Bioprinted 3D cell constructs as biomimetics for *in vitro* cancer drug screening

M. L. Blömer, R. A. Bank. July 2020

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1. Introduction

In this review, the application of 3D bioprinting for cancer drug screening is elaborated. Worldwide, cancer is a considerable health problem, and much is unknown about this disease. Innovations concerning cancer treatment are important to improve the life of cancer patients. The conventional treatment options for cancer safes lives (chemotherapy, radiotherapy, and surgery), although optimization is needed. Side effects of all therapies are considerable, especially in chemotherapy, and must therefore be reduced. Luckily, other forms of therapy are investigated, like targeted therapy. This works by identifying specific molecular targets in cancer cells, and by producing drugs aiming to these targets, like cancer cell division proteins. The challenges here are the "undruggable" but important targets. Another therapy is immunotherapy, which urges the immune system to attack cancer cells. The danger here is the autoimmune reaction which can damage healthy tissue. Thus, both therapies have points for improvements and only work for some patients. In addition, resistance can occur in chemotherapy and targeted drugs, which makes the mechanisms behind and how to overcome those important for investigation. Another difficulty is the diversity between the tumours of patients. Tumours differ because of various mutation sets which lead to tumour development. Even secondary tumours caused by metastasis can differ from the original tumour. As a result, medications may not work on similar types of tumours. Altogether, there are still many challenges regarding cancer treatment to overcome (National Cancer Institute, 2019). *Ex vivo* cancer models can help finding appropriate treatments for cancer. With the use of 3D bioprinters, it is believed that mimicking the complexity of a human tumour is possible. Hereby, various anticancer therapies can be tested in a realistic environment. In addition, patient-specific models can be used for individual treatment selection and resistance investigation (Yi, 2019). The goal in this review is to provide an overview of factors concerning 3D bioprinting and anti-cancer drug screening. Discussed topics are the materials used for printing, such as bioinks and bioprinting techniques. For elaboration on the significance of a 3D bioprinted culture, a comparison is made between 2D cultures and regular 3D cultures. Additionally, the use in tissue engineering and regenerative medicine is discussed. Hereafter, the use of the 3D printed constructs for drug screening in cancer is explained, as well as important factors to be monitored around drug screening like metastasis. Different ways to present, administer, and release drugs are elaborated, just as culture conditions concerning hypoxia. Eventually, several 3D printed research models are explained about the brain cancer glioblastoma multiforme. Finally, after the conclusion, future remarks are noted.

2. 3D bioprinting

Nowadays, 3D printing in X, Y and Z directions has become a general principle. In 3D bioprinting however, cells and biomaterials are used as ink. This way, it is possible to make *in vitro* tissues that are very similar to real human tissues. To produce accurate tissues, magnetic resonance imaging (MRI) or computer tomography (CT) are used to obtain information about the tissue structure, which is reconstructed with specialized computer software (Ma, 2018). In some cases, biomaterials are used to print only a construct, after which cells are seeded and allowed to form complex networks. In addition, it is possible to print biomaterial including living cells. This is called 3D bioprinting by which complex tissues are constructed *in vitro*. Multiple factors, like using different cell types, can be adjusted to obtain the desired biomimetic tissue. Furthermore, print resolution, biomaterial, and the inclusion of blood vessels into 3D structures must be set, which can be challenging (Hermida, 2020).

3D bioprinting can produce large numbers of similar models, which is useful for reproducible research. There are many medical applications for these 3D cultures, such as testing medicines or humane implantation to restore organ function. Figure 1 demonstrates a bioprinting progress, starting with merging cells and biomaterial, in this case gelatin methacryloyl (GelMA) (further explained below). This mixture is cultivated and printed using a 3D bioprinter. In addition, the printed material is illuminated with UV light for strengthening. This process is known as crosslinking and necessary for firmness when using GelMA. Finally, the 3D tissue is created (Hermida, 2020)

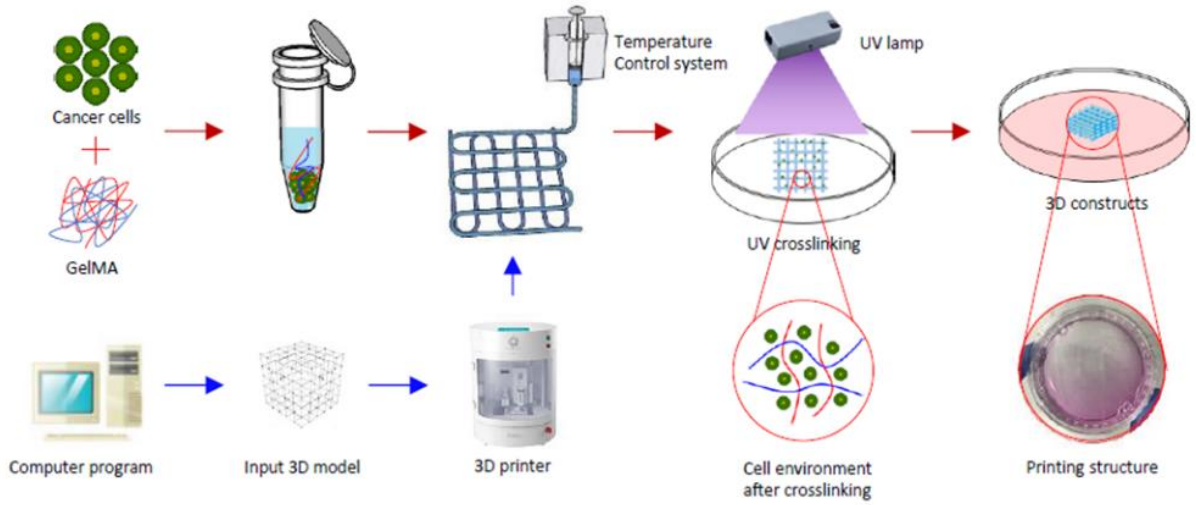

Figure: 1. Schematic illustration of the 3D bioprinting progress. Cancer cells are mixed with GelMA, cultured, and printed following a model derived from specialized software. After crosslinking with UV light, the 3D construct is created (Kim, 2019)

3. Biomaterials

Multiple materials are needed to print a functional biomimetic tissue, since native tissues consist of various molecules, proteins, and cell types. For example, the extracellular matrix (ECM) is very delicate and has a specific structure in different tissue types. Therefore, recreating the complexity of organs and tissues with 3D printing is challenging. A bioink is created by incorporating living cells in biomaterials, which can be used for printing. It is possible to print a structure concluding biomaterial and seed the cells afterwards. However, cells are distributed irregularly which results in low quality of biomimetic tissue. In addition, bioactive molecules like growth factors are crucial for cell functioning and differentiation, which can be added to a bioink. It can be concluded that, an ideal biomimetic tissue is developed by integrating biomolecules and multiple cell types into the biomaterial before printing. This composite ink is called a heterogeneous bioink (Ashammakhi, 2019).

A proper biomaterial has multiple properties to optimise cell attachment, viability, and proliferation. Cells must be integrated in a biocompatible material to grow and must survive during the bioprinting process. In addition, biomaterial must accept remodelling by cells, so a tissue-like situation is represented in which the ECM is biodegradable. Also, the material must be sturdy enough to retain shape after printing and without collapsing. There are many possible biomaterials some of which are discussed below.

3.1 Natural biomaterials

Materials derived from nature are desirable because of their complexity and similarity to the ECM. Because of their natural properties, an environment is created in which cells can live and grow exceptionally. A drawback is formed by the higher variation within the material itself. In addition, mechanically speaking these materials are relatively fragile (Ma, 2018). Examples of natural materials for bioprinting are gelatin, collagen, fibrin/fibrinogen, alginate, hyaluronic acid (HA), decellularized extracellular matrix (dECM), agarose, chitosan, cellulose, and silk (Gungor-Ozkerim, 2018; Ashammakhi, 2019).

3.2 Synthetic biomaterials

Synthetically derived materials are always identical, and properties can be decided upon, such as mechanical strength, biocompatibility, and degradability. The material can have built-in functions, such as cell-binding proteins. Synthetic materials are strong but lack the specificity to serve as ECM, simply lacking certain essential components. Additionally, when cells break down surrounding material, unwanted toxic products can be released, which is detrimental to the cells themselves (Ma, 2018). An example of a synthetic biomaterial is polyethylene glycol (PEG). PEG alone is unsuitable for bioprinting. Therefore, PEG is often mixed with other molecules such as

methacrylate (PEGMA), which enhances printability (Ashammakhi, 2019).

3.3 Composite biomaterials

The shortcomings of natural and synthetic materials can be overcome by using multiple materials for a bioink. Synthetic materials can serve for strength and certain specific proteins can be added for. Natural materials can determine specificity and biocompatibility (Ma, 2018). An example of a combination of synthetic and natural materials is GelMA, shown in figure 1. This is a hydrogel consisting of gelatin, which is widely used, and methacrylate. This bioink can be used when it is crosslinked with UV light (Kim, 2019) or combined with PEG (Ma, 2018). In addition, many other combinations are possible, also with only natural materials. For example, the combination of alginate with gelatin or fibrinogen. Alginate is highly biocompatible but lacks bioactivity. Fibrinogen improves cell interaction and gelatin is highly bioactive, making them a good combination. Therefore, one should select the appropriate natural and synthetic materials, depending on the function of the desired tissue (Ashammakhi, 2019).

4. Biomolecules

The presence of biomolecules is important in 3D structures, just like in human tissues. Biomolecules can modulate cell activity and regulation. It is possible to insert certain molecules into the bioink. The choice of biomolecules depends on the application of the 3D construct. Commonly used molecules are vascular endothelial growth factor (VEGF) for blood vessel formation, fibroblast growth factor (FGFs) for vital cell processes, and transforming growth factor (TGF) for certain tissue formation (Ashammakhi, 2019). Biomolecules can be added in gradients. Hereby, more control is created over the effect of the molecules. An example is a study using GelMA in combination with gradients of VEGF, creating small vasculature varying in density. This method can be applied using different cells, biomaterials, and biomolecules (Byambaa, 2017).

5. Bioprinting techniques

The application of the biomimetic tissue determines the choice of bioprinter. This also accounts for the type of biomaterial and cells being used. There are several types of bioprinting techniques, namely inkjet-based, extrusion based and light-assisted. The differences between these techniques are based on the type of biomaterial, printing time, resolution, and printing mechanism. It is possible to print with bioink, using living cells, although this is not necessary (Ma, 2018).

5.1 Inkjet-based bioprinting

When using this type of technique, there are multiple ways for printing. All of them deposit individual droplets of bioink. The nozzle of the printer moves along multiple directions and, because of a vapour bubble (figure 2A), droplets form. Eventually, the droplets connect, forming a close network. There are the thermal, piezoelectric, and electromagnetic types. The thermal inkjet printer (figure 2A) is generally used for 3D printing because of the easily operated and low costs system. The printing process can affect cell viability because shear forces can damage cells. After inkjet printing, cell viability is rather high. Inkjet printers have a speed range of hundreds of millimetres per second, which is relatively slow, and a resolution of 20 μm depending on the nozzle diameter and bioink type (Cui X. B., 2012). Printing can be optimised by using low viscosity materials to reduce the chance of blocking small diameter nozzles. Thus, this type of bioprinting is limited by nozzle blocking and slow speed range due to the deposition of droplets. Nevertheless, this technique has advantages concerning costs and is flexible in biomaterial choice (Ma, 2018).

5.2 Extrusion-based bioprinting

The extrusion-based technique (figure 2B) applies pressure on the bioink, which is printed in one continuous filament. The printer is controlled by a computer which determines the time and location of dispensing the material by moving the nozzle. Printing can be done through pressure-based, mechanical or solenoid (magnetically regulated) control. The finest resolution is 5μm but generally 100μm is used, depending on the biomaterials used. This technique is best for features lacking microscale properties, but it is

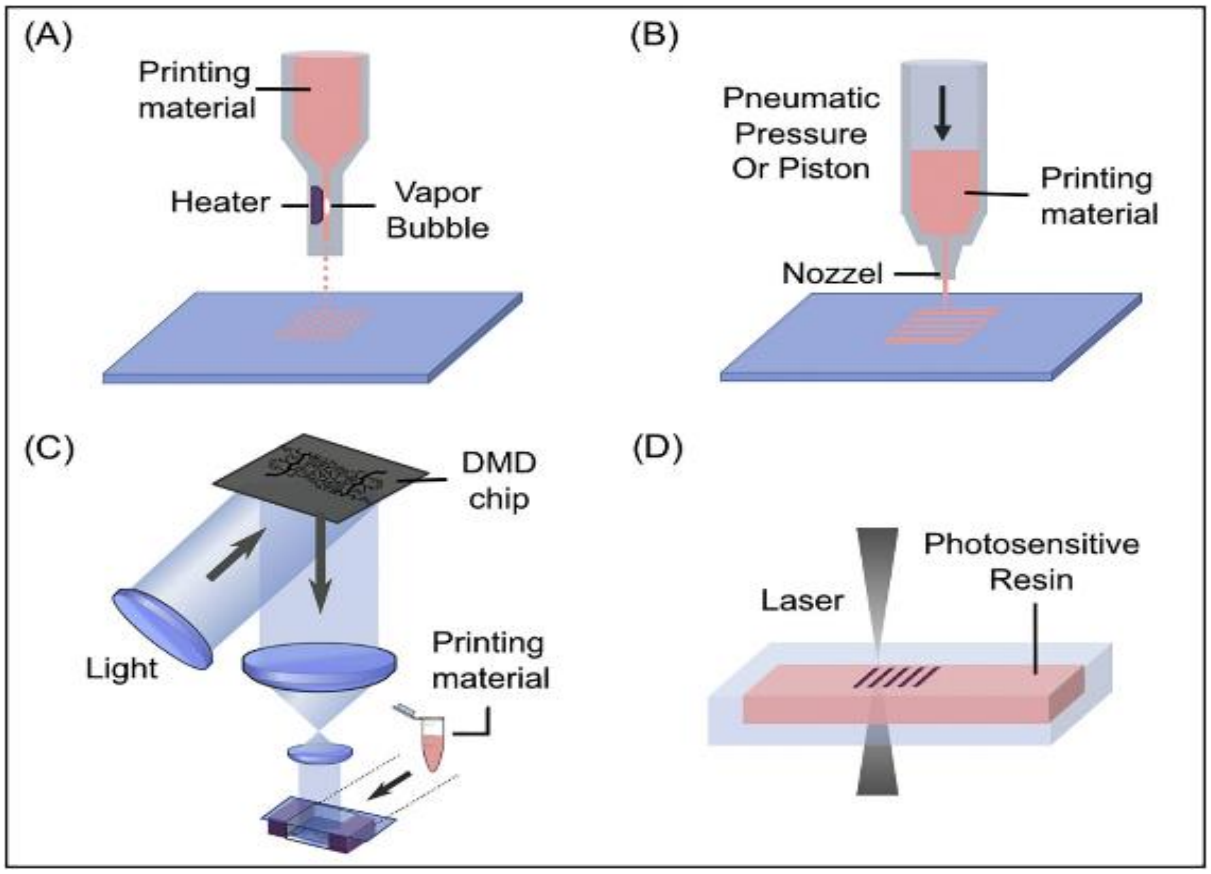

Figure. 2. Schematic approaches showing different printing techniques: (A) inkjet-based bioprinting systems, (B) extrusion-based bioprinting systems, (C) DLP-based bioprinting and (D) TPP-based bioprinting platforms (Ma, 2018).

appropriate for a high variety of biomaterials. This type of printing has the lowest speed of the discussed techniques, about 10 to 50μm/s (You, 2017; Hung, 2016).

5.3 Light-assisted: DLP-based bioprinting

This technique uses two million micro-mirrors which can be regulated independently to optimise light projection throughout printing. The light is projected on a prepolymer solution, which is the bioink. By light projection, the biomaterial polymerizes in a specific manner resulting in a firm 3D structure. The size of the light beam determines the resolution, which is usually at microscale. The printing speed is a couple cubic millimetres per second (the fastest technique) due to irradiating the material almost all at once. With DLP-printing (figure 2C) the entire pattern is projected using mirrors, so there is no interface between printed lines or dots. This results in qualitatively higher mechanical properties (Zhu W. M., 2016). This printing technique has advantages in pattern input, printing speed and resolution. Biomaterials must be photopolymerizable, like gelatin methacrylate (GelMA), but can be expanded by using photocurable materials. This technique is ideal to construct complex tissues for disease models. Vascular networks can be included as well (Zhu W. Q., 2017).

5.4 Light assisted: TPP-based bioprinting

This type of printing uses a laser to polymerize photo-selective monomers. In figure 2D, photosensitive resin is present. This is a photosensitive polymer, which means that after light exposure, properties like firmness enhance. Its 300nm resolution makes this technique ideal for nano- and microscale applications. The TTP-based printer (figure 2D) has a printing speed of 200-1600 mm/s (faster compared to extrusion- and inkjet printers). Various biomaterials can be used, like type I collagen and laminin. However, the choice of materials is limited by the free-radical polymerization method, which requires free-radical building blocks. Another limitation is the need of washing steps in between the printing process when there is a change of biomaterial or

cells. Despite this inconvenience, cell viability, resolution (single cell resolution is possible with this technique) and printing speed are high (Zhu W. M., 2016; Truby, 2016).

5.5 Multi-nozzle bioprinting

Many bioprinting techniques make use of a single nozzle. However, it is also possible to use multiple nozzles at the same time during printing. In this way, different biomaterials or bioinks can be printed simultaneously, which is desirable for producing heterogenous constructs. These constructs contain multiple cell types and/or biomaterials. Consequently, using multiple nozzles makes manufacturing heterogenous constructs obvious. An example of a multiple nozzle study is from Song et al (2011). In this study two biomaterials, sodium alginate and a 1% calcium chloride (CaCl2) solution, were printed simultaneously with different nozzles. Sodium alginate is water soluble and crosslinks through contact with CaCl2 which makes the entity firmer directly after printing. Through printing layer by layer, CaCl2 constantly following sodium alginate, a 3D hydrogel arises. Furthermore, adding cells and biomolecules to the biomaterial is feasible (Song, 2011).

6. Advantages compared to 2D cell cultures

Cell cultures are often in a 2D structure. In these cases, there is a monolayer of cells. Conversely, the cells in 3D models are multi-layered and contain many cell-cell and cell-ECM interactions. These conditions represent a more natural environment of cells, resembling cells in the human body. Almost all cells are surrounded by other cells and are part of complex structures. For example, cells in organs are all 3D oriented. Therefore, 3D models are ideal to simulate these situations. These models result ins a cell culture with higher complexity and specificity like living tissues compared to 2D cultures. In this way, a tumour can be mimicked and be studied in an environment more similar to the human body (Edmondson, 2014).

For example, the sensitivity of (cancer) cells to certain drugs can change by their cell-cell interactions. Therefore, cells in a 2D structure may react different to molecules compared to cells in a 3D structure. The 3D structure is a closer resemblance to the *in vivo* situation, making 3D models more reliable. In support of this, drugs tested in 3D models have a higher anticancer probability in real life (Kim, 2019).

Another major advantage of a 3D model is the possibility of including blood vessels. Blood vessels play a major role in tumours, especially in cancer cell metastasis. Cancer cells migrate from the primary tumour to the surrounding blood vessels, after which they can spread to other tissues in the body. This way, the cells can form new tumours. During this process, the interactions between endothelial cells and tumour cells (cell-cell interactions) are very important. These processes can be studied in a 3D model of a tumour, which is not the case in a 2D model. Blood vessels cannot be included in such a model. In cancer models, it is important to study blood vessels because they are different from vessels in healthy tissue. Tumour blood vessels are heterogeneous and hyperpermeable. In a 3D structure the involved processes and the effects of anti-cancer drugs can be studied whereas in a 2D culture these processes are inaccessible (Zervantonakis, 2012).

7. Advantages compared to 3D cultures

In general, 3D cultures are also used without being printed. These can be made by, for example, growing cells in small containers, so that cells can grow up to the edge. In this way, you cannot achieve the desired specificity of tissues, just like using 2D cultures. As for example, blood vessels are difficult to include into these cultures (Song, 2011). In addition, the shape, consistency, and the arrangement inside of the construct cannot be regulated. In contrast, 3D printed constructs can be made layer by layer, controlling every part by pre-programming the layout. Furthermore, cells can be distributed in desired densities. Consequently, 3D printed constructs can mimic human tissues (Khalil, 2009).

8. Use of one or multiple cell types

To mimic a human tissue in a model, several cell types are required like endothelial cells, immune cells as well as stromal cells (connective tissue cells). These cells contribute to the environment of a tumour. Therefore, integrating these cells,

like fibroblasts, into 3D printed tumour models, improves the significance of such studies. Consequently, tumour models preferably contain multiple cell types. Nevertheless, a model with a single cell type can be used to look at properties of tumours and effects of drugs on a smaller scale. Moreover, sometimes it is desirable to study a specific interaction between two cell types in one tumour (Meng, 2019).

An example of a study using a single cell type is from Kim et al. in 2019. This study uses a single bladder cancer cell type to examine the cell-cell and cell-ECM interactions in a 3D structure. Pathways regulating these interactions and the effects of certain drugs were examined. Given the above, using a single cell type makes it possible to study singular distinguishable aspects (Kim, 2019).

As mentioned before, using multiple cell types gives more realistic results. This is the case when studying, for example, metastasis and immunoreactivity. These specific processes, concerning multiple cell types, are involved in cancer. Both these processes are important to monitor while experimenting with anti-cancer drugs (Meng, 2019).

9. Tissue engineering and regenerative medicine

3D bioprinting has multiple applications in the field of regenerative medicine and tissue engineering. This printing technique can construct patient specific tissue in a controlled manner. Therefore, this biomimetic tissue can be used for several applications, like implantations in patients. After printing and insertion, the implant can regenerate tissue in patients to replace the defective or missing tissue. After this, the printed implant slowly degrades and is replaced by native tissue. An example is ear or nose replacement for cartilage regeneration in plastic surgery. Moreover, the printed constructs can be used for disease modelling, drug delivery in the body, personalized medicine, tissue regeneration and drug and toxicology research. Bioprinting entire organs is the optimal outcome here, but unfortunately, this has not yet been achieved (Maloney, 2020).

10. 3D bioprinting for drug screening in cancer

Normally, animal models are standard in drug screening. However, due to the difference in species, translation of the results of animal tests to humans is an issue. Luckily, 3D bio-printed constructs can replace or add to drug screening in animals. 3D tissues can be used before human (and animal) drug testing. This allows the testing of new drugs and the biocompatibility of materials. If a tumour model is constructed, the possibility arises to investigate drugs in human cancer cells. In addition, multiple manners of drug administration and delivery can be tested. Also, concentrations and amounts of medicines can be determined to use in practise (Meng, 2019).

Over the years, multiple successful cancer models have been developed to investigate anticancer drugs, 3D printed tissues being one of the most promising models. The possibility of creating cell-cell and cell-ECM interaction makes 3D bioprinting promising for cancer research (Hermida, 2020). One of the first 3D printed models for cancer research was made in 2014 by Zhao et al. This study used HeLa cells suspended in a combination of gelatin, alginate, and fibrinogen. Cell proliferation, protein expression, and anticancer drugs were investigated to examine chemoresistance on this 3D cervical cancer model. In this way, this kind of models were illustrated useful in cancer research. Besides chemoresistance, many other observations that come with cancer and anti-cancer drugs can be studied (Zhao, 2014).

10.1 Cellular characterizations

Depending on the study, the parameters to be monitored might differ. There are some general parameters, such as cell proliferation, viability, metabolic activity, and morphology. It has become standard to record these properties before and after anticancer drug application to investigate the drug's effects (Hermida, 2020). A useful method to characterize various cell properties is through fluorescent labelling of specific proteins. For example, the morphology of cells can be visualized by staining cell nuclei and F-actin (cytoskeleton protein). Through confocal imaging, cell morphology becomes visible (figure 3). The images can be compared before and after anticancer treatment (Duchamp, 2019). If multiple cell types are included in a model, it is

possible to distinguish between cell types using fluorescent labelling. To determine cell viability and proliferation rate, fluorescence can be a useful tool to visualize the number of living and dividing cells (Kim, 2019). The interactions between tumour cells and surrounding cells are crucial for tumour growth and ultimately cancer metastasis. Needless to say, these interactions are relevant to monitor. Interactions too are visualized using fluorescent protein specific markers. Scanning electron microscopy (SEM) is also used to study cellular interactions (Heinrich, 2019). Lastly, cell distribution, migration, metastasis, and colonisation can be visualized using fluorescent labelling (Duchamp, 2019).

10.2 Gene expression

Some characteristics that many tumours possess, like ECM remodelling and vascularization proteins, can be monitored through gene expression analyses (Heinrich, 2019). For example, in cancer, angiogenesis is an important process preceding metastasis (further explained below). Therefore, expression of proteins involved in the production of new vasculature for metastasis is enhanced in many tumour cells. These cells produce and secrete VEGF, which stimulates endothelial cells to form blood vessels. In this example, gene expression of VEGF (and other involved metabolites) can be monitored to provide clues about the metastasis process, for instance before and after drug testing (Cui H. E., 2019; Meng, 2019). Additionally, metabolic activity measurements can be used to study cell proliferation. This is important in cancer research because growth is a major problem in tumours. Metabolic activity can be assessed through gene expression patterns. This parameter can be used to investigate the effect of anticancer drugs and resistance (Zhao, 2014).

10.3 Metastasis

In cancer research, metastasis is of great importance. A metastasising tumour results in a poor prognosis in patients and is the main cause of death caused by cancer (Wang Y. D., 2020). Therefore, studying drugs that might influence metastasis of cancer cells from a primary to a secondary site is promising for treatment efficiency (Wang Y. D., 2020). An example of metastasis is the invasion of breast cancer cells into bone cells through vascularization. In breast cancer, metastasis of tumour cells to bone is seen in most cases. A study by Cui et al. (2019) used a triculture model were breast cells (BrCa) and vascularised bone tissue were printed in suitable bio inks. In this study, multiple aspects of metastasis were studied, like expansion of the cancer cells, migration, and the colonisation into bone tissue. In addition, the effect of anticancer medicines on metastasis was assessed. Multiple cells types were used in this model, namely breast cancer cells, osteoblasts, and endothelial cells. A schematic metastasis model is shown in figure 4. The migration of cells was visualized using fluorescent markers, and genetic analysis looking for expression of migratory proteins. This study helped to understand interactions between different cell types and tumour cells, which in turn drives identifying and testing different anticancer drugs (Cui H. E., 2019).

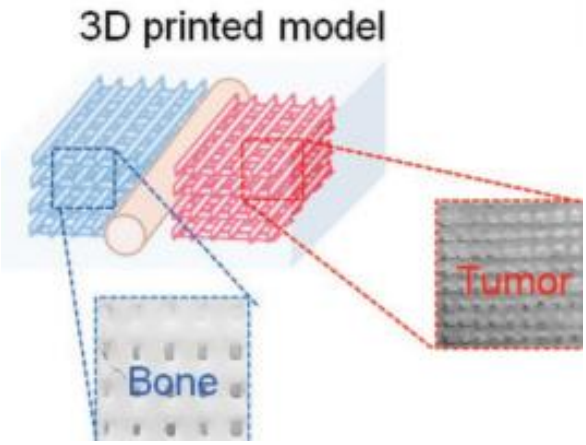

Figure 4. Schematic illustration of a 3D printed BrCa bone model for studying metastasis (Cui H. E., 2019).

10.4 Drug presentation

There are several possibilities to present anticancer drugs to the 3D constructs. The first one is by simply adding the drugs to the growth medium of the cells. The drug can reach the cells easily and examining the appropriate amount is possible by using different concentrations (Kim, 2019; Maloney, 2020). This method is simple and effective but does not entirely represent the human situation during drug administration. In the human situation, drugs often reach a tumour by blood vessels, which are not used when adding drugs to the medium. As mentioned before, vasculature and metastasis are important in cancer development. Using a 3D printed metastasis model, drug screening using vasculature is possible. In a study of Nie et al. (2020) a tumour model including vasculature was made to examine metastasis. Furthermore, they used this model for drug screening as well. Anticancer drugs were

added to the larger vessels of the model and they looked at drug dose-reactions. The effects on endothelial and tumour cells were studied. The drugs reached the tumour cells by diffusion, and the higher the concentration, the less tumour cells present (Nie, 2020). This technique is called organ-on-a-chip model but in this case, cancer-on-a-chip-model. The 3D construct is added to a so-called chip, connected to blood vessels. Delivery of nutrients and drugs to 3D constructs can be done by this vasculature. This method can be used to study anticancer drugs, proliferation, angiogenesis, migration, and intravasation of tumour cells (Shirure, 2018). Another method for drug administration is by inserting them into the polymers of the bioink. Cells constantly remodel the ECM surrounding them. By including drugs in the ECM, cellular reactions to ECM modification can be studied. Also, drugs can be released from the polymers at a constant rate. This is important for anticancer drug research. Making use of drug gradients improves insight in drug efficiency (Liang, 2017).

10.5 Drug administration

Various options are present to administer drugs to the 3D construct. First, there are many anticancer drugs with varying properties. Most of these drugs are hydrophobic. One example is paclitaxel, which is extensively used for treating ovarian and breast cancer. Delivering hydrophobic drugs is difficult because they repel watery surroundings. Therefore, reaching a target inside cancer cells is problematic. A solution is to enclose the drugs into nanocarriers, a nano-scale small material used for drug delivery. Nanocarriers must have an appropriate loading capacity and can be made of various materials, like biocompatible polymers. Hydrophilic anticancer drugs are commonly used as well. This group contains biomolecules, like nucleic acids. Hydrophilic drugs have difficulties such as rapid breakdown in the body and having a low stability. Additionally, permeating cell membranes is complicated. Luckily, encapsulating hydrophilic drugs by nanocarriers is possible as well. Nanocarriers can be presented to the 3D constructs as described above. Nanocarriers can be made of polymers, carbon, proteins, metals, and many more materials (figure 5). Variations in shape, size, and coating can also be made, as is shown in figure 5 (Sun, 2014). Another option for encapsulating drugs is by using microspheres, which are similar to nanoparticles, only larger: in the range of 1μm to 1mm (He, 2011).

In addition, drugs can be administered by including them into the biomaterial. Some materials create a strong binding to drugs which can be used for controlled release. A binding of a drug to biomaterial can also be too strong, so

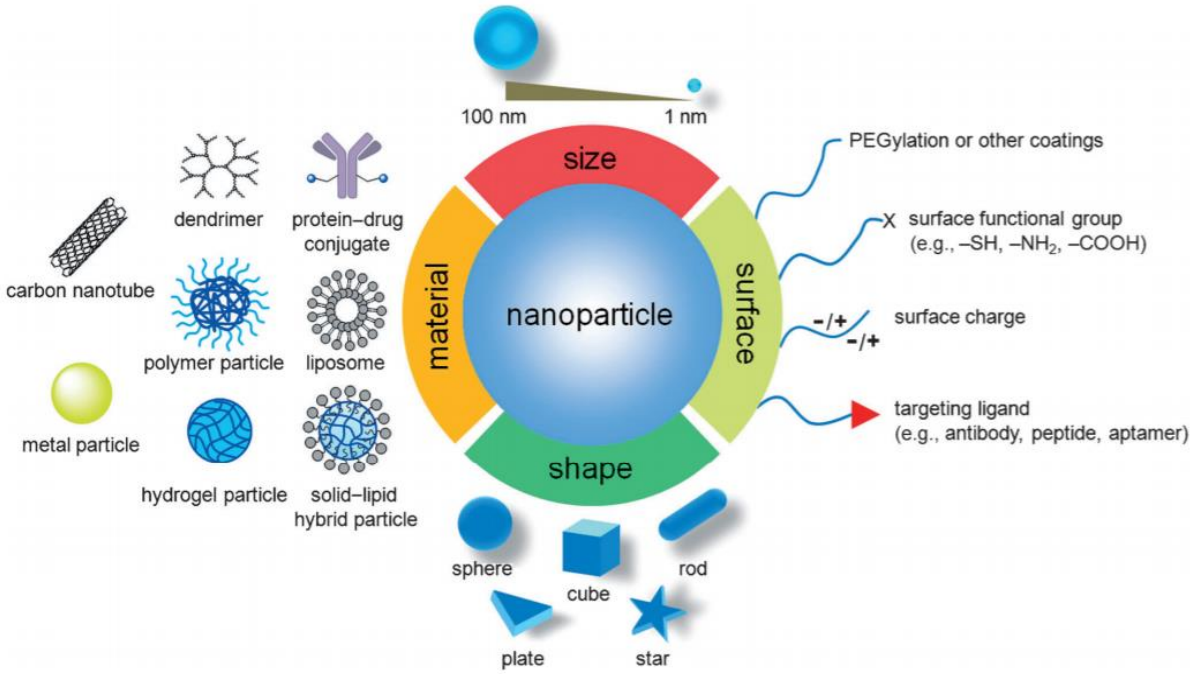

Figure 5. summary of nanoparticles that have been explored as carriers for drug delivery in cancer therapy, together with illustrations of biophysiochemical properties. Visualized are variations in material, size, surface, and shape (Sun, 2014).

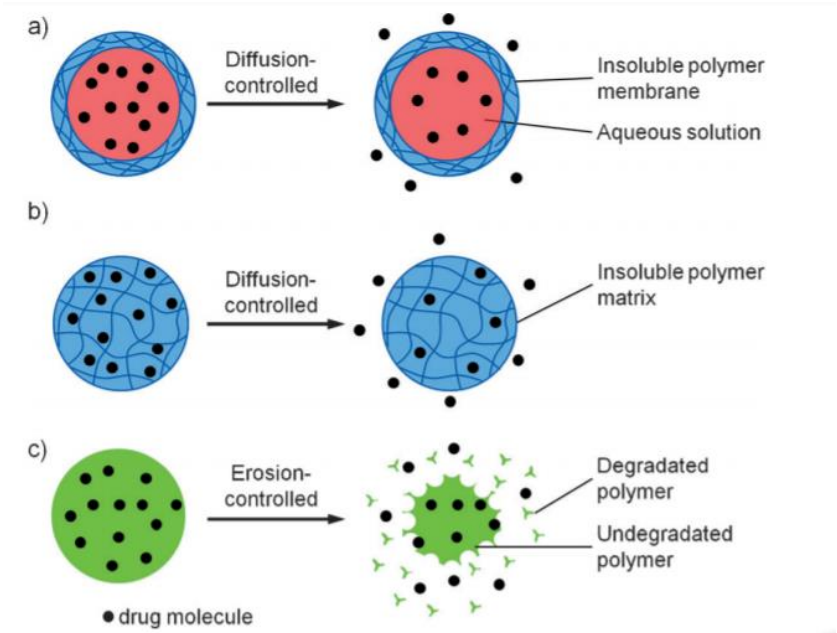

Figure 6. Three major mechanisms for controlled drug release. A: diffusion through an insoluble polymer, B: diffusion through an insoluble polymer matrix, C: erosion of a soluble polymer matrix (Sun, 2014).

strong that diffusion and the action of the drug is not sufficient to achieve results. Therefore, it is important that drugs can easily reach the cells in the 3D construct (Liang, 2017).

10.6 Release of the drug

When small particles like nanocarriers and microspheres are used, controlled drug release is a possibility. Anticancer drugs are slowly released for a relatively long time. For patients, this means an improved effect of a drug and reduces the discomfort which a drug can cause. In addition, the anti-cancer effect can last longer (He, 2011). Small particles degrade in different ways depending on the type of material they are made of. Diffusion of the material is a possibility of drug release. The medicines can also be released through erosion of the biomaterial. In figure 6, the three major mechanisms for controlled drug release are visualized, which are controlled depending on the type of material they are made of. Diffusion controlled is possible when insoluble polymers are used. When soluble polymers are used, they erode making erosion-controlled drug release possible (Sun, 2014). Gradients can be used when testing anti-cancer drugs. The medicines are being applied to different degrees in the 3D construct. This allows the correct amount and concentration of drugs to be determined. Multiple methods for controlled drug release are used. One example is a study of Meng et al. (2019) researchers used small particles with water inside of them. Inside these aqueous capsules, anti-cancer drugs were present. The shell of the particles consisted of biocompatible polymers and plasmonic gold nanorods (AuNRs). These golden nanoparticles had a photothermal response, which means they resonated when irradiated with the correct wavelength. When this happens to cancer cells, the particles are destroyed by the resonance and the encapsulated drugs are released. Consequently, the drugs get to exactly the right location whenever desired (Meng, 2019).

10.7 Culture conditions: hypoxia

A growing cancer inside the human body affects its environment. As mentioned before, blood vessels grow inside a tumour and are adjusted by tumour cells. These alterations result in hypoxia, in other words oxygen deficiency, because of the rapid proliferation of cancer cells. Hypoxia only exist in solid tumours, which excludes cancer in blood and bone marrow, and arises when blood supply is insufficient. Cancer cells can withstand the oxygen deficiency by adjusting their metabolism (Garcia-Bermudez, 2018). Additionally, hypoxia triggers a cascade in transcription and epigenetic factors, which results in a fastened tumour progression (Harms, 2019). Therefore, it may be interesting to include the factor hypoxia into culture conditions of 3D cancer constructs. This could be done by reducing oxygen in the culture. In addition, cancer cells in hypoxia are resistant to many anticancer therapies. For starters, radiotherapy works by inducing double strand breaks in the DNA by using oxygen. Secondly, some chemotherapy drugs have difficulties entering a hypoxic tumour due to the reduced blood vessel system. Thirdly,

the effect of immunotherapy can be reduced by alteration of the immune system by hypoxic cells, like suppression of T-cell (Harms, 2019). For this reason, it is important to study the effect of drugs when there is a lack of oxygen in tumours. Nevertheless, there are already several therapies and drugs targeting cells in hypoxia. An example are radiosensitizers, which increase the sensitivity for radiotherapy by reducing the overall cellular oxygen consumption. This can increase the overall oxygen availability (Ashton, 2016). Another anti-hypoxia strategy is by increasing systemic blood oxygen by, for example, pure oxygen breathing or adding oxygen nanobubbles to the blood (Owen, 2016). Unfortunately, hypoxia is still incompletely resolved (Harms, 2019) and therefore may be important to include as variable in culture conditions in 3D constructs. One possibility is to reduce oxygen levels in culture conditions to mimic hypoxia. Another option is to develop a 3D construct including vasculature were hypoxia evolves by itself when the tumour grows. Given the above, it may be important to induce hypoxia when culturing a 3D construct and testing anticancer drugs.

11. Model examples for glioblastoma

To illustrate all the discussed topics for 3D models, a specific type of cancer, namely glioblastoma multiforme (GBM), is used as an example. Several 3D models have been developed for glioblastoma since it is the most aggressive and common form of cancer arising in the brain (Ostrom, 2016). As a result, the 10-year survival rate is less than 1% (Tykocki, 2018). It follows that current treatment, consisting of radiotherapy, surgery and/or chemotherapy, is usually not optimal also because the quality of life has deteriorated considerably (Lee, 2019). There are three accepted cellular origins of GBM: neural stem cells, astrocytes, and oligodendrocyte precursor cells (Yao, 2018). A glioblastoma tumour consists of multiple cells, namely cancer, vascular and stromal cells (Hermida, 2020). The first example of a general 3D printed model for glioblastoma originates from a study of Hermida et al. (2020) in which glioblastoma stem cells, glioma associated stromal cells, and microglia were used for printing. For the bioink, the cells were added to alginate altered with collagen-1, hyaluronic acid, and cell adhesion peptides. 3D bioprinting was executed by an extrusion printer with a 200 μm nozzle. The printed constructs were crosslinked using calcium (specifically CaCl2 and BaCl2). Before printing, cells were adjusted with lentiviral transduction (using plasmids) to become fluorescent. Consequently, cell proliferation and viability were assessed. In addition, cellular protein kinases and RNA was analysed to study cell signalling. For drug testing, various concentrations of cisplatin and temozolomide (chemotherapy drugs) were added to the medium and viability was studied. As a result, a higher resistance to these drugs was found compared to 2D cultures. Altogether, this model is suitable for medicine testing and tumour-environment studying (Hermida, 2020).

11.1 Mini-brain

The second example for a 3D printed glioblastoma model is from Heinrich et al. (2019). In this study, glioblastoma cells and macrophages originating from mice were used. The crosstalk between these cells was the main concern since glioblastoma-associated macrophages (GMAs) have a major role in glioblastoma development. Therefore, cell-cell interactions, migration, invasion, and the interaction of macrophages and glioblastoma cells was examined by protein expression and fluorescence. The printing process used a custom-modified bioprinter and used two steps to eventually create a small brain. The first step used a bioink consisting of macrophages (RAW264.6) and printed a construct with a cavity, which was filled in step two with bioink containing glioblastoma cells (GL261) as can be seen in figure 7. Both the bioinks consisted of GelMA as biomaterial. In figure 7 A-E the structure, size, cross-section, and communication between cell types is visualized. Subsequently, the possibility of drug screening was tested by adding immunomodulatory and chemotherapy drugs to investigate cell growth. Concluding, the mini-brain study creates the possibility to observe interaction between cells and showed that glioblastoma cells turned macrophages into GMAs, which enhances glioblastoma progression. Additionally, anti-cancer drugs inhibited interaction between GMAs and glioblastoma cells which inhibited cell growth and improved chemotherapy affectability (Heinrich, 2019). Notably, for the translation to the human situation it would be an improvement to use human cells.

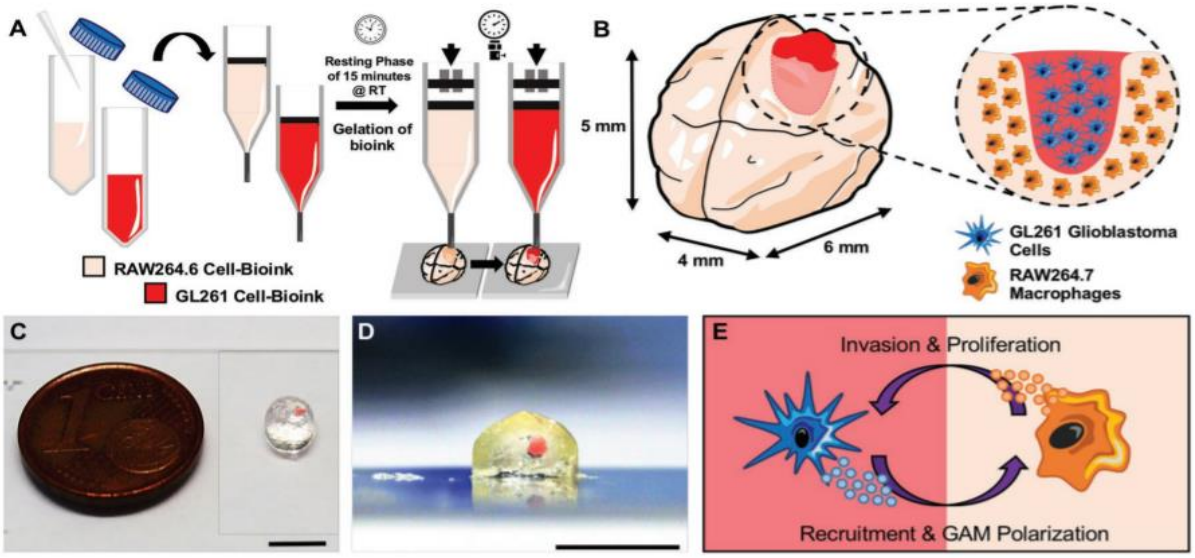

Figure 7. Bioprinting process and mini-brains schematic display. A, visualizing bioink with GelMA and (1) RAW264.6 (2) GL261 in the two-step printing process. B, cross-section of a mini-brain with glioblastoma cells and macrophages. C, mini-brain on the right of a 1 cent piece with glioblastoma cells in red (scale bar = 5mm). D, mini-brain with cross-section in frontal plain, glioblastoma cells in red (scale bar = 5 mm). E, interaction between glioblastoma cells (orange) and macrophages (blue).

11.2 Glioblastoma-on-a-chip

A third example is the human-glioblastoma-on-a-chip model from a study of Yi et al (2019). Here a 3D printed, multi-cellular and -material model is constructed. The aim of the researchers is to print using a patient's tumour cells and determine the correct drug combination. As biomaterial for the bioink, decellularized porcine ECM (BdECM) originating from the brain was used. BdECM is ideal for mimicking the original tissue of cells and in addition, cell proliferation enhances in dECM compared to a bioink of collagen. BdECM is made available by decellularization with enzymatic and chemical components. With the BdECM, two different bioinks were created by adding human endothelial cells to one, and human glioblastoma cells to a second. The printing process had several steps to create a spherical structure, since a human glioblastoma grows in recognizable regions, as is shown in figure 8a. With a multi-nozzle in-house 3D printing system, a chip wall was printed with permeable silicone ink. Inside of this structure the endothelial cells with BdECM were printed in a ring to create vasculature. In the middle of the ring, the glioblastoma cells plus BdECM were printed. This circular structure is shown in figure 8b. Automatically, solidification takes place after printing. Since the construct is spherical and oxygen is obtained from the medium, hypoxia is created in the centre of the chip and decreases peripherally, as it would in a human tumour. As a result, cancer cells migrated to relatively higher oxygen concentrations (towards the periphery). Experiments were executed with human cell lines and cells from patients with glioblastoma. 3D models were made with the cells of patients as described above, after which drug screening could take place. Drug resistance in patients was used for comparing individual responses to different treatments, namely chemoradiation (CCRT), which consists of radiotherapy combined with blocking the DNA repair mechanism with temozolomide or methoxamine (MX). The glioblastoma chips reproduced the resistance in the patients accurately. Overall, a patient specific drug combination can be identified using the glioblastoma-on-a-chip model. Finally, a model can be printed in 1-2 weeks, which is favourable given the fast progression of a glioblastoma (Yi, 2019).

11.3 Controlled drug release

The last glioblastoma model example is from a study of Mirani et al (2019) and is different compared to the previous examples, in the sense of drug delivery and bioprinting. Here, the initial printing process is without cells. The printing ink consist of GelMA, alginate and drug-loaded microspheres. The drug included in the microspheres is all-trans retinoic acid (ATRA or Vesanoid), used for various cancers like leukaemia and induces G1/G0 cell cycle arrest and mitochondria-induced apoptosis. ATRA is

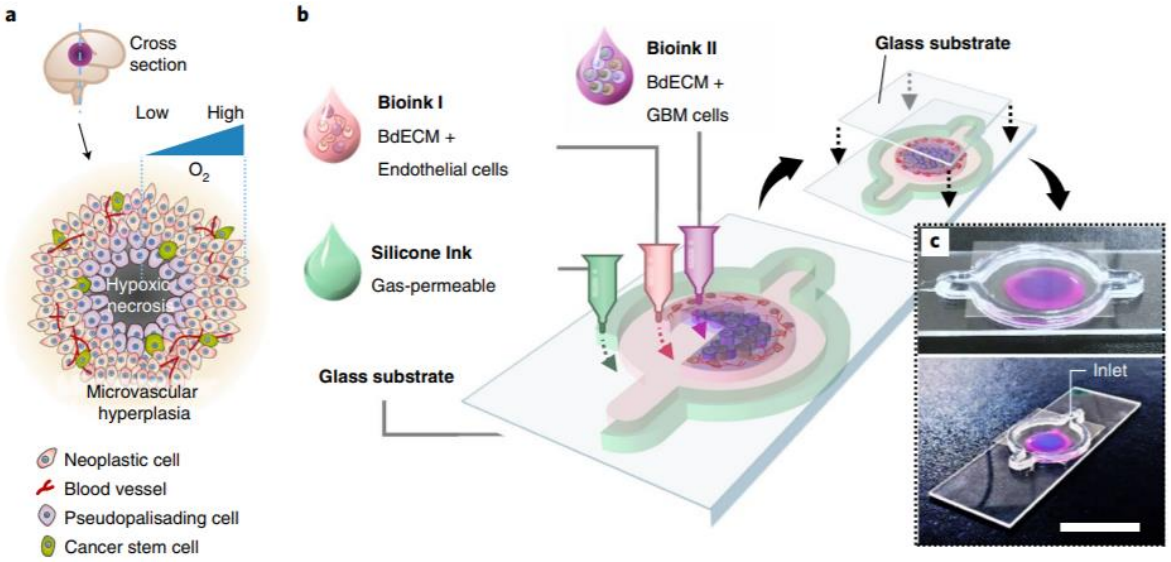

Figure 8. Schematic representation of glioblastoma and the glioblastoma-on-a-chip. A, schematic cross-section of a native glioblastoma structure with neoplastic cells, blood vessels, pseudopalisading cells, and cancer stem cells. B, representation of the bioprinting process with two different bioinks and various materials.

believed to be inhibitory for proliferating glioblastoma cells. The microspheres with ATRA further consists of polycaprolactone (PCL) which is biodegradable and hydrophobic. Normally, ATRA has a half-life of one hour in water, but PCL prevents fast degradation of this drug. This research group adjusted a commercial 3D bioprinter (Prusa i3) to an extruder printer with two nozzles. The ink is simultaneously printed with CaCl2, acting as crosslinker. In addition, UV light is used for crosslinking as well to improve the connection between the printed layers. The printed constructs were printed with a range of drug containing microsphere densities. These hydrogels were tested on 2D cultured glioblastoma cells and, separately, human primary astrocytes. By transferring the hydrogels to the cell cultures, the effect of ATRA on glioblastoma cells was assessed by viability. Glioblastoma viability was reduced, and an apoptotic morphology was seen in most of the cells. Furthermore, ATRA added to the medium was assessed to verify the effect, which was slight cell shrinkage. In astrocytes, ATRA did not have a cytotoxic effect. The objective of this study is to create the possibility of hydrogel implantation into the tumour. Direct drug delivery is made possible and has great advantages, like reduction of systemic drug effects (side effects), enhancing drug concentration at the tumour site, preserving drug concentration, and avoiding the blood-brain barrier. The current study shows a continuous drug delivery for 25 days, which is promising for implantation research of these drug-loaded hydrogels (Mirani, 2019). A proposed improvement would be to include an 3D printed cell culture to the hydrogel drug testing. For example, the hydrogel can be tested on the previously mentioned mini-brains, or another glioblastoma model to obtain results more comparable to the human tumour situation.

12. Overall conclusion

In this review, an overview is given about factors concerning 3D bioprinting and anti-cancer drug screening. Cancer therapies have challenges to overcome, like treatment finetuning, reducing side effects, specific drug targeting, and personalized treatment selection. 3D bioprinted tumour models can contribute to drug screening for cancer therapy research. In 3D bioprinting, an *in vitro* construct comparable to human tissues can be produced. Hereby, (cancer) cells and biomaterial are used to create a model for studying cancer. A biomaterial is selected by its resemblance to human ECM for optimal tissue mimicking and cell viability. The best biomaterial is a composite material, consisting of a combination of natural materials (fragile but similar to ECM) and/or synthetic materials (not specific but high selection of properties). For example, GelMA is a commonly used composite biomaterial, consisting of gelatin and methacryloyl. The inclusion of biomolecules, like VEGF, creates the possibility to induce gradients

and increases the similarity of the construct to the human situation. For the bioprinting process, various printing techniques vary in biomaterial type, printing time, resolution, and printing mechanism. The most used printing techniques are inkjet-based, extrusion-based, and light-assisted. A composite material is often printed using multiple nozzle bioprinters for simultaneously printing of different bioinks. A 3D printed construct has multiple advantages compared to 2D cultures, mostly due to the resemblance to human 3D tissues, plus the possibility of adding blood vessels. In addition, unprinted 3D cultures lack the specificity and the regulation of cell distribution needed for tissue mimicking. Inclusion of multiple cell types into a 3D printed construct, such as cancer cells, stromal cells, and vascular cells, improves the significance of such studies because of the higher resemblance to human tissue. 3D printing is used tissue engineering and regenerative medicine for implantation (regeneration of native tissue), drug delivery, and organ printing. Additionally, models for drug screening are made with 3D bioprinting, also used in cancer models to study anti-cancer drugs. Hereby, cellular characterisations can be studied, like cell interaction, distribution, migration, and metastasis by using fluorescent labelling and SEM. Gene expression monitoring in these models is important as well, for example in treatment determination and resistance. For the same reason, metastasis is a major focus point in research since it is the main cause of death in cancer. In drug screening, the presentation of a drug to the model is possible by including them to the growth medium, through vasculature, and by including them to the bioink. Drugs sometimes are encapsulated in nanocarriers or microspheres, or without anything around it, depending on the type of drug. By encapsulation, controlled drug release is possible, which is desired for reducing side effects and enhancing the local effect of a drug. In the culture conditions of 3D tumours, hypoxia may be important to induce for optimal tumour mimicking, although there are possible strategies for the reduction of hypoxia in tumours. To illustrate all the discussed topics, four example models used for studying and drug screening for glioblastoma multiforme were elaborated. Firstly, Hermida et al (2020) printed a model with glioblastoma stem cells, glioma associated stromal cells, and microglia, together with alginate altered with collagen-1, hyaluronic acid, and cell adhesion peptides. Cisplatin and temozolomide were tested via the growth medium. Secondly, Heinrich et al (2019) 3D printed a mini-brain with glioblastoma cells and macrophages with GelMA. For drug screening, they used chemotherapy and immunomodulatory drugs. Thirdly, Yi et al (2019) constructed a glioblastoma-on-a-chip model with BdECM, endothelial and glioblastoma cells. They experimented with chemoradiation and hypoxia was involved in their model. Lastly, Mirani et al (2019) printed with an ink consisting of PCL microspheres including ATRA as anti-cancer drug and GelMA with alginate. This construct was tested for controlled drug release on 2D cultures of glioblastoma cells and astrocytes for cytotoxicity. All these examples are suitable models for studying glioblastoma with slightly different goals. Nevertheless, there are some general improvements that can be made, such as using human cells in all experiments to improve translation to the human situation. Another important improvement is to test a drug delivery system, like in the last example, on a 3D printed tumour instead of a 2D cell culture. Altogether, important factors to include in a 3D printed tumour model are multiple human cell types, biomaterial representing human ECM, and probably hypoxia. It is essential to mimic a human tumour or tissue as close to reality as possible to achieve significant results for drug screening.

13. Future remarks

Overall, much has been achieved in the field of tissue engineering. Although, the possibilities can be considerably expanded, making improvements in factors concerning 3D bioprinting, such as the hard- and software of printers, and ECM originating bioinks. For example, models could be printed on a large scale as a standard for drug screening, development and personalized medicine (Maloney, 2020). Personalized drug screening in 3D printed constructs will have a major impact on the medical world and its concerning patients. In cancer, patient-specific constructs are probably important in choosing the most suitable treatment per patient. This can be done as follows, taking an example from a study already discussed about glioblastoma-on-a-chip from Yi et al (2019). As can be seen in figure 9, a biopsy of cancer cells is taken from a patient after which the cells are added to a bioink. The patient's cells are 3D printed and cultured, where after several drugs are tested on the model. The appropriate drug combination is selected and given to the patient following a treatment plan

(Yi, 2019). Ultimately, 3D bioprinting is a promising strategy for drug screening in cancer.

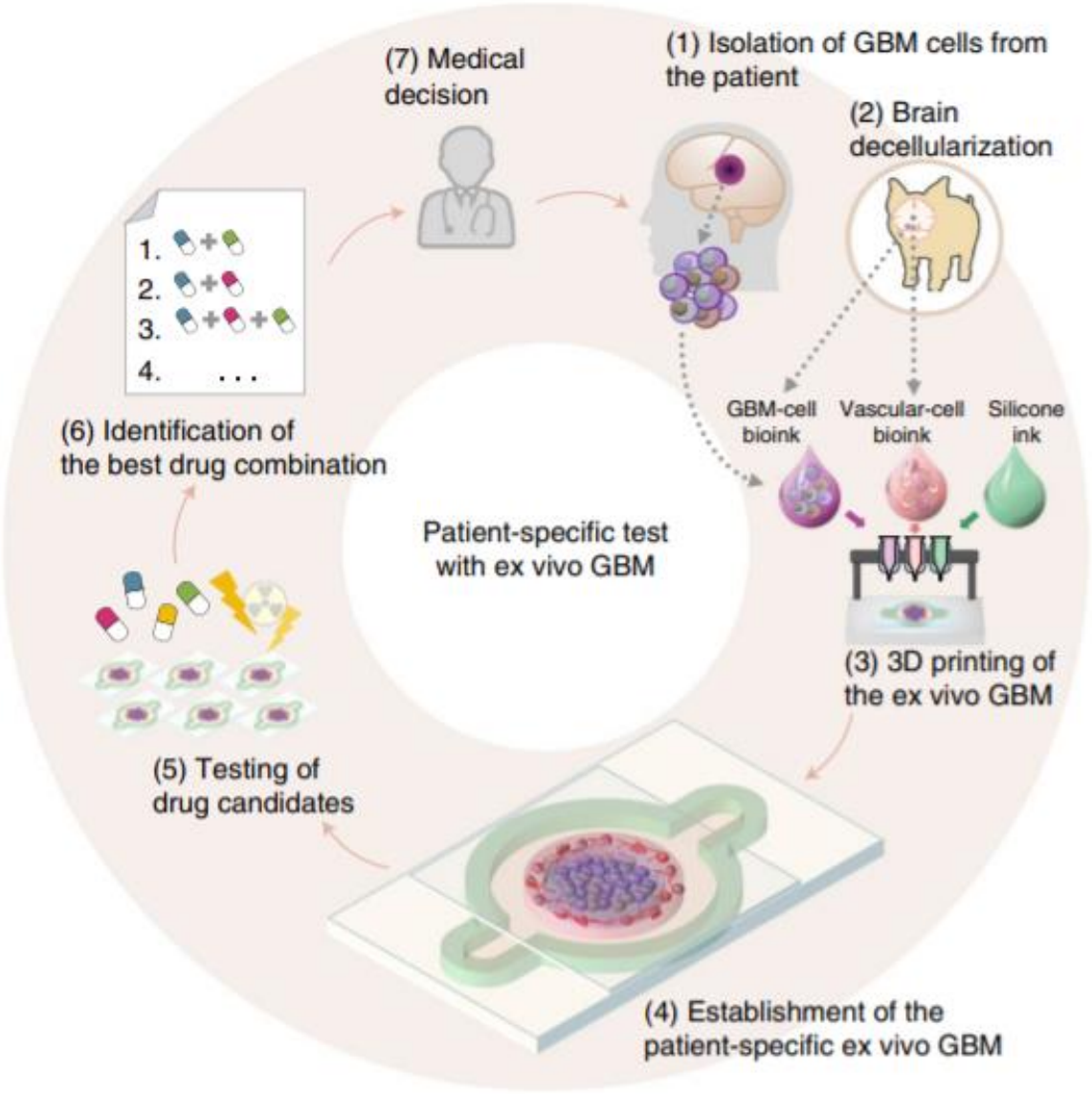

Figure 9. schematic representation of 3D bioprinting and patient-specific treatment selection using a glioblastoma-on-a-chip model. (1) surgical collection of glioblastoma cells. (2) collection of decellularized porcine ECM. (3) glioblastoma cells are 3D bioprinted with the BdECM, together with a vascular laden bioink, and silicon ink. (4) a cancer-on-a-chip is formed and cultured for 1-2 weeks. (5) different drugs are tested on the chip. (6) following best results, the best drug combination is selected. (7) a patient specific treatment plant is designed (Yi, 2019).

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