3D spheroids of lung (A-549), liver (HEPG2) and pancreatic (S2VP10) cancer cells to study uptake of nanoparticles. Gold nanoshells coated with alkanethiol and phosphatidylcholine exhibited significantly higher uptake compared to PEGylated nanoshells in all three tumor spheroids. Besides using cell spheroids in suspension, scaffolding materials have also been used to create a 3D environment for cancer cultures. Human lung cancer cells were cultured in alginate scaffolds, AlgiMatrix™, for this purpose [140]. Uptake of nebulized celecoxib encapsulated lipid nanocarriers was then evaluated. Results showed that uptake of the lipid nanocarriers were limited only to the peripheral cells of the tumor spheroids. Cell spheroids could also be produced by bringing cells together using paramagnetic particles. Ho et al [141] demonstrated this approach by biotinylating HeLa cells and conjugating streptavidin paramagnetic particles to them. With these magnetized cell spheroids, the authors tested the efficacy of pH-responsive nanocarriers synthesized by grafting L-phenylalanine with poly(L-lysine isophthalamide). They found that these nanocarriers, of hydrodynamic size 37 nm at pH 7.4, were able to penetrate to the interior of the magnetized HeLa cell spheroids and be internalized. Another variation of 3D culture for nanomedicine application is the combination of cell cancer cell spheroids with explant cultures of healthy tissue. Meng et al. cultured medulloblastoma-derived cell spheroids on healthy rat cerebellum slices and found that these cells internalized poly(glycerol-adipate) nanoparticles while the healthy brain cells in the cerebellum slices did not [142].

For imaging applications, such as in theranostics approaches, *in vivo* tumors are often the preferred choice to evaluate nanoparticle localization because they come complete with

vasculature and a mature stroma to allow appreciation of the real capabilities of the nanoparticle being studied. For example, Robinson et al [143] demonstrated the capability of phospholipid-PEG solubilized single-walled carbon nanotubes to localize to 4T1 murine breast tumors in Balb/c mice rapidly and effectively. Imaging was achieved by making use of the intrinsic fluorescence of the carbon nanotubes in the second near-infrared window combined with dynamic contrast imaging through principal component analysis. A similar approach was also explored using CdSe quantum dots conjugated with monoclonal anti-HER2 antibody, for imaging breast cancer cells in 3D tumors *in vivo* [144]. However *in vitro* 3D culture systems can be beneficial as well for purposes such as *in situ* imaging of nanoparticle uptake and localization within cells. For these purposes, imaging modalities such as a software for analyzing cell uptake of nanoparticles in 3D [145] and darkfield-confocal microscopy detection of nanoparticle internalization [146] have been developed.

4.3 Toxicological testing of nanomedicine in 3D

Emphasis is heavy on nanomedicine functionality in eliciting a particular therapeutic effect for obvious and understandable reasons. However, side effects like toxicity of the same nanomedicine on non-targeted cells need to be determined with equal tenacity. Therefore, as with the advocacy of testing nanomedicine in 3D, similarly, nanotoxicology studies for the purpose of developing nanomedicine approaches for cancer will also benefit from switching from 2D to 3D cultures *in vitro* [55]. It is known that even in 2D cultures, nanotoxicity can be influenced by cell density and culture formats [147]. It is therefore not surprising that the mode of nanoparticle interaction with biological systems varies between 2D and 3D environments, which can significantly affect nanotoxicity influences. Such discrepancies are in fact a source of confusion in the current literature with regards to contradictory toxicity

reports. For example, single-walled carbon nanotubes did not affect the viability of Schwann cells cultured in 3D Matrigel™ as much as in 2D monolayers [148]. In another tissue-mimetic model, produced by using an ultrasound standing wave trap to generate 3D cell aggregates of human monocytic leukemia cells (THP-1), both purified and oxidized single-walled carbon nanotubes did not exert significant toxicity after 24 hours exposure, compared to 2D cultures [149]. Inflammatory cytokine secretions were also not elevated in the 3D spheroids. In agreement with most nanotoxicology studies using 3D cultures, cadmium telluride quantum dots (QDs) were found to exert greater toxicity in dispersed, heterogeneous, 2D cultures of dorsal root ganglia cells compared to 3D dorsal root ganglia explant cultures, under the influence of α -lipoic acid as a cytoprotective antioxidant [150]. Explant cultures were evaluated based on their ability to maintain and extend neurite outgrowth. Similar differences between 2D and 3D spheroid cultures were also reported in liver cancer models made from human hepatocellular carcinoma cells (HepG2) [151]. For nanotoxicity screening studies, 3D cultures may be perceived to be at a disadvantage because of challenges in setting up high throughput approaches. Nonetheless, the feasibility of this was demonstrated by Luo et al [152], who produced 3D microtissue arrays by seeding HeLa and MG-63 cells in non-sticky microwells, and allowing them to aggregate into microtissues with defined size and shape. The authors tested the cytotoxicity influence of bismuth, zinc sulfate-coated cadmium selenide and iron oxide nanoparticles using classical metabolic and live/dead assays and concluded that toxicity levels were significantly lower in the 3D microtissues compared with 2D cultures. These studies generally outline the likelihood that nanotoxicity studies carried out on 2D cell cultures inherently exaggerate the real toxicity of the nanoparticle in question, presumably due to unrealistically direct access to the cell of interest in 2D.

Although 3D cultures are important in enhancing the relevance of *in vitro* studies in nanomedicine applications in cancer, they are limited in size to about a few hundred microns in diameter due to diffusion limits, which could restrict their versatility. Care is needed to prevent confounding factors such as diffusion limits, influence of vasculature and extracellular matrix protein composition from clouding our interpretation of data discrepancies between 2D and 3D cultures.

5. Conclusions and Future perspectives

Nanomedicine has the promising potential to significantly improve clinical practice. It has opened up new platforms of opportunities to provide more efficient and effective modalities in diagnostics, therapy and research. Nanomedicine applications in cancer management are particularly exciting because cancer remains a major threat to human health. Nonetheless, there remain significant challenges in bringing a nanomedicine strategy for managing cancer from research to clinical reality.

There are conflicting data on the potential effects of various nanomedicine. This is somewhat expected given the multitude of parameters that can affect the physical and biochemical behavior of these nanomedicine in different biological contexts. For accurate comparisons across different studies, the onus is therefore on scientists to report as much details as possible about the physico-chemical properties of the nanomedicine instead of just its efficacy. It is also important that these properties be measured in conditions that mirror that of the actual situation that the nanomaterials are exposed to the biological system studied.

For decades, since the first few cell lines were successfully derived from tumors, the 2D cell culture system has been the main *in vitro* approach used to answer important cell biology questions in more easily controlled experiments. Those efforts have enhanced our understanding to a great extent. As the call to improve on *in vitro* setups to better recapitulate the *in vivo* environment continues to grow louder, 3D cell culture systems have emerged as the front runner to help us move towards that direction. Cancer cell spheroids cultured in suspension or embedded in hydrogels are one of the most commonly used 3D culture models. Other 3D biomaterial systems, organotypic cultures and explant cultures are now increasingly being used. We envisioned that more *in vivo*-like 3D systems will materialize in tandem with our increasing knowledge of fundamental cancer cell biology and their interactions with their micro-, nano- and molecular- environments. These will give us greater ability to manipulate cellular behavior, image their phenotype (or change) quantitatively in real time. There might be increased integration of nanotechnology with 3D culture systems as we begin to solve the many technical issues associated with being 3D. Some of these enabling nano technologies that can resolve these issues could be ultrasensitive *in situ* nanosensors capable of monitoring the biochemistry of the cells in 3D, or engineered targeting-stimuli release nanomedicine capable of homing into specific cells within the heterogeneous cell population of a 3D culture system. Testing nanomedicine with 3D culture cancer models instead of using traditional 2D based cultures increases the reliability of the findings since the 3D models mimics the *in vivo* environment more closely. To test nanomedicine candidates at the industrial scale, the use of 3D cultures models allows integration into the existing high throughput liquid handling infrastructure ironically more easily than the current 2D culture models.

Given the complex, multifactorial nature of studying the efficacies of nanomedicine strategies in 3D cancer models, mathematical modeling is another useful tool to help with the process. Experimental data provide the parameters needed for advanced computer simulation techniques to solve the rigorous calculations needed to model nanoparticle behavior like diffusion or cellular uptake of nanoparticles in a 3D setting. These mathematical models can then be compared again with experimental 3D models to validate their accuracy. This convergence can be iteratively examined as hypotheses are first tested using mathematical models and further validated again with experimental data and vice versa [153]. Borrowing the idea of a multiscale road map for modelling cancer spheroids to understand cancer progression [153], we present here a road map for integrating nanoparticle configurations, 3D culture models, experimental techniques and mathematical modeling in order to advance cancer nanotherapeutics (**Figure 5**).

Overall, the elegant interactions between nanomedicine and 3D culture systems are synergistic towards the ultimate goal of improving cancer therapeutics. It is our belief and hope that this can herald in a new era in novel therapeutic discovery and their mechanistic understanding.

6. Acknowledgment

D.T. Leong would like to acknowledge funding support from Ministry of Education, Singapore (R-279-000-350-112) and NUS Department of Chemical and Biomolecular Engineering. K.W. Ng would like to acknowledge the funding support from the Nanyang Technological University (COE_SUG/RSS_20AUG10_10/14).

References

- [1] K.S. Smalley, M. Lioni, M. Herlyn, Life isn't flat: taking cancer biology to the next dimension, *In Vitro Cell Dev Biol Anim*, 42 (2006) 242-247.
- [2] A. Abbott, Cell culture: biology's new dimension, *Nature*, 424 (2003) 870-872.
- [3] J.S. Harunaga, K.M. Yamada, Cell-matrix adhesions in 3D, *Matrix Biol.*, 30 (2011) 363-368.
- [4] HTStec, 3D Cell Culture Trends 2011, Cambridge, UK, 2011.
- [5] D.W. Hutmacher, Biomaterials offer cancer research the third dimension, *Nat Mater*, 9 (2010) 90-93.
- [6] V.M. Weaver, O.W. Petersen, F. Wang, C.A. Larabell, P. Briand, C. Damsky, M.J. Bissell, Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies, *J. Cell Biol.*, 137 (1997) 231-245.
- [7] M.J. Bissell, A. Rizki, I.S. Mian, Tissue architecture: the ultimate regulator of breast epithelial function, *Curr. Opin. Cell Biol.*, 15 (2003) 753-762.
- [8] V. Wagner, A. Dullaart, A.K. Bock, A. Zweck, The emerging nanomedicine landscape, *Nat Biotechnol*, 24 (2006) 1211-1217.
- [9] Y. Yu, Q. Zhang, J. Xie, J.Y. Lee, Engineering the architectural diversity of heterogeneous metallic nanocrystals, *Nat Commun*, 4 (2013) 1454.
- [10] X. Yuan, B. Zhang, Z. Luo, Q. Yao, D.T. Leong, N. Yan, J. Xie, Balancing the rate of cluster growth and etching for gram-scale synthesis of thiolate-protected au25 nanoclusters with atomic precision, *Angewandte Chemie (International ed. in English)*, 53 (2014) 4623-4627.
- [11] G.R. Tan, S.S. Feng, D.T. Leong, The reduction of anti-cancer drug antagonism by the spatial protection of drugs with PLA-TPGS nanoparticles, *Biomaterials*, 35 (2014) 3044-3051.
- [12] C.Y. Tay, W. Fang, M.I. Setyawati, C.P. Sum, J. Xie, K.W. Ng, X. Chen, C.H.L. Hong, D.T. Leong, Reciprocal Response of Human Oral Epithelial Cells to Internalized Silica Nanoparticles, *Particle & Particle Systems Characterization*, 30 (2013) 784-793.
- [13] X.D. Zhang, J. Chen, Z. Luo, D. Wu, X. Shen, S.S. Song, Y.M. Sun, P.X. Liu, J. Zhao, S. Huo, S. Fan, F. Fan, X.J. Liang, J. Xie, Enhanced Tumor Accumulation of Sub-2 nm Gold Nanoclusters for Cancer Radiation Therapy, *Adv Healthc Mater*, 3 (2014) 133-141.
- [14] M.S. Muthu, D.T. Leong, L. Mei, S.S. Feng, Nanotheranostics - Application and Further Development of Nanomedicine Strategies for Advanced Theranostics, *Theranostics*, 4 (2014) 660-677.
- [15] X.-D. Zhang, Z. Luo, J. Chen, X. Shen, S. Song, Y. Sun, S. Fan, F. Fan, D.T. Leong, J. Xie, Ultrasmall Au₁₀₋₁₂(SG)₁₀₋₁₂ Nanomolecules for High Tumor Specificity and Cancer Radiotherapy, *Advanced Materials*, (2014).
- [16] Y. Yu, Z. Luo, D.M. Chevrier, D.T. Leong, P. Zhang, D.E. Jiang, J. Xie, Identification of a highly luminescent Au₂₂(SG)₁₈ nanocluster, *Journal of the American Chemical Society*, 136 (2014) 1246-1249.
- [17] Y. Zhao, J.L. Howe, Z. Yu, D.T. Leong, J.J. Chu, J.S. Loo, K.W. Ng, Exposure to titanium dioxide nanoparticles induces autophagy in primary human keratinocytes, *Small*, 9 (2013) 387-392.
- [18] C.Y. Tay, P.Q. Cai, M.I. Setyawati, W. Fang, L.P. Tan, C.H.L. Hong, X. Chen, D.T. Leong, Nanoparticles strengthen intracellular tension and retard cellular migration, *Nano letters*, 14 (2013) 83-88.

- [19] M.I. Setyawati, P.K.S. Khoo, B.H. Eng, S. Xiong, X. Zhao, G.K. Das, T.T.-Y. Tan, J.S.C. Loo, D.T. Leong, K.W. Ng, Cytotoxic and genotoxic characterization of titanium dioxide, gadolinium oxide, and poly(lactic-co-glycolic acid) nanoparticles in human fibroblasts, *Journal of Biomedical Materials Research Part A*, 101A (2013) 633-640.
- [20] W. Zhang, Y. Ji, X. Wu, H. Xu, Trafficking of gold nanorods in breast cancer cells: uptake, lysosome maturation, and elimination, *ACS applied materials & interfaces*, 5 (2013) 9856-9865.
- [21] M.I. Setyawati, C.Y. Tay, D.T. Leong, Exploiting cancer's antioxidative weakness through p53 with nanotoxicology, *Nanomedicine (London, England)*, 9 (2014) 369-371.
- [22] T. Mustafa, Y. Zhang, F. Watanabe, A. Karmakar, M.P. Asar, R. Little, M.K. Hudson, Y. Xu, A.S. Biris, Iron oxide nanoparticle-based radio-frequency thermotherapy for human breast adenocarcinoma cancer cells, *Biomaterials Science*, 1 (2013) 870-880.
- [23] X. Yuan, M. Setyawati, D. Leong, J. Xie, Ultrasmall Ag⁺-rich nanoclusters as highly efficient nanoreservoirs for bacterial killing, *Nano Res.*, 7 (2014) 301-307.
- [24] M. Giovanni, C.Y. Tay, M. Ingrid Setyawati, J. Xie, C.N. Ong, R. Fan, J. Yue, L. Zhang, D. Tai Leong, Toxicity profiling of water contextual zinc oxide, silver, and titanium dioxide nanoparticles in human oral and gastrointestinal cell systems, *Environmental Toxicology*, (2014).
- [25] M.I. Setyawati, C.Y. Tay, D.T. Leong, The gap between endothelial cells: key to the quick escape of nanomaterials?, *Nanomedicine*, (2014).
- [26] X. Yuan, M.I. Setyawati, A.S. Tan, C.N. Ong, D.T. Leong, J. Xie, Highly luminescent silver nanoclusters with tunable emissions: cyclic reduction-decomposition synthesis and antimicrobial properties, *NPG Asia Mater*, 5 (2013) e39.
- [27] K.W. Ng, S.P. Khoo, B.C. Heng, M.I. Setyawati, E.C. Tan, X. Zhao, S. Xiong, W. Fang, D.T. Leong, J.S. Loo, The role of the tumor suppressor p53 pathway in the cellular DNA damage response to zinc oxide nanoparticles, *Biomaterials*, 32 (2011) 8218-8225.
- [28] M.I. Setyawati, C.Y. Tay, D.T. Leong, Effect of zinc oxide nanomaterials-induced oxidative stress on the p53 pathway, *Biomaterials*, 34 (2013) 10133-10142.
- [29] M.I. Setyawati, W. Fang, S.L. Chia, D.T. Leong, Nanotoxicology of common metal oxide based nanomaterials: their ROS-y and non-ROS-y consequences, *Asia-Pacific Journal of Chemical Engineering*, 8 (2013) 205-217.
- [30] C.Y. Tay, D.T. Leong, Inorganic nanoparticles as tubulin binding agents for cancer therapy, *Nanomedicine*, (2014).
- [31] M.L. Etheridge, S.A. Campbell, A.G. Erdman, C.L. Haynes, S.M. Wolf, J. McCullough, The big picture on nanomedicine: the state of investigational and approved nanomedicine products, *Nanomedicine*, 9 (2013) 1-14.
- [32] P. Prabhu, V. Patravale, The upcoming field of theranostic nanomedicine: an overview, *J Biomed Nanotechnol*, 8 (2012) 859-882.
- [33] R. Tong, D.S. Kohane, Shedding light on nanomedicine, *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 4 (2012) 638-662.
- [34] X.Q. Zhang, X. Xu, N. Bertrand, E. Pridgen, A. Swami, O.C. Farokhzad, Interactions of nanomaterials and biological systems: Implications to personalized nanomedicine, *Adv Drug Deliv Rev*, 64 (2012) 1363-1384.
- [35] E.K. Chow, D. Ho, Cancer nanomedicine: from drug delivery to imaging, *Sci Transl Med*, 5 (2013) 216rv214.
- [36] B.D. Chithrani, J. Stewart, C. Allen, D.A. Jaffray, Intracellular uptake, transport, and processing of nanostructures in cancer cells, *Nanomedicine : nanotechnology, biology, and medicine*, 5 (2009) 118-127.
- [37] D.B. Peckys, N. de Jonge, Visualizing gold nanoparticle uptake in live cells with liquid scanning transmission electron microscopy, *Nano letters*, 11 (2011) 1733-1738.

- [38] L.C. Cheng, X. Jiang, J. Wang, C. Chen, R.S. Liu, Nano-bio effects: interaction of nanomaterials with cells, *Nanoscale*, 5 (2013) 3547-3569.
- [39] C.Y. Tay, Y. Yong, M.I. Setyawati, J. Xie, D.T. Leong, Presentation matters: identity of gold nanoclusters capping agent governs intracellular uptake and cell metabolism, *Nano Res.*, (2014).
- [40] C.Y. Tay, W. Fang, M.I. Setyawati, S.L. Chia, K.S. Tan, C.H.L. Hong, D.T. Leong, Nano-hydroxyapatite and Nano-titanium Dioxide Exhibit Different Subcellular Distribution and Apoptotic Profile in Human Oral Epithelium, *ACS Applied Materials & Interfaces*, 6 (2014) 6248-6256.
- [41] Y.L. Wu, N. Putcha, K.W. Ng, D.T. Leong, C.T. Lim, S.C. Loo, X. Chen, Biophysical responses upon the interaction of nanomaterials with cellular interfaces, *Acc Chem Res*, 46 (2013) 782-791.
- [42] M.I. Setyawati, X. Yuan, J. Xie, D.T. Leong, The influence of lysosomal stability of silver nanomaterials on their toxicity to human cells, *Biomaterials*, (2014).
- [43] D.Y. Huang, Y.L. Hou, S.M. Yang, R.G. Wang, Advances in nanomedicine for head and neck cancer, *Front Biosci (Landmark Ed)*, 19 (2014) 783-788.
- [44] L.S. Del Burgo, R.M. Hernandez, G. Orive, J.L. Pedraz, Nanotherapeutic approaches for brain cancer management, *Nanomedicine*, (2013).
- [45] V. Sanna, M. Sechi, Nanoparticle therapeutics for prostate cancer treatment, *Nanomedicine*, 8 Suppl 1 (2012) S31-36.
- [46] J. Chen, R. Shao, X.D. Zhang, C. Chen, Applications of nanotechnology for melanoma treatment, diagnosis, and theranostics, *Int J Nanomedicine*, 8 (2013) 2677-2688.
- [47] P.D. Sykes, J.P. Neoptolemos, E. Costello, C.M. Halloran, Nanotechnology advances in upper gastrointestinal, liver and pancreatic cancer, *Expert Rev Gastroenterol Hepatol*, 6 (2012) 343-356.
- [48] A.J. Thorley, T.D. Tetley, New perspectives in nanomedicine, *Pharmacol Ther*, (2013).
- [49] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, *Cancer Res*, 46 (1986) 6387-6392.
- [50] F. Yuan, M. Dellian, D. Fukumura, M. Leunig, D.A. Berk, V.P. Torchilin, R.K. Jain, Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size, *Cancer Res*, 55 (1995) 3752-3756.
- [51] M.I. Setyawati, C.Y. Tay, S.L. Chia, S.L. Goh, W. Fang, M.J. Neo, H.C. Chong, S.M. Tan, S.C. Loo, K.W. Ng, J.P. Xie, C.N. Ong, N.S. Tan, D.T. Leong, Titanium dioxide nanomaterials cause endothelial cell leakiness by disrupting the homophilic interaction of VE-cadherin, *Nat Commun*, 4 (2013) 1673.
- [52] E.S. Glazer, C. Zhu, A.N. Hamir, A. Borne, C.S. Thompson, S.A. Curley, Biodistribution and acute toxicity of naked gold nanoparticles in a rabbit hepatic tumor model, *Nanotoxicology*, 5 (2011) 459-468.
- [53] P.J.A. Borm, D. Robbins, S. Haubold, T. Kuhlbusch, H. Fissan, K. Donaldson, R. Schins, V. Stone, W. Kreyling, J. Lademann, J. Krutmann, D. Warheit, E. Oberdorster, The potential risks of nanomaterials: a review carried out for ECETOC, *Particle and fibre toxicology*, 3 (2006) 11.
- [54] K. Donaldson, V. Stone, C.L. Tran, W. Kreyling, P.J. Borm, *Nanotoxicology, Occup Environ Med*, 61 (2004) 727-728.
- [55] M.H. Kathawala, S. Xiong, M. Richards, K.W. Ng, S. George, S.C.J. Loo, Emerging in vitro models for safety screening of high-volume production nanomaterials under environmentally relevant exposure conditions, *Small*, 9 (2013) 1504-1520.
- [56] L. Gonzalez, L.C. Thomassen, G. Plas, V. Rabolli, D. Napierska, I. Decordier, M. Roelants, P.H. Hoet, C.E. Kirschhock, J.A. Martens, D. Lison, M. Kirsch-Volders,

- Exploring the aneugenic and clastogenic potential in the nanosize range: A549 human lung carcinoma cells and amorphous monodisperse silica nanoparticles as models, *Nanotoxicology*, 4 (2010) 382-395.
- [57] R.K. Srivastava, A.B. Pant, M.P. Kashyap, V. Kumar, M. Lohani, L. Jonas, Q. Rahman, Multi-walled carbon nanotubes induce oxidative stress and apoptosis in human lung cancer cell line-A549, *Nanotoxicology*, 5 (2011) 195-207.
- [58] I.M. Paino, V.S. Marangoni, C. de Oliveira Rde, L.M. Antunes, V. Zucolotto, Cyto and genotoxicity of gold nanoparticles in human hepatocellular carcinoma and peripheral blood mononuclear cells, *Toxicol Lett*, 215 (2012) 119-125.
- [59] J. Petkovic, B. Zegura, M. Stevanovic, N. Drnovsek, D. Uskokovic, S. Novak, M. Filipic, DNA damage and alterations in expression of DNA damage responsive genes induced by TiO₂ nanoparticles in human hepatoma HepG2 cells, *Nanotoxicology*, 5 (2011) 341-353.
- [60] J. Pelka, H. Gehrke, A. Rechel, M. Kappes, F. Hennrich, C.G. Hartinger, D. Marko, DNA damaging properties of single walled carbon nanotubes in human colon carcinoma cells, *Nanotoxicology*, 7 (2013) 2-20.
- [61] V. Sanna, N. Pala, M. Sechi, Targeted therapy using nanotechnology: focus on cancer, *International Journal of Nanomedicine*, 9 (2014) 467-483.
- [62] W. Dai, T. Yang, Y. Wang, X. Wang, J. Wang, X. Zhang, Q. Zhang, Peptide PHSCNK as an integrin $\alpha 5 \beta 1$ antagonist targets stealth liposomes to integrin-overexpressing melanoma, *Nanomedicine*, 8 (2012) 1152-1161.
- [63] C.L. Tseng, W.Y. Su, K.C. Yen, K.C. Yang, F.H. Lin, The use of biotinylated-EGF-modified gelatin nanoparticle carrier to enhance cisplatin accumulation in cancerous lungs via inhalation, *Biomaterials*, 30 (2009) 3476-3485.
- [64] P. Palekar-Shanbhag, S.V. Jog, M.M. Chogale, S.S. Gaikwad, Theranostics for cancer therapy, *Curr Drug Deliv*, 10 (2013) 357-362.
- [65] S. Svenson, Theranostics: are we there yet?, *Mol Pharm*, 10 (2013) 848-856.
- [66] O. Diou, N. Tsapis, E. Fattal, Targeted nanotheranostics for personalized cancer therapy, *Expert Opin Drug Deliv*, 9 (2012) 1475-1487.
- [67] J.W. Kim, E.I. Galanzha, D.A. Zaharoff, R.J. Griffin, V.P. Zharov, Nanotheranostics of circulating tumor cells, infections and other pathological features in vivo, *Mol Pharm*, 10 (2013) 813-830.
- [68] T.H. Kim, S. Lee, X. Chen, Nanotheranostics for personalized medicine, *Expert Rev Mol Diagn*, 13 (2013) 257-269.
- [69] L.S. Wang, M.C. Chuang, J.A. Ho, Nanotheranostics--a review of recent publications, *Int J Nanomedicine*, 7 (2012) 4679-4695.
- [70] L. Vannucci, E. Falvo, M. Fornara, P. Di Micco, O. Benada, J. Krizan, J. Svoboda, K. Hulikova-Capkova, V. Morea, A. Boffi, P. Ceci, Selective targeting of melanoma by PEG-masked protein-based multifunctional nanoparticles, *Int J Nanomedicine*, 7 (2012) 1489-1509.
- [71] N. Han, Y.Y. Yang, S. Wang, S. Zheng, W. Fan, Polymer-based cancer nanotheranostics: retrospectives of multi-functionalities and pharmacokinetics, *Curr Drug Metab*, 14 (2013) 661-674.
- [72] N. Ahmed, H. Fessi, A. Elaissari, Theranostic applications of nanoparticles in cancer, *Drug Discov Today*, 17 (2012) 928-934.
- [73] A. Singh, S.K. Sahoo, Magnetic nanoparticles: a novel platform for cancer theranostics, *Drug Discov Today*, 19 (2014) 474-481.
- [74] T.P. Gustafson, Y.H. Lim, J.A. Flores, G.S. Heo, F. Zhang, S. Zhang, S. Samarajeewa, J.E. Raymond, K.L. Wooley, Holistic assessment of covalently labeled core-shell

- polymeric nanoparticles with fluorescent contrast agents for theranostic applications, *Langmuir*, 30 (2014) 631-641.
- [75] K.W. Ng, D.T.W. Leong, D.W. Huttmacher, The challenge to measure cell proliferation in two and three dimensions, *Tissue Engineering*, 11 (2005) 182-191.
 - [76] V.H. Ho, W.M. Guo, C.L. Huang, S.F. Ho, S.Y. Chaw, E.Y. Tan, K.W. Ng, J.S. Loo, Manipulating magnetic 3D spheroids in hanging drops for applications in tissue engineering and drug screening, *Adv Healthc Mater*, 2 (2013) 1430-1434.
 - [77] F. Pampaloni, E.G. Reynaud, E.H.K. Stelzer, The third dimension bridges the gap between cell culture and live tissue, *Nat Rev Mol Cell Biol*, 8 (2007) 839-845.
 - [78] J. Lee, M.J. Cuddihy, N.A. Kotov, Three-dimensional cell culture matrices: state of the art, *Tissue Eng Part B Rev*, 14 (2008) 61-86.
 - [79] C.Y. Tay, M. Pal, H.Y. Yu, W.S. Leong, N.S. Tan, K.W. Ng, S. Venkatraman, F. Boey, D.T. Leong, L.P. Tan, Bio-inspired Micropatterned Platform to Steer Stem Cell Differentiation, *Small*, 7 (2011) 1416-1421.
 - [80] C.Y. Tay, H.Y. Yu, M. Pal, W.S. Leong, N.S. Tan, K.W. Ng, D.T. Leong, L.P. Tan, Micropatterned matrix directs differentiation of human mesenchymal stem cells towards myocardial lineage, *Experimental Cell Research*, 316 (2010) 1159-1168.
 - [81] L.M. McCaffrey, I.G. Macara, Epithelial organization, cell polarity and tumorigenesis, *Trends in cell biology*, 21 (2011) 727-735.
 - [82] L.E. O'Brien, M.M. Zegers, K.E. Mostov, Opinion: Building epithelial architecture: insights from three-dimensional culture models, *Nat Rev Mol Cell Biol*, 3 (2002) 531-537.
 - [83] W. Hua, D. Sheff, D. Toomre, I. Mellman, Vectorial insertion of apical and basolateral membrane proteins in polarized epithelial cells revealed by quantitative 3D live cell imaging, *The Journal of cell biology*, 172 (2006) 1035-1044.
 - [84] D.M. Bryant, K.E. Mostov, From cells to organs: building polarized tissue, *Nat Rev Mol Cell Biol*, 9 (2008) 887-901.
 - [85] H. Lodish, A. Berk, S. Zipursky, Actin and Myosin in Nonmuscle Cells, in: W.H. Freeman (Ed.) *Molecular Cell Biology*, New York, 2000.
 - [86] T.T. Chang, M. Hughes-Fulford, Monolayer and spheroid culture of human liver hepatocellular carcinoma cell line cells demonstrate distinct global gene expression patterns and functional phenotypes, *Tissue engineering. Part A*, 15 (2009) 559-567.
 - [87] M.H. Zaman, L.M. Trapani, A.L. Sieminski, D. Mackellar, H. Gong, R.D. Kamm, A. Wells, D.A. Lauffenburger, P. Matsudaira, Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis, *Proc Natl Acad Sci U S A*, 103 (2006) 10889-10894.
 - [88] E. Cukierman, R. Pankov, D.R. Stevens, K.M. Yamada, Taking cell-matrix adhesions to the third dimension, *Science (New York, N.Y.)*, 294 (2001) 1708-1712.
 - [89] D. Huh, G.A. Hamilton, D.E. Ingber, From 3D cell culture to organs-on-chips, *Trends in cell biology*, 21 (2011) 745-754.
 - [90] F. Zhang, R. Xu, M.J. Zhao, QSG-7701 human hepatocytes form polarized acini in three-dimensional culture, *Journal of cellular biochemistry*, 110 (2010) 1175-1186.
 - [91] D.E. Discher, P. Janmey, Y.L. Wang, Tissue cells feel and respond to the stiffness of their substrate, *Science (New York, N.Y.)*, 310 (2005) 1139-1143.
 - [92] K. Ren, T. Crouzier, C. Roy, C. Picart, Polyelectrolyte multilayer films of controlled stiffness modulate myoblast cells differentiation, *Adv Funct Mater*, 18 (2008) 1378-1389.
 - [93] J.S. Park, J.S. Chu, A.D. Tsou, R. Diop, Z. Tang, A. Wang, S. Li, The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta, *Biomaterials*, 32 (2011) 3921-3930.

- [94] W.P. Daley, S.B. Peters, M. Larsen, Extracellular matrix dynamics in development and regenerative medicine, *J Cell Sci*, 121 (2008) 255-264.
- [95] S. Zhang, F. Gelain, X. Zhao, Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures, *Seminars in cancer biology*, 15 (2005) 413-420.
- [96] J.E. Nichols, J. Cortiella, J. Lee, J.A. Niles, M. Cuddihy, S. Wang, J. Bielitzki, A. Cantu, R. Mlcak, E. Valdivia, R. Yancy, M.L. McClure, N.A. Kotov, In vitro analog of human bone marrow from 3D scaffolds with biomimetic inverted colloidal crystal geometry, *Biomaterials*, 30 (2009) 1071-1079.
- [97] T.M. Walker, A.J. Woodrooffe, Cytochrome P450 activity in control and induced long-term cultures of rat hepatocyte spheroids, *Toxicology in vitro : an international journal published in association with BIBRA*, 15 (2001) 713-719.
- [98] D.T. Leong, W.M. Khor, F.T. Chew, T.C. Lim, D.W. Hutmacher, Characterization of osteogenically induced adipose tissue-derived precursor cells in 2-dimensional and 3-dimensional environments, *Cells Tissues Organs*, 182 (2006) 1-11.
- [99] D.T. Leong, M.C. Abraham, S.N. Rath, T.C. Lim, F.T. Chew, D.W. Hutmacher, Investigating the effects of preinduction on human adipose-derived precursor cells in an athymic rat model, *Differentiation*, 74 (2006) 519-529.
- [100] D.T. Leong, W.K. Nah, A. Gupta, D.W. Hutmacher, M.A. Woodruff, The osteogenic differentiation of adipose tissue-derived precursor cells in a 3D scaffold/matrix environment, *Curr Drug Discov Technol*, 5 (2008) 319-327.
- [101] V.I. Khaoustov, G.J. Darlington, H.E. Soriano, B. Krishnan, D. Risin, N.R. Pellis, B. Yoffe, Induction of three-dimensional assembly of human liver cells by simulated microgravity, *In Vitro Cell Dev Biol Anim*, 35 (1999) 501-509.
- [102] R. Montesano, G. Schaller, L. Orci, Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors, *Cell*, 66 (1991) 697-711.
- [103] M.D. Amatangelo, D.E. Bassi, A.J. Klein-Szanto, E. Cukierman, Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts, *The American journal of pathology*, 167 (2005) 475-488.
- [104] R. Wang, J. Xu, L. Juliette, A. Castilleja, J. Love, S.Y. Sung, H.E. Zhau, T.J. Goodwin, L.W. Chung, Three-dimensional co-culture models to study prostate cancer growth, progression, and metastasis to bone, *Seminars in cancer biology*, 15 (2005) 353-364.
- [105] D.T. Leong, M.C. Abraham, A. Gupta, T.C. Lim, F.T. Chew, D.W. Hutmacher, ATF5, a possible regulator of osteogenic differentiation in human adipose-derived stem cells, *Journal of cellular biochemistry*, 113 (2012) 2744-2753.
- [106] F. Hirschhaeuser, H. Menne, C. Dittfeld, J. West, W. Mueller-Klieser, L.A. Kunz-Schughart, Multicellular tumor spheroids: an underestimated tool is catching up again, *J Biotechnol*, 148 (2010) 3-15.
- [107] A. Hofmann, U. Ritz, S. Verrier, D. Eglin, M. Alini, S. Fuchs, C.J. Kirkpatrick, P.M. Rommens, The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds, *Biomaterials*, 29 (2008) 4217-4226.
- [108] S. Chung, R. Sudo, P.J. Mack, C.R. Wan, V. Vickerman, R.D. Kamm, Cell migration into scaffolds under co-culture conditions in a microfluidic platform, *Lab on a chip*, 9 (2009) 269-275.
- [109] L.G. Griffith, M.A. Swartz, Capturing complex 3D tissue physiology in vitro, *Nat Rev Mol Cell Biol*, 7 (2006) 211-224.
- [110] J.B. Kim, Three-dimensional tissue culture models in cancer biology, *Seminars in cancer biology*, 15 (2005) 365-377.

- [111] M. Radisic, J. Malda, E. Epping, W. Geng, R. Langer, G. Vunjak-Novakovic, Oxygen gradients correlate with cell density and cell viability in engineered cardiac tissue, *Biotechnology and bioengineering*, 93 (2006) 332-343.
- [112] G. Mazzoleni, D. Di Lorenzo, N. Steimberg, Modelling tissues in 3D: the next future of pharmaco-toxicology and food research?, *Genes & nutrition*, 4 (2009) 13-22.
- [113] K.M. Yamada, E. Cukierman, Modeling tissue morphogenesis and cancer in 3D, *Cell*, 130 (2007) 601-610.
- [114] A. Astashkina, B. Mann, D.W. Grainger, A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity, *Pharmacol Ther*, 134 (2012) 82-106.
- [115] L.A. Kunz-Schughart, J.P. Freyer, F. Hofstaedter, R. Ebner, The use of 3-D cultures for high-throughput screening: the multicellular spheroid model, *Journal of biomolecular screening*, 9 (2004) 273-285.
- [116] A. Rangarajan, S.J. Hong, A. Gifford, R.A. Weinberg, Species- and cell type-specific requirements for cellular transformation, *Cancer cell*, 6 (2004) 171-183.
- [117] H. Vorsmann, F. Groeber, H. Walles, S. Busch, S. Beissert, H. Walczak, D. Kulms, Development of a human three-dimensional organotypic skin-melanoma spheroid model for in vitro drug testing, *Cell death & disease*, 4 (2013) e719.
- [118] B. Marrero, R. Heller, The use of an in vitro 3D melanoma model to predict in vivo plasmid transfection using electroporation, *Biomaterials*, 33 (2012) 3036-3046.
- [119] J. Debnath, S.K. Muthuswamy, J.S. Brugge, Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures, *Methods (San Diego, Calif.)*, 30 (2003) 256-268.
- [120] C. Fischbach, R. Chen, T. Matsumoto, T. Schmelzle, J.S. Brugge, P.J. Polverini, D.J. Mooney, Engineering tumors with 3D scaffolds, *Nature methods*, 4 (2007) 855-860.
- [121] G.Y. Lee, P.A. Kenny, E.H. Lee, M.J. Bissell, Three-dimensional culture models of normal and malignant breast epithelial cells, *Nature methods*, 4 (2007) 359-365.
- [122] Y. Yoshii, A. Waki, K. Yoshida, A. Kakezuka, M. Kobayashi, H. Namiki, Y. Kuroda, Y. Kiyono, H. Yoshii, T. Furukawa, T. Asai, H. Okazawa, J.G. Gelovani, Y. Fujibayashi, The use of nanoimprinted scaffolds as 3D culture models to facilitate spontaneous tumor cell migration and well-regulated spheroid formation, *Biomaterials*, 32 (2011) 6052-6058.
- [123] G.L. Semenza, Evaluation of HIF-1 inhibitors as anticancer agents, *Drug Discov Today*, 12 (2007) 853-859.
- [124] H. Dolznig, C. Rupp, C. Puri, C. Haslinger, N. Schweifer, E. Wieser, D. Kerjaschki, P. Garin-Chesa, Modeling colon adenocarcinomas in vitro a 3D co-culture system induces cancer-relevant pathways upon tumor cell and stromal fibroblast interaction, *The American journal of pathology*, 179 (2011) 487-501.
- [125] H. Yamamoto, J.W. Soh, T. Monden, M.G. Klein, L.M. Zhang, H. Shirin, N. Arber, N. Tomita, I. Schieren, C.A. Stein, I.B. Weinstein, Paradoxical increase in retinoblastoma protein in colorectal carcinomas may protect cells from apoptosis, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 5 (1999) 1805-1815.
- [126] R.M. Sutherland, B. Sordat, J. Bamat, H. Gabbert, B. Bourrat, W. Mueller-Klieser, Oxygenation and differentiation in multicellular spheroids of human colon carcinoma, *Cancer Res*, 46 (1986) 5320-5329.
- [127] L. Hlatky, P. Hahnfeldt, J. Folkman, Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us, *Journal of the National Cancer Institute*, 94 (2002) 883-893.

- [128] L. Vermeulen, E.M.F. De Sousa, M. van der Heijden, K. Cameron, J.H. de Jong, T. Borovski, J.B. Tuynman, M. Todaro, C. Merz, H. Rodermond, M.R. Sprick, K. Kemper, D.J. Richel, G. Stassi, J.P. Medema, Wnt activity defines colon cancer stem cells and is regulated by the microenvironment, *Nature cell biology*, 12 (2010) 468-476.
- [129] B.M. Holzapfel, F. Wagner, D. Loessner, N.P. Holzapfel, L. Thibaudeau, R. Crawford, M.T. Ling, J.A. Clements, P.J. Russell, D.W. Hutmacher, Species-specific homing mechanisms of human prostate cancer metastasis in tissue engineered bone, *Biomaterials*, 35 (2014) 4108-4115.
- [130] L. Thibaudeau, A.V. Taubenberger, B.M. Holzapfel, V.M. Quent, T. Fuehrmann, P. Hesami, T.D. Brown, P.D. Dalton, C.A. Power, B.G. Hollier, D.W. Hutmacher, A tissue-engineered humanized xenograft model of human breast cancer metastasis to bone, *Dis Model Mech*, 7 (2014) 299-309.
- [131] A.V. Taubenberger, V.M. Quent, L. Thibaudeau, J.A. Clements, D.W. Hutmacher, Delineating breast cancer cell interactions with engineered bone microenvironments, *J Bone Miner Res*, 28 (2013) 1399-1411.
- [132] L. Thibaudeau, V.M. Quent, B.M. Holzapfel, A.V. Taubenberger, M. Straub, D.W. Hutmacher, Mimicking breast cancer-induced bone metastasis in vivo: current transplantation models and advanced humanized strategies, *Cancer Metastasis Rev*, (2014).
- [133] M. Mitra, C. Mohanty, A. Harilal, U.K. Maheswari, S.K. Sahoo, S. Krishnakumar, A novel in vitro three-dimensional retinoblastoma model for evaluating chemotherapeutic drugs, *Molecular Vision*, 18 (2012) 1361-1378.
- [134] S. Talukdar, S.C. Kundu, A non-mulberry silk fibroin protein based 3D in vitro tumor model for evaluation of anticancer drug activity, *Advanced Functional Materials*, 22 (2012) 4778-4788.
- [135] X. Jiang, X. Sha, H. Xin, X. Xu, J. Gu, W. Xia, S. Chen, Y. Xie, L. Chen, Y. Chen, X. Fang, Integrin-facilitated transcytosis for enhanced penetration of advanced gliomas by poly(trimethylene carbonate)-based nanoparticles encapsulating paclitaxel, *Biomaterials*, 34 (2013) 2969-2979.
- [136] H. Cheng, C.J. Kastrup, R. Ramanathan, D.J. Siegwart, M. Ma, S.R. Bogatyrev, Q. Xu, K.A. Whitehead, R. Langer, D.G. Anderson, Nanoparticulate cellular patches for cell-mediated tumor tropic delivery, *ACS Nano*, 4 (2010) 625-631.
- [137] H.H. Chen, C.C. Chien, C. Petibois, C.L. Wang, Y.S. Chu, S.F. Lai, T.E. Hua, Y.Y. Chen, X. Cai, I.M. Kempson, Y. Hwu, G. Margaritondo, Quantitative analysis of nanoparticle internalization in mammalian cells by high resolution X-ray microscopy, *J Nanobiotechnology*, 9 (2011) 14.
- [138] A.H. Abouzeid, V.P. Torchilin, The role of cell cycle in the efficiency and activity of cancer nanomedicines, *Expert Opin Drug Deliv*, 10 (2013) 775-786.
- [139] C.G. England, T. Priest, G. Zhang, X. Sun, D.N. Patel, L.R. McNally, V. van Berkel, A.M. Gobin, H.B. Frieboes, Enhanced penetration into 3D cell culture using two and three layered gold nanoparticles, *Int J Nanomedicine*, 8 (2013) 3603-3617.
- [140] C. Godugu, A.R. Patel, U. Desai, T. Andey, A. Sams, M. Singh, AlgiMatrix™ Based 3D Cell Culture System as an In-Vitro Tumor Model for Anticancer Studies, *PLoS ONE*, 8 (2013).
- [141] V.H.B. Ho, N.K.H. Slater, R. Chen, PH-responsive endosomolytic pseudo-peptides for drug delivery to multicellular spheroids tumour models, *Biomaterials*, 32 (2011) 2953-2958.
- [142] W. Meng, P. Kallinteri, D.A. Walker, T.L. Parker, M.C. Garnett, Evaluation of poly (glycerol-adipate) nanoparticle uptake in an in vitro 3-D brain tumor co-culture model, *Exp Biol Med (Maywood)*, 232 (2007) 1100-1108.

- [143] J.T. Robinson, G. Hong, Y. Liang, B. Zhang, O.K. Yaghi, H. Dai, In vivo fluorescence imaging in the second near-infrared window with long circulating carbon nanotubes capable of ultrahigh tumor uptake, *Journal of the American Chemical Society*, 134 (2012) 10664-10669.
- [144] M. Takeda, H. Tada, H. Higuchi, Y. Kobayashi, M. Kobayashi, Y. Sakurai, T. Ishida, N. Ohuchi, In vivo single molecular imaging and sentinel node navigation by nanotechnology for molecular targeting drug-delivery systems and tailor-made medicine, *Breast Cancer*, 15 (2008) 145-152.
- [145] J. Blechinger, A.T. Bauer, A.A. Torrano, C. Gorzelanny, C. Brauchle, S.W. Schneider, Uptake kinetics and nanotoxicity of silica nanoparticles are cell type dependent, *Small*, 9 (2013) 3970-3980, 3906.
- [146] E.A. Gibbs-Flournoy, P.A. Bromberg, T.P. Hofer, J.M. Samet, R.M. Zucker, Darkfield-confocal microscopy detection of nanoscale particle internalization by human lung cells, *Part Fibre Toxicol*, 8 (2011) 2.
- [147] B.C. Heng, X. Zhao, S. Xiong, K.W. Ng, F.Y. Boey, J.S. Loo, Cytotoxicity of zinc oxide (ZnO) nanoparticles is influenced by cell density and culture format, *Arch Toxicol*, 85 (2011) 695-704.
- [148] B.L. Behan, D.G. DeWitt, D.R. Bogdanowicz, A.N. Koppes, S.S. Bale, D.M. Thompson, Single-walled carbon nanotubes alter Schwann cell behavior differentially within 2D and 3D environments, *Journal of Biomedical Materials Research - Part A*, 96 A (2011) 46-57.
- [149] D. Movia, A. Prina-Mello, D. Bazou, Y. Volkov, S. Giordani, Screening the cytotoxicity of single-walled carbon nanotubes using novel 3D tissue-mimetic models, *ACS Nano*, 5 (2011) 9278-9290.
- [150] M.P. Jain, A.O. Choi, K.D. Neibert, D. Maysinger, Probing and preventing quantum dot-induced cytotoxicity with multimodal α -lipoic acid in multiple dimensions of the peripheral nervous system, *Nanomedicine*, 4 (2009) 277-290.
- [151] J. Lee, G.D. Lilly, R.C. Doty, P. Podsiadlo, N.A. Kotov, In vitro toxicity testing of nanoparticles in 3D cell culture, *Small*, 5 (2009) 1213-1221.
- [152] Y. Luo, C. Wang, M. Hossain, Y. Qiao, L. Ma, J. An, M. Su, Three-dimensional microtissue assay for high-throughput cytotoxicity of nanoparticles, *Anal Chem*, 84 (2012) 6731-6738.
- [153] D. Loessner, J.P. Little, G.J. Pettet, D.W. Hutmacher, A multiscale road map of cancer spheroids--incorporating experimental and mathematical modelling to understand cancer progression, *Journal of cell science*, 126 (2013) 2761-2771.
- [154] K.W. Ng, W. Tham, T.C. Lim, D. Werner Hutmacher, Assimilating cell sheets and hybrid scaffolds for dermal tissue engineering, *Journal of biomedical materials research. Part A*, 75 (2005) 425-438.
- [155] N.S. Bhise, J. Ribas, V. Manoharan, Y.S. Zhang, A. Polini, S. Massa, M.R. Dokmeci, A. Khademhosseini, Organ-on-a-Chip Platforms for Studying Drug Delivery Systems, *J Control Release*, (2014).
- [156] C.M. Hwang, A. Khademhosseini, Y. Park, K. Sun, S.H. Lee, Microfluidic chip-based fabrication of PLGA microfiber scaffolds for tissue engineering, *Langmuir*, 24 (2008) 6845-6851.
- [157] D. Mueller, L. Kramer, E. Hoffmann, S. Klein, F. Noor, 3D organotypic HepaRG cultures as in vitro model for acute and repeated dose toxicity studies, *Toxicology in vitro : an international journal published in association with BIBRA*, 28 (2014) 104-112.
- [158] M. Brandenburger, J. Wenzel, R. Bogdan, D. Richardt, F. Nguemo, M. Reppel, J. Hescheler, H. Terlau, A. Dendorfer, Organotypic slice culture from human adult ventricular myocardium, *Cardiovasc Res*, 93 (2012) 50-59.

Figure Captions

Figure 1. Publication numbers recorded in PUBMED from 1995 to 2013, based on the search term “3D cell culture”.

Figure 2. Examples of cell spheroids as the simplest and most convenient model of 3D cell culture. A) Human embryonic kidney cells (HEK293) in 2D culture. B) HEK293 cells in suspensions added to non-adherent agarose molds of defined size to encourage cell-cell adhesion, resulting in spheroids of sizes defined by the mold diameter. C1) Human fibroblasts (BJ) in 2D and in C2) 3D spheroid cultures. Note the drastic change in morphology. D) For easy tracking, BJ cells were labelled with 5 μ M of CellTrackerTM Orange CMFDA (Life-Technologies, USA) for 45min before spheroid formation. E) Jiang et al. [135] used U87MG glioma spheroids as a 3D model for visualizing 12h uptake of RGD peptide functionalized poly(trimethylene carbonate)-based nanoparticles labelled with coumarin-6, on a confocal laser microscope. *Reprinted with permission*; All scale bars: 100 μ m.

Figure 3. An overview of representative scaffold-based 3D cell culture systems. A) A microporous sponge made from human hair keratins. B1) Scanning electron microscopy image of human dermal fibroblasts cultured on a 3D textile mesh made by knitting 20 μ m fibers of poly(lactic-co-glycolic acid) (PLGA; 90:10). B2) Confocal laser microscopy image showed that the fibroblasts anchored well onto the PLGA fibers. *green: F-actin; red: PLGA (fibers) and cell nuclei (elliptical spots)*. C) L929 mouse fibroblasts suspended in a human keratin hydrogel proliferated over 3 days to form multi-cell clusters. D1) Macroscopic view of a 3D organotypic skin culture assembled by culturing keratinocytes on a fibroblast-

populated collagen disc and exposed to air. D2) Histological assessment of a skin organotypic culture, showing normal anatomical arrangement of the epidermis and dermis.

Figure 4. A schematic representation of representative approaches for using engineered nanoparticle systems to target, detect, and deliver drugs to cancer cells in 3D cell culture models.

Figure 5. A road map for integrating mathematical modelling with experimental modelling, to build a holistic approach towards profiling nanoparticle based strategies in 3D cancer models. Nanoparticles of various configurations and materials could be combined and measured using conventional techniques. This experimental data could be used as input data for mathematical modelling or for validating results from mathematical modelling. Subsequently after a few iterative convergence steps, it is then possible to arrive at a predictive model to correlate *in silico* and *in vitro* experiments for a particular combination of nanoparticle and 3D cancer model.

Table 1. Summary of the different 3D cell culture systems developed to date.

3D culture system	Advantages	Disadvantages	Matrix material needed	Reference
Cell spheroids	Easy and quick to produce. Cheap. High-throughput possible. No additional materials needed. Easy to image/harvest samples. Easy to track nanoparticles.	Limited spheroid size. Heterogeneity of cell lineage. Lack of matrix interaction. Potentially unrealistic nanoparticle uptake.	Cells' native ECM composition in response to 3D microenvironment	[76], [86], [97], [106], [114], [115], [117], [122], [126], [141]
Scaffolds - Films - Gels - Sponges - Fibres (micro and nano) - Additive manufacturing - Microcarriers	Larger size possible. Customizable physical, mechanical, biochemical environment. Relatively easy to track nanoparticles. Ready-to-use commercial products available.	Additional processing steps. Possible batch variations. May not be transparent. May be difficult to harvest samples for analysis. Change in scaffold properties over time. (e.g. degradation)	Natural or synthetic polymers*	[75], [95], [96], [95-97], [107], [108], [120], [154]
Organ-on-a-chip	High throughput possible. Minimal variations between samples. Precise control of environment possible. Real-time analysis possible. Easy to image.	Skill intensive. Matrix replication limited. Potentially expensive.	Microfluids chip; Natural or synthetic polymers*	[89], [155, 156]
Organotypic cultures	Closest to real tissue/organ. Realistic anatomy. Possible to recreate tissue compartmentalization.	Skill intensive. May need multiple supplements/growth factors. Expensive. Time consuming. Low throughput. May be difficult to image. Relatively difficult to track nanoparticles.	Natural or synthetic polymers*	[55], [117], [157]

Explant cultures	Native matrix. Realistic cell-cell interactions. Realistic cell compartmentalization.	Limited supply. Limited experiment time. Large genetic variations between samples. Low throughput. Difficult to harvest samples for analysis. Difficult to image. Difficult to track nanoparticles.	None	[142], [150], [158]
-------------------------	---	---	------	---------------------

*Natural polymers:

Collagen, Fibrin, Elastin, Gelatin, Fibronectin, Vitronectin, Hyaluronic acid, Alginate, Glycosaminoglycan, Chitosan, Silk, Keratin

*Synthetic polymers:

Poly(glycolic acid) (PGA), Poly(lactic acid) (PLA), Poly(ϵ -caprolactone) (PCL), Poly(ethylene glycol) (PEG), Poly(vinylalcohol) (PVA), Poly(hydroxyalkanoate) (PHA), Poly(propylene fumarate) (PPF), Poly(acrylic acid) (PAA) synthetic peptides, synthetic DNA and the co-polymers of any of these.

Figure 1

Figure 2

Figure 3

Core/shell
nanoparticle with
cancer drugs or
fluorophore

Nanorods &
nanotubes

Nanoparticles
decorated with
cell targeting
ligands

Figure 4

Figure 5