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Final Thesis

Evaluation of synthetic alternatives scaffolds for three dimensional culture method for liver organoids

Supervisor
Ch. Prof. Flavio Rizzolio

Graduand
Elena Bagnara
849964

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“Dans la vie, rien n'est à craindre, tout est à comprendre. Il est temps à présent de comprendre davantage, afin que nous puissions moins avoir moins peur. "

Marie Curie

Summary

ABSTRACT

In recent years, biology has made great strides in scientific progress. The discovery of cellular structures that grow in three dimensions, the so called organoids, has led to the development of interesting models for different applications in the biomedical fields.

Until recently, biomedical studies were mainly based on tests on two dimensional cell cultures then integrated by tests on living organism. The advent of these three dimension cell cultures gives the ability to create cellular models that resemble structures that actually exist in vivo, and it can be applied for different purposes such as drug screening, disease modeling and regenerative medicine.

In order to obtain organoids culture, it is necessary to recreate a matrix that is suitable for their growth and therefore it mimics those microenvironments, or niches, found in nature. The optimal microenvironment for cell growth must therefore mimic the Extra cellular matrix (ECM) as closely as possible (McKee, et al., 2019). Cultrex® matrix is the perfect example. It is a purified basement membrane, which derives by Engelbreth-Holm-Swarm (EHS) tumor.

The naturalness of this product, however, despite giving optimal results in morphology and vitality of the organoids, can present various problems that should not be underestimated. Precisely, due to its natural origin, problems related to the variability between the various batches may arise, thus compromising the obtained data. For these reasons, researchers are focusing their attention on the creation of well-defined scaffolds from a chemical and physical point of view. Nowadays synthetics scaffolds, that can be obtained in laboratory, are functionalize with peculiar membrane proteins that promote their attachment, their growth and differentiation. In this way scaffolds with high tuning capabilities were obtained. A further advantage linked to these synthetic scaffolds is the low cost of production, making them accessible to a wider research market. Unfortunately, the challenges of these synthetic scaffolds on their applications are still high (Aisenbrey & Murphy, 2020).

This thesis is focused on a synthetic alternative for liver organoid formation, and it unfolds in six main chapters. In the first chapter, a general explanation will be given on what organoids are and their origin, together with other basic concepts to fully understand this topic.

The second chapter that will follow, gives an overview of the various characteristics

related to Cultrex®. Other types of scaffolds will then be analyzed as alternatives to Cultrex® with their respective case of studies. The following chapter, the third, will illustrate the purpose of this thesis. In chapter four, the materials and methods used throughout this thesis will be described in detail. The fifth chapter will face all the results and discussion obtained for all the tested scaffold. The results will be analyze, firstly in a qualitative way and then in a quantitative way. The final chapter, the sixth, will detail the various results obtained and it will be devoted to provide a conclusive overview of the scaffolds tested.

1 . ORGANOIDS

1.1 Brief history of organoids

The formation of an *in vitro* model of a three dimensional (3D) cell culture was a crucial discovery of the last decade in bioengineering research since, thanks to their use, it is possible to study and recreate models for the development of an innumerable applications in many different fields, for instance: regenerative medicine, cancer therapy, drug discovery and organ development. As far as their 3D structure is concerned, the cluster, defined by the scientists as "organoids" in Figure 1, bears, in some respects, a striking resemblance to an human organ. The Organoids show micro-anatomic peculiarities but at the same time they are less complex making it easier to approach the study of this topic. Thus the use of Organoids in science was a very important turning point in the history of research due to their important representativeness, which is better than the simply monolayer of cell culture used a lot in the past years (Corrò, et al., 2020).

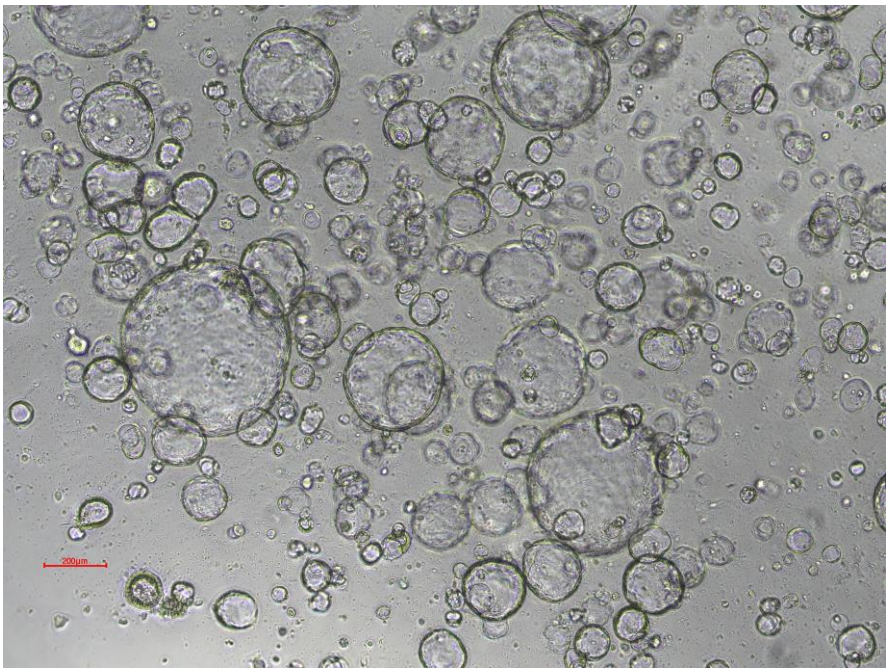


Figure 1. Liver organoids (4X enlargement)

In order to understand the importance of the role of organoids it is necessary to offer an insight into the history. During the first half of the 20th century, it was demonstrated by Henry Van Peters Wilson that the sponge, once dissociated, can easily organize itself to

recreate an entire organism. The years went by and several scientists studied with different approaches the aggregation, after a dissociation, of some embryos' organs, until, in 1964, Malcolm Steinberg elaborated a theory that implied the use of thermodynamics to understand the self-organization. At the end of the century, exactly in 1981, the things got interesting due to the first isolation of Pluripotent Stem Cells (PSCs) from mice's embryos. Only after 6 years great results were obtained just tuning the cell culture features mimicking *in vivo* conditions and they understood the power of the presence of ECM matrix that interact and create a network with the tissues. Therefore Li, et al., 2005 noticed that breast epithelial in presence of an Extracellular matrix were able to produce and emit milk proteins. This was the landmark, which delimitate the passage from a two dimensional approach to a three dimensional one, so it was possible to create a cerebral cortex from Embryonic stem cell (ESCs) (Eiraku, et al., 2008). Thanks to all these discoveries, in 1998 it was possible to separate and culture ESCs which comes from human blastocysts. Afterwards, the attention was moved to Induced Pluripotent Stem cells (IPCS) of both human and mouse which increased a lot the knowledge of organoids' research (Corrò, et al., 2020). Consequently, the study in this field continues to develop and grow through the years.

All the steps previously described, are highlighted in the following diagram:

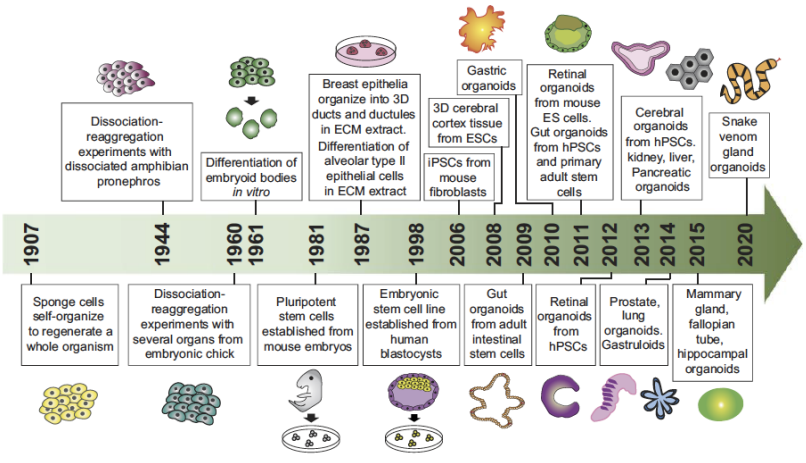


Figure 2. Time scale of the events which leads to the development of a 3d model or organoids (Corrò, et al., 2020)

*Lancaster et al i*n their papers (Lancaster & Knoblich, 2014) offers a precise definition of organoids: first of all these three dimensional structures must contain more than one type of cells that compose the organ in question, furthermore it must show specific function of this organs and last the self-organization of the cells must reproduce the spatial disposition of the resemble organ. It's important to specify that the formation process of an organoid must show a similarity with the self-assembled of an organ during its development, which is called "organogenesis" and rely on cell-sorting and lineage commitments. The cell sorting represents the capacity of cells to self-organize and the ability of them to recreate structures as the ones present in vivo. In addition, it is responsible for the cell's movement which are sensitive to different adhesion properties given by adhesion proteins present in cell membrane. The tissue morphogenesis is influenced by lineage commitment which represent the pathway's differentiation process starting from the parental cell. Hence the organogenesis is influenced by numerous cues pathways which are mediated by intrinsic or extrinsic environment (Lancaster & Knoblich, 2014).

The main applications of organoids are in biomedical fields due to the fact that they possess a three dimensional structure which resemble a real organ. The most important future development, in the fields of organoids technology, is about medical transplantation in order to replace damage part of organs using healthy cells. Unfortunately, these applications have yet to been used. Therefore, further studies and experiments have to be done to achieve this goal (Hofer & Lutolf, 2021). During these years it was noticed, that with organoids' culture it is difficult to obtain a total maturity, like in real organs. In addition a total functionality of organoids, it is very difficult to achieve. So none of organoids obtained in vitro, in comparison with the real organs, is able to show all their functional repertoire and its complexity and they do not possess specialized cells. The fact that organoids are widely different from real organs can be partially explained by the lack of a vascular system and mesenchymal compartment. Scientists anyway have generated organoids, which create a multi-compartment system, but also in this case was not a complete success because organoids didn't have typical cellular organizations (Park, et al., 2011). The experimental improvement with an air flow, which interferes with a mechanical stimulus to obtain a total maturation of the cell in vitro conditions, still remain a big challenge. A further limitation of organoids' culture

is time. The organoids cultured in vitro condition possess a limited life time (~ 10 days), after this time they will die , and this fact doesn't allow a total survival of these organoids in natural environment. The limited accessibility of this organs will influence their life, precisely when an organoid grows in vitro, its dimension increase and the nutrients diffusions and waste elimination will be highly inefficient and this leads to have problems to control different compartments which organoids possess (Hofer & Lutolf, 2021). Another limitation, which derive from organoids' culture is the heterogeneity. In vitro organoids possess a very high variability in morphology, in their formation efficiency and functions. This high variability is due to the fact that a cultured system of organoids can be considered a stochastic system (it means that the system behaves in a non-deterministic way and its subsequent state is dependent mainly by random elements). Saying that, it is necessary to reduce this variability in order to exploit organoid's potentiality to regenerative medicine , drug screening and disease modeling (Hofer & Lutolf, 2021). A future development to reduce this kind of variability could be, for instance, the use of well-defined scaffold with an appropriate growth medium combined with engineering strategies.

1.2 Stem cells for organoids formation

After this brief summary of the history of organoids, it was possible to understand what the genesis of the organoids it comes from the ability of stem cells that are able to self-assemble. These cell cluster can be originated from embryonic stem cell (ESCs), from Induced pluripotent stem cells (iPSCs) or from adult or neonatal stem cells (ASCs). Starting from these type of cells they are able to self-organize and differentiate in the presence of different cues (figure 3)

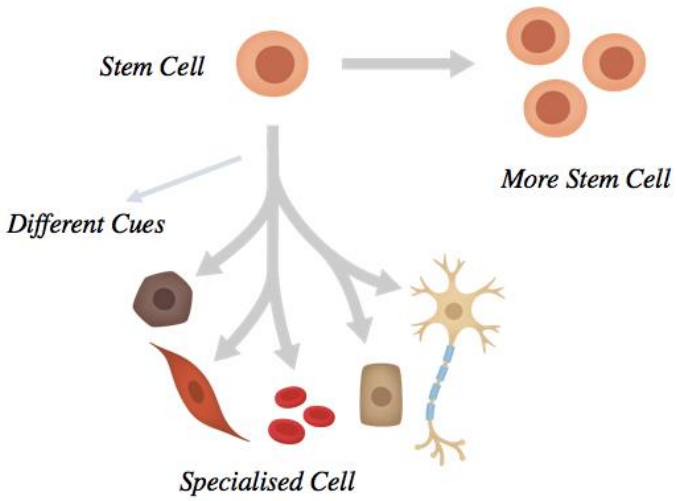


Figure 3. Stem cell differentiation (the grey arrows represent different signal)

Stem cells are the only type of cells which are able to replicate themselves in an antisymmetric way. It means that, starting from a staminal cell it is possible to obtain either a differentiate cell or a stem cell again (figure 3). But not all the stem cells are equal between each other: there are the Totipotent stem cells which are able to give different cell types (in example the cells in bone marrow can generate simultaneously erythrocytes, leukocytes, lymphocytes and thrombocytes) and from this cell is possible to obtain all the possible tissues of an organism. There are then, the Unipotent stem cells which can generate only one type of cells (as muscles cells) and the Pluripotent stem cells which can differentiate in any tissue but not in the whole organism.

The origin of embryonic stem cells (Pera, et al., 2000) comes from the embryos, that is a type of cell which is typical of the first week of the embryos. Furthermore, it is interesting

to say that, while the embryos develop, there is the formation of germinal stem cells that remain effective in adult life of the organism to promote the cellular regeneration. The renewal of cells comes from the differentiate offspring and this fact, in the last decade, can partially explain the origin of cancer. It is supposed to come from stem cells or their offspring, so it arise from a disparity between the rate of production of cells and their rate of differentiations or death. Having ascertained that, the knowledge and manipulation of stem cells is a fundamental factor to treat and control cancer therapy, and thanks to the versatility of stem cells, they are used on a large scale in biomedical research. Picking up on the origin of organoids from stem cells: for those who arise from Pluripotent stem cells it is possible to say that considering these types of stem cells which are able to produce ectoderm, mesoderm and endoderm, are able to originate all type of specific tissues as well as the type of functionality mature cells which derive from them. As of today organoids, which derive from PSCs, have been established for several organs including liver which were used for all the experiments in this thesis (Figure 1). For example, by focusing on the evolution of liver organoids it is possible to mention numerous examples of scientific studies.

The organoids, which derive from Adult stem cells present in a somatic adult tissue, assume a considerable importance in scientific research. Thanks to their ability of self-renewal and differentiation into different cell types, the organoids are able to preserve their genetic integrity. Comparing the organoids which derive from PSCs with the ones coming from ASCs, it is possible to notice that some differences emerge. Those that who come from ASCs show problems mainly due to the inability to recreate the correct signals of the cellular microenvironment in culture. They can only be induced to form organoids by mimicking culture conditions which are created during the physiological self-renewal of the tissue itself. Thanks to the peculiar and features property of the stem cells combined with an extracellular matrix as a scaffold with growth medium, it was possible to achieve the creation of organoids in vitro deriving directly from an organism.

1.3 Liver organoids

Focusing on the evolution of liver organoids it is possible to give as example numerous scientific studies. Concerning the biology beyond liver, it's possible to say that they are composed by some epithelial cells as hepatocytes and cholangiocytes. These epithelial cells work simultaneously with complex formed by: stromal, endothelial and mesenchymal cells. This complex plays a fundamental role to reach the body homeostasis. (Prior, et al., 2019) During hepatogenesis, a progenitor cell (called hepatoblast) divides from the anterior portion of the primary gastrointestinal tract, which arises from the endodermis, to form a condensed mass of tissue called liver bud. This mass is then vascularized. This process is led by factors responsible of signaling, which derived from the neighboring mesenchyme (FGF, BMP and Hepatocite growth factors HGF). From liver bud grow hepatoblasts, which are the precursors of hepatocytes and epithelial cells biliary while fibroblasts and stellate cells derive from the adjacent mesoderm. In vivo, starting from a single Lgr5+ hepatoblast, it is possible to obtain both hepatocity and cholangiocytes as a consequence of the bi-potency of this kind of cells depending on signaling. In general a liver, to support the normal functionality, as in vivo occurs, must be maintained constant during the body homeostasis. The cellular turnover, which characterize this type of organ, is considered very slow. Just considerate that in mice the replication is more or less 60 days for cholangiocytes and 150 days for hepatocytes so a very long time. But despite this fact, liver shows a great ability to self regenerates in case of damages. In addition to this, hepatocytes respond inducing signaling such as TNFa and iterleukin-6 in order to restore the damage mass in more or less one week. Even in the case of the proliferation of the majority of hepatocytes, which are compromised by some diseases, liver is able to regenerate itself. That's why liver transplantation it is considered a quite easy procedure that most of the time solve the patient's problems. Finally, the development of the liver represents a complex interaction of tissues deriving from both the endoderm and the mesoderm. Weiss et al. (Weiss & Taylor, 1960) in the second half of 20th century tested in a laboratory the possibility to create secretory unit with their functional bile starting from dissociated a chick embryo hepatic tissue. This study was taken as inspiration by Hunch et.al to recreate in vitro a three dimensional liver culture using Matrigel scaffold. They see that cells were able to differentiate forming functional hepatocytes. (Huch, et al., 2013) Liver organoid cultures

are composed mainly by cells which are the progenitor of bile ducts and hepatocyte markers, that if they are transplanted in an organism (mice in example) they differentiate in functional hepatocytes. This experiment was applied in 2018, by two different groups (Peng, et al., 2018) (Hu, et al., 2018). Both research groups obtained good result in long term expansion of hepatocytes derived from mouse and human, creating so a three dimensional organoid's culture. A vascularized human liver has been created by (Takebe, et al., 2014) using iPSCs. The starting point of this study is to cultivate in vitro a two dimensional culture of human PSCs combined with mesenchymal SC and endothelial cells. Moving from a 2D to 3D, the culture previously mentioned was able to generate three dimensional vascularized aggregates.

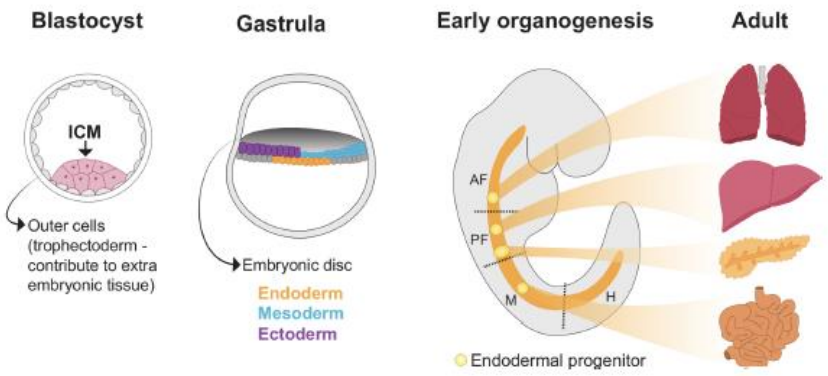


Figure 4. Different steps (organogenesis) to isolate the progenitor of liver organoid. Blastocyst= two distinct layers are formed: Outer and Inner cell mass (ICM). ICM is composed by Pluripotent stem cells. Gastrula= ICM differentiate into Endoderm, Mesoderm and Ectoderm, the 3 germ layers. From Gastrula starts the Early Organogenesis in which start to be born organs and Tissues. (Prior, et al., 2019)

1.4 Organoids and reverse engineering

Behind the creation of a 3D culture of organoids there is the reverse engineering. This is a process which extract some basic design information starting from a model to arrive to a new one which is improved respect to the previous model. The main concept behind the reverse engineering is the analysis in a detailed way of the various component, which compose the system based on the disassembling and identification of the fewest fundamental compartments. They are able to reproduce the entire system one reconstituted it. Organs are complex structures which are surrounded by a vascular system to promote oxygen diffusion and nutrient diffusion and they show mechanical forces as well. For this reason, exploiting the reverse engineering to recreate in vitro a functional organ is very difficult. But all these characteristics were taken as an inspiration to develop the so called "organ –on- chips" (Ingber, 2016). These organ-on-chips are, in some respect, a microsystem which recreates in vitro three dimensional cultures which mimics not only the physiologic response of a real organ but also they mimic the mechanical functionality. Some examples of the building block of a typical biological system could be the type of cells, the microenvironment, the shape and flow and other physical parameters. Applying the concept of the reverse engineering, it's possible to solve problems related to an in vitro condition such as the reproduction of the heterogeneity between cells (Lou & Leung, 2018). Here, the extracellular matrix plays a fundamental role in the birth of organoids in vitro conditions, which extrude not only the essential growth factors but also enzymes and small molecules that all together will produce a complex intertwining of chemical and physical signals to promote the morphogenesis of tissues, and organs as well.

In the previous chapter it was seen that the formation of three dimensional organoid culture comes by staminal cells. But despite the fact that the stem cell can reproduce in vitro condition the building block of a real organ, there are still huge differences between in vitro conditions and in vivo conditions. This could be partially explained by alteration of the microenvironment surrounding organoids. The science of bio and Nanomaterials combined with the bio engineering allow to analyze in a detailed way the natural system with their complexity, peculiarities and heterogeneity. These features are related to the microenvironment, which surround them in order to replace it in vitro conditions in a

controlled way. All the techniques related to this field allow a very strictly control on biochemistry and biophysical environment of organoids and in general of cells as the organogenesis and morphogenesis. The development in the biomaterial fields have, therefore, brought innumerable advantages to control in a detailed way the time-space relation on the cellular microenvironment, giving the possibility to study the effects of the isolated environmental components and isolate quickly the models, which give rise to a synthetic culture for each type of organs. Integrating all the features and characteristics listed so far, it is possible to improve in vitro model getting better and better results over years.

1.5 Organoids in biomedical application

Considering the fact that a culture of organoids could come from a defined organism, exactly from ASCs, this can be applicable in a lot of biomedical applications to succeed in clinical trials. Nowadays, the results of in vitro toxicology tests are really unreliable in comparison with animal test. But not all the laboratories or research groups could exploit easily the animal model and furthermore a lot of ethical question arises in this modern world to avoid an over usage of animals. In any case, also the animal models express different reactions to the biomedical test because of the variability in specific species and the age of animals in question. Having said that, some important entities as EPA (Environmental Protection Agency), DARPA (Defense Advanced Research Projects Agency) and the NIH (National Institute of Health) focused on develop patient-specific cellular models to evaluate the efficiency of drugs and other chemical substances (Kleinstreuer, et al., 2014). The Defense Advanced Research Projects Agency stress out to create approaches for the synthesis of three dimensional in vitro model based on human native tissues in a bioengineered substrate. Therefore, it is possible to understand the importance of the development of an efficient protocol based on human tissue samples. Due to the fact that organoids' culture have characteristics to possess a three dimension architecture and it is possible to obtain different kinds of biological tissues starting from stem cells, they can be applied for several biomedical applications. In addition, it is possible to maintain them in a long period of time, for these reasons are considered a powerful tool not only for medical and clinical research but also in biological fields. And knowing that they can derive directly from Adult stem cells it is possible to design and

create therapies suitable for each patient's need. Following the main possible applications of organoids will be described in a detailed way (Lou & Leung, 2018). Thanks to organoids technology the goals has been reached to obtain semi-functional organoids that resemble to real organs, but not all the type of organs were created. The reason why this totality of creation has not been achieved, depends on the fact that there was a lack of strong differentiation process. Examples of these types of tissues are the ones which are involved in the incorporation of the ectoderm derived cell-types (skin eyes and so on) (Lou & Leung, 2018). As far as the internal organs are concerned, it was observed that the neurovascular network is composed mainly by autonomous neurons, vascular and smooth muscles cells and endothelial cells. So introducing a functional neurovascular network in organoid systems, it is possible to obtain a real and precise model for the diseases, precisely applicable for genetic diseases in which the neurovascular development is non-functional (Workman, et al., 2016). This is an another demonstration of the powerful characteristic, which possess the progenitors of organoids that give the possibility to create tissue in vitro condition just using the self-organization.

Drug screening :

The pathway which underlies the drug development is firstly very expensive because for a new drug the cost is almost 1,7 billion dollars and secondly it is as very long process, and it takes more or less fourteen years. A common problem related to the majority of pharmaceutical industry is the high desertion of drugs due to the lack of models which are able to evaluate safety and drug toxicity. Precisely, it is exactly by the lack of this toxicological model that it can happen that commercialized drugs are withdrawn from the market as they are responsible for adverse reactions in patients (Primohamed, et al., 2004). Some of the common adverse reactions in patients are: liver injury, cardiovascular toxicity and neurotoxicity.

For the reasons listed before, it is necessary to find a suitable and efficient model to predict the toxicity and efficacy of drugs. The potentiality of organoids' culture could be a good solution. Thanks to their tree dimensionality, the cellular heterogeneity and the simplicity in cultivation, will generate a similar human model, which can be more applicable in comparison to two dimensional' cultures. Organoids can be used as a screening to test the efficacy of pharmaceutical treatment. Furthermore injuries deriving from drugs toxicity could be direct (it means that there is an immediate reaction once in

contact with the drug) or indirect (it means that show a long term effect compromising more organs). As a confirmation of this, tests based on liver human culture were made. It is not a case the choice of liver culture, indeed liver is one of the first organs which is able to metabolize the injected drugs. But this test is not enough to predict the toxicity, because some drugs could show a kind of toxicity in vivo condition instead in vitro condition it is not toxic to hepatocytes. This is a further proof which confirm that it is no sufficient an in vitro test. (Godoy, et al., 2013) Knowing that the non-parenchymal cells of liver, as Kupffer cells and hepatic stellate cells, are responsible of the inflammatory reaction that may have in liver (due to a damage generate by a drug), can be applied to liver organoids culture. In such a way, the model composed by liver organoids, Kupffer cells and hepatic stellate cells will be more predictable respect to the previous model based only on the hepatocytes culture. Unfortunately, nowadays pharmaceutical industry still use two dimensional model because of the technical difficulties related to organoids culture. It is so necessary to shift the attention from a 2D system to a 3D one because thanks to this it is possible to optimize the preclinical studies.

As a future development for toxicological tests, the organoids' culture are one of the crucial point of this modern age because are representative of a real organ in which it is possible to perform accurate tests increasing the possibilities of never using animals for clinical test, which create countless ethical problems.

Organoids for help bioengineering approaches:

Combining the features of organoids with the capacity of bioreactors, it is possible to have a huge quantity of material, in comparison with others techniques, in order to obtain engineered tissues for biomedical application as for examples intestinal tissues (Kim, et al., 2007). In 2015, Ramachandran, et al. tested the self-organization on the adult liver cell populations inserting simultaneously in a bioreactor the different cells which compose a liver organ. (Ramachandran, et al., 2015)

In addition, using organoids in combination with organ-on-chip it is possible to predict the outcomes of the drugs' activity to speculate on it (Lou & Leung, 2018).

Organoids for diseases modeling:

Organoids are employed in the treatment of genetic diseases. The first successfully

therapy, based on organoids model, was developed in 2018 in Netherlands by Berkers et (Berkers, et al., 2019). In their study they withdrew a sample of cells coming from a patient which was affect by cystic fibrosis, a genetic disease caused by an alteration of the CFTR gene (transmembrane conductance regulator chloride channel). Using the protocol written by Dekkers et al (Dekkers, et al., 2013) in 2013 they were able to reproduce in vitro organoids characterize by F508del-CFTR mutation. In these organoids was tested a drug, which shows very promising results. An interesting application of the CRISPR in this case of study is the modification of the PDOs (primary patient derived organoids) in order to modify the CFTR mutation exploiting also a gene editing technique. Another interesting example of organoids in genetic diseases (neurodegenerative) concerns the Alzheimer diseases (AD). AD is one of the common neurodegenerative types of dementia. This neurodegenerative disease is characterized by a deposition misfolded amyloid A, which contains a sort of plaque and NFTs (neurofibrillary tangle) (Selkoe & Hardy, 2016). In 2016, a group of scientists was able to recreate in vitro brain organoids coming from a patient affected by Alzheimer disease. (Raja, et al., 2016) The use of these organoids for specific drugs, based on secretase inhibitors, strongly reduce the pathology. The organoid technology not only can be applied to genetic diseases but it could be applied on the case of infectious diseases. Moreover, these models offer a very suitable approach to treat and study the in interactions, which occur between the host and pathogens (bacteria, viruses and also protozoa). An example of a case of study is the application of brain organoids to Zika virus. (Qian, et al., 2016) The use of intestinal organoids, instead, was applied to the study the Middle East respiratory syndrome, known as MERS-CoV, which was the responsible of a lot of victims in 2012 and predecessor of COVID-19. (Zhou, et al., 2017)

Organoids in cancer therapy:

Organoids are also used in cancer therapy, creating models that allow to recreate in vitro the conditions present in patients affected by this disease. Until now, the principal approach to study cancer was based on immortalized cell lines derived from human patients affected by it, but these types of approaches are not enough to obtain a predictable model. Nowadays, in order to study the types of cancers, different types of organoids are employed. A group of scientists in 2017 derives from a patient a liver cancer organoids

in order to expand it in vitro and to obtain the 3 main common types of cancer related to liver (cholangiocarcinoma, hepatocellular carcinoma and the combination of hepatocellular and cholangiocarcinoma) (Broutier, et al., 2017). Sachs et al were able to reproduce perfect breast cancer organoids, which exactly behaves as a natural breast cancer. This breast organoids' culture was able to replicate the perfect morphology, all the mutational landscape, the hormones receptors and also the histopathology (Sachs, et al., 2018). This is a fundamental turning point in cancer research because, if it is possible to replicate an identical cancer model in comparison with the real one, it is possible to obtain good results in drug test in vitro.

Organoids coming from tumors, most of the time are able to maintain the heterogeneity present usually in tumors in comparison with a two dimensional approach, which is not able to maintain this kind of heterogeneity. Furthermore, organoid technology was applied to obtain a sort of Phylogenetic trees fundamental to reconstruct a wider view in cancer research (Corrò, et al., 2020).

Bio banking of organoids:

Thanks to the fact that organoids can be maintained in culture for a long period of time they can be used for the bio banking of a disease derived from organoids. The constitution of a furnished biobank will be an important resource for clinical application for precise medicine (Block, et al., 2021). A biobank was created by Valchongians et al with the aim of predicting drug response and clinical outcomes in patients affected by metastatic gastrophageal and colorectal cancers (Vlachogiannis, et al., 2018). Another case of study made up by Broutier et al found that the screening of patient derived organoids forms hepatocellular carcinoma helped in the identification of ERK signaling as a potential therapeutic target for liver cancer (Broutier, et al., 2017).

Organoids for regenerative medicine:

The high request of demands related to tissue transplantations has increased in the last years and to face these high requests, new technologies and new approaches must be applied. Most of the organs requested is for kidney, it was estimated that a percentage bigger than 80% of people, registered in organs transplantation's list, was only for kidney. This high rate of requests is followed by a 10% of people, which are waiting for a liver

transplant and a 5% of people for hearth (Lou & Leung, 2018). The high request is not only related by internal organs, but also for tissue repair (bones, muscles, skin and so on). The common way to have an organ replacement is the allogenic transplantations (means that organs come from other donors). A field not yet explored in transplantation, is to use the organoids, derived from the patients (Yui, et al., 2012). Another interesting characteristic to exploit organoids in transplantation application is the possibility to combine those with different scaffolds. The scaffolds could be synthetic or derived by biological decellularized (Schweinlin, et al., 2016). Also, in this case the combination of organoid technology with gene modification could lead to a potential therapy for degenerative diseases. It easy to understand the potentiality that organoids possess for the regenerative medicine. Here in this diagram, figure 5, all the concepts previously seen are listed and summarized (Hofer & Lutolf, 2021).

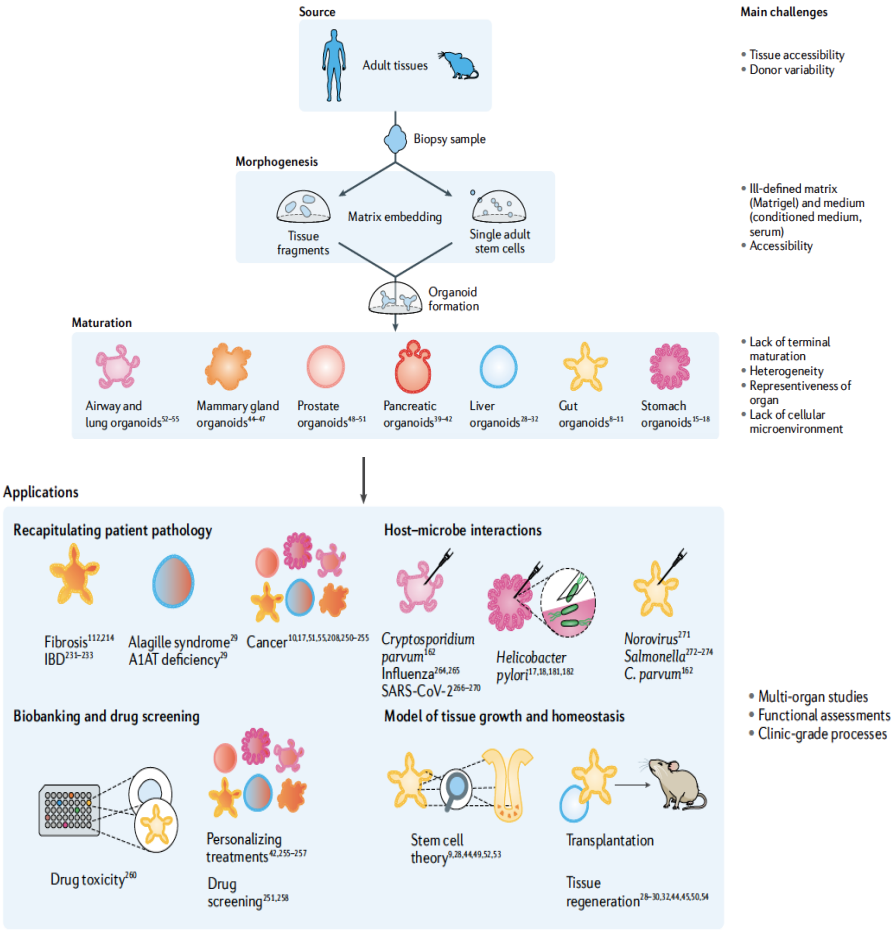


Figure 5. overview of types of organoids and their applications (Hofer & Lutolf, 2021)

2. SCAFFOLDS

In 2006 in a study performed by Mina Bessel et al (Nelson & Bissel, 2006) it was understood the strong influence which have extracellular matrix (ECM) to a culture of mammary cells. These cells are able to self-organize directly interacting with integrin, a glycoprotein in cell membrane that interacts with fibronectin in ECM .

2.1 Extracellular matrix (ECM)

The extracellular matrix is a peculiar three-dimensional structure which influence many characteristic features of cells, for example the morphology (cytoskeleton), the growth and the differentiation. The Extracellular matrix appear as a structure strong and stable under epithelia and it covers all the connective tissue. There is a continuous exchange and interactions between cells and their respective extracellular matrix, furthermore different extracellular matrix composed by different types of cells can interact (Saheli, et al., 2017). The exocytosis is the way thorough which the main components are introduced and then integrated in the extracellular matrix made up off resident cells. ECM is a complex structure generated by a mixture of fibrous protein connected of glycosaminooglycans (GAG), disposed in a sort of net. These GAG, which are carbohydrates, are involved in the formation of proteoglycans. Proteoglycans allow a continuous hydration of ECM and cells thanks to its capacity to attract positive ions, such Na^+, because of its negative charged. In addition, it is able to retain growth factors inside its structure. Four main types of proteoglycans have been identified in extracellular matrix environment.

1. Heparan sulfate (HS) → it becomes proteoglycans when two chains of it interact with ECM proteins. It is linear chain of polysaccharides. HS's main activity is the angiogenesis.
2. Chondroitin sulfate → Its main activity is related to the flexibility of cartilage.
3. Kertan sulfate → It is characterized by a not fixed content of sulphate.
4. Hayaluronic acid (HA)→ It is a non-proteoglycan polysaccharide (alternation of D-glucuronic with N-acetylglucosamine functional groups). HA is responsible for the turgor's cell because it is able to retain a high quantitative of water giving to cells the possibility to undergo to mechanical stress. Saying that, it is easy to understand that in cells HA is in a very abundant quantity (McKee, et al., 2019).

Not only carbohydrate such as glycosaminoglycans are involved in the creation of extracellular matrix, also proteins paly a fundamental role in this incredible structure. Collagenous proteins are, indeed, the main constituent of extracellular matrix (in example collagen is present in a percentage around 90% in bones tissues). The first characteristics attributed to these proteins is the one related to the structural conformation. During years, it was seen that the collagen fibres are also involved in cells attachment and cells differentiations (Bunyaratavej & Wang, 2001). The structural activity of collagen can be partially explained by the fact that the amino acids, which composed it, are disposed in a triple helix structure, called also collagenous domain. This domain consists in three well separated Alpha chains. These sequences are disposed in a left-handed helix. In addition, these helices are folded again together, and this will give the typical shape to collagen molecules. Furthermore, this triple helix is better stabilized by the formation of hydrogen bonds. The weak hydrogen bonds are the first bonds that will break when will be a chemical o thermal reaction in collagen molecules leading to an unfolded molecule (figure 6).

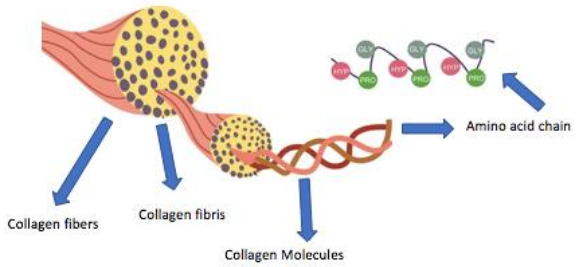


Figure 6. Schematic representation of collagen and its components

Scientists identified in nature different types of collagens in according with their structure (exactly were classified fourteen types). The first structure, which comprise the collagen type I, II, III, V and XI, is marked by their fibrillary structure. To the fibers structure belongs, indeed, collagen type IX, XII and XIV. Following it was possible to classify a Short Chain structure (collagen type VIII and X) and other types which are similar to this last structure (collagen type VI, VII, XIII). In the end, biologists identified a Basement membrane which is characterize by collagen type IV. The basement membrane is a thin layer belonging to ECM and it gives support to cells and tissues (figure 7).

Another interesting component of the extracellular matrix is the Elastin. Elastin, which is an insoluble protein, is fundamental in cell biology because it provides elastic proprieties to cells and tissues. Moreover, it is fundamental in blood vessels and skin to preserve their elasticity (Huleihel, et al., 2016).

The connection between cells and the extra cellular matrix are mediated by connection proteins as Fibronectin and Laminin. The first one is a glycoprotein and it is the responsible for the intra-connection between cells and the extracellular matrix. Thanks to this interaction, cells are free to move within ECM. The collagen fibers in ECM are connected to Fibronectin and this simultaneously bind Integrins (a glycoprotein). The mechanism behind integrin – ECM network relies on a precise site of recognition sequence. This sequence is a tripeptide RGD, which promotes the cell adhesion. The Laminin, instead, is able to generate a sort of network in the basal lamina able to cope with mechanical stresses and they participate to cell adhesion (Yan-Ru & Leung, 2018).

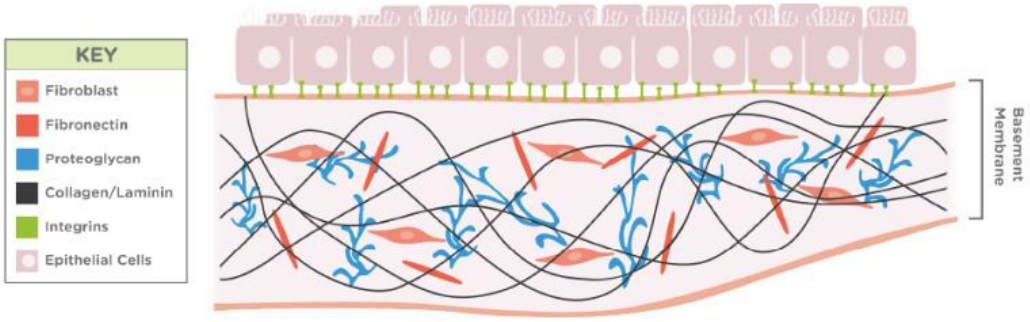


Figure 7.Schematic representation of Basement Membrane (Manchester, 2021)

2.2 Hydrogels

Among the most promising and interesting scaffolds for the cultivation of organoids in three dimensions there are hydrogels. Hydrogels are composed of hydrophilic polymers, which create a three dimensional network once they undergo to a change of phase, from gel to sol, after chemical or physical stimuli. The interesting features associated to this Hydrogels will characterize the future growth's cells. Two interesting peculiarities of these types of scaffolds are: the stiffness, which allow a suitable environment for the

growth and the permeability that allow the passage of oxygen and nutrients and the degradability.

Matrigel (or Cultrex®) is widely used in research for three dimensional cell culture, it is a basement membrane matrix which derived from Engelbreth-Holm-Swarm (EHS) mouse purified sarcoma (Aisenbrey & Murphy, 2020). This matrix, due to the fact that derives from a murine tumor, which generates an huge quantity of ECM proteins. Furthermore, it is very similar to a natural ECM. For these reasons good results were obtained in organoids' culture using this scaffold. The composition of Matrigel is given by a high quantitative of Laminin (~ 60%) followed by a high content in collagen type IV (~30%) accompanied with a small amount of Entactin (~8%) and a small amount of heparin sulfate proteoglycan perlecan (~ 2/3%). Matrigel is able to produce a gel structure when it is subjected to a temperature between 22-37 °C. During the process of a jellification, to form the hydrogel, the Entactin will be the cross linker between Laminin and Collagen IV.

Although Matrigel mimics in a perfect way the natural ECM providing a suitable environment or organoid's growth, it could generate some impediment during the culture. These impediments could be associated to some variabilities related to different batches. This variability generates such a big problem during the cultivation. The variability associated to different batches is because the natural origin of this hydrogel, and it is known that in nature the variability is predominant. This limitation can have strong negative effects during cultivation because of the deficiency in reproducibility. In addition, the complex structure, and the not known composition, makes this matrix very expensive and so it is not easy to reproduce by own in laboratory. Another deficit related to the use of Matrigel hydrogels is that it is not suitable to be tuned to obtains specific organoids' niche in order to achieve specific organoids culture.

For this reason,, in the last years, the research has tried to change its horizons by focusing mainly on the creation of synthetic hydrogels. Therefore, working with synthetic Hydrogels, it is possible to obtain perfectly reproducible cell cultures or organoid cultures avoiding variabilities, as could be for natural Hydrogels (Kaur, et al., 2021).

2.3 Natural based Hydrogels

Firstly, natural scaffolds were evaluated as alternative to matrigel. Natural alternatives to matrigel show an analogy with in vivo ECM as the chemical composition and the structural morphology. For these reasons, the natural hydrogel gives very good results in cultures methods. Usually, these natural alternatives are composed mainly of polysaccharides, different proteins and animal derived components. The polysaccharides possess a fast gelation, generating so, a three-dimensional architecture suitable for cell cultivations with different shapes. The common polysaccharides involved are: Hyaluronic acid, agarose, chitosan, pectin, alginate, cellulose, gelatine, heparin and dextran. Considering, instead, the proteins involved in these matrices are collagen, fibrin, poly-l-lysine and silk fibroin. All the proteins listed before mediate the interactions between cells and the physiological microenvironment (Kaur, et al., 2021). Some scaffolds made up by components as Hyaluronic acid, heparan collagen, laminin or directly from a whole decellularized ECM matrices, are involved in biocompatibility and activity of specific actions, which are crucial for the life maintenance. An interesting characteristics attributes to natural scaffold is the biodegradability. The concept of biodegradability is not to be underestimates as, once organoids are implanted in vivo. Indeed, it is important that the matrix degrades without leaving toxic residues.

But also in this case, natural scaffolds possess several limitations related to mechanical properties and this result in deficits in the stability of the scaffold. This problem can be solved using a chemical crosslinker, in such way this leads to “immobilize” the scaffolds. But the use of this chemicals could cause toxicity in organoids. Natural Hydrogels, also called smart hydrogels, are based on peptide or protein technology undergoing to a conversion from sol to gel just tuning temperature or pH (so due to external stimuli). One disadvantage related to these types of scaffolds is that they can be affected by poor mechanical properties. (Katyal, et al., 2020)

In recent years, exactly in 2019 a study conducted by G. Giobbe et al (Giobbe, et al., 2019) describes the potentiality of an extracellular matrix, derived from decellularized tissues, used as a three-dimensional scaffolds suitable for organoids culture. Also, in this case the high variability found in nature will lead to a heterogeneous result.

2.4 Synthetic based Hydrogels

Due to the high variability showed in natural based hydrogels, scientists feel to focus their attention on creating chemically defined hydrogel scaffolds. For this reason, were employed synthetic polymers as PEG (polyethylene glycol), PLGA (poly lactic-co-glycolic acid), PLLA (poly-L-Lactide) and other polymers belonging to this group. The most common used PEG hydrogels (biocompatible and biodegradable) are suitable for the growth of organoids or cells through an encapsulation of it in the scaffold. In 2019, a group of scientists, directed by Funfak, developed synthetic scaffold based on soft PEG, which allow the formation of cyst organoids. PEG based scaffold is characterize by an elastic module that is more or less 1kPa. The results obtained, with this synthetic matrix in this study, were compared to the ones obtained with Matrigel. They understand, comparing the two different scaffolds, that to obtain a cystic morphology it is needed to be considered the parameters related to biophysics and biochemistry of the matrices (Gjorevski, et al., 2016).

The interesting features, related to mechanical characteristics of these synthetic scaffolds, have mean that could be interesting tools for biomedical applications. The paying attentions to the biocompatibility and the toxic effects that could generate in vivo conditions, it must not be underestimated. A way to improve the bioactivity and the biocompatibility of these types of scaffolds is its functionalization with peculiar functional groups. In that way, it will get scaffolds which increase the cell adhesion, proliferation and the growth just modifying its chemical surface features. Some of the modifications in their chemical structure could be the use of oxyl, hydroxyl, carboxyl or aromatic groups, in such way it is possible to play with the hydrophilicity or hydrophobic properties. Another technique to improve the efficiency of the synthetic scaffold is modifying the structure with signalling proteins, in a controlled way (Marusina, et al., 2019). The most used proteins for this purpose are: Collagen type IV, fibronectin, Laminin, Hyaluronic acid and RGD (Hernandez-Gordillo, et al., 2020).

In a general view, engineering synthetic based hydrogels lead to modify the morphology and the biochemical features for possible future in vivo applications.

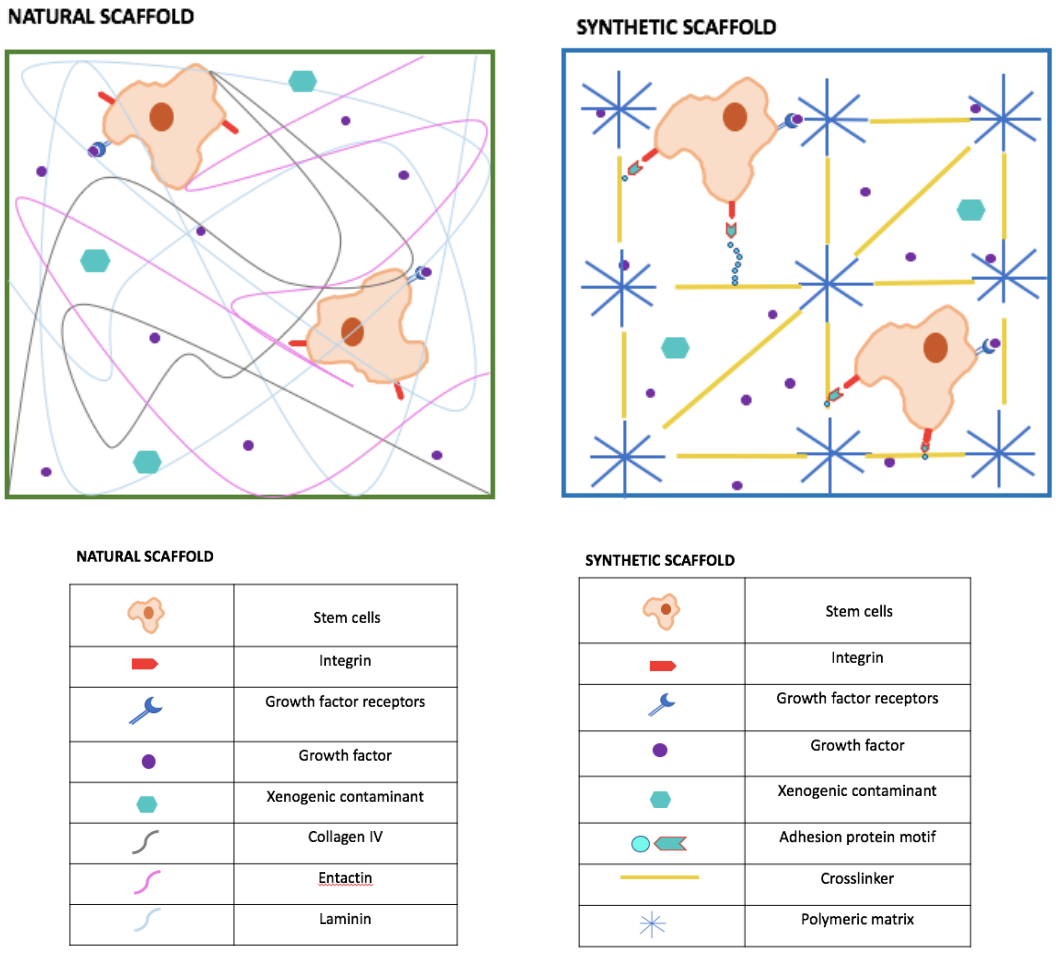


Figure 8. Comparison schemes of natural scaffold with synthetic scaffold

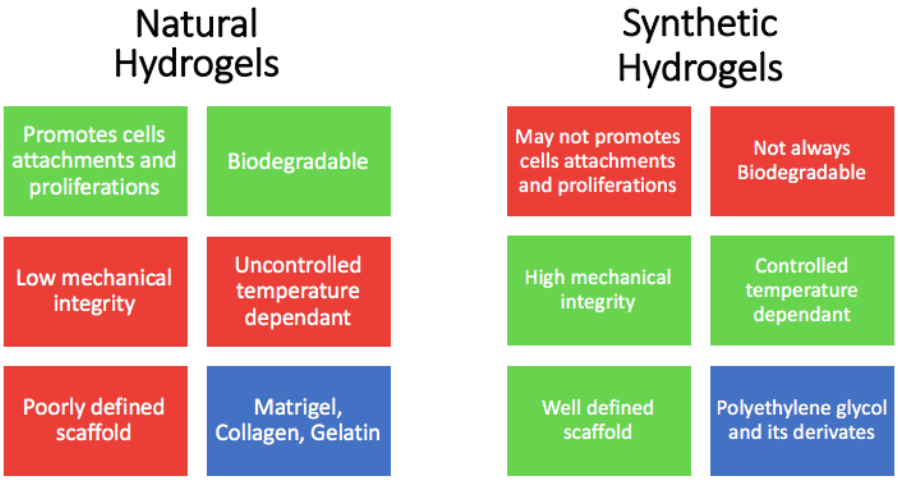


Figure 9. Advantages (green) Disadvantages (red) and characteristics (blue) of synthetic and natural scaffolds

2.5 Hybrid based Hydrogels

The synthetic hydrogels seen so far are not enough to satisfy all the requirements needed for in vitro culture. In addition, natural hydrogels show deficits in mechanical features.
A solution to address these issues involves the use of peculiar hydrogels: the hybrid hydrogels. With this kind of hydrogel, it is possible to achieve features which are in common of the aforementioned scaffolds in a single scaffold. The mechanism behind these hybrid scaffolds is based on the co-polymerization and chemical modification of their components. Many combinations of hybrid scaffolds can be generated as a natural one with another natural scaffold, or natural with synthetic or synthetic with synthetic.
Speaking about the generation of hybrid hydrogels coming from two natural scaffolds: they are formed through a mixing of polymer taking as example the native extracellular matrix (Sang , et al., 2018). The hybrid hydrogel deriving from two synthetic scaffolds, indeed, are generated by the simultaneous polymerization of 2 or more polymers in order to tune the biodegradability and their mechanical features. Also, in the case of hybrid hydrogels the main goal is to achieve a scaffold which mimic as more as possible the natural ECM, employing a huge number of polymers and crosslinking procedures.
Alginate combined with synthetic polymers will lead to enhance the mechanical features (Kaur, et al., 2021).
A case of studied of Klotz et al foresaw the use of PEG in combination with gelatine (functionalized with lysine proteins) to obtain a three-dimensional scaffold for liver tissue engineering. The results obtained are promising, in fact the efficacy was improved in comparison to matrigel scaffold also enhancing the growth and differentiation of organoids (Klotz, et al., s.d.).
The possibility of combine different types of scaffolds to get a hybrid one is an attractive choose to enhance the similarity between organoids and real organs and furthermore to substitute animal derived hydrogels.

2.6 Non-Hydrogels scaffolds:

Other types of scaffolds were investigated so far to promote the cell growth and differentiations in vitro conditions: Non-hydrogel scaffolds. These new types of scaffolds are made up by porous microspheres. The network, which is formed by the

interconnections of these porous microspheres is very complicated and extended, in such a way it creates a very high specific surface area. The width of this surface area allows the proliferation of cells, which are able to generate the Extracellular matrix, furthermore it promotes the cell attachment (Cassereau , et al., 2015). Some examples of polymers, used to obtain a high homogeneous porosity, are PLLA, PCL and PLGA. In addition, the scaffold obtained with these polymers are biodegradable, which is fundamental for biomedical applications. The high porosity, that is created by the porous microsphere, is responsible for the transport of nutrient (very efficient), of oxygen and also waste product made up by cells. Non-hydrogel scaffolds provide, so, an high dominance in the physical and spatial features in comparison with the other scaffolds (Pelin, et al., 2019).

Many cases of study are present in literature in which groups of study tested the growth of organoids with porous PPMS polymers and PLGAMS biocompatible and biodegradable microspheres (generated from Poly lactic-co glycolic acid PLGA (Lih, et al., 2016). Liu et al in 2014 developed a scaffold which derive by the combination of hydrogels and porous microspheres. This combination is called “Macroporous cryogel”. Thanks to these scaffolds it is possible to achieve a high surface area and also an optimal microenvironment suitable for organoid growth. This microenvironment will generate “micro-niches” with the possibility to tune their chemical and physical features, promoting, in such a way, the proliferations and viability of organoids (Liu, et al., 2014). A recent study, in 2020, saw the creation of a three-dimensional structure deriving from the interaction of the self-assemble between cell-laden gelatine microcryogels. The interaction that occurs between cells and between cells and their extra cellular matrix leads to the generation of the quite real microenvironment to promote the viability of cells. The cells and organoids which grown in these scaffolds have demonstrated an increment in maintenance and a reduction in senescence. In in vivo applications, indeed, the results obtained were very satisfying because this type of system induce the reparation of cartilage tissues without the implementation of extrinsic factors. The future applications of this interesting scaffold are very promising for biomedical fields (Xing, et al., 2020).

3. PURPOSE OF THE THESIS

This thesis will make this judgement looking at a new scaffold, which allow the liver organoids growth in order to substitute the use in research application of animal derived extracellular matrix. The need to find a new synthetic scaffold lies in the fact that Cultrex® or other animal-derived scaffolds, can cause several problems in the continued reproducibility of in vitro experiments.

Therefore, natural derived scaffolds can be responsible for numerous problems due to the heterogeneity typical of in vivo conditions. Some of these limitations are, for instance, a difficult control in the jellification process and the lack in reproducibility in vitro conditions. In addition, ethical problems emerge for the use of animal derivation scaffolds.

Firstly, preliminary bibliographic research was necessary to try to understand which scaffold could be suitable for possible biomedical applications. Four different types of scaffolds, each of them composed by different sub-type, were tested in comparison with Cultrex®. The efficiency of Cultrex® has been extensively tested in many different studies, for this reason it was chosen as positive control.

The procedure to do the so called maintenance, for liver organoids, was taken from the protocol written and tested by (Broutier, et al., 2016). All the scaffolds were analyzed with an optical microscope, in a qualitative way. In addition, to verify the viability of liver organoids in the different scaffolds, a quantitative analysis of Resazurin presto blue assay was performed. Moreover, inclusions in paraffin were made in order to test the hematoxylin and eosin staining to better understand the peculiarities of the liver organoids morphology.

Finally, several tests were taken. In addition, only the scaffolds which give more promising result were selected, to better evaluate if they could replace the Cultrex® used so far.

4. MATERIALS AND METHODS

Here in this section there will be an explanation of the protocols used during the period of experimental section of this thesis. All the experiments were performed at the Scientific Campus of Ca'Foscari University Venice, at the Professor Flavio Rizzolio's research laboratory, at the Gamma building.

Consumables and standard laboratory devices used on experiments are the following Materials needed:

- Falcon® 15 mL tubes
- Falcon ® 50 mL tubes
- Flat bottom 24 multiwell (IWAKI microplate code 3820-024)
- 96 Multiwell Falcon®
- Fumhood (LABOGENE model: Mars)
- Centrifuge (Thermo scientific: Multifuge X1R)
- Micropipets (2-20μL ; 20-200μL ; 100-1000 μL → RAININ Pipet-Lote XLS)
- Incubator (PHCBI) 37°C, 5% CO_2
- Syringe 500 mL
- Filter 0.22 μm (Merck Millipore Vented Millex)
- Eppendorf 2 ml
- Cryovials 2 ml
- Heatbath VWR
- Analytic balance Eternity Gibertini
- Sonicator Elmasonic S 10H (Elma)
- Vortex ZX3 advanced Vortex Mixer (VELP scientifica)
- Litmus paper

4.1 Mouse's liver organoids for three-dimensional culture maintenance

Mouse's liver organoids culture protocol is inspired by the work of Broutier et al. published in 2016 (Broutier, et al., 2016) and it was optimized based on the laboratory test.

Solutions needed:

- HBSS 1X→ Hank's Balanced salt solution $CaCl_2$ $MgCl_2$ (Gibco Ref 14175-053 ; Lot 22423399)
- Cultrex Stem cell Qualified RGF BME → (Lot 1545531 , Catalog # 3434-005-02, 5 ml, store at ≤ -70 °C for research use only 3043435.00 , Origin : USA Minneapolis, MN 554138003437475. R&D SYSTEMS)
- FBS → Fetal Bovine Serum (Sigma Aldrich)
- DMSO → Dimethyl Sulfoxide (Sigma Aldrich)
- Washing medium for organoids : Dulbecco's modified Eagle's → medium/nutrient mixture F-12 Ham with 15 mM HEPES and Sodium bicarbonate, without L-glutamine and phenol red, liquid sterile-filtered + 10% FBS (Fetal bovine serum) +1% P/S (Peniicillin-Streptomycin) . Lot# RNBG9027
- Trypsin 0.05 % (Euroclone)
- Ovarian Mouse culture medium
- PBS: Dulbecco's Phosphate Buffered Saline →modified without calcium chloride and magnesium chloride, liquid sterile filtered. Lot# RNBK0697
- FBS Sigma Aldrich
- DMSO → Dimethyl Sulfoxide

Defrosting procedure:

Mouse's liver organoids were taken out from liquid nitrogen (T = -195,8 °C) and quickly thaw in a 37°C water bath. As soon as the organoids melt, organoids are transferred in a 15 mL tube filled with 5-7 mL of washing medium. The latter is centrifuged at 1500 rpm for five minutes, then the supernatant is discarded and the organoid's pellet is gently suspended, in order to avoid bubbles formation, with the proper amount of matrix. Hence, the organoids are plated in a 24-well plate distributed in 10 μL drops. The plate is then inserted in the incubator upside down for approximately half an hour and finally, 500 μL of filtered culture medium was added to each single well and left in the incubator overnight (37°C 5%CO_2) . The culture media is replaced every 24 hours.

Maintenance procedure:

Before starting the procedure of the maintenance it is important to take out from freezer (-20 °C) the Cultrex® and store it on ice until it is melted. Subsequently, in a 15 mL Falcon® tube a 5-7 ml of washing medium is inserted. The culture medium is removed from the wells using the vacuum pump, then 1 mL of HBSS is added in order to eliminate the cell's debris and Ca^{2+} and Mg^{2+} ions which can inhibit trypsin. Hence, HBSS is removed, 200 µL of trypsin was added and the matrix shattered using pipette tips. Therefore, these are incubated for five minutes in the incubator and then the trypsin is inactivated by transferring the organoids in a 15 mL tube containing washing medium. All this passages must be performed quickly to avoid the death of organoids. Hence, the 15 mL tube is stored on ice for fifteen minutes to completely dissolve the matrix . Subsequently it is centrifuged, the supernatant discarded and the organoids gently suspended. . If the organoids' pellet is full of debris, coming from the dead organoids, it is suggested to add again a 3-4 mL of washing medium and centrifuge it again. Therefore, the organoids are suspended with the matrix and distributed in 10 µL drops on a 24-well plate and then incubated for thirty minutes. Finally, 500 µL of culture media is added and the organoids are stored again in the incubator (figure 10).

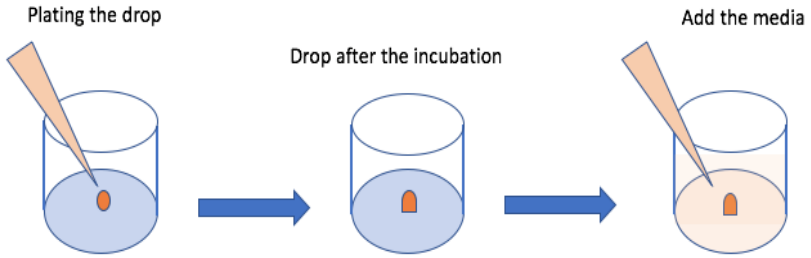


Figure 10. Plating procedure

Procedure for freezing:

After the obtaining of organoids pellet, a freezing solution is made: firstly 225μL of culture media, secondly 225 μL of FBS (mixing it to ensure an homogeneous diffusion) and then, 50 μL of DMSO are added. The solution is mixed to promote the complete homogenization and then it is transferred in a properly labelled cryovial. The latter is firstly put in a freezing container and it is transferred to a -80°C fridge. After one week it is transferred in liquid nitrogen.

4.2 Matrices tested

The tested scaffolds are Manchester Biogel Peptigels®, Biogelx™ Hydrogels and HyStem™. Their characteristics will be listed and described below with their respective procedures and protocols:

4.2.1 Cultrex Stem cell Qualified RGF BME:

The Cultrex® SC matrix is used as a positive control due to the high efficiency and the wide usage in literature. All the steps to manage this type of scaffold are previously described in 4.1. Paragraph. Cultrex® SC matrix is produced by R&D Systems which is a biological product company based in Minnesota. It is made up of a succession of small peculiar protein's sheets which are involved in the layer among tissues and their nearest stroma. These types of scaffolds are widely used for the creation of three dimensional scaffolds made up of stem cells. The origin of Cultrex® SC comes from Engelbreth-Holm-Swarm (EHS) tumor. This extract is then purified and its main components are ~ 60% laminin, ~30 collagen type IV, ~8 Entactin and ~ 2/3 % heparin sulfate proteoglycan perlcan. Cultrex® SC shows a very quick gelation in fact after 30 minutes it polymerizes at 37°C. It maintains its integrity and stability until about 10 days and, of course, it is sterile. The data sheet of this product is visible on their website (R&D, 2021).

4.2.2 Biogelx™ Hydrogels for 3D cell culture:

The first scaffold tested is the Biogelx™ Hydrogels. The project of these hydrogels was conceived in 2013 at the University of Starthclyde in Glasgow and the team developed these scaffolds suitable for 3D cell culture entirely composed of synthetic peptide which mimics an environment typical of extracellular matrix to promote the proliferation of cells in three dimensions. Since the composition of these scaffolds is exactly defined and

doesn’t show any kind of natural variability, it could be used to obtain cells culture which are not influenced by the matrix as it often happens, for instance, in a natural-derived scaffold.

The science beyond the Biogelx™ rely on very easy self-assembling peptides: the first peptide, Fmoc-diphenylalanine, acts as an hydrophobic substances which promotes the gelation in presence of Fmoc-serine which is considered, in some respect, as a hydrophilic surfactant. They both are able to self-assembling to form a two peptides system in an aqueous environment promoting the formation of nano-fibers thanks to the presence of Ca^{2+} leading to the formation of this 3D network composed by a percentage bigger than 95% of water mimicking, in this way, the natural ECM and consequently they are suitable for the cell culturing (figure 11).

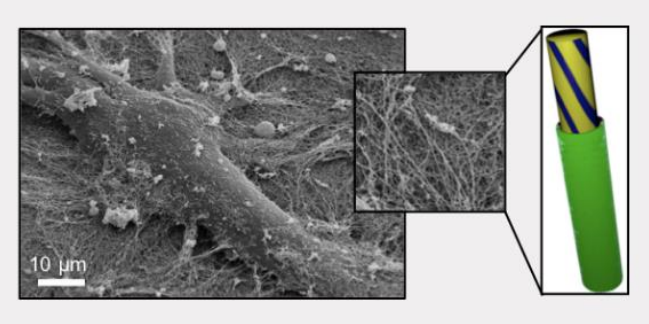


Figure 11. SEM image of core-shell synthetic peptide (Alakpa, et al., 2016)

Moreover, the hydrophilic component can be easily functionalized with different peptide sequences in the extracellular matrix as Fibronectin, Laminin and Collagen to increase the cell adhesion to this artificial matrix.

So in the end there will be a formation of a nano-fibrous network similar to ECM with the advantage to tune chemical and mechanical properties as the stiffness of the hydrogel. (Alakpa, et al., 2016)

The Biogelx™ screening kit was purchased by the official site of the company (Biogelx™, 2021). This kit is composed of four different types of hydrogel of 50 mg of contents:

- Biogelx™ S → Standard Hydrogel
- Biogelx™ RGD → Fibronectin functionalized Hydrogel
- Biogelx™ GFOGER→ Collagen functionalized Hydrogel

- Biogelx™ IKVAV→ Laminin functionalized Hydrogel

All these powders must be storage at -20°C for about one year.

Figure 12. Vials of Biogelx™ (S; GFOGER; IKVAV; RGD)

	Biogelx™ S	**Biogelx™ RGD**	**Biogelx™ GFOGER**	**Biogelx™ IKVAV**
Catalog number	A0W-0000	A	A5W-000	A2W-
Batch	FFS052RM	FFS	FFSGFOG	FFS
Lot	#125	#115	#113	#111

Table1. Features of Biogelx™ (S; GFOGER; IKVAV; RGD)

4.2.2.1 Procedure for the creation of the Hydrogels for 3D cell culture:

The formation of the four types Biogelx™ hydrogel is the same for each of them and it will be explained below. The only difference between S, RGD, GFOGER and IKVAV is given by the quantitative weighed to allow the formation of the gels.

Extra materials needed: Ultra-pure milli-Q water

The first step consists in taking out from the fridge the glass vials allowing them to reach room temperature. Once the temperature is stabilized, the vials can be opened and under sterile conditions weighing the desire quantitative of powder according to the following tables :

Biogelx™ S :

Stiffness range (kPa)	Milligrams of Biogelx™ S (mg) for 1 mL volume
Light : 0.5-1.5	4.4 mg
Medium : 2.0-4.5	8.8 mg
High : 6.0-10.0	13.2 mg

Biogelx™ RGD:

Stiffness range (kPa)	Milligrams of Biogelx™ RGD (mg) for 1 mL volume
Light : 0.5-2.5	5.0 mg
Medium : 5.5-8.0	9.9 mg
High : 9-15.0	14.9 mg

Biogelx™ GFOGER

Stiffness range (kPa)	Milligrams of Biogelx™ GFOGER (mg) for 1 mL volume
Light : 0.5-2.5	5.8 mg
Medium : 4.5-7.0	11.6 mg
High : 7.5-10.5	17.4 mg

Biogelx™ IKVAV:

Stiffness range (kPa)	Milligrams of Biogelx™ S (mg) for 1 mL volume
Light : 0.8-1.1	4.9 mg
Medium : 3.0-4.0	9.8 mg
High : 8.0-9.2	14.7 mg

Table2. Stiffness range of Biogelx™ (S; GFOGER; IKVAV; RGD)

Once the amount of requested powder is weighed, under sterile condition it is important to keep the powder in the bottom of 2 mL Eppendorf to avoid any kind of powder's loss. At this point, it is added the suitable quantitative of ultrapure milli-Q water to obtain the

Pregel solutions. In order to be sure of the total dissolution of the pregel, it is recommended to vortex for at least 30 seconds and if some air bubbles are still there sonication is suggested for 30 seconds (figure 13).

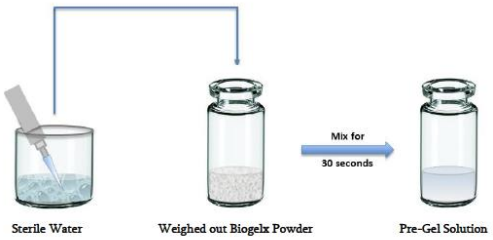


Figure 13. Biogelx™: Preparation of Pre-Gel Solution (Biogelx™, 2021)

To eliminate any type of bacterial or fungal contamination, it is possible to leave the Pregel solution for about 30- 40 minutes under UV-light. Following these steps to obtain the 3D oganoids' culture it's necessary to proceed by putting in the incubator separately the Ovarian mouse growth media and the desired volume of pregel solution (requested for the organoids culture) until they reach the 37°C. The organoids which come from the maintenance are trypsinized following the standard protocol applied for Cultrex®. So after centrifugation (5 min 1500 rpm) and discarding the supernatant, the whole pellet is suspended in a quantitative of washing medium such as to obtain 1 Eppendorf for each condition with the same number of organoids inside. Then the 4 Eppendorfs obtained are centrifuged again (5 min 1500 rpm) and brought dry from the supernatant (it is allowed to leave ~ 10% of washing medium). At this point is added a quantitative of pregel at 37°C, previously removed from the incubator, such that to obtain a 100 μL of hydrogel for each condition plated in a 96 multiwell. The Eppendorfs with the pellet are filled with the pregel solution and, with the help of a micropipettes, they are carefully mixed to avoid any bubbles formations and to allow even distribution of organoids. As a result, a homogeneous mixture with organoids was obtained. The solution is plated into the bottom of a 96 multiwell with a drop volume of 100 μL and the plate is left in the incubator (37 °C, 5% CO_2) for 30 minutes. After the time required for the process has passed , the culture medium (at 37°C previously warmed in the incubator) is added (150 μL) gently dropwise in the center of the wells, making sure that the tip of the micropipette doesn't touch the gel. Visibly it is possible to distinguish two separate layers due to the presence

of Hydrogels and the growth medium. After 2 hours the media is replaced with a fresh one, and leave the plate in the incubator overnight. Several tests were performed to verify the effectiveness of these Hydrogels. In chapter "Results and discussion" are reported all the details.

4.2.3 Manchester BIOGEL Peptigels® for 3D cell culture:

Peptigels® are created by the company Manchester Biogel. This company was established in 2002 in the United kingdom, and it is ranked among the bests ones for the creation of Hydrogel suitable for the three dimensional bio printing. Recently they develop a mechanically and chemically well-defined Hydrogel to promote the creation of 3D culture of cells. Their Hydrogels tries to mimic the microenvironment that is created around cells. Peptigels ® are fully synthetic scaffolds composed mainly of synthetic peptides so they are animal friendly and free of any kind of contamination. Furthermore, due to the fact that they are synthetic and chemically and mechanically defined, it is impossible to find variability between batches, as it can happen for natural matrices. The gels made by this company are biocompatible and biodegradable so this product is also considered environmental friendly. Thanks to all these features Peptigels® are suitable for clinical researches and for the creation of a personal model for the patient to create target therapies. Since they do not polymerize at room temperature they are very easy to handle.

In this thesis the starter pack of this company is tested. The latter is composed of five different type of gels, each of these five formulations consists of 2 mL of product. The five types present in this kit are : Alpha1, Alpha2, Alpha 2-RGD, Alpha4 and Gamma 2.These Gels usually are purchased with their respective Peptisols ® which are specific solutions used to tune the stiffness of the gels. Unfortunately due to a problem of shipment these solutions did not arrive and therefore were not tested different stiffness of these gels.

Furthermore, the company did not include the chemical peculiarities of these scaffolds in their website and at the moment of the purchase.

	PeptiGel® Alpha 1	PeptiGel® Alpha 2	PeptiGel® Alpha 2-RGD	PeptiGel® Alpha 4	PeptiGel® Gamma 2
Vial number	210209B15	191014B5	200914B11	191029C16	191203C5

Table3. Features of Manchester biogel Peptigels® Vials (Alpha1, Alpha2, Alpha 2RGD, Gamma 2 and Alpha4)

Figure 14. Manchester biogel Peptigels® Vials (Alpha1, Alpha2, Alpha 2RGD, Gamma 2 and Alpha4)

4.2.3.1 Procedure for the creation of the Peptigels ® for 3D cell culture:

In this section, it will be explained how to obtain a peptigels using the starter pack with no other extra materials. The protocol in question has been modified from the original one, to make it more adaptable to Flavio Rizzolio's laboratory's research needs.
In addition, the quantities of material required were also modified according to the needs of the experiments. All the entire protocol is described on their website (Manchester, 2021). Before starting with the entire procedure, it is important to verify if there are some air bubbles inside the vials. In this case it is suggested to centrifuge the vials for 1 minute at 3000 rpm. Having done that, it is required to wait until the vials reach room temperature in order to get a greater ease to handle. This protocol was written to have a 60 μL volume of Peptigels ®: five different Eppendorf has been filled with 60 μL of the PeptiGels ® for each conditions. Starting from the maintenance (see paragraph 4.1.), 5 Eppendorfs can be obtained with the respective pellet. The pellet in the Eppendorf has been suspended in 12 μL of Growth medium a gently mixed to obtain an homogeneous solution. This cells

suspension is inserted in the Eppenderf previously prepared with the Peptigels® inside and mixed carefully to avoid any kind of bubbles formation. A trick to avoid a bubble formation is to mix carefully without taking out the tips of the micropipette from the solution. Here, at this point, the protocol was a little modified in order to have a single drop inside the 96 multiwell plate. Therefore a drop of 30 μL was plated in the center of the well (the original protocol requires the insert in which spread the gels). All the passages previously described must be performed in a short range of time to avoid any kind of polymerization. At this point the multiwall plate is inserted upside-down in the incubator for 5 minutes, and after the growth medium was added for each well with a volume of 150 μL . Furthermore the media is changed 2-3 times in the span of one hour to obtain a better diffusion of nutrients inside the drop and then, it was left in the incubator overnight. Culture medium must be change every day in the day that follows. All the steps are visible in the picture figure 15 below:

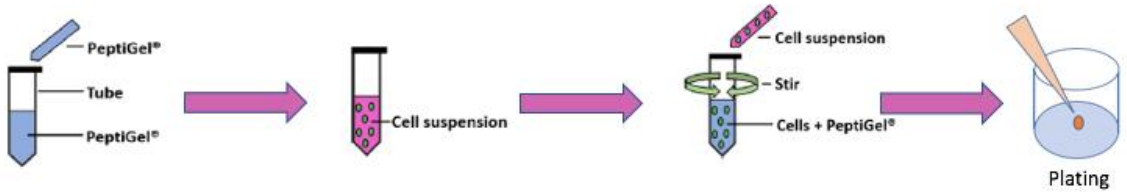


Figure 15. Peptigels® preparation's steps

4.2.4 Hystem™ and Hystem-C™ for 3D cell culture:

The house of production of Hystem ™ and Hystem-C is the Advanced Biomatrix company. This company propose solutions which are environmental friendly, biodegradable and biocompatible hence suitable for biomedical applications. The hydrogels which they propose try to mimic the environment usually present in Extrcellular matrix in cells, so it results suitable for three dimensional culture. This type of scaffold is fully synthetic and xeno-free. The extralink solution, which is composed of polyethylene glycol dyacrilate, is created by matching up acrylate groups with the extremity of PEG polymer (polyethylene glycol). PEG, of course, is completely inorganic and animal friendly. The components of Hyaluronic acid, to create the Hystem™, stem

from bacterial fermentation. The bacteria in question is the *Bacillus Subtilis*, so also in this case we deal with an animal friendly chemicals.

The Hyaluronic acid (HA), which represent the major components of the Extracellular matrix, in this scaffold represent the majority of the components. Chemically speaking, it is classified as the simplest glycosaminoglycan which is not sulfated. The hyaluronic acid is composed of a polysaccharide chain forming a non-branched linear structure. The peculiarity of these substances is to have a lot of hydrogen bonds which are connected with glycosidic bonds . The aforementioned bonds stabilize the conformation of the structure. It's interesting to stress out that, at physiologic pH (7.34—7.45), the carboxyl groups are ionized (becomes Hyuloronan acid) generating in this way an high polarity of the molecule. Consequently this means a very high solubility in the presence of water. This is the reason why it's is one of the major component of extracellular matrix, which is composed mainly of water. Thanks to all these features, the HA play a fundamental role in compression strength. Then, the HA lubricates and forms the main scaffold hydrating the Extra cellular matrix. The cell adhesion, the motility and the cell proliferation are some of the other functions of this incredible molecule. Hystem-C ™ is slightly different from Hystem ™ because of the presence of Gelin-s ™ which is a thiol-modified denaturized collagen. Having said that, the Hystem™ and Hystem-C ™ hydrogels are created with the view of recreating the Extracellular matrix's environment. The chemistry below these hydrogels relies on the chemical reactions between thiol-modified, hyaluronic acid, in presence of a crosslinker that is a thiol reactive for Hystem™. Concerning Hystem-C ™, the chemical reaction which occurs are between thiol-modified, thiol reactive cross linker and with Gelin-s™ . This chemical reaction will produce an hydrogel that easily gelify obtaining in this manner a suitable scaffold for cellular culture which is biocompatible bioresorbable and with the possibility to tune precisely the rigidity and viscosity of the hydrogel. In figure16 are shown, in a schematic way the three main components of this scaffold:

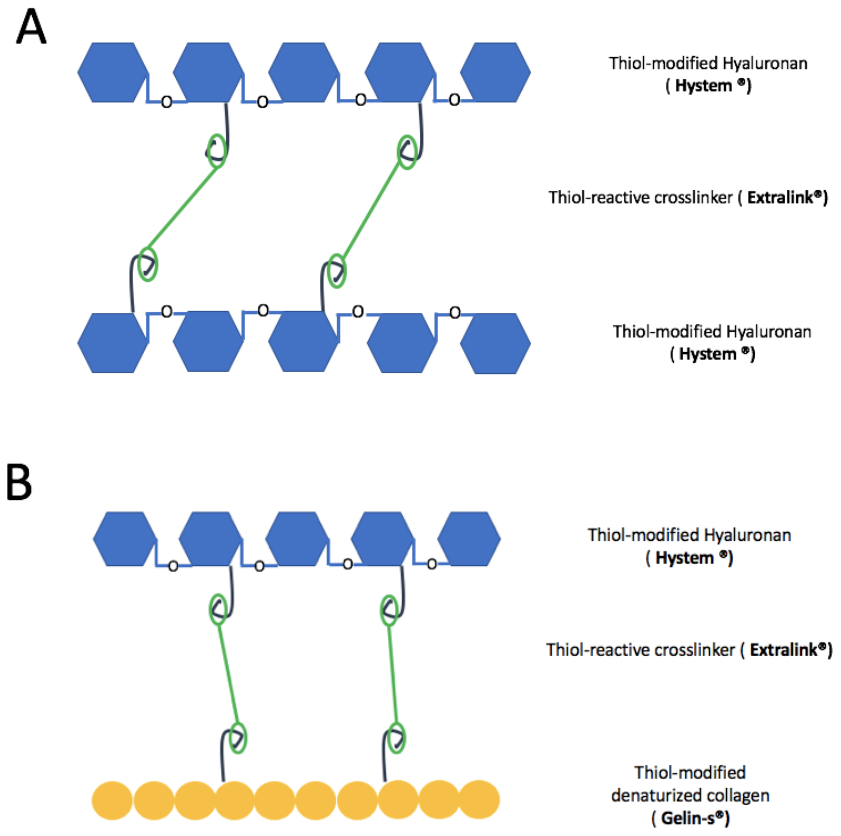


Figure 16. A: Schematic representation of Hystem™. B: Schematic representation of Hystem-C™

The product is presented in sterile vials pre-measured:

- Hystem™

Catalog number: HYS010

- 2 vials of 1mL volume: each is composed by 10 mg of Hystem and 9.6 mg of phosphate buffered saline (PBS)
- 1 vials of Extralink of 0.5 mL volume, composed by: 5 mg of extralink and 4.8 mg of phosphate buffered saline (PBS)
- 1 vials of degassed water of 10 mL volume, composed by: deionized water with 9.6 of PBS.

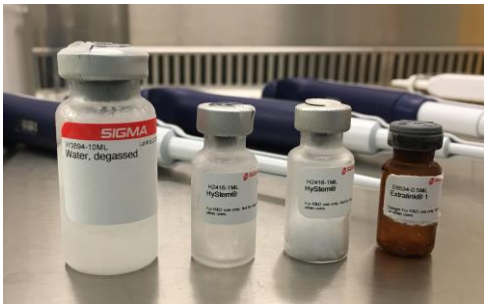


Figure 17. Hystem™ vials

- Hystem-C™

Catalog number: HYSC010

- 1 vials of 1mL volume: it is composed by 10 mg of Hystem and 9.6 mg of phosphate buffered saline (PBS)
- 1 vials of 1mL volume: it is composed by 10 mg of Gelin-S and 9.6 mg of PBS salt
- 1 vials of Extralink of 0.5 mL volume, composed by: 5 mg of extralink and 4.8 mg of phosphate buffered saline (PBS)
- 1 vials of degassed water of 10 mL volume, composed by: deionized water with 9.6 of PBS.

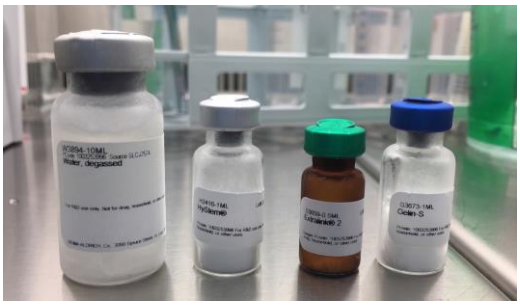


Figure 18. Hystem-C™ vials

Once the vials are mixed together (for both Hystem™ and Hystem-C ™) with the deionized water, they immediately start to form a transparent hydrogel at room temperature without the necessity to modify the pH. By Varying the amount of crosslinker, it's possible to tune the gelification and the rigidity of the hydrogel. The gelification depends also on the pH of the solutions mixed with the cells. This means that the higher is the pH the faster will be the gelification. The type of the used growth medium

can influence the total pH of the solution. An interesting feature of this type of scaffold is the high contents of water (bigger than 98%in weight). Meaning that this type of hydrogel is very permeable to the Oxygen and nutrients. Hystem™ is suitable for the adhesion ad survival of the cells. The mouse's liver organoids are encapsulated in this matrix during the crosslinking reaction where they remain attached inside the scaffold, degrading slowly the implanted matrix and replacing it with their natural extracellular matrix. (AdvancedBiomatrix, 2021)

4.2.4.1 Procedure for the creation of Hystem™ and Hystem-C ™ for 3D cell culture:

The first step is in common for both matrices so: it is needed to take out the Hystem™ and Hystem-C ™ kits from the fridge (- 20 °C) and to allow it to reach room temperature.

- Hystem™ :

  With the help of a syringe, 1 mL of degassed water is added to each Hystem™ vial. Immediately after, the vials must be positioned on a shaker for a total dissolution which comes in less than 30 minutes. The resulting mixture appears so clear and slightly viscous. Then 0.5 mL of degassed water is added to the Extralink vials and shaken upside down several times to promote a complete diffusion. At this point, the 2 vials of Hystem are mixed together. The organoids pellet, which comes from the maintenance, are suspended in the Hystem™ solution. In the end, in order to obtain the hydrogel it is needed a combination of 0.5 mL of extralink with 2.0 ml oh Hystem stock solution. After this, the gelation occurs in less than 20 minutes. A drop of 10 µL is plated in 96 multiwell and incubated for 30 minutes at 37°C 5%CO_2. After this time 150 µL of growth medium is added gently touching the wall of the wells.

- Hystem-C ™ :

Also in this case with the help of a syringe, 1 mL of degassed water is added to Hystem™ and vial and Gelin-s ™ vial and also 0.5 ml of degassed water in extralink™ vial. Immediately after the vials must be positioned on a shaker for a total dissolution which comes in less than 30 minutes. The resulting mixtures appear so clear and slightly viscous. At this point the Hystem vials are mixed with Gelin-s one to create one homogeneous

taking care to avoid the entrance of oxygen which promote the gelification. The organoids pellet, which comes from the maintenance, are suspended in the Hystem™ and Gelin-s™ solution. In the end, in order to obtain the hydrogel it is needed a combination of 0.5 mL of extralink with 2.0 ml of Hystem+Gelin-s stock solution. After this, the gelation occurs in less than 20 minutes. A drop of 10 μL is plated in 96 multiwell and incubated for 30 minutes at 37°C 5%CO_2 . After this time 150 μL of growth medium is added gently touching the wall of the wells

4.2.5 Modification of scaffolds with collagen:

In order to increase the efficiency of these scaffolds, a protocol to include a 30% of collagen to each drop plated in the multiwell plate was applied. This protocol took inspiration from Li one (Li, et al., 2016), but some modifications will be applied to make it suitable for the purpose of this thesis.

Extra materials needed:

- Collagen type I solution from rat tail (lot# SLBS8676, Sigma Aldrich)
- Ham F-12 → Nutrient mixture powder with L-glutamine and without Sodium bicarbonate, Sigma Aldrich (lot# SLCB6501)
- Ultrapure milli-Q water
- $NaHCO_3$ → molar mass :84, 007 g/mol (lot# SLCG3879)
- NaOH → molar mass : 39,997 g/mol (lot # SLBZ399T)

4.2.5.1 Procedure for the modification

This procedure involves the formation of three distinct solutions which will then be combined to obtain the collagen to be applied in these scaffolds. The first solution, called SOLUTION A, is made up by pure Collagen type I, it will appear jelly and transparent. The second solution , SOLUTION B, is composed by Ham F-12 10X combined with 1.176g/L of $NaHCO_3$. In order to obtain Ham F-12 10X solution is requested to mix the Ham F-12 powder vials with 100 mL of ultra-pure milli-Q water, previously sterilized with the help of a 0.22 μL filter to eliminate any chance of contaminations. In Ham F-12 is also present the phenol red which acts as pH indicator. At this point considering the

volume of Ham F-12 is 100mL is necessary to adjust the value of $NaHCO_3$ to add, which becomes ~ 0.118 g . The SOLUTION C, also called "Reconstitution solution" is made up for instance by 45 μL of collagen combined with 5 μL of NaOH. This last solution is fundamental for the right polymerization because the collagen appears liquid at room temperature under acidic condition, instead increasing the pH it can easily polymerized, also a 37°C influence positively the polymerization, this is the reason why there is the addition of 5 μL of a strong base. At this point taking into account the Li procedure (Li, et al., 2016) : 8 part of solution A is added to 1 part of solution B and the added 1 part of solution C. Following these steps: mixing solution A with solution B the resulting mixture becomes light yellow, due to the acidic condition. If in this last solution will add the solution C the resulting color will change and becomes light pink, becoming a basic pH.

4.3 Inclusion in Paraffin

To analyze in a microscopic way tissue coming from living organism, the inclusion in paraffin is a useful technique to prevent the decomposition of living tissues. Biological tissues, once removed, tend to decompose very quickly due to the variation of physical and chemical properties (temperature pH). So try to block this degeneration is fundamental to obtain good results during analysis with the inclusion in paraffin. This sample preparation was made in the lab CRO's Laboratories (National cancer institute) located in Aviano (PN) Italy.

4.3.1 Protocol:

This is a general set up to include tissue in paraffin. The first step is to remove the medium present in the organoids culture, with the help of 1mL of a catted tip withdraw of a drop of PBS and use this to detach the matrix from the wells exploiting also a gently mechanical pressure lifting the edges to obtain an entire drop of matrix. At this point with the tips enlarged by the cut previously done, aspirate the drop of matrix and transfer it into the cup Tissue-Tek Cryomold, previously slightly filled with a layer of BioAgar. Following this, another layer of BioAgar was deposited over the cup, which is deposited in a cup filled of formaline. At this point, the sample must be prepared for the inclusion in paraffin removing the BioAgar block and inserting it into Cell Block case like a sandwich structure with first a layer made up by a sponge pre wetted with milli-Q water,

the layer of BioAgar with inside the matrix drop and further layer of sponge pre-wetted by milli-Q water, closed together in the Cell Block case. This complex is then kept for 24 hours in 10% formalin (→ 4% formalin in 10% final Buffer) following included in paraffin and after these two passages a thin slight of the sample is cut with the microtome with a width of ~ 2.5 μm, to be applicable to histological analysis as Hematoxylin & Eosin experiment.

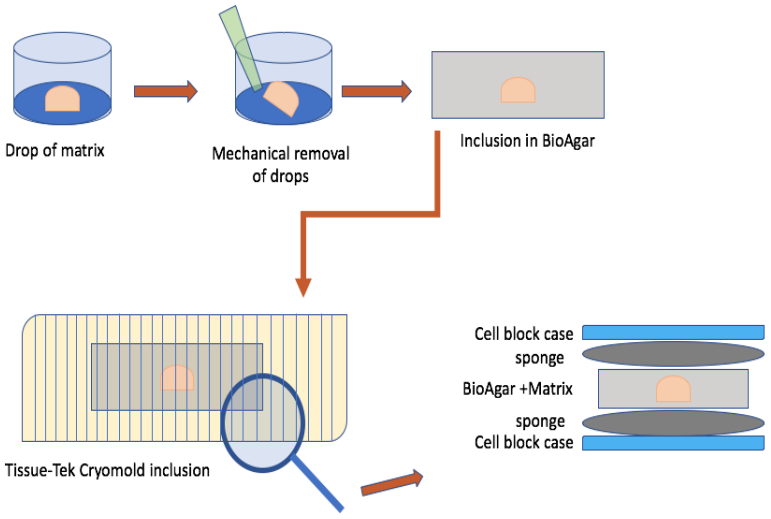


Figure 19. Scheme of inclusion in paraffin of samples

4.4. Analysis Technique

Analysis techniques have been applied to each of the matrices described above in order to verify the growth of mouse's liver organoids within them, to understand the temporal duration of the scaffold and try to identify a synthetic alternative to Cultrex®. The cultures of mouse liver organoids were followed for a prolonged period of time thanks to the aid of the Optical microscope which through it was able to photograph them, study them and understand how they grew. Furthermore, a Presto Blu HS assay was made to verify the viability of the organoids included in the scaffolds. Morpho-phenotypic tests were then performed on some scaffolds using coloration Hematoxylin & Eosin protocol.

4.4.1 Microscope

The analysis based on the direct visual observation of the various scaffolds was performed with the help of the Optical Microscope Nikon Eclipse Ts2R in addition to this, it has

been combined with the use of the microscope Evos FL Auto 2. With Eclipse it was be possible to study and follow the different growth of different scaffold and to evaluate, during days, the quality, the morphology and dimensions of Organoids in culture. Nikon Eclipse allows to take photos in a precise point of the scaffold and to move through the different planes of the three dimensional cell culture with different enlargement : 2X, 4X, 10X, 20X. In comparison with Microscope Evos its possible to obtain a photos in an automatic way of the totality of the well and not only in a precise point with different enlargements 4X,10X, 20X,40X and 60X . The resulting photos are by "Celleste" software.

4.4.2 Resazurin Presto blue HS assay

Once the organoids have been plated within different scaffolds to estimate the viability, therefore the number of active and surviving cells, Resazurin presto Blue Hs assay provides a homogeneous effective and fluorimetric method. This assay relay on the use of resazurin. This molecule is a dye, precisely is a phenoxazine dye, it's is interesting because doesn't show any kind of toxicity on cells and it is permeable to them. In addition it is weakly fluorescent and feels the reduction and oxidation's reactions. Resazurin molecule appears dark blue at pH ~ 6.5 and Presto Blue HS reagent is a buffer solution which contains an high purified resazurin which is optimal to test the cell viability. The excitation range of resazurin is 579 nm, instead the exact emission range is 584 nm. The maximum absorbance or resazurin is set to 605 nm , instead the resorfurin has the maximum absorbance set in 573 nm. Thanks to the fact which resazurin presto blue HS is a redox sensitive it is possible to obtain a measurements of the metabolic capacity of organoids because when they are active and viable they are able to reduce resazurin into resorfurin showing an high fluorescence , in the figure below show the reaction barely described : figure 20.

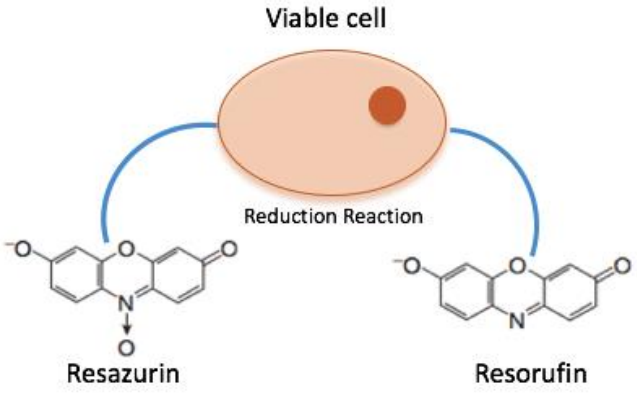


Figure 20. Reduction of Resazurin which becomes Resorfurin

When this reduction occurs the visible light is characterized by a Blue shift. Instead non-viable organoids are not able to reduce resazurin into resorfurin and so they will not generate a signal based on fluorescence. The time of incubation of resazurin vary from different type of cells and depend on their metabolic capacity. It was tested that 1hour is enough to evaluate the viability of liver organoids. This Assay can be applicable for several day and thanks to this it's possible to obtain a general overview to monitoring the cycle life of organoids. The analysis was performed with SYNERGY H^1 microplate reader (BioTek) which is modular and multimode. It possesses a filter based optics and a monochromator-based optics. This type of instrument is based on hybrid technology for this reason is very sensitive and flexible for the analysis.

Procedure

The plates with different scaffolds were taken out of the incubator. The recommended quantity of resazurin is 5 µL in 45 µL of culture medium, so knowing that it's present 150 µL of growth medium in each scaffold's condition is important to eliminate the excess and leave in each single well 45 µL of growth medium. At this point when all the wells are "normalized" to the required condition, under sterile condition in fume hood, with light off to each well are added 5 µL of resazurin. Furthermore to each 96 multiwell plates in an empty well is added 45 µL of growth medium with 5 µL of resazurin, this will act as a control to determine the background fluorescence that may be present during analysis. Once added the resazurin all the plates are left in incubator at 37°C 5% CO_2 for exact 1 hour.

Here were reported in a schematic way the passage just described : (Figure 21)

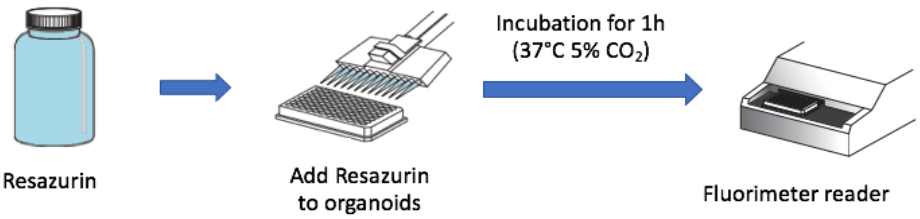


Figure 21. Resazurin steps procedure

All the tests were done in triplicate for each condition for each scaffold. The data obtained will be described in Chapter 5.

The waiting time has elapsed so the plates are inserted in the instrument which is set to "Fluorescent intensity" and programmed it to excitation = 560 and emission =590. All the data are processed with: "Gen5 ™ microplate reader and imager software" (version 3.05). Different time points were fixed for the period of organoids 'culture: T_0 = 24h T_1=48h T_2= 72h T_3=96h. All the data were normalized in respect of their T_0.

4.4.3 Comparative ratio method:

This normalization method was necessary to develop more critical assumptions about the quality of the tested matrices. It allows to compare the single matrices with Cultrex®. The data provided in chapter 5 will be shown in a table like this (Table 4.):

	24h	48h	72h	98h
Cultrex®	Y	Y'	Y''	Y'''
Matrices	X	X'	X''	X'''
Ratio (X/Y)	Z	Z'	Z''	Z'''
Results	Z/Z	Z'/Z	Z''/Z	Z'''/Z

Table 4. General set-up to calculate the comparative ratio

The values obtained for the respective time points, are entered in the individual boxes (X, Y; X ', Y' and so on). The values of the Matrices of each time points were divided by the

value of Cultrex® of the same time-points in order to see how much the Matrices varies compare to Cultrex® (X / Y = Z).

Then, to get an overview of how the individual time points vary with respect to the starting point T0 (24h), a further ratio (Z/Z) is applied for each ratio obtained before. If the result given will have a value of 0, it means that the tested matrix will not be considered optimal for the growth of organoids. If, on the other hand, the matrix is equal to 1 or greater than it, it will be considered an excellent matrix.

4.4.4 Growth curve

These graphics are representative of the increase in the size of the organoids in culture. "Imagej" software has been used to calculate the area of all the organoids of all the matrices at every time-points. Once this is done, the program automatically averages the dimensions and provides the data. The data are then normalized to T0.

5. RESULT AND DISCUSSION

In this section were reported all the results obtained for the different scaffold's conditions. Each scaffold will be exanimated individually in different sections. At the end, all the results were compared between each other to better evaluate which of this will give positive results for liver organoids culture.

5.1 Cultrex®:

Before starting to introduce the different scaffolds taken in consideration for this thesis, it is important to introduce the results obtained by Cultrex®. First, it is analysed by a qualitative point of view and secondly a viability assay is taken to better understand from a quantitative point of view.

5.1.1.Qualitative analysis:

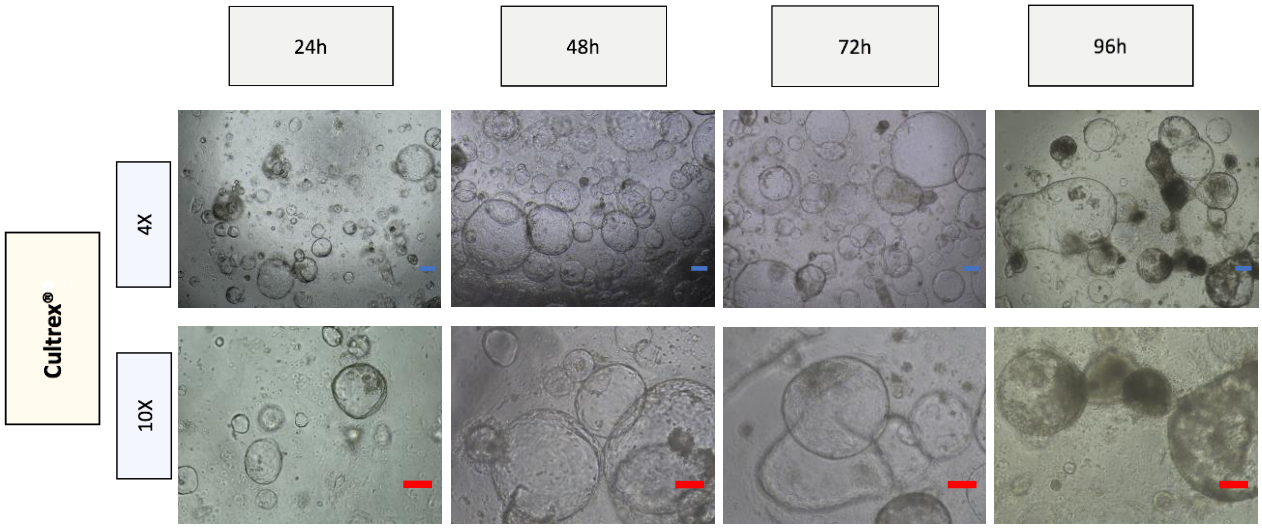


Figure 22. Table of liver organoids in Cultrex® (4X and 10X enlargement) at different time points: 24, 48, 72 and 96 hours. The red bar lower left represents the scale bar of 200 μm, the blue one of 100 μm.

The Figure 22 represents the typical growth of liver organoids in an efficient scaffold. During the first 24 hours, small organoids with a spherical structure are formed in Cultrex®. The average dimension is more or less 50-100 μm, this dimension shows a significant increase from 24 to 72 hours in which they arrive to measure 500 μm.

After 72 hours, organoids start to die, this increase in death is due to the fact that organoids will grow too much and inhibit their own vitality. This is very evident at 96 hours, because organoids appear dark and more irregular in shape, clear sign of the incoming death. So, this suggest that the quantity of organoids, during the splitting, will influence the duration of their viability. The right splitting ratio is a fundamental to avoid an overgrowth in the first 24-48 hours. Two different enlargements were taken into account to have a better panoramic on the trend of liver organoids. To make Cultrex® a very good scaffold, it is due to its ease of use, because its stiffness remains manageable during all the procedure described in previous chapter 4 "Material and Methods".

5.1.2.Quantitative analysis:

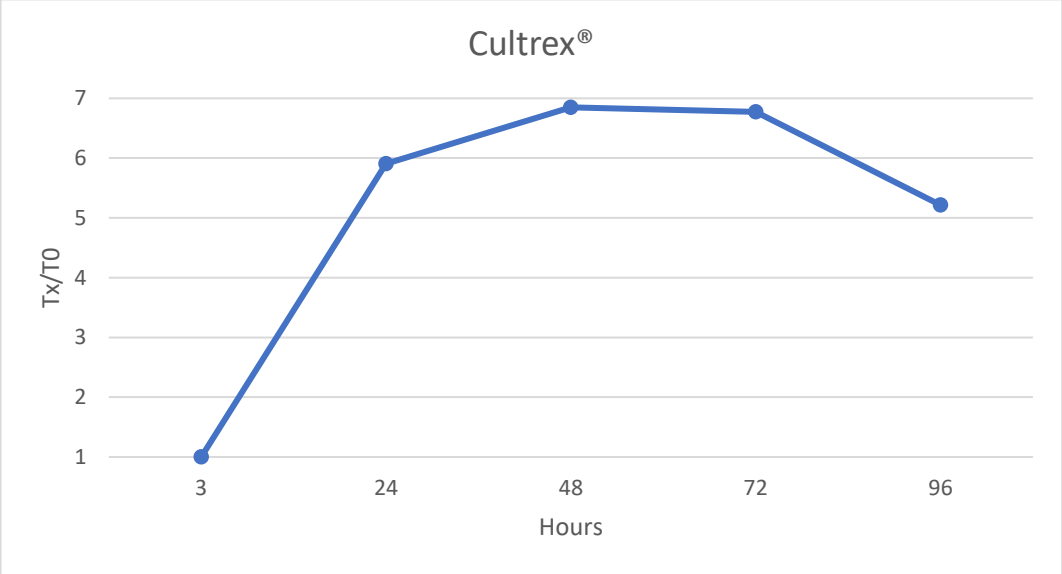


Figure 23. Graphic of Resazurin presto blue assay to evaluate the viability of liver organoids in Cultrex®

This graph in figure 23 represents the trend of the viability of liver organoids. Five time points were chosen for this test: T_0=3h, T_1= 24, T_2=48, T_3=72 and T_4=96h.

All these values were normalized with respect to their T_0. The culture of liver organoids in Cultrex®, in the period of time ranging from 3 to 24 hours, shows an exponential

increase in viability. The viability continues to grow up to 48 hours where it reaches a plateau up to 72 hours of incubation. In the period of time ranging from 72 to 96 hours there is a decrease in their vitality. This is due to the fact that organoids, now of considerable size, due to their high quantity begin to die from the lack of oxygen and nutrients in culture.

Only in the case of Cultrex®, it was possible inserting the first time point at T_0=3h. The other scaffolds tested will instead be analyzed from 24h onwards, since there were problems during the first hours of plating in the addition of resazurin. The addition of resazurin led to a dissolution of the freshly plated matrix, which does not happen after 24 hours.

5.1.3.Growth curve:

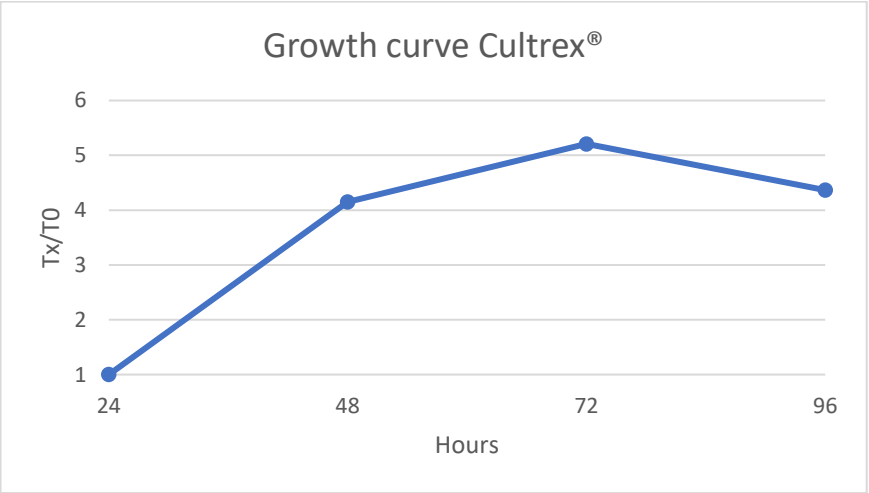


Figure 24. Graphic of Cultrex®'s growth curve at 24, 48, 72 and 96 hours

As demonstrated in this graphic (figure 24) in which it is reported the average dimension of the diameter of the organoids, there is an increasing in their size up until the 72h. After this time point, the huge grow of the organoids will determine their death because the nucleus of organoids goes into necrosis, due to a lack of oxygen and not sufficient nutrients to allow their continuous growth. The starting point of this graphic was settled at 24h to compare it with the other tested scaffolds.

5.2 Biogelx™:

This scaffold is divided in four different sub-types: –S, -RGD, -GFOGER and –IKVAV. All the features and protocols were described previously in chapter 4. Moreover, three different stiffness were tested in according with the procedure described in materials and methods. All the results were compared with the ones obtained by Cultrex®. Here will be reported, firstly a qualitative analysis of the three stiffness and secondly a quantitative analysis (Resazurin) for all the sub types.

5.2.1.Qualitative analysis.

Biogelx™ -S:

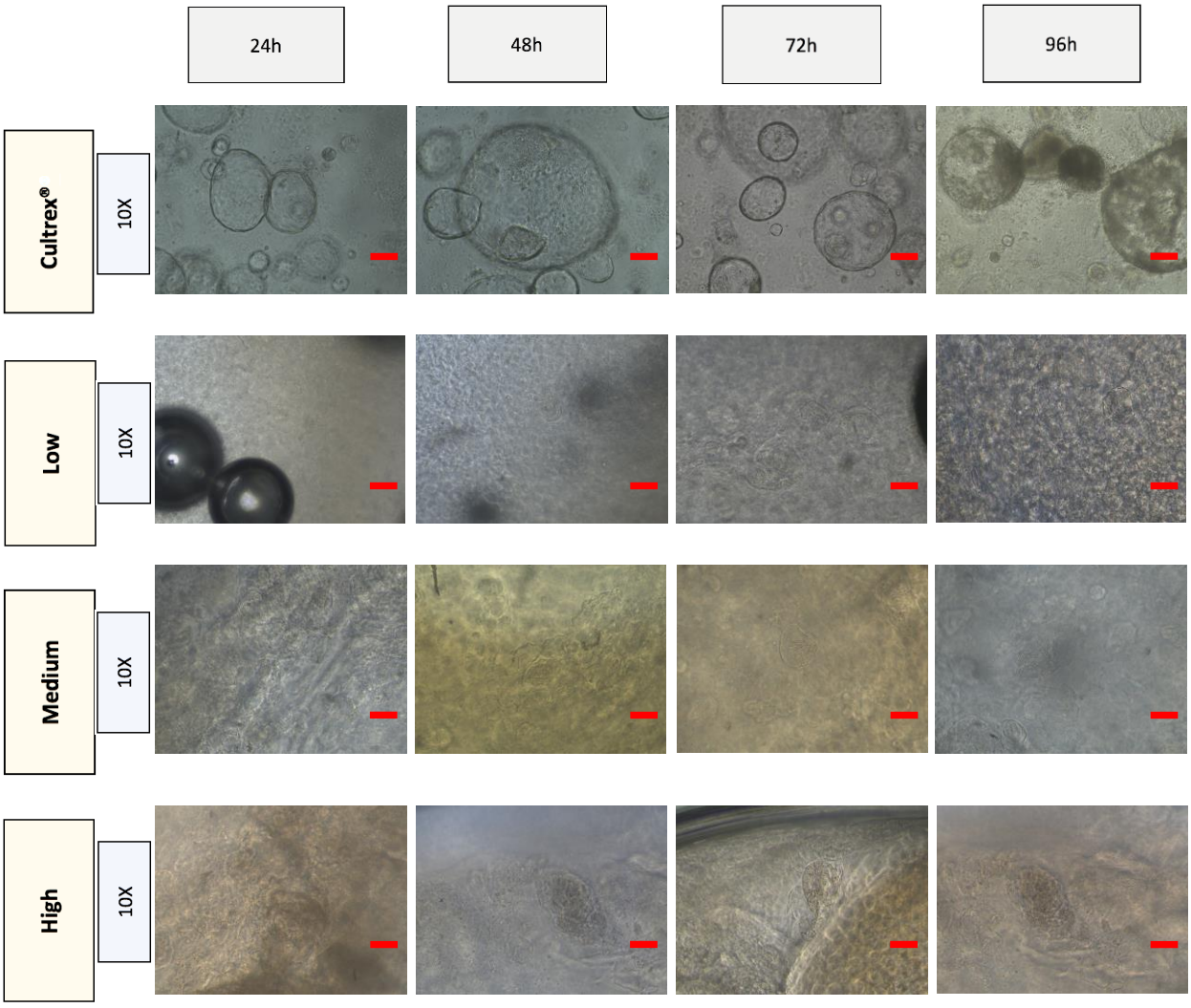


Figure 25. Biogelx ™-S light, medium and high stiffness compared with Cultrex® (10X enlargement). The red bar lower left represents the scale bar of 200 μm.

Biogelx™ -RGD:

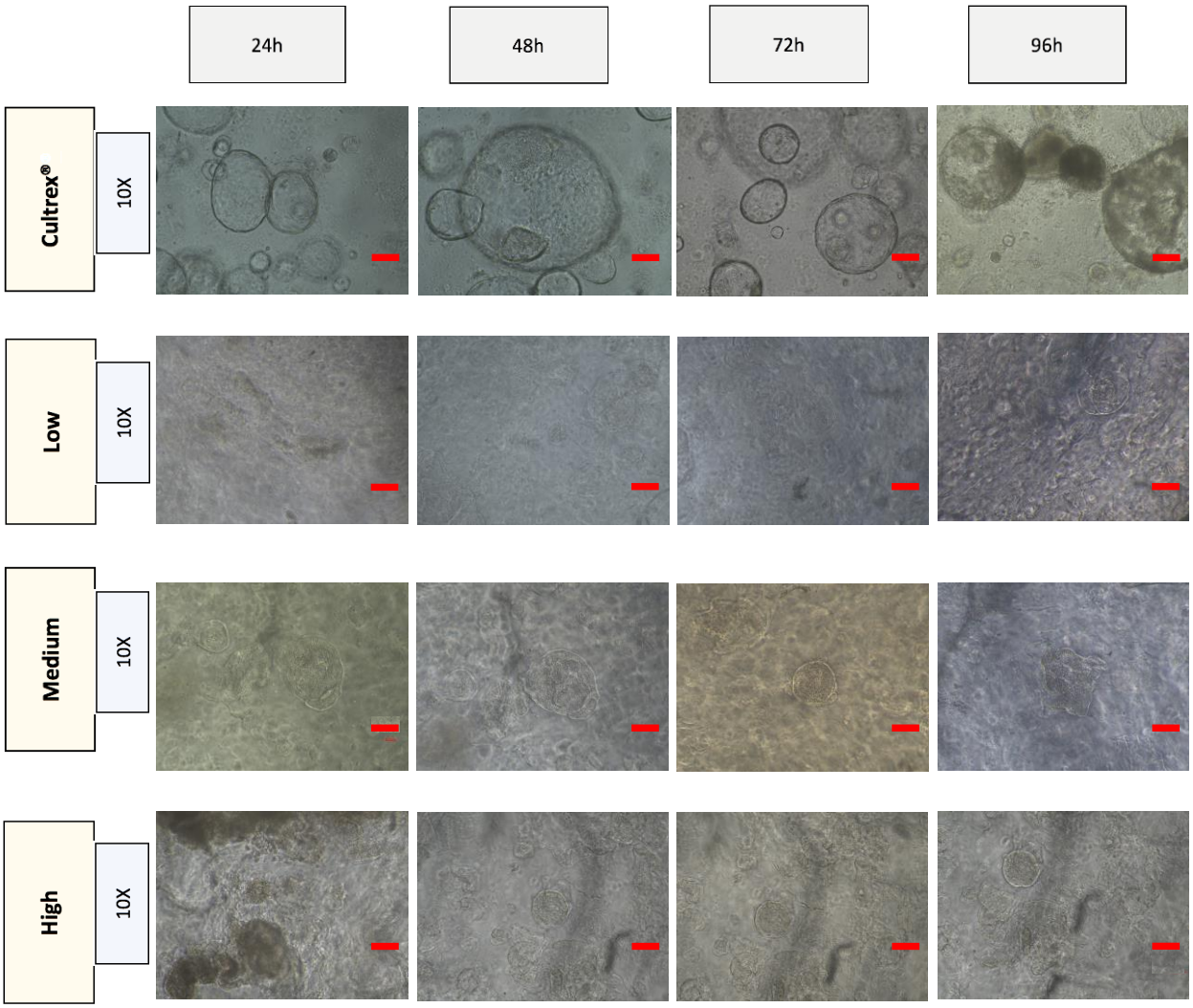


Figure 26. Biogelx ™--RGD light, medium and high stiffness compared with Cultrex® (10X enlargement). The red bar lower left represents the scale bar of 200 μm.

Biogelx™ -GFOGER:

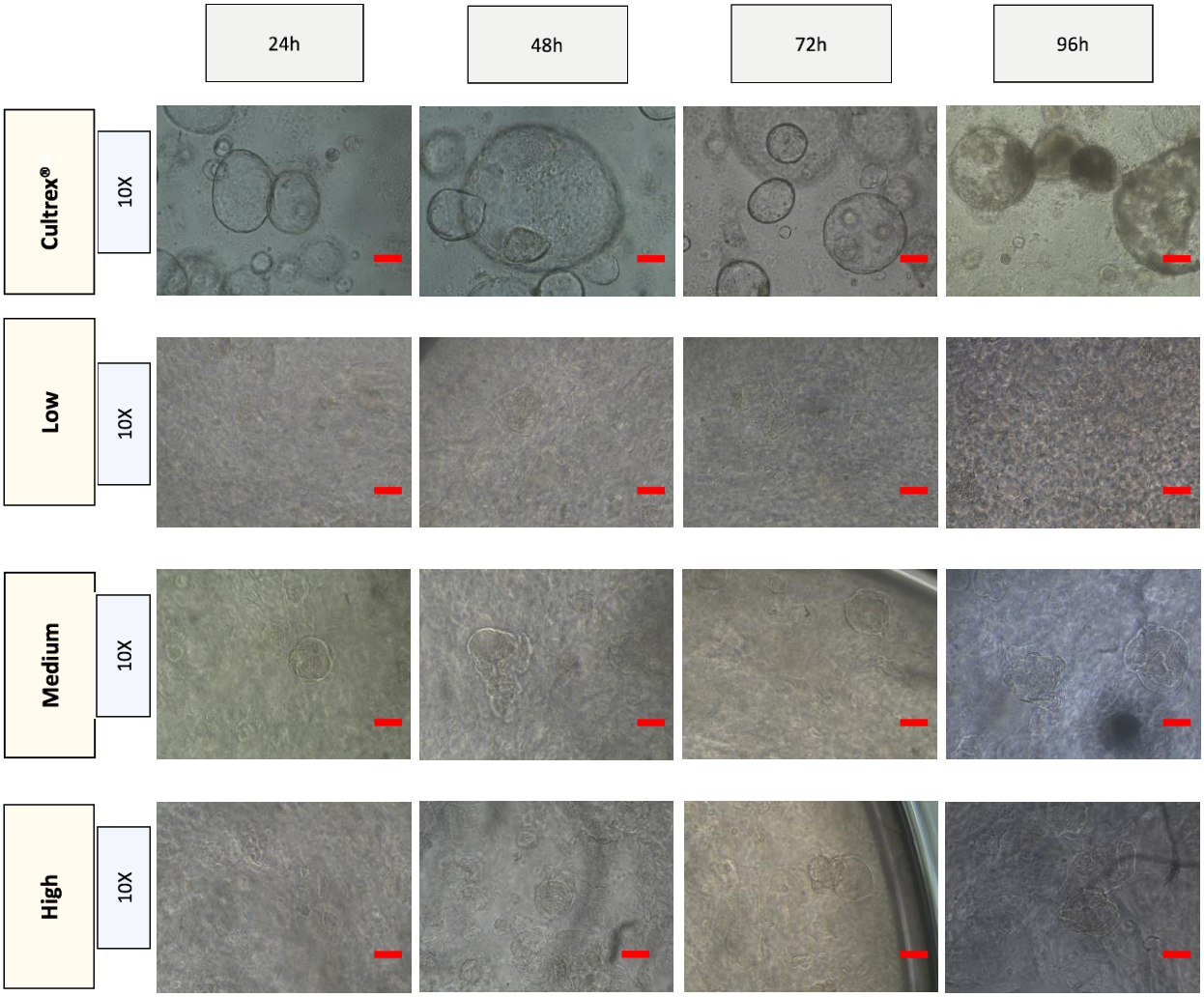


Figure 27. Biogelx ™-GFOGER light, medium and high stiffness compared with Cultrex® (10X enlargement). The red bar lower left represents the scale bar of 200 μm**.**

Biogelx™ -IKVAV:

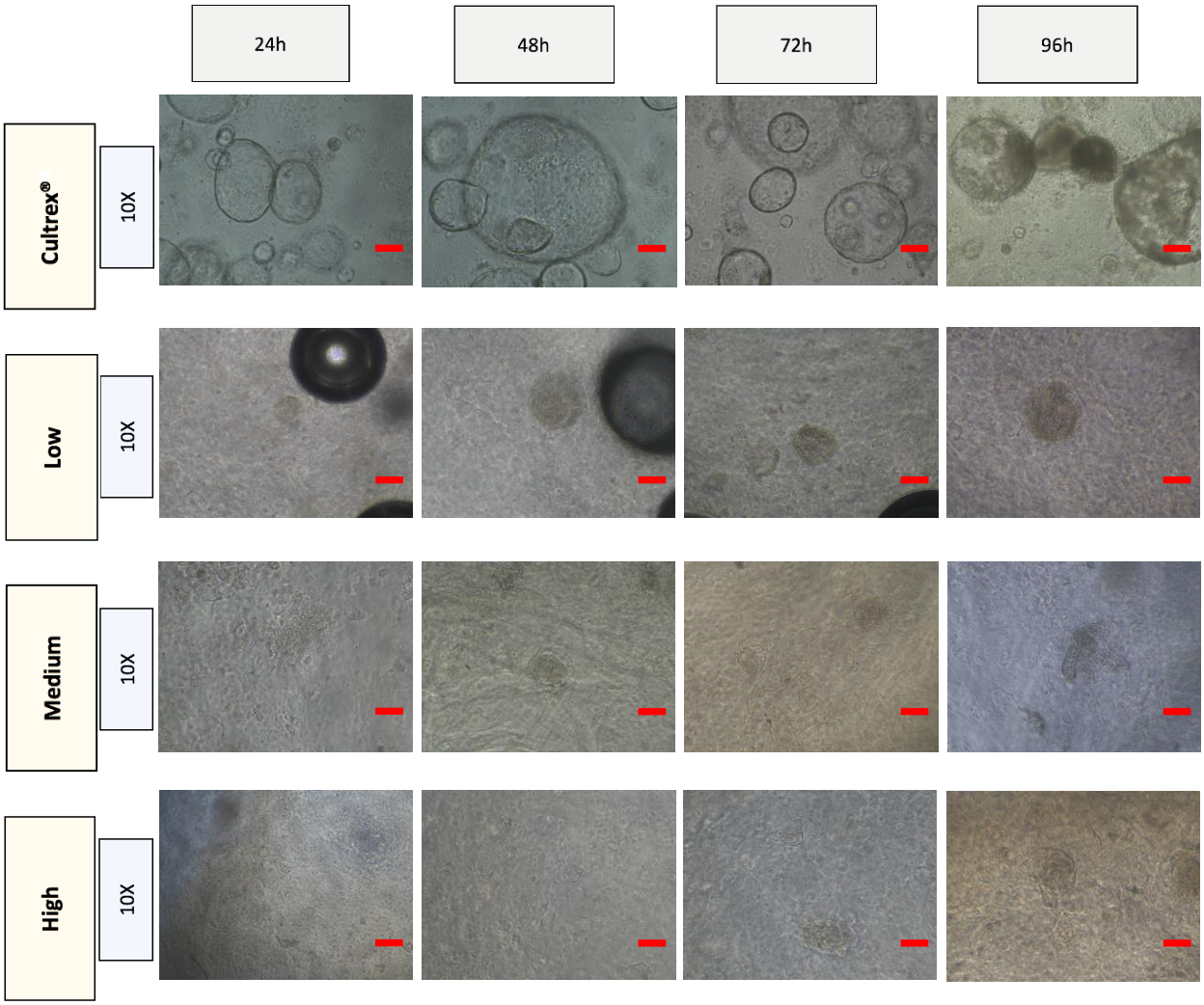


Figure 28. Biogelx ™-IKVAV light, medium and high stiffness compared with Cultrex® (10X enlargement). The red bar lower left represents the scale bar of 200 μm.

These results, coming from the qualitative analysis of the four different sub-types of Biogelx™, tested in three different stiffness, are not properly satisfying the desired conditions for a three-dimensional culture needed. Furthermore, no substantial differences emerged between the various types of scaffolds. All the samples were analysed with the microscope in 2 different enlargements (4X and 10X). In figure 25, 26, 27 and 28 were reported only the results in 10X because in 4X the cells appear very small and not so visible in photos.

As regarding the impression during the handle of these matrix, it is possible to say that

there were no problems during plating and preparation. The Hydrogel appears clear and transparent and easy to use, but once it has been plated and incubated overnight it appears very cloudy and blurred.

Looking in details Biogelx™-S (figure 25), which represents the standard hydrogel of this company without any kind of functionalization in the structure, it is possible to observe that there are no organoids formation, but cells aggregate in a form of clusters. Moreover, no differences emerge between the three-different stiffness, and this leads to think that it is not the density that influences the growth of the organoids, but precisely the matrix that is not suitable to support their growth.

As concerning the Biogelx™-RGD (figure 26), which is a hydrogel functionalized by fibronectin, it doesn't show any kind of improvement in culture with respect to Biogelx™-S. Organoids in the presence of fibronectin should be grow in an easier way, because fibronectin is involved in the binding of integrin receptors, which are present in the surfaces of the cells. In this case, very weak differences emerge in the stiffness: the medium and the high stiffness produce clusters of the cells. This could be partially explained by the fact that cells need to be in proximity of each other to form a signalling network to form the links between integrin receptors and fibronectin, which is in the scaffold.

Biogelx™ -GFOGER (figure 27) (scaffold functionalized by collagen) in comparison with Biogelx™-S and Biogelx-RGD shows greater roundness in organoid formation in high stiffness. Although the result is still not acceptable, the shape of these organoids is a further confirmation of how fundamental the presence of collagen is inside the scaffold.

The last sub-types scaffold checked taking advantages from microscope is the Biogelx™-IKVAV (figure 28). This last type is functionalized with Laminin. Laminin is a glycoprotein, which acts in the same way as the fibronectin, promoting cellular growth and differentiation, for these reasons the results are quite similar to the ones obtained from Biogelx™-RGD.

At the end, looking all the organoids, or better, clusters of cells formed in the four different sub-types of Biogelx™ scaffolds, compared with the ones obtained by Cultrex®, it is possible to make some considerations. In the Cultrex®, the organoids appear healthy with a typical spherical shape of liver organoids, indeed, in Biogelx™ the organoids formed are quite spherical and not clear inside. The dimension of organoids cultivated in

Cultrex® were in an around of 400-500 µm instead in Biogelx™ the organoids appear smaller: (~100 µm) and not perfectly spherical. Moreover, in Cultrex® conditions, the organoids possess a homogeneous distribution in comparison with Biogelx ™ which is characterized by a totally random distribution. No substantial differences emerge between the three different stiffness, and this leads to think that, in this case, it is not the density that influences the growth of the organoids, but precisely the matrix that is not suitable to support their growth.

5.2.2 Quantitative analysis:

In the graphic below, figure 29, were reported the results obtained from the Resazurin presto blue assay, to evaluate the viability of organoids in these scaffolds.

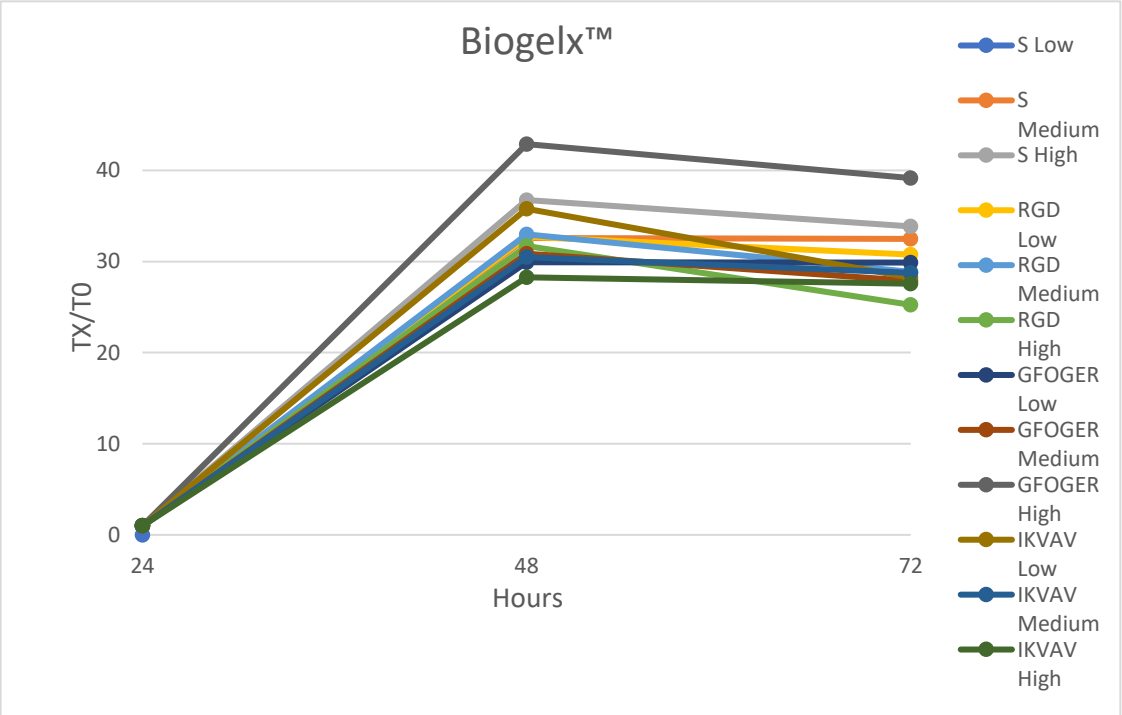


Figure 29. Graphic of viability test of three stiffness of Biogelx™ in 24h, 48h, 72h and 96h

By carefully observing the values obtained from the graphic (Figure 29) of Biogelx™ scaffolds, results which are completely not in accordance with the ones obtained from

qualitative analysis are shown.

The time passed between 24 and 48 hours shows a dramatically increase in the values of the fluorescence, which are not correspondent to the qualitative analysis made so far. Indeed, in qualitative analysis it is possible to say that the scaffold is not suitable for organoids cultivation. For this reason, the viability assay was stopped at 72 hours of incubation. The high value obtained in fluorescence could be partially explained by three main hypotheses. Biogelx ™ is composed by two synthetic peptide components, which together participate in the formation of the matrix. The formed complex shows an emission in a range until 600 nm, and this could partially enhance the background noise leading to a modification of results. (Alakpa, et al., 2016)

A second hypothesis, for the explanation of these high values obtained could be that the scaffolds, due to a different conformation of the protective group of F-Moc (Fluoroenylmethyloxycarbonyl), generates a fluorescence, which modify the results.

Finally, a third hypothesis for these high values could be that cells in the matrix do not form a three-dimensional structure, but a wide two dimensional substrate of cells.

To prove all these ideas, two tests were made. Firstly, it was plated the scaffolds without cells (Figure 30).

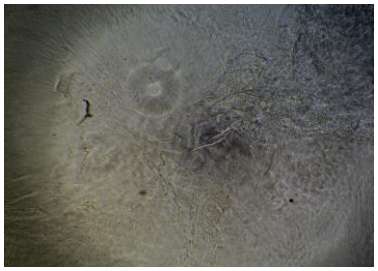


Figure 30. Biogelx™ without cells

In this figure 30, in fact, it is possible to note that the matrix without cells appears different in morphology resulting quite clear and transparent, instead in qualitative analysis in paragraph 5.2.1, the scaffolds appear not transparent and not clear.

Moreover, a second test was made, to understand if the matrix interferes with the analysis or not. All the Biogelx™ matrices were plated without cells, and checked with resazurin presto blue assay:

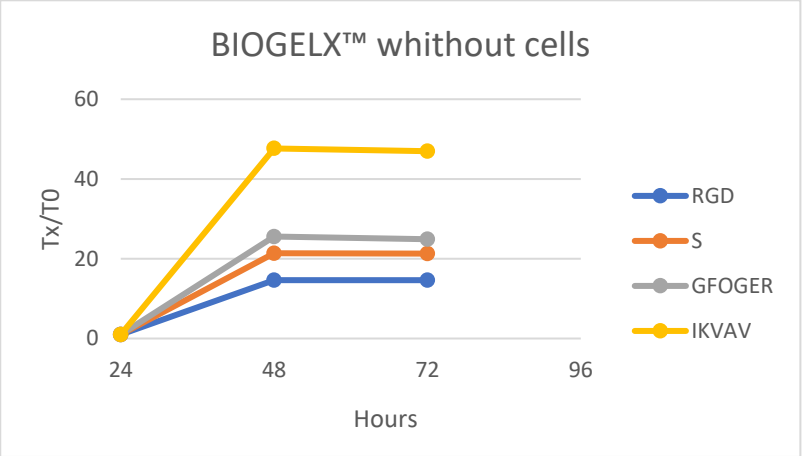


Figure 31. Graphic of viability assay of Biogelx™ without cells

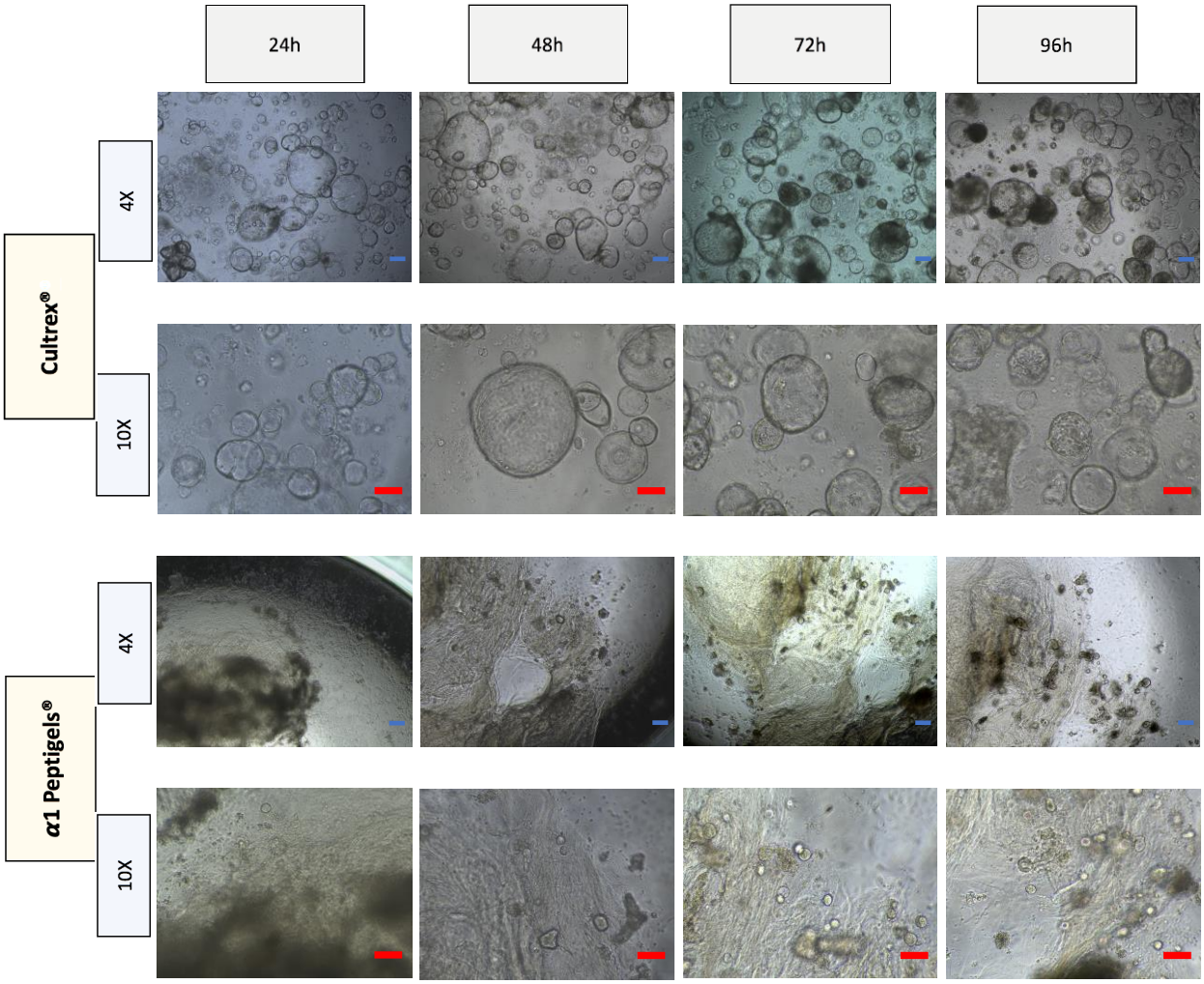
This graphic, in figure 31, confirms the speculations made so far. Biogelx™ shows a high increase in fluorescence without cells.

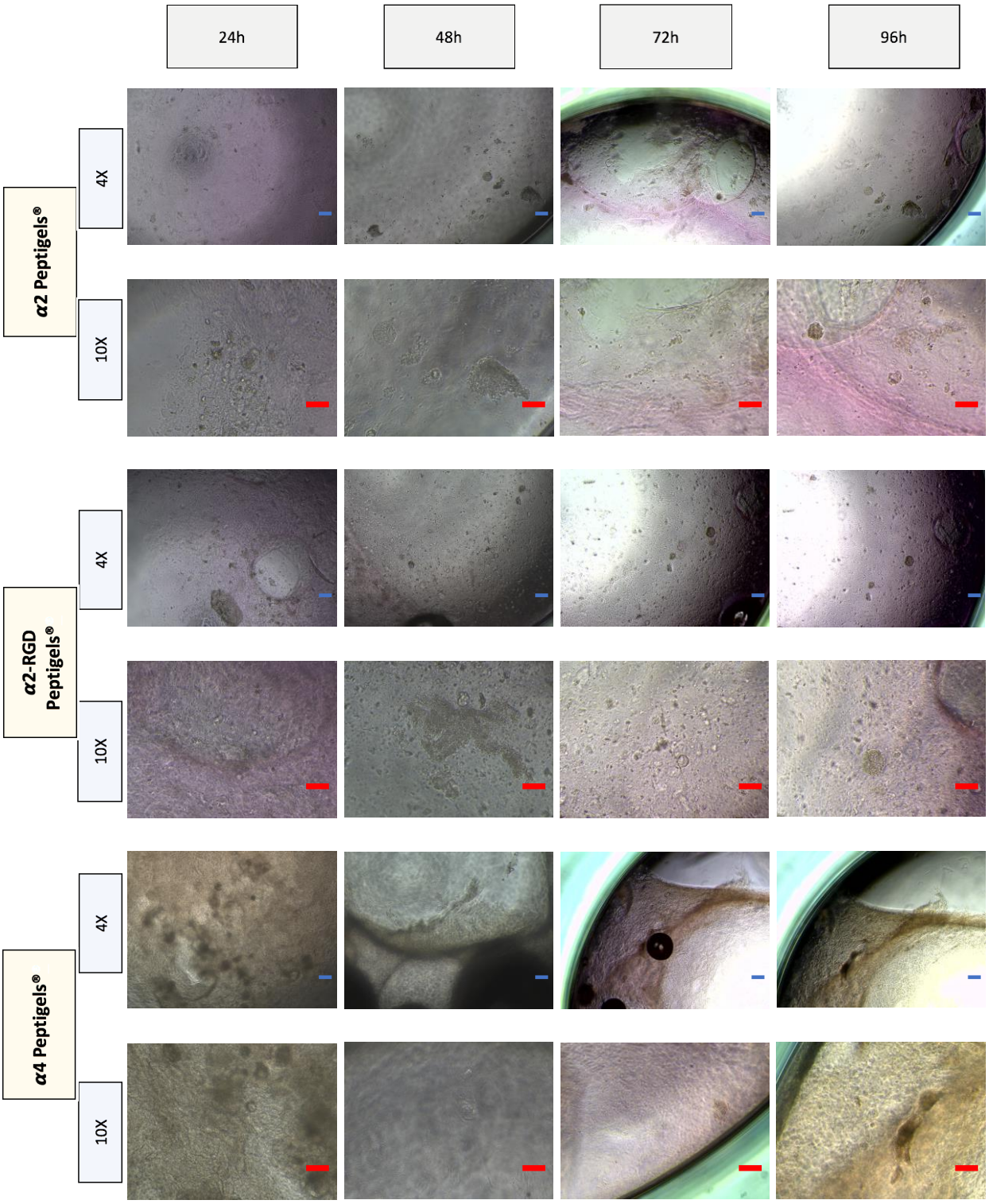
The experiments were repeated several times, in different passages of maintenance. But, despite the huge repetitions of the tests, the results remain constant, and this suggests that Biogelx™ is not suitable to obtain a three-dimensional culture because it generates a cell substrate. For this reason, all these scaffolds were no used anymore.

5.3 Manchester BIOGEL Peptigels®:

This scaffold is divided in five different sub-types. All the features and protocols of this scaffolds were described previously in chapter 4. All the results were compared simultaneously with the ones obtained by Cultrex®. More repetitions were performed for each conditions. Here will be reported, firstly a qualitative analysis of the three stiffness and then a quantitative analysis (Resazurin) for all the 5 types of Manchester biogel Peptigels®.

5.3.1 Qualitative analysis:




24h
48h
72h
96h
α2 Peptigels®
4X
10X
α2-RGD Peptigels®
4X
10X
α4 Peptigels®
4X
10X

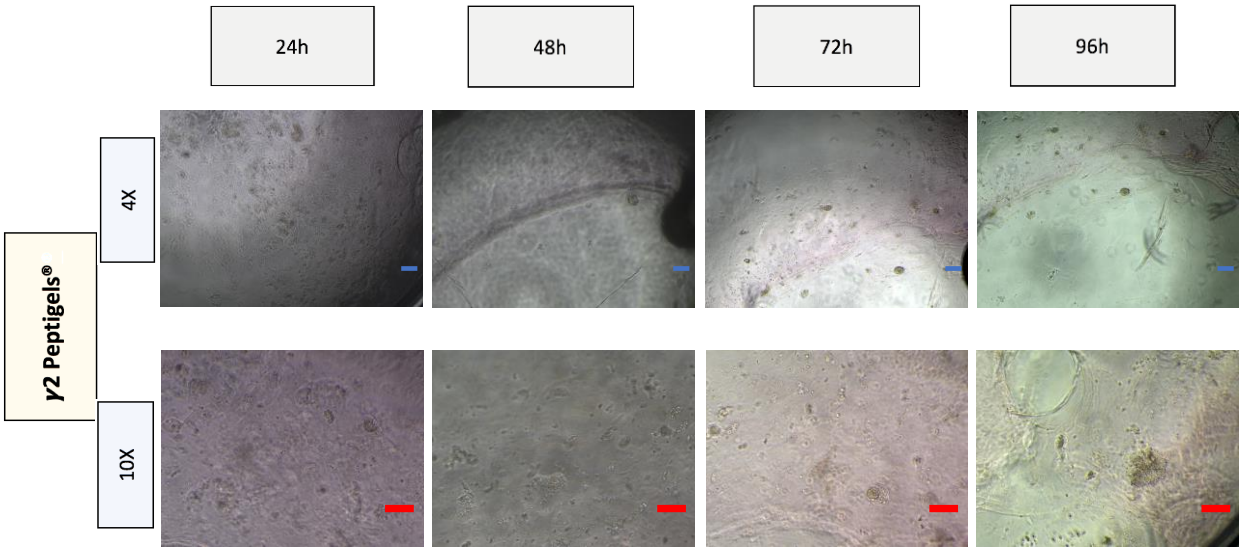


Figure 32. Manchester BIOGEL Peptigels® compared with Cultrex® (4X and 10X enlargement). The red bar lower left represents the scale bar of 200 μm, the blue bar lower left represents scale bar of 100 μm

In figure 32 it was reported all the 5 types of Manchester Biogel Peptigels® in two different enlargement : 4X and 10X. The reference scaffold is always Cultrex®, which is plated simultaneously with the five types of Peptigels®. Analyzing the photos obtained from the optical microscope, it is possible to observe that big differences emerge between the scaffolds. Unfortunately, the exact composition of these hydrogels is unknown, but it can be done some hypothesis on it, just comparing the qualitative results obtained with Biogelx™ in qualitative analysis.

The first scaffold plated is the Alpha1 (α1) Peptigels®. During the plating this scaffold appears very viscous and difficult to manage, furthermore when it is added in the solution whit organoids, suspended in growth medium, it quickly polymerized. For this reason, it is suggested to plate very quickly avoiding so the undesired polymerization. This scaffold gives quite promising results because after 48 hours some organoids appear with the typical morphology, in accordance with the ones cultivated in Cultrex®. If organoids obtained by α1 Peptigels® are compared with the ones plated in Cultrex® it is possible to note that organoids in Cultrex® possess a dimension in an around of 200-400 μm, organoids in α1 Peptigels®, indeed, possess dimensions of 20-80 μm. The small dimensions obtained cannot be considered a disadvantage. It is actually considered an

advantage because it is possible to maintain organoids in a longer period of time in culture without split it several times. In 10X enlargement of α1 Peptigels®, organoids can be seen distinctly with spherical shape and clear inside. During days, they seem to grow increasing their dimension. It would be interesting to test a dilution in this scaffold to see how they behaves in a matrix with low stiffness, but due to a problem of shipment, the Peptisol® solution never arrived. The high density of α1 Peptigels® not generate a homogeneous sample, indeed if it is compared the homogeneity with Cultrex® emerges a very huge difference. Putting all these data and considerations together, this scaffold was thought to be functionalized with collagen because organoids grow with a typical spherical shape and because it results very viscous during plating as collagen.

Manchester Peptigels® Alpha 2 (α2) and Alpha 2-RGD (α2-RGD) from a qualitative observation under the microscope are quite similar between each other. Both of them do not create three dimensional structures, except of few organoids formed, but the overall culture results in two dimension cluster of cells. It was supposed that Alpha 2 could be functionalized by laminin because it gives results quite similar to the ones obtained by Biogelx™-Ikvav (functionalized indeed by laminin). Alpha 2-RGD, instead, as the name suggest could be composed by a synthetic peptide which is functionalized by fibronectin. RGD, indeed, is an amino acidic sequence, which increases the cell adhesion. This fact is confirmed by the photos in figure 32 in which organoids or cell cluster are more attached to the matrix respect Alpha 2, but with a non-defined structure.

The fourth scaffold tested is Manchester Peptigels® Alpha 4 (α4). The results obtained from this matrix were not exactly satisfying because of the fewest presence of organoids in the matrix. This could be deriving from the type of the synthetic scaffold employed in this matrix that could not promote the cell adhesion and growth.

Finally, the last scaffold tested is Manchester Peptigels® Gamma2 (γ2). Also, in this case the results obtained are quite similar to Alpha 2-RGD because organoids appear not well defined in shape and there are a lot of cell clusters. For this reason, this scaffold could be functionalized with laminin or fibronectin. Among all these five-different types of tested scaffolds, Alpha 1 is the one, which gives quite satisfying results because organoids appear in their typical morphology with a quite homogeneous distribution.

5.3.2 Quantitative analysis:

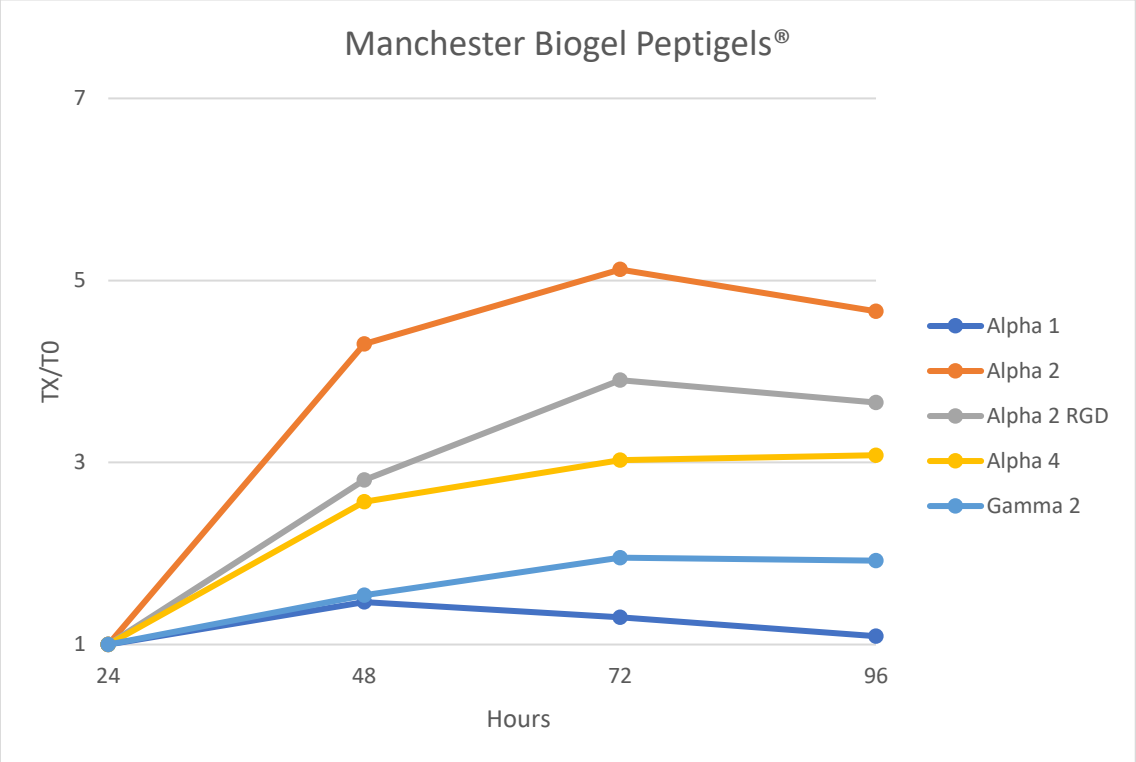


Figure 33. Graphic of viability of Five type of Manchester Peptigels® at 24, 48, 72, 96 hours

In this graphic, figure 33, were reported the value obtained by viability assay Resazurin presto Blue of the five different scaffolds of Manchester Peptigels®. All the values obtained by the analysis of the data are not so much in accordance with the interpretation given before in the qualitative observations of sample with optic microscope. All the data obtained with this analysis were normalized respected to T_0 which is set to 24h of incubations. Alpha2, Alpha2-RGD and Alpha 4 show a bias similar with the one obtained by Biogelx™ analysis, confirming, also in this case, what supposed from the previous experiment with Biogelx™: could be the background noise of the matrix that enhance the fluorescence signal. So, these three scaffolds are not able to promote a three-dimensional culture as it is possible to see in qualitative analysis.

Another speech can be done, indeed for the Alpha1 and Gamma2 scaffolds. Even though Gamma 2 doesn't show a high formation of organoids, but only forms some three-dimensional aggregates, the values obtained by the Resazurin presto blue assay are in accordance with the ones obtained by Alpha 1, which give organoids. Alpha1, as for qualitative analysis, seems to be a promising scaffold to obtain a three-dimensional

culture. Indeed, the values obtained follow, in a reduced way, the trend showed in Cultrex® matrices.

COMPARATIVE RATIO:

To better understand the difference between the tested Manchester Peptigels® and the control matrix Cultrex®, will be applied the calculation "comparative ratio" procedure explained before in materials and methods (Paragraph 4.4.3)

Here will be listed the 5 different tables with their respective analyses:

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Alpha 1	167692	245915.3333	217707.3333	183188.6667
Ratio (X/Y)	0.44828803	0.567858059	0.510916053	0.563151466
Results	1	1.266725901	1.139704874	1.256226863

Table 5. Comparative ratio Manchester biogel Peptigels® Alpha1

The values obtained are quite in accordance with the ones obtained by qualitative analysis, suggesting that this could be a promising synthetic scaffold. It would be interesting to test it with different stiffness because, as mentioned before, the organoids which grow within this matrix seems to be forced in their morphology.

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Alpha 2	3437.333333	14790	17598.66667	16024
Ratio (X/Y)	0.009188962	0.034152489	0.04130059	0.049260357
Results	1	0.076184253	0.092129585	0.109885506

Table 6. Comparative ratio Manchester biogel Peptigels® Alpha2

The results suggest that Alpha 2 in comparison with Cultrex® show a strong negative difference which suggest that it is not suitable to organoid culture. The same considerations can be done for Alpha2-RGD.

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Alpha 2 RGD	31251.66667	87771	122015	114301.6667
Ratio (X/Y)	0.083544523	0.202677357	0.286345073	0.351381733
Results	1	0.452114139	0.638752439	0.783830282

Table 7. Comparative ratio Manchester biogel Peptigels® Alpha 2RGD

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Alpha 4	81149.33333	208558	245575.6667	249948
Ratio (X/Y)	0.216935064	0.481593968	0.576317519	0.768380409
Results	1	1.074295844	1.285596493	1.714032848

Table 8. Comparative ratio Manchester biogel Peptigels® Alpha 4

Looking the table of Alpha 4 (table 8), it is possible to note value >1. This is not an indicator on the quality of the matrix because in this condition there is a formation of 2-dimensional substrate and so the recorded fluorescence will be high.

As regarding Gamma 2, the results are not totally negative:

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Gamma 2	108861.3333	167905.6667	212788.3333	209336.6667
Ratio (X/Y)	0.291017059	0.387721191	0.499372133	0.643534629
Results	1	0.864893027	1.113953751	1.435538284

Table 9. Comparative ratio Manchester biogel Peptigels® Gamma2

The quite good results obtained from alpha1 and Gamma 2 are then tested again in the comparative analysis (Paragraph 5.6).

5.3.3.Growth curve:

Here in this section will be reported the growth curve of Alpha 1

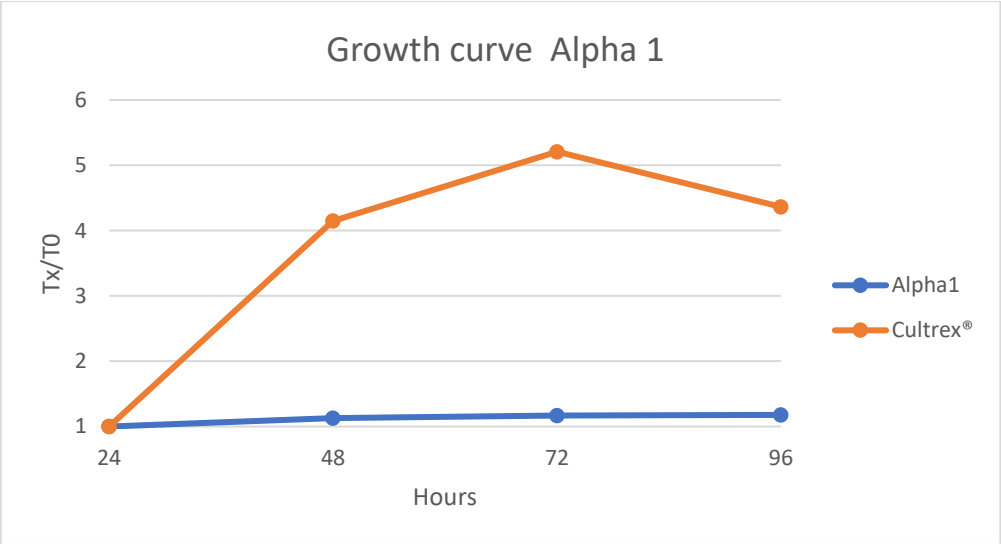


Figure 34. Growth curve of Alpha 1compared with Cultrex®

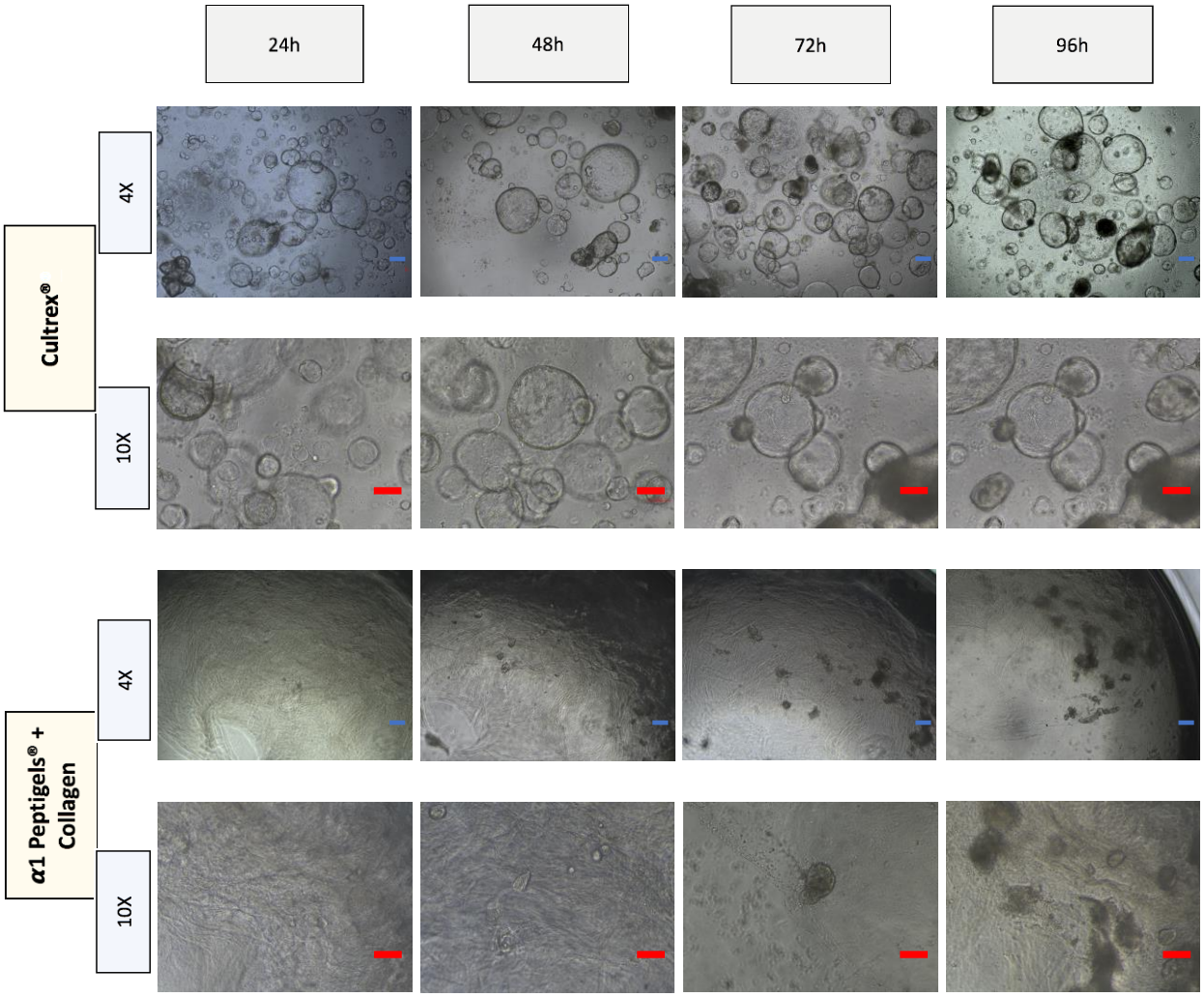
It was possible to analyse and create the growth curve only for Alfa 1 (with ImageJ) because was the only one which give results at least comparable to Cultrex®.

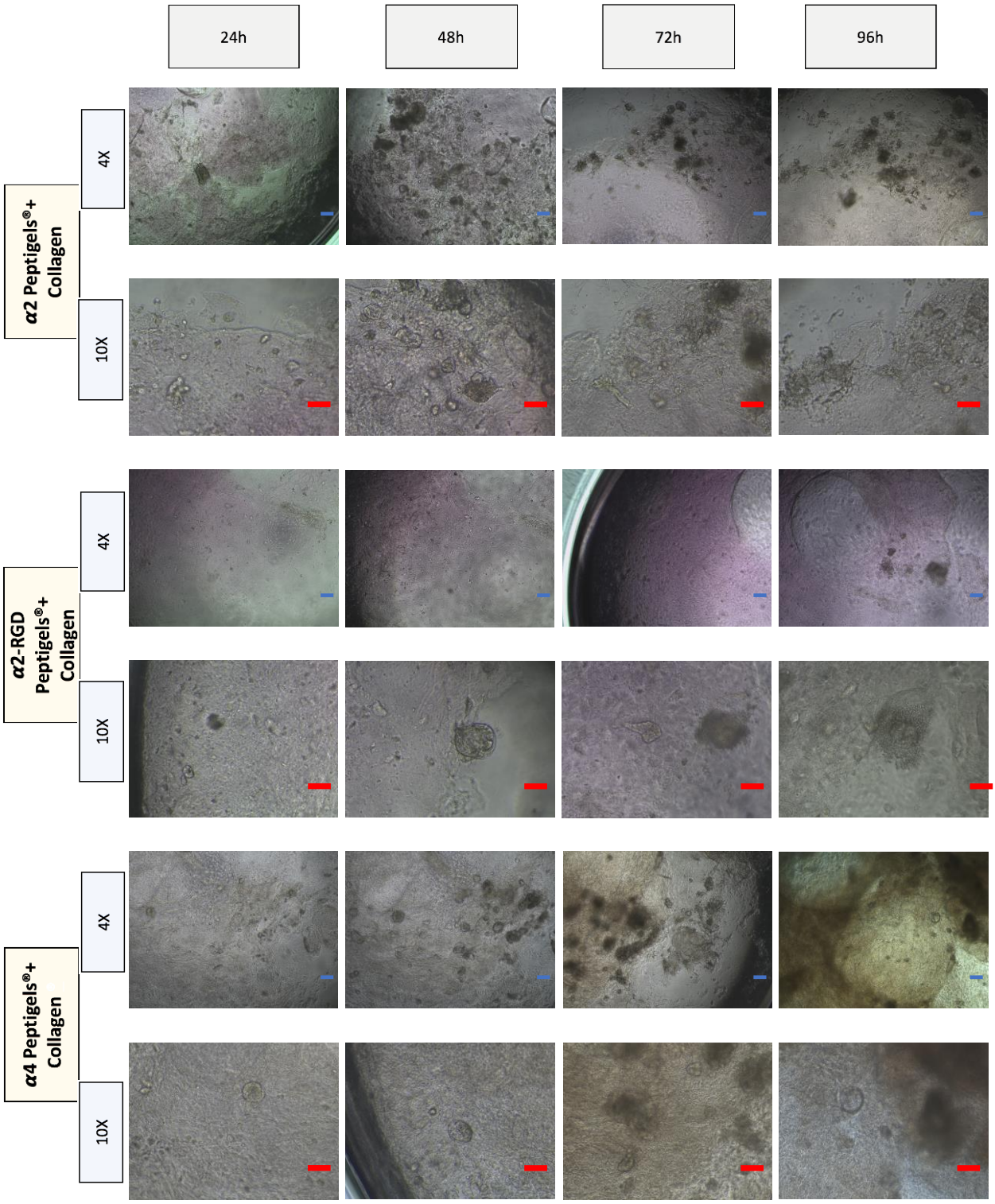
There is a marked difference in the organoids formed in two different matrices. In Cultrex®, the organoids during the 72 hours show a very high increase in dimension (Red line), and decreasing at 96 hours. Instead, Alpha1 (Blue line) shows a very weak increase in dimension, that still remain constant for all the 96 hours.

5.4 Manchester BIOGEL Peptigels® modified with collagen:

Due to the fact that the composition of Manchester Peptigels® is unknown, it was chosen to modify their structure adding a 30% of collagen in order to try to improve the efficacy and to modify the stiffness. The procedure to modify these scaffolds is reported in Paragraph 4.2.5 in materials and methods.

5.4.1.Qualitative analysis:




24h
48h
72h
96h
α2 Peptigels®+ Collagen
4X
10X
α2-RGD Peptigels®+ Collagen
4X
10X
α4 Peptigels®+ Collagen
4X
10X

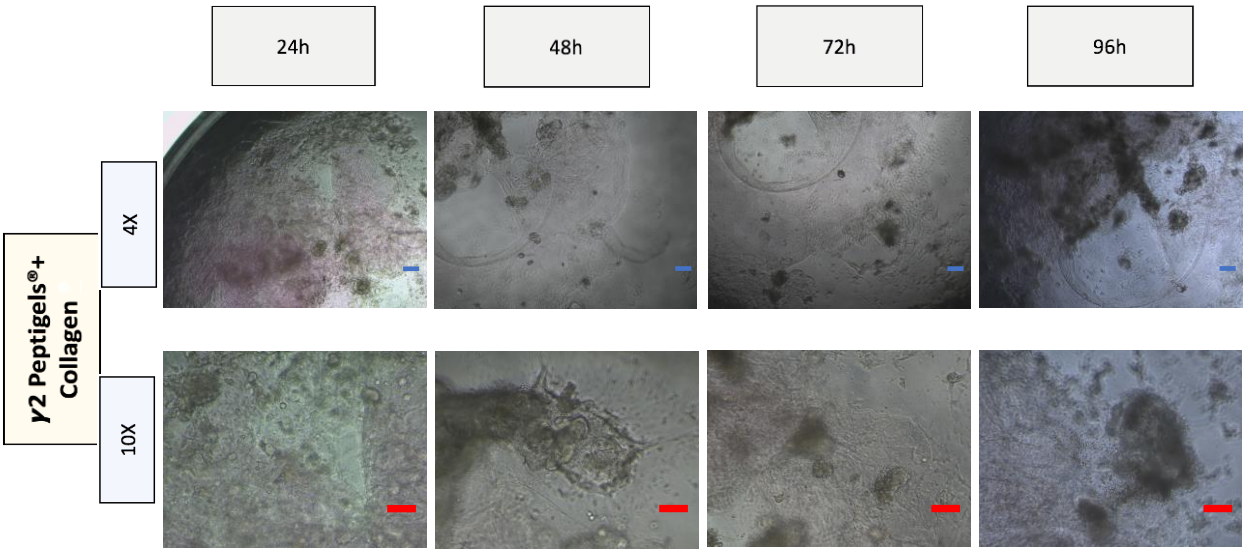


Figure 35. Manchester BIOGEL Peptigels® + 30% of collagen compared with Cultrex® (4X and 10X enlargement). The red bar lower left represents the scale bar of 200 μm, the blue bar lower left represents scale bar of 100 μm

The observation of the samples in figure 35 with an optic microscope was made at two magnification: 4X and 10X.

The total amount of collagen needed to modify the scaffold was in accordance with the percentage of its quantity in Cultrex® matrix (30%). After a general observation of all the samples, it can be said that there has been an actual improvement in the formation of three dimensional structures and organoids. But the differences between Cultrex® conditions and Manchester biogel Peptigels® still remain very marked. In Manchester Peptigels® the organoids, grown in the five different types of matrices, possess an average dimension of 30 μm which are a tenth of order smaller than Cultrex® conditions. Peptigel® Alpha 1 (α1) with the addition of a 30% of collagen doesn't show a significate improvement on its capacity to generate a three-dimensional cell culture. On the contrary seems that the Alpha 1 lost its capacity to generate small organoids, but this could be associated to an error during plating. Another supposition could be due to the increase in stiffness of this matrix. This high stiffness in the matrix density could reduce the growth and, so, the formation of the organoids. Indeed, the organoids in Alpha 1 lost their spherical morphology forming structures more elongated respect to the ones obtained in Alpha 1 without the addition of collagen. Moreover, also during the plating it was possible

to observe that the stiffness of the matrix, after the addition of collagen, was increased resulting in a difficult plating of the drops.

Alpha 2 + Collagen (α2) shows a very high increase in the formation of organoids in comparison with the results obtained by Alpha 2 (α2). In a quick observation in 4X enlargement are not so much visible but, if it is switched to a 10X enlargement, it is possible to see a huge number of organoids within the matrix. The matrix results also in this case very compact, for this reason organoids in 4X are not so much visible. The organoids, which grow in these scaffolds possess more or less the typical shape of the one obtained by Cultrex® conditions, but it is possible to note that they are in a certain sense forced in their morphology due to the high stiffness of this matrix. The dimensional range of these organoids are of 40 μm. In the first day of incubation organoids appear very small and quite clear inside (20 μm of diameter). After three days, organoids duplicate their dimension to reach, indeed, 40 μm. In the fifth day of incubation they start to die, observing, in figure 35, that a lot of them becomes dark inside which is a clear marker of their death.

As regarding Alpha 2-RGD + collagen, no a net difference emerges respect to Alpha 2 without collagen, also in this case seems that there is no formation of organoid structure but seems that a no homogeneous two-dimensional substrate is formed.

The modification with Collagen of Alpha 4 shows a slightly improvement in the capacity of the matrix to form three dimensional aggregates and organoids. The density of this scaffold remains very high and in this case, this influences the morphology of the organoids. After three days of cultivation in a 10X enlargement, it is possible to note a big organoid in figure 35 with a dimension of 50 μm. After 5 days, also in this case, organoids start to die, forming a black cluster.

The last scaffold modified with collagen is the Gamma 2 (γ2), as for Alpha2-RGD, no net difference has been observed in this scaffold in comparison with Gamma 2 without collagen. Since this types of Peptigels® is more manageable respect the other types, the plating was easier also with the collagen addition. The three dimensional cluster formed grow linearly until the third day of incubation. In the fifth day they start to die.

5.4.2.Quantitative analysis:

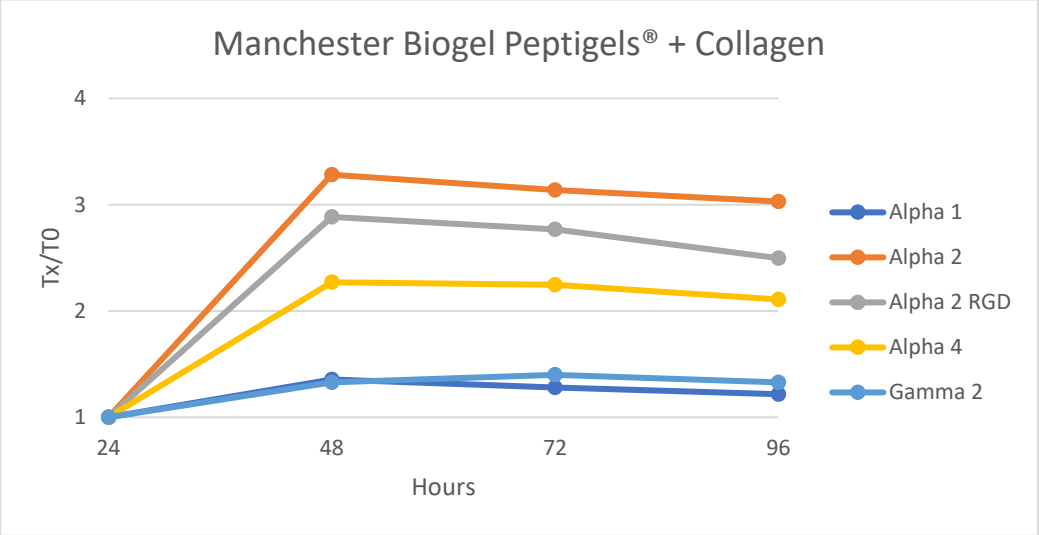


Figure 36. Graphic of viability of Five type of Manchester Peptigels®+ 30% of collagen

Here in this graphic (figure 36) were reported the results obtained by Resazurin presto Blue assay of the five different types of Manchester Biogel Peptigels® modified with collagen. Alpha 1 and Gamma 2 give results quite similar between each other. They are in accordance with the previous results obtained by the viability assay of the Manchester Peptigels® not modified with collagen.

The other scaffolds tested: Alpha2, Alpha2RGD and Alpha4 show a trend very similar to the previous test. This suggest that inside the synthetic matrix it could be some synthetic peptide which, with its fluorescence, could modified the real data.

All the obtained data, from this quantitative analysis are so not in accordance with the ones showed in qualitative analysis.

To better understand if the scaffolds give good results or not, it was applied, also in this case, the calculation described in chapter 4 paragraph 4.4.3:

COMPARATIVE RATIO:

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Alpha 1 + Collagen	161917	139961	215296	179829
Ratio (X/Y)	0.432849826	0.323192461	0.50525713	0.55282331
Results	1	0.720948228	1.117081466	1.033187755

Table 10. Comparative ratio Manchester biogel Peptigels® Alpha1+Collagen

Cultrex®	24	48	72	96
Alpha 2 + Collagen	374072	433057.75	426111.75	325292
Ratio	3867.73	15036	169237.5	16793
Results	0.010339534	0.034720542	0.39716694	0.051624387
	1	0.077451415	0.885963741	0.815158968

Table11.Comparative ratio Manchester biogel Peptigels® Alpha2+Collagen

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Gamma2 + Collagen	113512	168416	220142	219421
Ratio (X/Y)	0.303449603	0.388899633	0.516629734	0.674535494
Results	1	0.867521787	1.102450432	1.094692182

Table 12. Comparative ratio Manchester biogel Peptigels® Gamma2 + Collagen

Also modifying Manchester biogel Peptigels®, the results from this ratio are aligned with the one obtained by the scaffolds not modified. Alpha1 seems to give quite good results, indeed in qualitative analysis it is possible to see a small formation of organoids. Gamma 2 shows value bigger than 1, but not so much organoids are formed (figure 35). The high value is because of the growth of cells in two dimensions. An interesting result was obtained by Alpha2 modified with collagen. From figure 35 in 10X enlargement it is possible to note the formation of small organoids. The value obtained by this comparative ratio is in accordance with the qualitative analysis.

As regarding the two remaining scaffolds: Alpha2-RGD and Alpha4, their results suggest that the two types are not acceptable. Alpha2-RGD gives values lower than zero suggesting that this is not suitable for the organoids growth. Alpha4 instead show values bigger than 1 but this is due to the formation of two dimensional substrates:

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Alpha 2RGD + Collagen	30582.1	87131	119639	119672
Ratio (X/Y)	0.081754582	0.201199494	0.280769071	0.367891003
Results	1	0.448817457	0.626314003	0.820657653

Table 13. Comparative ratio Manchester biogel Peptigels® Alha2-RGD + Collagen

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Alpha 4 + Collagen	82156.2	211261	252791	260142
Ratio (X/Y)	0.219626703	0.487835629	0.593250479	0.799718407
Results	1	1.088219173	1.323368994	1.583938792

Table 13. Comparative ratio Manchester biogel Peptigels® Alpha 4 + Collagen

5.4.3.Growth curve:

The only growth curve that was possible to do was the Alpha2, which is compared with the one obtained by Cultrex® conditions.

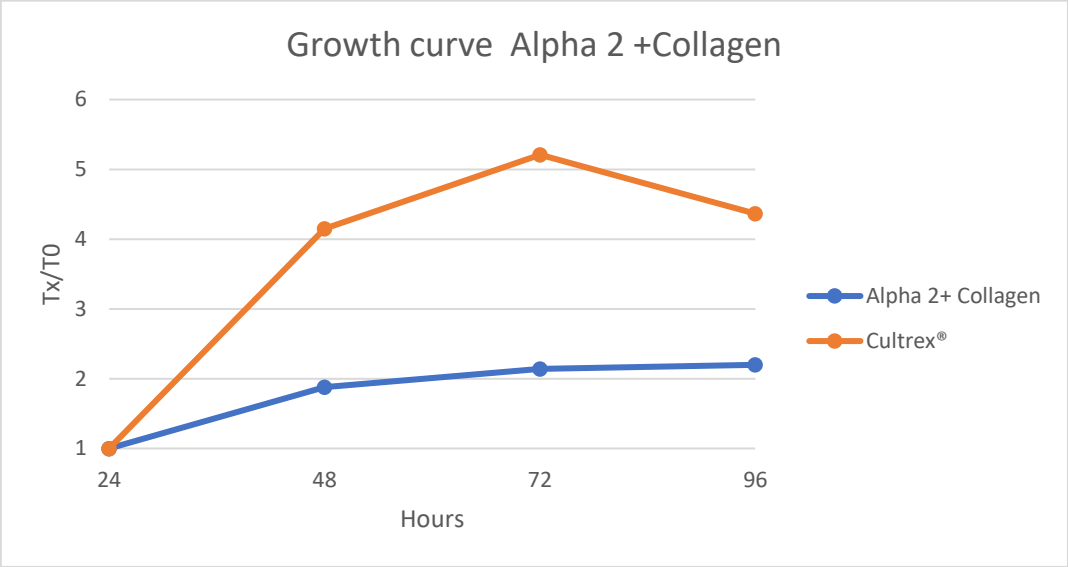


Figure 37. Growth curve of Alpha 2+ 30% collage in comparison with Cultrex®

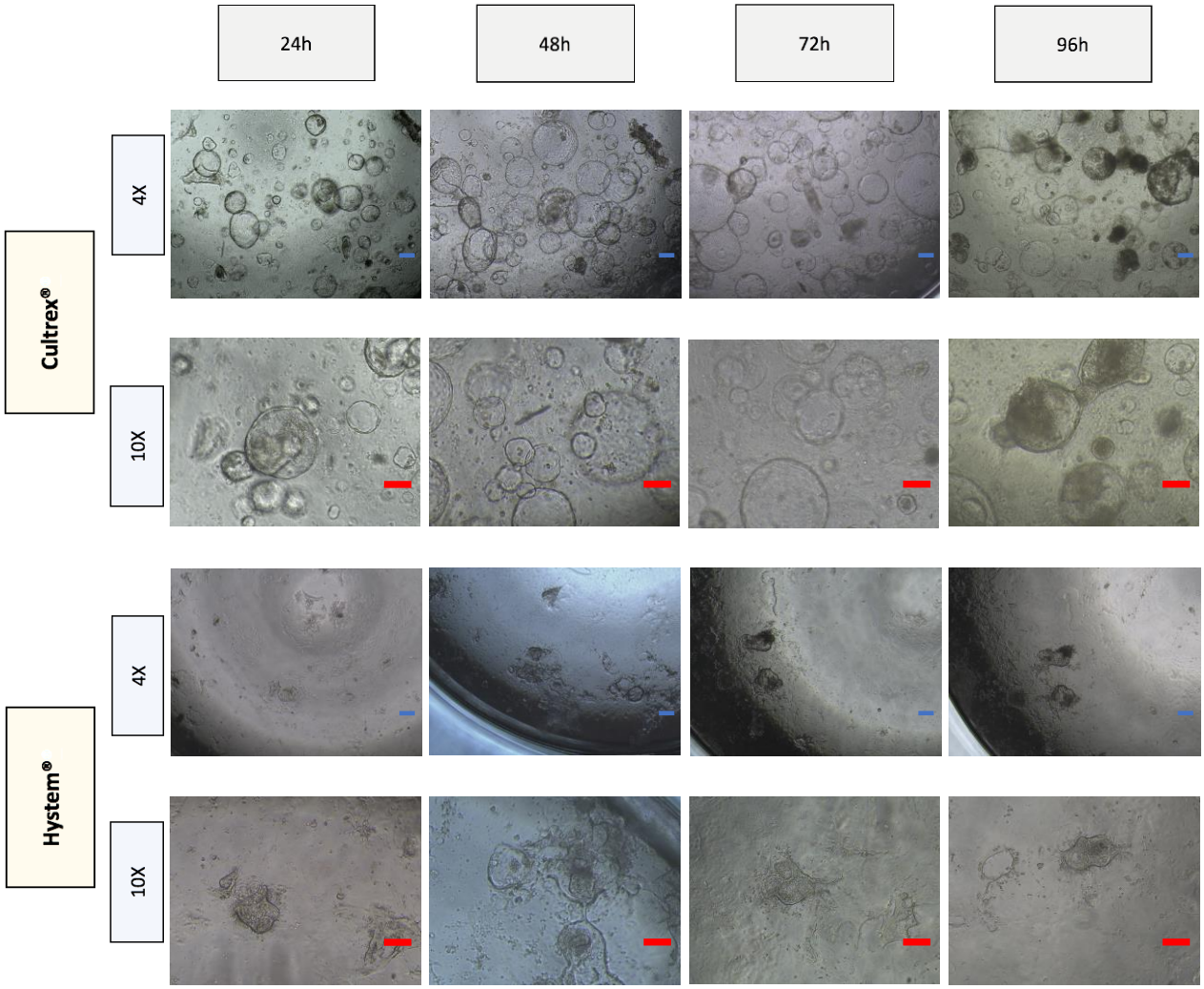
Also in this case, Cultrex® gives better results respect to Alpha2 modified with collagen. The dimension of organoids obtained in Cultrex® condition are higher than the ones obtained by Alpha2 modified with collagen. An interesting feature, that is possible to note, is that in Cultrex® at 72 hours the dimension starts to diminish, instead in Alpha2 still increases.

5.5 Hystem™ an Hystem -C™:

Here are reported the results and discussion of the Hystem™ and Hystem-C™ matrix. Firstly a qualitative analysis is made through the observations of the sample with an optical microscope in comparison with Cultrex®, and then will be reported the Resazurin presto blue assay's data to have also a quantitative analysis.

All the procedure of plating is explained in the previous section: "material and methods" in paragraph 4.2.4 .

5.5.1.Qualitative analysis:



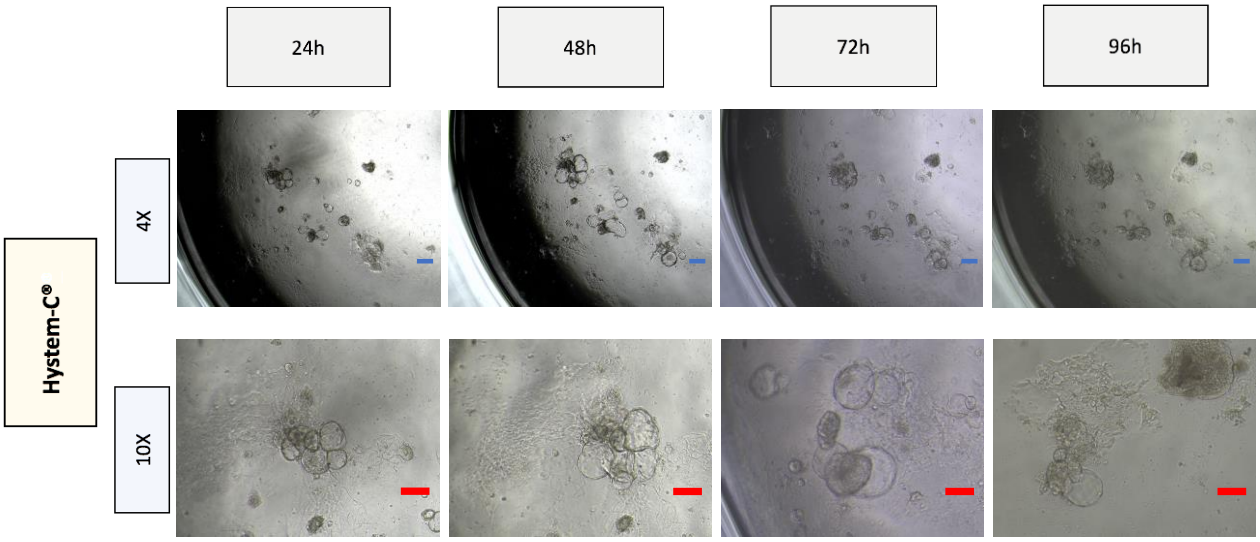


Figure 38. Hystem™ and Hystem-C™ compared with Cultrex® (4X and 10X enlargement). The red bar lower left represents the scale bar of 200 μm, the blue bar lower left represents scale bar of 100 μm.

In this qualitative analysis, made up by optical microscope, in a simultaneous way Hystem™ and Hystem-C™ in comparison with Cultrex® are analysed. The photos in Figure 38 are in two enlargements, 4X and 10X.

A very high difference emerges between Hystem™ and Hystem-C™. Hystem™ during the 96 hours generate principally cluster of cells and not organoids. In 48 hours of incubations seems that a sort of organoids is formed, but this doesn't possess the typical characteristics of liver organoids, as it is for organoids which grows in Cultrex® conditions. The major trend of this scaffold is to form a two-dimensional substrate of cells and not a three dimensional one. This is because Hystem™ is not able to provides the necessary factors, which generate a suitable niche for organoid formations. On the other hand, very interesting results were obtained by Hystem-C ™. Barely during the first 24h, organoids start to form with an optimal structure and grow in a linear way. In the first 24 hours, they reach a dimension of 100 μm, in 48 hours the dimension is increasing up to 150 μm arriving at 200-300 μm in 72 hours and 96 hours. This matrix shows a slow growth of organoids in comparison with Cultrex® that, after 48 hours reach high value of viability rate.

In a general observation of Hystem-C™, in comparison with Cultrex®, few organoids are shown, indeed the control (with Cultrex®) shows a huge quantity of them. For this reason,

the culture of Hystem-C™ could resist more days, because of this small amount of liver organoids. Indeed, in Cultrex® after 96 hours is reached the confluency determining their death.

The interesting composition of Hystem-C™ leads to achieve good results for organoid formation. This matrix mimics in an efficient way extracellular matrix with its high content of water (~98%) allowing an easy passage of oxygen and nutrients, making it very permeable. Moreover, the Gelin-S, which is a form of denaturized collagen type IV, in combination with Hyaluronic acid (HA) provide an efficient site for bind the cells. This is a further proof on how it is important the presence of collagen to recreate an artificial niche suitable for a three-dimensional culture.

5.5.2.Quantitative analysis:

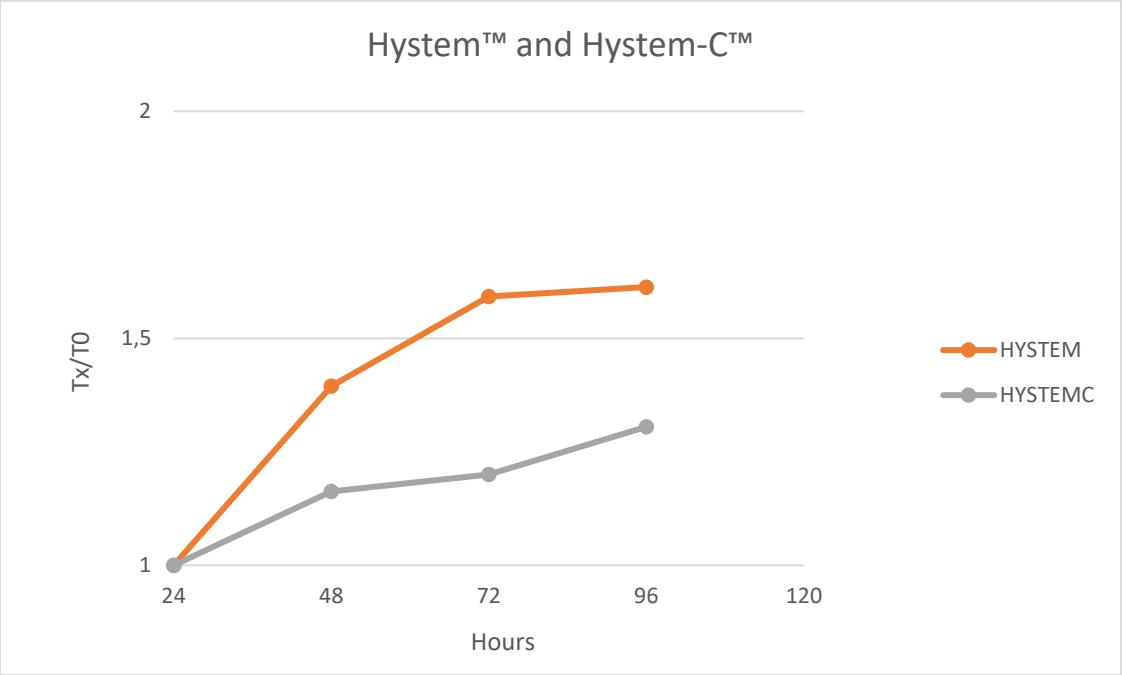


Figure 39. Graphic of viability of Hystem™ compared with Hystem-C™

Looking only at the graphic in figure 39 itself, without comparing it to the qualitative analysis, it would seem that Hystem™ grew better than Hystem-C™. But the high values of Hystem™ are due to the formation of a cell substrate which, obviously, increases the

viability in the scaffold. Therefore, looking at figure 38 it is possible to notice that the organoids were formed only in Hystem-C™.

Moreover, the values obtained for this scaffold with Resazurin presto blue assay are in agreement with the photos in figure 38. Organoids start with slow growth until the growth rate is increased during the 96 hours of incubation. The data obtained from both analysis (qualitative and quantitative), therefore, confirm that this scaffold is good for supporting cell growth in three dimensions.

COMPARATIVE RATIO:

	24	48	72	96
Cultrex®	374072	433057.75	426111.75	325292
Hystem™	282764.3333	394390	450094	447161
Ratio (X/Y)	0.755908845	0.910709946	1.056281597	1.374644934
Results	1	2.031528581	2.356256528	3.066432387

Table 14. Comparative ratio Hystem™

	24	48	72	96
Cultrex	374072	433057.75	426111.75	325292
Hystem-C™	153882.6667	178897.5	184690.2	200800.8
Ratio (X/Y)	0.411371786	0.413103102	0.433431371	0.617294
Results	1	0.921512676	0.966859122	1.377003085

Table 15. Comparative ratio Hystem-C™

Even in this case, if it are looked at the results without the context obtained from the images in the qualitative analysis (figure 39), the value obtained by Hystem™ would be better than Hystem-C™. Obviously, this is not the case. The high values (> 1) are due to the presence of the cell substrate which develops in two dimensions. This suggests that this scaffold type is not suitable for organoid culture.

As regards the comparative ratio of Hystem-C™, it is possible to note that during the 4 time points of the 96 hours it shows an increase in the values. In fact, looking at the photos in figure 38, it is possible to see that the organoids, which grow in it, increase in size and vitality hour by hour.

5.5.3 Growth curve:

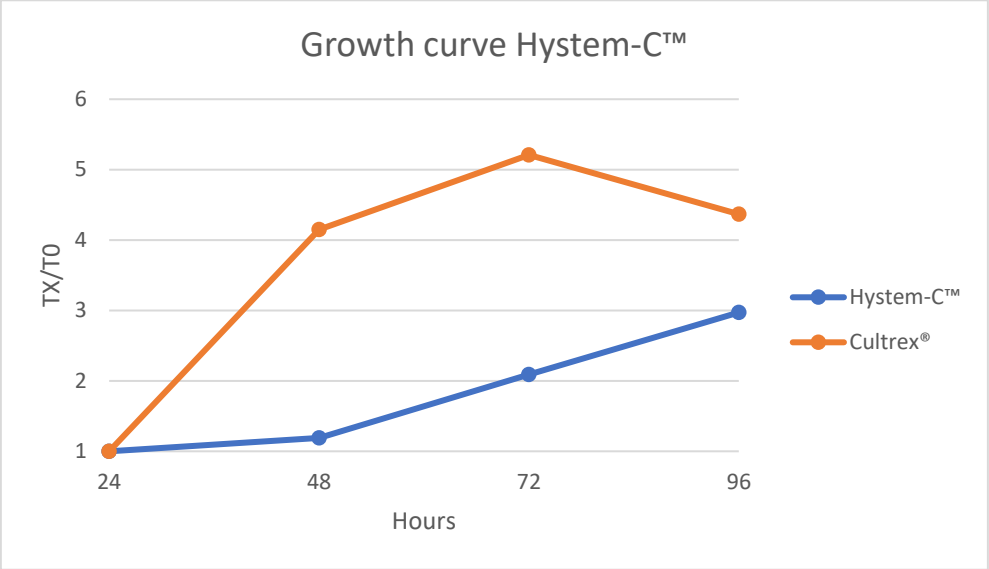


Figure 40. Graphic of growth of Hystem-C™ compared with Cultrex®

From the graphic above (figure 40) it is possible to see the trend of the growth curve of Hystem-C™ in comparison with Cultrex®. Obviously, Hystem™ was not included because it forms only two-dimensional structures.

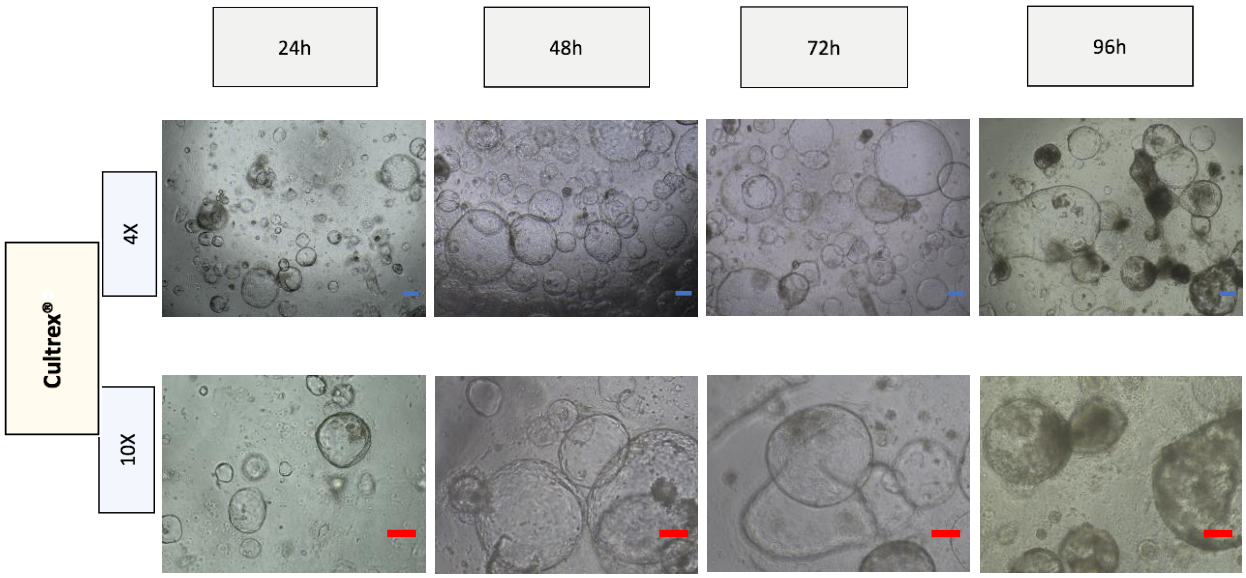
Analyzing this graphic in detail, it can be seen that Cultrex® increases its size up to 72 hours and then begins to decrease due to mortality. On the contrary, Hystem-C™ shows an exponential growth in its dimensions reaching almost the dimensions of organoids in Cultrex®. The explanation for this exponential growth is due to the fact that in Hystem-C™ the quantity of organoids obtained is significantly lower than those obtained with Cultrex® which are forced to die.

5.6 Comparative analysis

Here, in this section were reported in a comparative analysis the most promising scaffolds tested so far. Also in this case, there will be a qualitative analysis of the samples with optical microscope in two different enlargement 4X and 10X followed by a quantitative analysis with Resazurin presto Blue assay. The scaffold chosen for this comparative analysis are: Manchester Biogel Peptigels® Alpha1 (α1) and Gamma 2 (γ2), Hystem™-C, always compared with Cultrex®.

All of these matrices were plated at the same passage of the maintenance. Even though the Manchester biogel Peptigels® scaffolds modified with collagen give some results, it was chosen to not insert it in this comparative analysis because its hardworking procedure. Thus, for research applications it is better to use matrices which are easier to prepare.

5.6.1.Qualitative analysis:



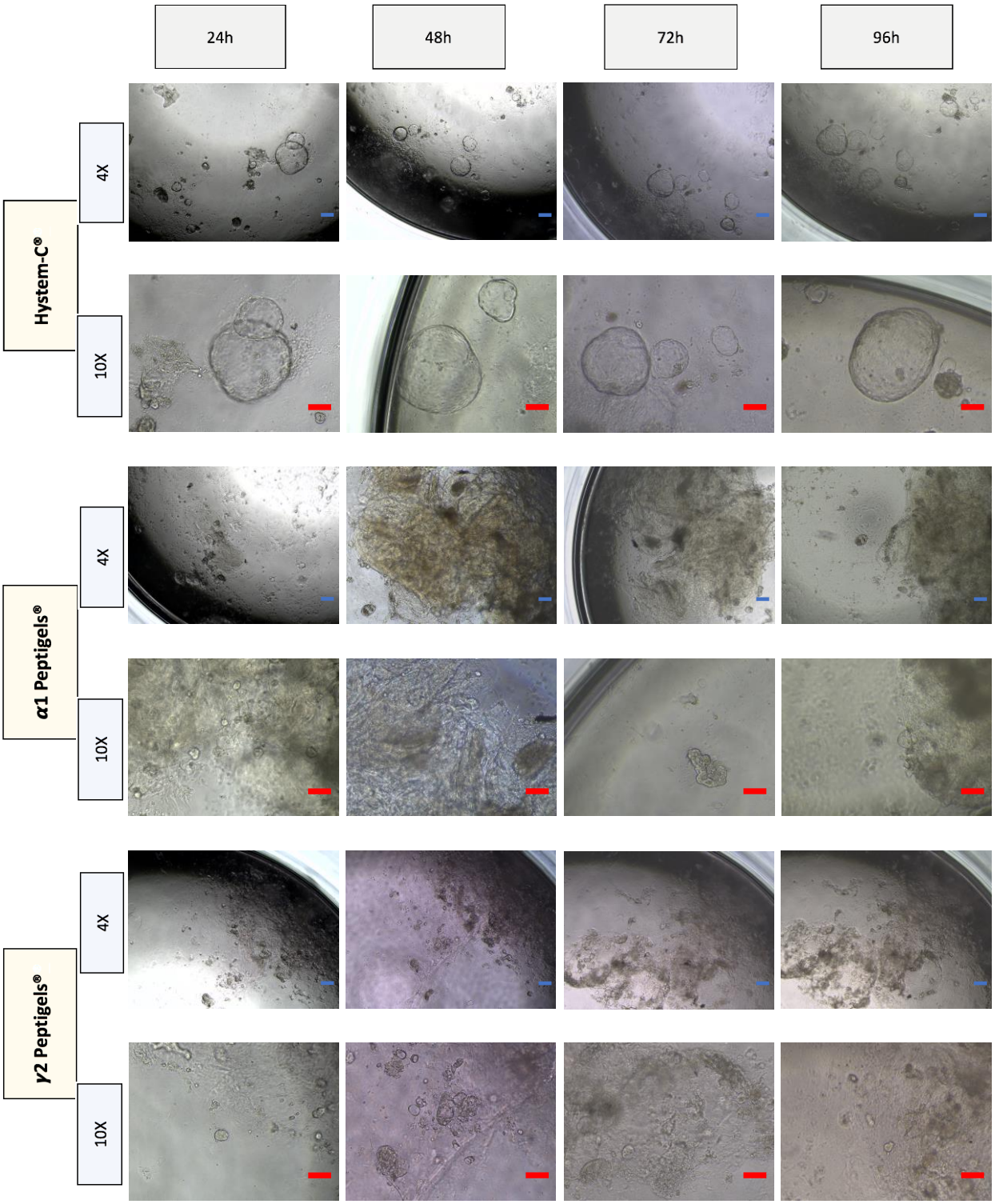


Figure 41. Hystem-c™, Manchester BIOGEL Peptigels® Alpha1, Gamma 2 compared with Cultrex® (4X and 10X enlargement). The red bar lower left represents the scale bar of 200 μm, the blue bar lower left represents scale bar of 100 μm

Here in figure 41 were reported in a comparative way the most promising scaffolds tested so far. Cultrex® was compared simultaneously in a triplicate with Manchester Biogel Peptigels® Alpha 1 (α1) and Gamma 2 (γ2), and with Hystem-C™. Among all the scaffolds tested Hystem-C™ gives the best results, even though the homogeneity is not comparable to the one obtained in Cultrex®. This could be observed especially in 4X enlargements. In 10X, indeed, it is possible to note that liver organoids in Hystem-C™ during the 96 hours still grow and reach bigger dimension (~600nm) than the one obtained by Cultrex®. Alpha 1 (α1) respects to the previous test (paragraph 5.3.1) gives worse results, this could be due to a mechanical error during plating where probably fewer starting organoids have been added coming from the split passage.

Gamma 2 (γ2) respect to the previous experiment produce more liver organoids. Also in this case, they are smaller than the ones obtained by Cultrex®. In addition, during days, it is possible to see an increase in growth in the different hours of incubation. The shape is quite regular and the dimensional range is ~ 100/200 nm.

Among these scaffolds it is possible to assert that the better scaffold is still the Cultrex®, followed by Hystem-C™. To better improve the efficacy of Alpha 1 and Gamma 2, it would be interesting to test it in different stiffness diluting them with Peptisol®.

5.6.2. Growth curve:

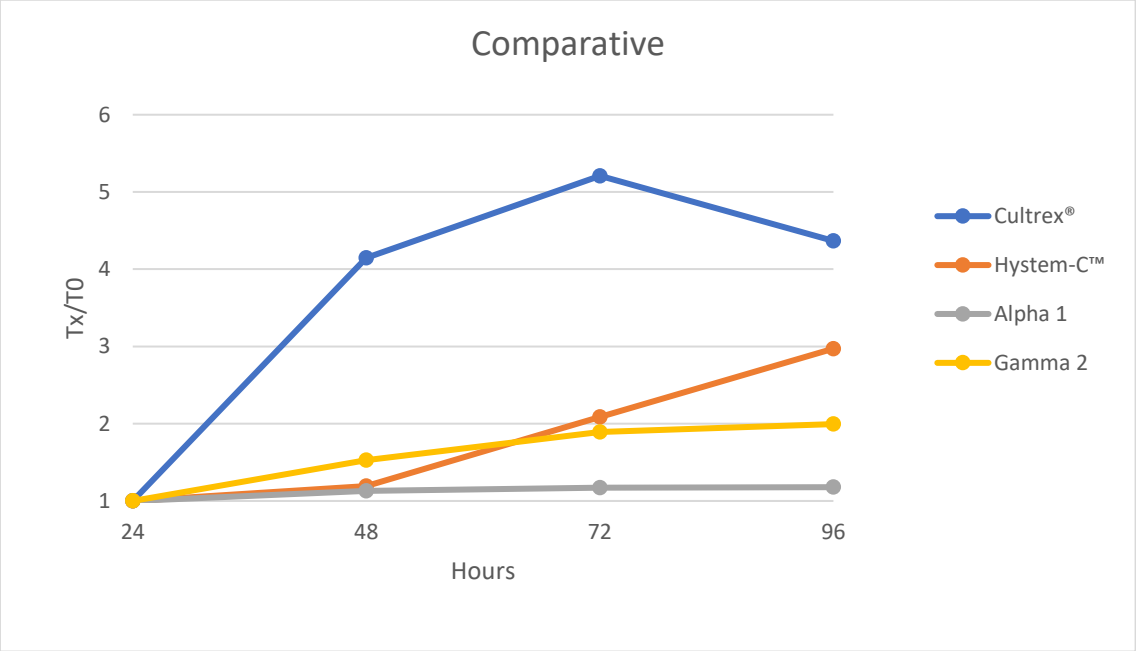


Figure 42. Comparative graphic of the growth curve of Cultrex®, Hystem-C™, Alpha 1 and Gamma2

From this graph, where the three best scaffolds are represented in comparison to Cultrex®, it is possible to see the differences between them.

Manchester biogel Peptigels® Alpha 1, in this case, is the worst of the four scaffolds because do not show a significant increase in the size and volume of its organoids. An interesting result was obtained by gamma 2. Unlike the previous test, the organoids are rounded and clear inside, which was not obtained in the previous analysis. The reason why they were not born in the previous analysis may be due to a plating error.

Hystem-C™, also in this case, is among the best of the tested synthetic scaffolds. The presence of collagen in its structure allows to mimic the composition and morphology of the Extra cellular matrix. Thus, providing an optimal substrate for the growth of organoids in cell cultures in three dimensions.

5. CONCLUSION

The use of organoids technology in biomedical and research fields saw a very increase during this last 30 years. The interesting characteristics related to organoids technology allow to obtain models which closely mimic the conditions that may have in vivo conditions. Cultrex® scaffolds is the matrix that is mainly used so far to obtain cell cultures in three dimensions. But, despite its ability to obtain highly efficient three dimensional culture, it has several disadvantages because of its natural derivation. One of the most important disadvantages may be that, since Cultrex® is a natural matrix, could generate substantial variability in different batches, thus producing false results (Kaur, et al., 2021). To overcome these problems, scientists are focusing their attention on the creation of synthetic hydrogels that mimic the typical characteristics of the Extracellular matrix, thus providing a microenvironment capable of supporting their growth in three dimensions. (Aisenbrey & Murphy, 2020)

This thesis therefore attempts to test different scaffolds alternatives to Cultrex®, trying to create a three dimensional culture with liver organoids.

In summary, the results obtained were not satisfactory. The first scaffold tested was the Biogelx™. This matrix, already from the qualitative analysis of the optical microscope, did not provide a good environment for organoids formation, but was suitable only for small clusters of cells that grew in two dimensions. Although it is divided into 4 different subtypes, functionalized with collagen, laminin and fibronectin, did not give acceptable results. In addition, problems related to the quantitative analysis based on Resazurin Presto blue assay also emerged. During the measurements, the values have risen exponentially, providing data that are absolutely not align with the qualitative analysis. Furthermore, the results were almost identical for the four different scaffolds. The high values obtained are thought to be mainly due to three facts: a presence of a two dimensional substrate, an undesirable chemical modification of the scaffold (by growth medium or cells) or, that the synthetic polymer, of which it is composed (fluorenylmethoxycarbonyl protecting group, Fmoc) generates a background fluorescence until 600 nm, thus influencing the results. Biogelx™ was tested in three different stiffness: Low (~ 05-2.5 kPa), Medium (~2-8 kPa) and High (~ 6.0-15 kPa). Despite the different stiffness, the results were all equals each other, suggesting that this matrix is not suitable for organoids formation.

The second scaffold tested in this thesis is the Manchester biogel Peptigels®. Also in this case it is divided into 5 subtypes and qualitative analysis were accompanied by quantitative analysis. Some of these subtypes Alpha 1 (α1) and Gamma 2 (γ2) have given rise to very small liver organoids. These, however, were limited in the growth due to the high viscosity of the matrix. It would have been interesting to dilute this scaffold with its Peptisol®, (product provided by the company to lower the stiffness) but due to a shipping problem it was not delivered. Indeed, the stiffness and viscosity of a general substrate affects the cell growth. For instance, when cells feel the rigidity of extra cellular matrix (in vivo conditions) change their morphology adapting their own cytoskeleton (Zhong & Ji, 2013). Also in this case the results of the viability test (Resazurin presto blue) gave results that did not agree with the qualitative analysis. Since the composition of Peptigels® is completely unknown, it was thought that, also in this case, it is composed of a peptide that emits in the emission range of resazurin (560-590 nm). Thanks to the birth of organoids in Alpha 1 and Gamma 2, it was possible to make growth curves which were then compared to Cultrex® matrix. Although liver organoids grew during the 96-hours of incubation in the synthetic scaffold, the results of the growth curve are not at the level of those obtained in Cultrex®.

An additional modification was performed on this scaffold by adding a 30% of collagen to see if the results were improved. The quantity of collagen to be added is in agreement with the percentage of it present in Cultrex®. Although improvements in subtype Alpha 4 were obtained, the overall results do not provide further improvements to the original scaffold, either in qualitative or quantitative analysis.

The last two scaffolds tested were Hystem™ and Hystem-C™. The first, in qualitative analysis generated only cell cultures in two dimensions, confirmed also by the quantitative tests. As for the second, organoids of shape and size comparable to those in Cultrex® were born. Thus, making Hystem-C™ the best among the scaffolds tested. But a limitation is the low homogeneity of the sample. The explanation of its superiority over the others may be due to the presence of collagen inside it.

What all this means, Cultrex® still remain the best choice to obtain a three dimensional culture of organoids. With Cultrex® were obtained homogeneous and spherical liver organoids as it was not possible to have them in the other scaffolds.

Future directions:

Trying to define a matrix that allows cell growth in three dimensions is of fundamental importance for clinical studies.

The simultaneous use of bio-printing, for the formation of synthetic matrices, the cell cultures and organoids technology could provide interesting ideas for positively increasing the characteristics in this field. In this way, complex cellular systems could be obtained, mimicking the environment found in vivo (in a simplified manner) in order that they can be applied for therapeutic treatments.

The use of three dimensional printers would simplify the homogeneity of the synthetic matrices by creating more superimposed planes that allow a better growth and dispersion of organoids in vitro. The difficulty in plating organoids was, indeed, widely found during the tests of this thesis.

The advent of bio-printing of synthetic scaffolds can therefore shift the attention to more eco-sustainable alternatives that do not require the use of laboratory animals, still obtaining high quality three dimensional organoids cultures.

Bibliography

Acevedo, L. M. et al., 2015. hESC Differentiation toward an Autonomic Neuronal Cell Fate Depends on Distinct Cues from the Co-Patterning Vasculature. *Cell Press*, Volume 4. DOI: 10.1016/j.stemcr.2015.04.013

AdvancedBiomatrix, 2021. *Advanced Biomatrix.* [Online]
Available at: https://advancedbiomatrix.com/hystem.html
[Consultato il giorno 2021].

Agrawal, T. et al., 2021. Recent advances in chemically defined and tunable hydrogel platforms for organoid culture. *Bio-Design and Manufacturing.* DOI: https://doi.org/10.1007/s42242-021-00126-7

Aisenbrey, E. A. & Murphy, W. L., 2020. Synthetic alternatives to Matrigel. *Nature*, Volume 5. DOI: https://doi.org/10.1038/s41578-020-0199-8

Alakpa, E. V., Jayawarna, . V., Lampe, A. & et al, 2016. Tunable Supramolecular Hydrogels for Selection of Lineage-Guiding Metabolites in Stem Cell Cultures. *Cell Press.* DOI*:* https://doi.org/10.1016/j.chempr.2016.07.001

Bahlmann, L. C., Fokina, A. & Schoichet, M. S., 2017. Dynamic bioengineered hydrogels as scaffolds for advanced stem cell and organoid culture. *MRS Comunications.* DOI: https://doi.org/10.1557/mrc.2017.72

Berkers, G. et al., 2019. Rectal Organoids Enable Personalized Treatment of Cystic Fibrosis. *Cell Press*, February. DOI: https://doi.org/10.1016/j.celrep.2019.01.068

Biogelx™, 2021. *Biogelx™.* [Online]
Available at: https://www.biogelx.com/wp-content/uploads/2021/04/PRO.BGX_.001-Biogelx-Powder-Preparation-and-Guidelines-for-Use-v17.pdf
[Consultato il giorno 2021].

Block, H. et al., 2021. Tracing Clonal Dynamics Reveals that Two- and Three-dimensional Patient-derived Cell Models Capture Tumor Heterogeneity of Clear Cell Renal Cell Carcinoma. Volume 7. DOI: 10.1016/j.euf.2019.06.009

Broutier, L. et al., 2016. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. *Nature Protocols.*
Broutier, L. et al., 2017. Human primary liver cancer–derived organoid cultures for disease modeling and drug screening. *Nature medicine,* 13

November. DOI: 10.1038/nprot.2016.097
Bunyaratavej, P. & Wang, H.-L., 2001. Collagen Membranes: A Review. *Journal of periodontology*. DOI: **https://doi.org/10.1902/jop.2001.72.2.215**

Cassereau , L. et al., 2015. A 3D tension bioreactor platform to study the interplay between ECM stiffness and tumor phenotype. *Journal of Biotechnology*, Volume 193. DOI: 10.1016/j.jbiotec.2014.11.008

Clevers, H., 2016. Modeling Development and Disease with Organoids. *Cell*, Volume 165. DOI: https://doi.org/10.1016/j.cell.2016.05.082

Corrò, C., Novellasdemunt, L. & S.W. Li, V., 2020. A brief history of organoids. *American Journal of Physiology-Cell Physiology*, May. DOI:https://doi.org/10.1152/ajpcell.00120.2020

Dekkers, J. F. et al., 2013. A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nature medicind*. DOI: https://doi.org/10.1038/nm.3201

Dong, C. & Lv, Y., 2016. Application of Collagen Scaffold in Tissue Engineering: Recent Advances and New Perspectives. DOI: https://doi.org/10.3390/polym8020042

Drost, J. & Hans, C., 2018. Organoids in cancer research. *Nature Review*. *DOI:* https://doi.org/10.1038/s41568-018-0007-6

Duzagc, F. et al., 2021. Microfluidic Organoids-on-a-Chip: Quantum Leap in Cancer Research. *MDPI*. DOI: https://doi.org/10.3390/cancers13040737

Eiraku, o. et al., 2008. elf-Organized Formation of Polarized Cortical Tissues from ESCs and Its Active Manipulation by Extrinsic Signals. *Cell Stem Cell*, Volume 3. DOI: 10.1016/j.stem.2008.09.002

Fischer, A. H., Jacobson, K. A., Rose, J. & Zeller, R., 2014. Hematoxylin and Eosin Staining of Tissue and Cell Sections. *Cold Spring Harbor Protocols*. DOI: 10.1101/pdb.prot073411

Funfak, A. et al., 2019. Biophysical Control of Bile Duct Epithelial Morphogenesis in Natural and Synthetic Scaffolds. *Frontiers in bioengineering and biotechnology*, December. DOI: https://doi.org/10.3389/fbioe.2019.00417

Giobbe, G. G. et al., 2019. Extracellular matrix hydrogel derived from decellularized tissues enables endodermal organoid culture. *Nature comunications*. DOI: https://doi.org/10.1038/s41467-019-13605-4

Giobbe, G. g. et al., 2019. Extracellular matrix hydrogel derived from decellularized tissues enables endodermal organoid culture. *Nature comunications.* DOI: https://doi.org/10.1038/s41467-019-13605-4

Gjorevski, N. et al., 2016. Designer matrices for intestinal stem cell and organoid culture. *Nature,* 16 November. DOI: https://doi.org/10.1038/nature20168

Godoy, P. et al., 2013. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of Toxicology volume,* Volume 87. DOI: 10.1007/s00204-013-1078-5

Hernandez-Gordillo, V. et al., 2020. Fully synthetic matrices for in vitro culture of primary human intestinal enteroids and endometrial organoids. *Biomaterials,* Volume 254. DOI: 10.1016/j.biomaterials.2020.120125

Hofer, M. & Lutolf, M., 2021. Engineering organoids. *Nature reviews,* 19 February. DOI: https://doi.org/10.1038/s41578-021-00279-y

Hu, H. et al., 2018. Long-Term Expansion of Functional Mouse and Human Hepatocytes as 3D Organoids. *Cell Press,* 29 November. DOI: https://doi.org/10.1016/j.cell.2018.11.013

Huch, M. et al., 2013. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature,* 2013 January. DOI: 10.1038/nature11826

Huleihel, L. et al., 2016. Matrix-bound nanovesicles within ECM bioscaffolds. *Science Advances,* 2(6). DOI: 10.1126/sciadv.1600502

Ingber, D. E., 2016. Reverse Engineering Human Pathophysiology with Organs-on-Chips. *Cell Press,* 16 March. DOI :https://doi.org/10.1016/j.cell.2016.02.049

Katyal, P., Mahmoudinobar, F. & Montclare, J. K., 2020. Recent trends in peptide and protein-based hydrogels. *Current Opinion in Structural Biology,* Volume 63. DOI: https://doi.org/10.1016/j.sbi.2020.04.007

Kaur, S. et al., 2021. Non-matrigel scaffolds for organoid cultures. *Cancer Letters. DOI: https://doi.org/10.1016/j.canlet.2021.01.025*

Kim, S. S., Penkala, R. & Abrahimi, P., 2007. A Perfusion Bioreactor for Intestinal Tissue Engineering. *Journal of Surgical Research,* October.142(2).

DOI: 10.1016/j.jss.2007.03.039

Kitano , K. et al., 2017. Bioengineering of functional human induced pluripotent stem cell-derived intestinalgrafts.*NatureComunications. DOI:https://doi.org/10.1038/s41467-017-00779-y*

Kleinstreuer, N. C. et al., 2014. Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. *Nature Biotechnology,* 2018 May. DOI: https://doi.org/10.1038/nbt.2914

Klotz, B. J. et al., s.d. A Versatile Biosynthetic Hydrogel Platform for Engineering of Tissue Analogues. *Advanced Healthcare materials,* Volume 8. DOI: https://doi.org/10.1002/adhm.201900979

Kopper, O. et al., 2019. An organoid platform for ovarian cancer captures intra- and interpatient heterogeneity. *Nature medicine.DOI: https://doi.org/10.1038/s41591-019-0422-6*

Krüger, M. et al., 2020. Cellulose Nanofibril Hydrogel Promotes Hepatic Differentiation of Human Liver Organoids. *Advanced Health Care Materials. DOI: https://doi.org/10.1002/adhm.201901658*

Lancaster, M. A. & Knoblich, J. A., 2014. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science,* Volume 345. DOI: 10.1126/science.1247125

Li, C., Uribe, D. & Daling, J., 2005. Clinical characteristics of different histologic types of breast cancer. *Nature. DOI: https://doi.org/10.1038/sj.bjc.6602787*

Li, X., Ootani, A. & Kuo, C., 2016. An Air–Liquid Interface Culture System for 3D Organoid Culture of Diverse Primary Gastrointestinal Tissues. *Gastrointestinal Physiology and Diseases: Methods and Protocols, Methods in Molecular Biology. DOI: 10.1007/978-1-4939-3603-8_4*

Lih, E. et al., 2016. Biomimetic Porous PLGA Scaffolds Incorporating Decellularized Extracellular Matrix for Kidney Tissue Regeneration. *Applied Materials & Interfaces. DOI: https://doi.org/10.1021/acsami.6b03771*

Lih, E. et al., 2016. RETURN TO ISSUEPREVRESEARCH ARTICLENEXT Biomimetic Porous PLGA Scaffolds Incorporating Decellularized Extracellular Matrix for Kidney Tissue Regeneration. *ACS Pubblications.*

Liu, W. et al., 2014. Microcryogels as injectable 3-D cellular microniches for site-directed and augmented cell delivery. *Acta Biomaterials,* Volume 10. DOI: DOI: 10.1016/j.actbio.2013.12.008

Lohrasbi , S. et al., 2019. Collagen/cellulose nanofiber hydrogel scaffold: physical, mechanical and cell biocompatibility properties. *Pharmaceutical sciences reaserch Center. DOI: https://doi.org/10.1007/s10570-019-02841-y*

Manchester, B., 2021. *Manchester biogel Peptigels®.* [Online]
Available at: https://manchesterbiogel.com/
[Consultato il giorno 2021].

Marusina, A. I. et al., 2019. Tunable hydrogels for mesenchymal stem cell delivery: Integrin-induced transcriptome alterations and hydrogel optimization for human wound healing. *Stem Cell Journal. DO*I: https://doi.org/10.1002/stem.3105

McKee, T. J., Perlman, G., Morris, G. & V., K. S., 2019. Extracellular matrix composition of connective tissues: a systematic review and meta-analysis. *Nature,* 22 July. DOI: https://doi.org/10.1038/s41598-019-46896-0

Michielin, F., Giobbe, G. G. & Luni, C. E. a., 2020. The Microfluidic Environment Reveals a Hidden Role of Self-Organizing Extracellular Matrix in Hepatic Commitment and Organoid Formation of hiPSCs. *Cell Press. DOI:10.1016/j.celrep.2020.108453.*

Mostajo-Radji, M. A., Schmitz, M. T., Torres Montoya, S. & Pollen, A. A., 2020. Reverse engineering human brain evolution using organoid models. *Brain Research. DOI: 10.1016/j.brainres.2019.146582*

Nelson, C. & Bissel, M. J., 2006. Of Extracellular Matrix, Scaffolds, and Signaling: Tissue Architecture Regulates Development, Homeostasis, and Cancer. *Annual Review of Cell and Developmental Biology,* Volume 22.
Olayanju, A. et al., 2021. Self-Assembling Peptide Hydrogels - PeptiGels® as a Platform for Hepatic Organoid Culture. *bioRxiv.*

Park, J. S. et al., 2011. The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-β. *Biomaterials,* Volume 32. DOI: 10.1016/j.biomaterials.2011.02.019

Park, S. E., Georgescu, A. & Huh, D., 2019. Organoids-on-a-chip. *Science,* Volume 364. DOI: 10.1126/science.aaw7894

Pelin, S.-M., Sultan, G.-I. & Avcid, C., 2019. Bioengineering-inspired three-dimensional

culture systems: Organoids to create tumor microenvironment. *Gene*, Volume 686. DOI: https://doi.org/10.1016/j.gene.2018.11.058

Peng, W. C. et al., 2018. Inflammatory Cytokine TNFα Promotes the Long-Term Expansion of Primary Hepatocytes in 3D Cultur. *Cell Press*, 29 November. DOI: https://doi.org/10.1016/j.cell.2018.11.012

Pera, M., Reubinoff, . B. & Trounson, A., 2000. Human embryonic stem cells. *Journal of Cell Science. DOI: https://doi.org/10.1242/jcs.113.1.5*

Primohamed, M. et al., 2004. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. *thebmj*, 11 june. DOI: 10.1136/bmj.329.7456.15

Prior, N., Inacio, . P. & Huch, M., 2019. Liver organoids: from basic research to therapeutic applications. *BMJ journals,* June. DOI: 10.1136/gutjnl-2019-319256

Qian, X. et al., 2016. Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. *Cell press.*

R&D, S., 2021. *R&D Systems.* [Online]
Available at: https://www.rndsystems.com/products/cultrex-stem-cell-qualified-reduced-growth-factor-basement-membrane-extract_3434-010-02
[Consultato il giorno 2021].

Raja, W. K. et al., 2016. Self-Organizing 3D Human Neural Tissue Derived from Induced Pluripotent Stem Cells Recapitulate Alzheimer's Disease Phenotypes. *Plos One. DOI: https://doi.org/10.1371/journal.pone.0161969*

Ramachandran, S. D. et al., 2015. In Vitro Generation of Functional Liver Organoid-Like Structures Using Adult Human Cells. *Plos One.*
Roberts, D., Rochas, C., Saiani, A. & Miller, A., 2012. Effect of Peptide and Guest Charge on the Structural, Mechanical and Release Properties of β-Sheet Forming Peptides. *Langmuir. DOI: 10.1021/la303328p*

Ryan, K. et al., 2015. Nanoscale piezoelectric properties of self-assembled Fmoc-FF peptide fibrous networks. *ACS, Applied Material and interfaces. DOI: https://doi.org/10.1021/acsami.5b01251*

Sachs, N. et al., 2018. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. *Cell Press. DOI:* https://doi.org/10.1016/j.cell.2017.11.010

Saheli, M. et al., 2017. Three-dimensional liver-derived extracellular matrix hydrogel

promotes liver organoids function. *Journal of cellular biochemistry. DOI:* https://doi.org/10.1002/jcb.26622

Sang , Z. W. et al., 2018. 3D bioprinted functional and contractile cardiac tissue constructs.*ActaBiomaterial,*Volume70.DOI:https://doi.org/10.1016/j.actbio.2018.02.007

Schweinlin, M. et al., 2016. Development of an Advanced Primary Human In Vitro Model of the Small Intestine. *Tissue Engineering,* Volume 22. DOI: 10.1089/ten.TEC.2016.0101

Selkoe, D. J. & Hardy, J., 2016. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO molecular medicine.*

Streuli, C. H., Bailey, N. & Bissell, . M. J., 1991. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity.. *Journal of cell Biology. DOI: 10.1083/jcb.115.5.1383*

Takebe, T. & Wells, J. M., 2019. Organoids by design. *Science.*

Takebe, T. et al., 2014. Generation of a vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature protocol,* 23 January. DOI: 10.1038/nprot.2014.020

Vlachogiannis, G. et al., 2018. Patient-derived organoids model treatment response of metastaticgastrointestinal cancers. *Science,* Volume 359. DOI: 10.1126/science.aao2774

Wang, Z. et al., 2018. 3D bioprinted functional and contractile cardiac tissue constructs. *Acta Biomaterial,* Volume 70. DOI: https://doi.org/10.1016/j.actbio.2018.02.007

Weiss, P. & Taylor, A. C., 1960. RECONSTITUTION OF COMPLETE ORGANS FROM SINGLE-CELL SUSPENSIONS OF CHICK EMBRYOS IN ADVANCED STAGES OF DIFFERENTIATION. *National accademy of Science.*

Workman, M. J. et al., 2016. Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system. *Nature Medicine,* 21 November.

Xing, D. et al., 2020. Engineering 3D functional tissue constructs using self-assembling cell-laden microniches. *Acta Biomaterial,* September.Volume 114. DOI: 10.1016/j.actbio.2020.07.058

Yan-Ru, L. & Leung, A. W., 2018. Next generation organoids for biomedical research

and applications. *Biotechnology Advances. DOI: 10.1016/j.biotechadv.2017.10.005*

Ye, S. et al., 2020. A Chemically Defined Hydrogel for Human Liver Organoid Culture. *Advanced functional Biomaterials. DOI: https://doi.org/10.1002/adfm.202000893*

Yeung, T. et al., 2004. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cytoskeleton*, 60(1). DOI: 10.1002/cm.20041

Yui, S. et al., 2012. Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5+ stem cell. *Nature medicine. DOI: https://doi.org/10.1038/nm.2695*

Zarembinski, T. I. & Skardal, A., 2018. HyStem®: A Unique Clinical Grade Hydrogel for Present and Future Medical Applications. *Intech Open.*

Zhang, M., Chen, Y., Li, G. & Du, Z., 2010. Rheological properties of fish skin collagen solution : Effects of temperature and concentration. *Rheology Journal Korea-Australia*, 22(2).

Zhong, Y. & Ji, B., 2013. Impact of cell shape on cell migration behavior on elastic substrate. *International society of biofabrication.*

Zhou, J. et al., 2017. Human intestinal tract serves as an alternative infection route for Middleastrespiratorysyndromecoronavirus.*Science*,November.DOI:10.1126/sciadv.aao4966