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Development of microfluidic devices using 3D printing for cell culture and drug testing

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ABSTRACT

CHAPTER 1: Advanced multiwell devices for cell culture and drug testing

1.1 CELL CULTURE: background and challenges

Cell cultures are systems in which cells are isolated from their natural environment, and continue their life process within a well-defined parameters. This cell-life condition, known as "in vitro", represent a fundamental tool for the study and for understanding of cell biology, tissue morphology, the mechanisms of various diseases and the action of drugs.

The history of cell cultures traces its roots back to 1907, when Harrison performed the first cell cultures during his research on the origin of nerve fibers. Since then, the advancement of biotechnologies, materials (both natural and artificial), and various culture media has led to the improvement and refinement of the method, making it possible to observe cell growth and differentiation outside the body. [1]

Nowadays, it is possible to classify cell cultures into two main families: two-dimensional culture systems and three-dimensional culture systems.

2D culture systems are characterized by a relatively simple structure composed of a single layer of cells (monolayer) placed in a specific nutrient-rich basal medium that allows for their proliferation. Specifically, the cells under investigation are placed inside a glass or plastic sterile container, e.g. Petri dish, that provides mechanical support. A fundamental aspect of cell cultures is the maintenance of culture conditions: it is necessary to ensure thermal stability, pH level and complete sterility of the medium. This latter aspect is crucial to avoid the presence of microorganisms that could secrete toxic substances harmful to the cells.

Growth in two-dimensional monolayers ensures that cells receive nearly identical amounts of nutrients, which guarantees homogeneous development. This characteristic, combined with simplicity and high efficiency, makes the use of such models very appealing in biomedical research.

2D in vitro models can be used for various applications. It is possible to study certain cellular processes that, under specific controlled conditions, can provide information about the behaviour of the cell line. They can also be employed in the study of the molecular mechanisms of a pathology, with the aim of identifying the characteristics underlying the onset of the disease and the subsequent progression of a specific illness. [2]

Finally, they can be used in the field of "drug discovery," which involves testing the effects of various chemical and pharmaceutical compounds on specific cell types.

Despite their various applications, 2D cell cultures remain a relatively limited tool because it is very difficult to mimic the immense complexity of the human body with such simple models. In vivo, the cellular microenvironment is rich in factors that influence cellular behavior, such as pH, the presence of ions, soluble factors, and more. Due to their simplicity and the lack of a complex, information-rich environment, 2D systems cannot replicate the cellular development processes observable in physiological contexts. [4] Therefore, they serve as a good starting point for obtaining information that remains general and must be supplemented by further observations made in more specific models, such as 3D models or directly in vivo. [3] Nevertheless, although these limitations are real and challenging to overcome, 2D models continue to be used in various research areas due to their cost-effectiveness and ease of reproducibility. [5]

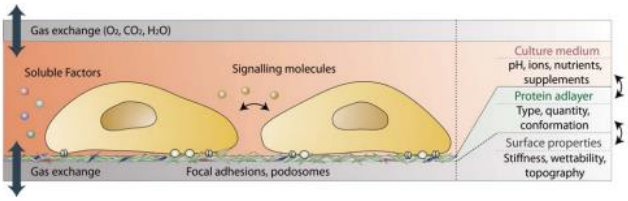

Figure 1. Cellular microenvironment in vitro

It is precisely because of these limitations that the introduction of more complex tools, such as 3D models, is becoming of paramount importance. This transition has been of crucial in the field of biomedical research because these models allow for cell culture growth that more closely resembles what occurs in vivo. [6]

Cells within our bodies aggregate to create tissues, which in turn form organs. Therefore, the ability to represent such structures is essential for conducting more specific studies of what happens in a particular tissue and/or organ when a certain physiological or pathological condition develops or spreads. Reproducing the human body remains a challenge for physicians, biologists, and bioengineers who have been striving for years to represent the mechanisms occurring in both healthy and diseased tissues. [7] 3D cell culture aims exactly at this goal.

An important aspect for the development of systems capable of achieving tissue-like cellular organization is the presence of various factors influencing cellular behavior, such as the architecture of the extracellular matrix (ECM), its composition, mechanical properties, and numerous interactions among these elements. In standard two-dimensional models, cells are cultured on a surface with higher rigidity (e.g., plastic Petri dish, approximately 105 kPa) compared to the ECM rigidity of tissues, which ranges from 1 to 25 kPa. Different rigidity induces different cytoskeletal conformations, affecting cellular polarity, metabolism, and protein expression. The turning point in 3D culturing was therefore determined by the ability to produce systems that mimic tissue-specific ECM, inducing cellular differentiation and organization resembling that of the tissue under study. [5]

From the initial discovery of the possibility to reproduce a specific environment mimicking the in vivo model, the 3D approach has immediately begun to capture the attention of the researchers. A 3D culture can be defined as "a culture that can mimic the organization and microarchitecture of a living organ," making it indeed a much closer tool to the in vivo model compared to the previously mentioned 2D models.[8]

In these systems, cells can grow within a three-dimensional structure using various supports, which can be synthetic, such as ceramics, metals and polymers, or natural, such as polysaccharides, proteins, ECM derivatives or hydrogels. Alternatively, cells can also grow freely in systems called spheroids and organoids. [6]

The 3D models, besides being innovative in the field of biomedical research, also represent an innovative method in the pharmaceutical and cosmetic industries. Among the potential applications, "drug discovery" is certainly one of the most relevant. In this context, three-dimensional cultures offer the possibility of obtaining more specific and reliable results compared to the exclusive use of two-dimensional systems. The additional dimension of 3D cultures not only influences the spatial organization of surface cellular receptors but also imposes physical constraints on the cells themselves. These spatial and physical aspects in 3D cultures affect signal transduction from the outside to the inside of the cells and, consequently, gene expression and related cellular behaviours. This leads to obtaining pharmacological treatment responses more like what occurs in vivo. [9]

The following table shows the differences between a 2D culture system and a 3D culture system, highlighting the advantages and disadvantages of using both types of systems:

Features	**2D Culture System**	**3D Culture System**
Cell shape	Flat and elongated Mono-layer	Normal cell shape Aggregates/Spheroids Multiple layers
Exposure to the medium	All cells receive the same amount of nutrients and growth factors	Not all cells receive the same amount of nutrients and growth factors Possible necrotic core

Cell junction	Less common and accurate than real cell junctions	Common and allow communication between cells
Cell differentiation	None	Well differentiated
Drug sensitivity	Drugs are poorly metabolized Low resistance to drug treatment	Better drug metabolism Greater resistance to drug treatment Better representation of drug effects
Cell proliferation	Cell prolifarate at an unnatural rate	Realistic proliferation rate
Expression levels	Gene and protein expressions different from those in in vivo models	Gene and protein expressions similar to those in in vivo models
Costs	Cheaper	More expensive
Cell apoptosis	Greater apoptotic effects	Lesser apoptotic effects

Table 1. Differences between 2D Culture System and 3D Culture System

1.2 In vitro DRUG TEST: background and challenges

Over the past 30 years, the process of drug discovery and development has become increasingly expensive and risky.

Generally, for the commercialization of drugs (or medical devices), it is necessary to follow specific sequential processes to validate them.

The drug must pass through two phases to be marketed: the preclinical phase and the clinical phase. In the preclinical phase, simplifications are made compared to the human body. In this phase, studies on the drug to be commercialized are conducted outside the physiological environment: animal models and in vitro models are used. In the clinical phase, we can distinguish four sub-phases:

1. Phase 1 involves studying the safety of the drug in a limited population of healthy individuals.

2. In Phase 2, the efficacy of the drug is evaluated in a larger population.

3. Phase 3 entails a more thorough study of the drug's efficacy and safety, maximizing the number of participants.

4. Phase 4 involves long-term observation of the product to identify any late side effects (clinical follow-up). [10]

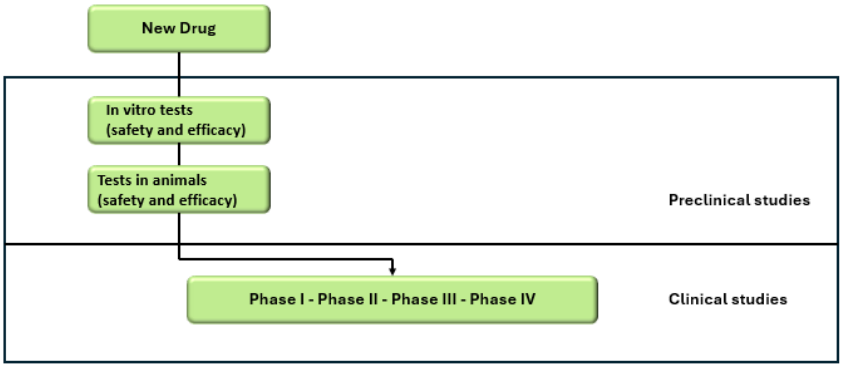

Figure 2. Diagram of drug development

Initially, the main and most widely used method for studying the onset of diseases and testing new drug therapies relied on animal models: indeed, they have a better organization compared to that which can be found in a two-dimensional in vitro construct. However, there are three main issues associated with the use of animal models:

1. The most significant issue is the *ethical concern*. The use of animals for experimentation in pharmacology and toxicology dates back to the last century and often involves the sacrifice of the animals at the end of the study.
   The main idea to address this issue is to follow the principle of the 3R.
   This principle was formulated by British scientists William Russel and Rex Burch in 1959 with the idea of replacing the use of animals in experiments. Since this goal was not immediately achievable, the two scientists encouraged the use of all possible means to improve animal welfare through reduction and refinement. When we talk about the 3R principle, in fact, we are referring to:
   1. **R**eplacement: replacing animal material with human material.
   2. **R**eduction.
   3. **R**efinement.
   This principle has been included in Directive 2010/63/EU on the protection of animals used for scientific purposes as a legal obligation to be applied to all aspects of the care and use of animals. Guidelines have been provided for EU funding requests for research projects involving animal experimentation. Detailed information must be provided on why live animals need to be used and why that particular choice was made.[11]
2. *Efficacy issue*: Using animal models involves simplifications. The use of animal models involves studying models that have differences both physiologically and anatomically compared to an individual. This is a problem of significant relevance, as especially in the validation of new drugs, many characteristics are lost in the

transition from the animal model to the human model, which affects the efficacy of the finding under examination, thus leading to a failure in transitioning to the subsequent phases of the clinical trial, hence not achieving validation.

3. Problem concerning *disease development*. While it is true that some animals can develop certain diseases associated with humans (diseases for which treatments are sought), it is also true that there are other diseases that some animals are unable to develop (such as neurodegenerative and tumor diseases). For this reason, animals requiring genetic modifications to enable them to develop specific diseases should be implemented. However, this often results in outcomes that are not like what would occur in humans. [10]

For the reasons mentioned above, efforts have been made to explore alternatives to the use of animal models (where possible) by developing experimental 2D and 3D models that exhibit biomimetic characteristics, aiming to achieve models that are increasingly similar to the in vivo environment.

In the biomedical field, the most commonly used tools are cell cultures. They can be of human or animal origin and, by seeking to faithfully reproduce tissue at a physiological level, they offer the possibility of studying and investigating various aspects of cellular functions.

Cellular assays thus become the key tool used to assess the potential efficacy of a new compound in drug discovery. To obtain more reliable results, the culture model used as a testing platform must function similarly to cells in vivo.

In the case of 2D cultures, they are only able to represent a "small portion" of the human body, mimicking the disease and how it is intended to be treated to a minimal extent. Therefore, the results of experiments with this type of tool have limitations:

a. While cells grow well in the laboratory, they are not representative of the variety of cells that make up a tissue.

b. Cells in culture do not receive signals from other cells in the body that influence their behavior.

Due to these limitations, three-dimensional cellular culture systems are gaining increasing interest in the field of drug discovery. They allow for more specific and reliable results compared to 2D systems. [9]

Several studies have shown that the way cells in three-dimensional systems respond to pharmacological treatments is similar to what happens in vivo, unlike in 2D systems.[12]

Several studies have highlighted a greater similarity in responses to pharmacological treatments in 3D cultures compared to 2D cultures, considering what happens in vivo. Numerous studies have shown that cells cultured in three-dimensional models are more resistant to anticancer drugs compared to 2D cultures. For example, the survival and proliferation of ovarian cancer cells in 3D cultures after treatment with paclitaxel

Commentato [IR1]: Ma qua l'hai scritto la riga sopra

Commentato [IR2]: E anche qua

were reduced by 40% to 60% in cell spheroids, while the same treatment led to an 80% reduction in cell viability in the 2D cell monolayer. [13]

Although 3D cell cultures have gained increasing attention in drug screening, many currently available 3D culture techniques require a lot of time, are costly, and lack reproducibility. Scientists are thus engaged in developing new rapid standard protocols for the use of three-dimensional cultures in pharmacological screening.[14]

1.3 MICROFLUIDIC AND 3D PRINTING FOR CELL COLTURE AND ADVANCED DRUG TESTING

Microfluidics deals with the study of fluids (gases and liquids) on a microscopic scale.

Commentato [IR3]: Secondo me questa non è la definizione vera. E poi ci fva un riferimento

In the last decade, the use of microfluidics has become increasingly important in biochemical and clinical applications. In particular, one of the areas where it seems to be attracting more attention is drug screening, and this is because nowadays drug discovery is a multibillion-dollar endeavor that requires a significant initial investment in terms of both time and capital, without guaranteeing any success.

Current studies indeed show that the development and study of a drug take approximately 15 years. Despite the high time and costs involved, data indicate that 7 out of 10 drugs do not recoup the research and development (R&D) costs incurred.

Commentato [IR4]: Riferimento?

This translates into a growing interest in the development of new devices that allow for biochemical experimentation/analysis of drugs on individual living cells using small amounts of fluid. These devices are called microfluidic devices. Using such devices brings the advantages of reducing cell consumption, enabling the automated addition of reagents, and reproducibly mixing reagents with cells. [15]

The Reynolds number (*Re*) is one of the most important parameters describing the flow in these systems. It is defined as the ratio of inertial forces to viscous forces:

$$Re = \frac{\rho v L}{\mu}$$

With ρ $\left(\frac{kg}{m^3}\right)$ fluid density

v $(\frac{m}{s})$ velocity

L (m) characteristic length and

μ $(Pa * s\ o\ \frac{N*s}{m^2}\ o\ \frac{kg}{m*s})$ dynamic viscosity.

The Reynolds number provides information about the flow regime. We can speak of laminar flow and turbulent flow: in the former case, the flow lines are parallel, while in the latter case, the flow lines are chaotic, and mixing is rapid, uncontrollable, and difficult to calculate.

In microfluidic devices, the Reynolds number is often less than unity, so laminar flows can be observed throughout the device.[16]

For the realization of such devices, microfabrication techniques are employed.

This type of production techniques is suitable for creating structures with defined shapes and positions on a micrometer scale that can be used to position cells and tissues, controlling the shape and function of cells in a controlled manner.

Microfabrication has found extensive applications across various domains in biology and medicine. These include the development of tools for molecular biology and biochemistry, advancements in cell biology instrumentation, the creation of medical devices, and the innovation of biosensor technologies.[17]

Soft lithography is the primary technique for manufacturing microfluidic devices. It replaces conventional photolithography, which is still used for creating devices larger than 100 nm in size, in order to achieve micro- and nanofabrication.[18]

Commentato [IR5]: E in cosa consiste la soft lithography?

Today, 3D printing represents a promising alternative to soft lithography. It allows for the creation of structures with well-defined geometries at a lower cost. The use of this type of technology, for example, eliminates the need to work in cleanrooms. Recent advancements in 3D printing technologies have enabled the production of highly complex microfluidic devices, which can be obtained in a single step or in multiple steps, assembling various components printed in 3D.

This alternative represents a rapid and economical production technique, making microfluidics more accessible to users. [19]

The use of 3D printers for PDMS microfluidic devices is particularly promising for cell culture applications. The printing process does not alter the properties of PDMS, such as biocompatibility and oxygen permeability, resulting in reduced overall costs and fabrication time.

Commentato [IR6]: E questo come è saltato fuori?

The low cost, easy surface modification and high gas permeability are the key properties of PDMS that make it a highly popular material for microfluidic-based systems. Its transparency is necessary and useful for users to observe what happens inside the device. [20]

Besides PDMS, 3D printing can be used with other types of resins, both commercial and non-commercial. However, for use in biomedical applications, the microfluidic devices produced must be made from materials that do not react with or absorb protein reagents and nucleic acids. The resins must also be biocompatible, so that when cells come into contact with them, they do not undergo cell death. Currently, many commercial resins are already cytocompatible. [21]

The increasingly widespread adoption of microfluidics is indeed due to the discovery and development of new materials.

With the continuous development of materials and the resulting increase in interest in microfluidics, attention has also been focused on a new concept, that of the organ-on-a-chip.

The term "organ-on-a-chip" refers to a biotechnology that currently represents one of the most promising and exciting developments in the field of microfluidics.

These devices aim to miniaturize and mimic a specific organ. They enable the simulation of crucial physiological signals, such as vascularization, thereby improving the emulation of in vivo physiological conditions for the study of various biological processes.

The main advantages lie in the ability to recreate a controlled and dynamic environment capable of mimicking the mechanical and physiological signals originating from cell cultures, making it a complex environment similar to what we find in the human body. Additionally, these systems have relatively low costs and rapid fabrication times. [11]

These biochips, therefore, emerge as promising candidates to replace animal models currently used in predicting human responses, given the inherent species differences and ethical debates surrounding them. The development of these microchips is associated with the idea that in the future, they may serve as the starting point for drug toxicity testing, leading to a reduction in the number of failures that occur when transitioning from preclinical to clinical studies in drug development. [6]

1.4 EXAMPLE OF MULTIWELL PLATES WITH INTEGRATED MICROFLUIDIC: FABRICATION AND APPLICATIONS

The continuous development of new microfluidic devices and their small dimensions has led to their use in cell culture, testing new drugs, and studying interactions between cells and specific molecules that can occur at the physiological level.
Lee et al. have developed, using stereolithography, a complete device that does not require additional components.

Commentato [IR7]: In che senso?

Specifically, they created a device for the detection of pathogenic bacteria consisting of a helical microchannel. The authors used clusters of magnetic nanoparticles functionalized with antibodies capable of binding to E. coli. The device features a trapezoidal cross-sectional shape to prevent the accumulation of particles adjacent to the inner walls of the channels.
By utilizing functionalized particles, the authors were able to separate clusters of individual particles from clusters of particles bound to the bacteria based on particle size, achieving a detection limit of 10 cfu/ml in a buffer solution and 10 cfu/ml in milk. [19]

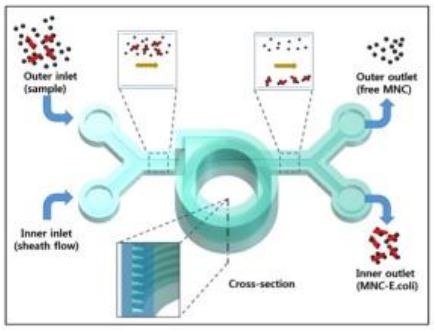

Figure 3. 3D-printed microfluidic system for bacteria detection

In addition to simple cell culture or drug testing, microfluidic devices can also be used to support malfunctioning organs. To address liver dysfunction in patients with liver pathologies, Park et al. developed a bioartificial liver device (BAL) that allows long-term survival of hepatocytes. They designed a radial flow bioreactor to protect the seeded hepatocytes from shear stresses due to metabolic exchange that could be harmful. This device consists of a stack of circular glass substrates with concentric microgrooves in a polycarbonate housing. A peristaltic pump was used to circulate culture medium between the reservoir, oxygenator, and bioreactor. Once the critical shear stress value of 0.33 dyn/cm^2 was identified, it was observed that with a flow rate of 18 ml/min, this threshold was still not exceeded. This device demonstrated that after 36 hours of perfusion, the viability of hepatocytes was around 95% at various radial distances observed (20, 12, and 5 mm) on substrates with microgrooves, while in those without microgrooves, cellular viability ranged from 98% to 0% depending on the observed distances.

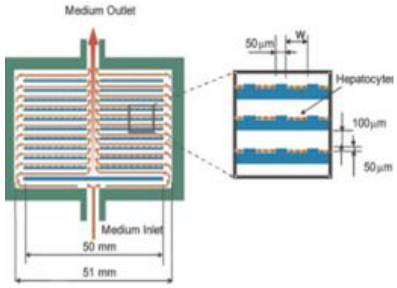

Figure 4. Schematic diagram of the radial-flow bioreactor with microgrooves for 2D culture of Hepatocytes

Based on the architecture of hepatic organs, Carraro et al. developed a two-layer microfluidic device featuring a network of channels mimicking blood vessels in one layer and a parenchymal chamber in the other. The layers were separated by a nanoporous polycarbonate membrane allowing metabolite transfer but protecting hepatocytes from excessive shear stress.

The width of the main inlet and outlet channels is 1650 μm, gradually branching and decreasing to 35 μm.
The fluid was injected into the system using a syringe pump with a flow rate of 0.5 ml/h. With this flow, the device demonstrated the ability to maintain viable hepatic cells, their proliferation, and functionality for 14 days.
Carraro et al. then considered how to further improve the number and vitality of cells by coating the polycarbonate membrane with collagen that mimics the extracellular matrix.
Budoin et al., instead of focusing on the vitality of liver cells, developed a device for studying the cultivation of the HepG3/C3A hepatocarcinoma cell line. The device consists of two layers of PDMS, characterized by microstructures designed to enhance 3D cell culture, on the lower layer. These microstructures consist of microchambers and microchannels placed within a cell culture chamber with a geometry suitable for uniform flow. Inlets and outlets were present in the upper layer.
This device was used to study the metabolic activity of cells at different flow rates of the medium (0.10 and 25 μl/min) to conduct a toxicological analysis. [15]

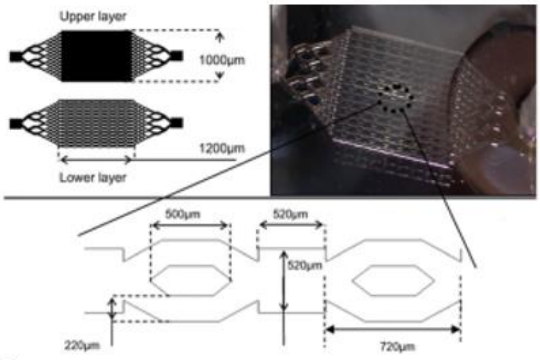

Figure 5. Design of the microfluidic bioreactor for hepatocarcinoma cultivation in 3D cell layers

CHAPTER 2: ADDITIVE MANUFACTURING AND 3D PRINTING

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