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### **DIPLOME DE DOCTORAT**

*Spécialité : Sciences pour l'ingénieur – Acoustique* 

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### **Charlène BOUYER**

# ACOUSTIC CELLS MANIPULATION FOR TISSUE ENGINEERING / MANIPULATIONS ACOUSTIQUES DE CELLULES POUR L'INGENIERIE TISSULAIRE

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# Abstract:

Genetic or physical cells manipulation aspires to be new challenges in tissue engineering. Current technologies to generate tissues, such as micro-scale hydrogels (microgel) assembly, scaffold seeding, molding or bio-printing suffer from the difficulty to control cells organization, multi-steps time consuming procedures and/or potentially cytotoxic side effects. In this PhD, we aimed at developing cell-friendly and rapid techniques, easily transferable to biological laboratories, for two broadly challenging applications: bone healing and neural tissue engineering, for which the above-mentioned techniques cannot yet provide widely reliable models.

In case of a bone critical size defect, external help is often needed for bone healing, and goldstandard for care is bone autograft. Alternatively, the fracture healing process can be stimulated and restored by the implantation at the fracture site of hydrogels embedding growth factors. Both technologies suffer however from side effects such as donor site morbidity or cells over-proliferation in the hydrogel proximity. Moreover, the kinetic of growth factors release cannot be temporally controlled. In this work, we aim at developing an alternative method using ultrasound to spatially and temporally control growth factors release within a biocompatible material: fibrin hydrogels. Towards this goal, we encapsulated, in lipoplexes, plasmids that are under the control of a heat-shock promoter. We then transfected cells, stimulate the production of the targeted protein by heat shock and reported its expression. We also optimized an encapsulation protocol for cells within fibrin gels. This proof of concept demonstrates the feasibility of transfection by lipoplexes with a plasmid under control of heat shock, and pave the way for future developments of *in situ* transfection of autologous cells, for a tight temporal and spatial control of therapeutic proteins expression using ultrasound-induced hyperthermia.

The second application aimed at neural tissue engineering, with the development of an *in vitro* cerebral cortex model. Conventional 3D bioengineering cannot control cell-cell interactions or cell-packing density and recapitulate the specific human neuronal multilayer structure. In the realm of neuro-engineering, several strategies have been reported but some limitations hamper their wide applications in biological labs due to the required multistep operations which are time consuming and may need for special expertise. Here, we demonstrate a novel, bioacoustic levitation (BAL) assembly method (BAL), based on near field standing waves, to generate 3D multilayer neural tissue constructs mimicking the 6-

layers architecture of the cortex. The primary acoustic radiation force and very weak secondary acoustic radiation force generated by the piezoelectric ceramic drive cells in multiple parallel planes, while limiting the appearance of radial patterns. Cells are then trapped in a stable polymer network by controlling the gelation dynamics of fibrin, which can be achieved through optimization of the prepolymer concentrations. Fibrin was chosen as extracellular matrix of the 3D constructs because its unique, tunable gelation properties and viscoelasticity make it a very suitable substrate for 3D tissue engineering purposes, as well as for clinical applications. By adjusting the fibrinogen and thrombin concentrations, the stiffness of the engineered 3D tissue was optimized to match that of a range of native tissues. Here, we optimized these conditions to create fibrin gels with mechanical properties mimicking that of the brain. This technology could also be used for cell patterning and separation, and to bioengineer different tissue types with a multilayered organization.

<u>Key words:</u> ultrasound, tissue engineering, cells transfection, neural construct, 3D dimensional tissue structure, 3D cells manipulation.

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# Résumé substantiel en Français:

Manipuler génétiquement ou physiquement des cellules présente un très grand intérêt pour l'ingénierie tissulaire mais soulève encore de nombreux challenges. Les technologies actuelles pour la fabrication de tissus, comme l'assemblage de micro-gels, le remplissage de matrice 3D, le modelage ou l'impression biocompatible sont limités dans leur capacité à organiser spatialement des cellules, souffrent d'un temps de manipulation conséquent, d'effets secondaires potentiellement cytotoxiques et d'une grande complexité de mise en œuvre, empêchant leur utilisation à grande échelle. Nous nous sommes intéressés dans cette thèse à développer des techniques biocompatibles, faciles à implémenter, rapides et facilement transférables dans des laboratoires de biologie. Nous les avons orientées vers deux applications stimulantes car en grand essor et pour lesquelles les techniques actuelles ne permettent pas encore une utilisation grande échelle : la réparation osseuse et l'ingénierie tissulaire neuronale.

Pour réparer un défaut osseux non consolidés, comme cela peut se produire après une fracture osseuse conséquente, la technique de référence est de réaliser une autogreffe osseuse. Une autre alternative est de stimuler localement le processus de régénération osseuse par le comblement du site de fracture avec des hydrogels contenant des facteurs de croissance. Ces techniques sont souvent accompagnées d'effets secondaires importants tels que la morbidité du site donneur dans le cas de l'autogreffe, ou la sur-prolifération de cellules à proximité du lieu d'injection de l'hydrogel. De plus la délivrance des facteurs de croissances n'est pas temporellement contrôlable. Le but de notre projet est de développer une technologie utilisant les ultrasons pour permettre le contrôle temporel et spatial du relargage de facteurs de croissance en utilisant un matériel biocompatible: la fibrine. Comme preuve de concept, des cellules ont été transfectées avec un plasmide possédant un promoteur « heat-shock » et la production de la protéine fabriquée après choc thermique a été étudiée. Un protocole d'encapsulation des cellules dans des gels de fibrine a aussi été établi. Ces travaux ouvrent la voie vers une transfection *in situ* de cellules autologues, pour une activation contrôlée par ultrasons de l'expression de la protéine cible dans un milieu 3D.

Une deuxième application en ingénierie tissulaire neuronale est ici mise en avant : la manipulation cellulaire pour la création de modèle tissulaire 3D reproduisant la structure du cortex cérébral. L'ingénierie tissulaire 3D actuelle ne permet pas de contrôler l'interaction cellules-cellules, la densité cellulaire et donc de récapituler l'organisation spécifique de tissus

tels que le cortex cérébral. Dans le domaine de l'ingénierie neuronale, plusieurs stratégies d'assemblage ou de formation d'organoïdes ont été mises en place mais des limitations demeurent. En effet, ces techniques sont très longues, demandent une expertise considérable et sont difficilement utilisables par des laboratoires non experts. Nous présentons une nouvelle stratégie simple, rapide, unique et biocompatible pour générer une structure récapitulant l'organisation des neurones au sein du cortex cérébral. Elle repose sur l'utilisation d'ultrasons pour l'assemblage de cellules embryonnaires en multicouches, sous l'effet d'une force de radiation primaire, puis en la culture des multicouches ainsi générés dans un milieu neuro-différentiant. Les cellules sont stabilisées dans un gel de fibrine dont la durée de polymérisation est contrôlée. Le choix de la fibrine comme hydrogel de support a été dictée par la possibilité de contrôler ses propriétés mécaniques, permettant ainsi d'optimiser sa composition pour obtenir un gel aux propriétés mécaniques similaires à celle du cortex cérébral. Cette technologie ne se limite pas à la création de structure neuronale, et pourra à l'avenir aussi être utilisée pour la manipulation cellulaire et l'organisation de différents modèles de tissus.

<u>Mots clefs</u>: Ultrasons, ingénierie tissulaire, transfection cellulaire, régénération osseuse, tissue neuronal, tissue 3 dimensions, manipulation cellulaire en 3D

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"It always seems impossible until it's done." Nelson Mandela

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# Introduction

When a tissue is damaged, the native surrounding organs and tissues may not be able to generate new functional tissues by themselves to repair or regenerate the damaged part. In such cases, tissue engineering solutions can be introduced to help the tissue regeneration or to replace the missing tissue part. In 1993, Langer & Vacanti<sup>[1]</sup> introduced tissue engineering as a strategy where the principles of biology and engineering are applied to the development of functional substitutes for damaged tissues. The general idea is that cells, embedded in scaffolds, can be cultured and expanded to ultimately be grafted into the patient. To obtain a bioengineered tissue with specific shape and properties, according to the aimed application, cells have to be manipulated to finally generate a native-like tissue construct. In that aim, controlling cell-cell interaction, by controlling their patterning and their 3D microenvironment is required. Scaffold properties such as porosity, size, geometry or elasticity are important parameters to control in order to obtain construct with similar properties than native tissues and within which cells can grow and/or differentiate.

Being able to genetically or physically manipulate cells opens many opportunities toward the fabrication of useful tissue-like structures and is currently a huge challenge in tissue engineering. Accurate cells positioning is required for tissue engineering applications where random seeding cannot reproduce the complexity of the spatial organization of tissues. Several technologies are being developed to control cell patterning within scaffolds, such as micro-scale hydrogels (microgel) assembly, scaffold seeding or bioprinting. Those commonly used techniques suffer from the difficulty to control cells organization, cell-cell interactions or cell-packing density, and from multi-steps time consuming procedures, adverse effects and the necessity of complex experimental apparatus and expertise. For wide applications in biological laboratories, such a patterning technology method should be simple, easy to implement and affordable. One of the goals during this PhD was therefore to overtake those challenges and to develop acoustic methods for easy, biocompatible and contactless manipulation of cells.

In this PhD, we aimed at two broadly challenging applications, for which the current abovementioned techniques cannot yet provide widely usable models: bone healing and neural bioengineering. Our objective was to develop ultrasound-based methods to 1) control gene expression, and 2) pattern cells in 3D. To control cell fate in 3 dimensions, a 3D microenvironment as to be chosen to fit with the desired application. Different materials are available to generate those scaffolds which are more or less tunable, bio-compatible, easy to manipulate and are accordingly specialized for a type of application. Fibrin is one of the most used one as it is biocompatible, its stiffness and size are tunable, and it is already used in clinical applications. The properties of fibrin are tunable, by tuning the pre-polymer solution concentrations, which is very useful to obtain hydrogels with native-like tissue properties. Because of all these characteristics, fibrin was chosen as reference material for all the experiments made during my PhD.

After a large bone fracture, external help is often needed for bone healing. The gold-standard for care is autograft. Alternatively, external growth factors can be delivered to the fracture site by implanting an embedded hydrogel. Those technologies suffer from side effects such as graft donor site morbidity or cells over-proliferation in the hydrogel proximity as the release of growth factors is difficult to temporally control. In this work, the idea was to develop an alternative method using acoustic to spatially and temporally control growth factors release within a biocompatible material: fibrin hydrogels. As a proof of concept, we encapsulated plasmids, under control of a heat-shock promoter, in lipoplexes. When cells integer those plasmids and are under a heat-shock, they induce the expression of the gene for which the plasmid encodes. The plasmid embedded lipoplexes were then put in contact of cells to allow cells transfection (cellular integration of the plasmid) and we stimulated the production of the targeted reporter gene by heat shock and detected its presence. We also determined an encapsulation protocol for cells within fibrin gels, to obtain 3D scaffold embedded with cells, which will be useful for 3D applications. This demonstrates the feasibility of transfection by lipoplexes with a plasmid under control of heat shock. Perspectives for further studies are considered: cells could be transfected in situ before activation, for example with a tight spatial 3D control using ultrasound-induced hyperthermia.

The second application aimed at here is neural tissue engineering, with the development of an *in vitro* cerebral cortex model. Conventional 3D bioengineering methods cannot control cellcell interactions or cell-packing density and recapitulate the specific human neuronal multilayer structure. In the realm of neuro-engineering, several strategies have been reported but some limitations hamper their wide applications in biological labs due to the required multistep operations which are time consuming and may need for special expertise. Here, we present a unique biocompatible strategy, based on near field standing waves, to generate a neural construct, mimicking brain cortex structure, in a facile and rapid manner by bioacoustic levitational (BAL) assembly of human embryonic stem cells (ESCs) derived neural progenitor cells (NPCs). The primary acoustic radiation force and very weak secondary acoustic radiation force generated by the piezoelectric ceramic drive cells in multiple parallel, horizontal planes while limiting the appearance of radial patterns. Cells are then trapped in a stable polymer network by controlling the gelation dynamics of fibrin, which can be achieved through optimization of the pre-polymer concentrations. Fibrin properties were optimized to match mechanical properties mimicking that of the brain. This bio-acoustic levitational technology could also be used for cell patterning and separation and could be widely used to adapt to any type of tissue needed or cell type detection.

The rest of the manuscript is divided as follow. Chapter 1 is an overview of the current status of tissue engineering for the two applications we are pursuing: bone healing and neural tissue engineering. Chapter 2 presents the progress made for the cells transfection with a transgene under the control of a heat-shock promoter: the establishment of protocols for cells transfection, as well as for activation and detection of the reporter gene, and also for cells encapsulation into fibrin gels. Those results open perspectives for in situ transfection and subsequent activation by ultrasound. In Chapter 3 the development of an acoustic device for cells manipulation is described, including all the characterization performed to optimize the device for a neural tissue engineering application. This application is developed in Chapter 4, where the optimization processes to establish the culture and differentiation of Embryonic Stem Cells into neuron-like cells in 3D microenvironment is presented as well as a time course of cells differentiation within this construct. Conclusion and perspectives are presented in Chapter 5.

# Chapter 1 – State of the art: outlines and challenges in tissue engineering

Tissue engineering can be described as the combination of engineered biomaterials and cells to produce biocompatible constructs with suitable biological and/or physiological functions to allow the improvement or replacement of native organs.

It can be used for broad applications such as repair or replacement of tissues such as skin<sup>[2,3]</sup>, liver<sup>[4]</sup>, heart<sup>[5]</sup>, bone<sup>[6]</sup>, cartilage<sup>[7]</sup>, or bladder<sup>[8]</sup>, and it is also used to produce organ models to help scientists to better understand some organ aspects that are still not fully understood such as their developments, cell-cell interactions within an organ or the influence of the microenvironment surrounding the cells.

In this chapter, I will summarize the history of tissue engineering, cells and scaffolds nowadays available, and will focus on some applications that are intensively studied: bone and cartilage repair, and tissue models.

## 1.1. Tissue engineering history

The history of tissue engineering started in ancient Egypt<sup>[9]</sup>. Human tissue exchange such as blood transfusion was already described in ancient Egyptian and Old Testament. A mummy was also found with intra-vital imputation of big toe<sup>[10]</sup>, suggesting that ancient Egyptian already practiced surgery and needed to replace a defected or lost organ. Many cultures referred the replacement of nose (skin and cartilage) since 1000BC. Then skin graft was extending to other tissues than nose since 1800's<sup>[9]</sup>.

Tissue engineering was then developed to restore, maintain or improve tissue function or whole organ<sup>[1]</sup> ranging from skin, liver, heart grafts to bone repair but was also extending to fabrication of tissue models, mimicking native tissue to help repairing tissue or better understand their functioning. In 1993, Langer and Vacanti<sup>[1]</sup>defined tissue engineering as an interdisciplinary field, applying "the principles of biology and engineering to the development of functional substitutes for damaged tissue", attempting to "provide solutions to tissue engineering solutions as every year millions of patients suffer from loss or end-stage organ failure and the cost for related health care is estimated to more than 400 billion per year in the USA. Three tissue engineering strategies were so described:

- Isolation of cells or cell substitutes taking from patient and re-introduce later on the healing site.
- Tissue-inducing substances: matrices embedded with components helping the tissue recovery (growth factors, anti-inflammatory drugs...) introduced at the patient's healing site.
- Cells placed on or within matrices: matrices embedded with cells which can colonize the patient healing site and help in the recovery

During the last decades tissue engineering was expanded to many applications as many improvements were done in the available technologies<sup>[11]</sup>. Internal organs such as bladder<sup>[12]</sup> or pancreas<sup>[13]</sup>, and more superficial tissues such as skin<sup>[14,15]</sup>, bone/cartilage<sup>[16,6]</sup>, or corneal<sup>[17]</sup> can now be engineered.

Some engineered tissues are already in an advanced stage as they were already implanted in human, such as artificial airway<sup>[18]</sup>, cartilage or heart. In fact, development of heart tissue model went through development of *in vitro* myocardial cells culture to the fabrication of an artificial heart implantable in patient<sup>[19]</sup>. The artificial heart graft went well but the first patient died after few weeks due to multiorgan failure. Many other tissues are not yet transferable to clinical stage but many developments were made to increase tissue construct biocompatibility. For example, development of cartilage tissue implantable improved from cells embedded scaffold to a generation of a scaffold free cartilage, only made of cells and cells production and without any chemical construct<sup>[20]</sup>, which enable a better biocompatibility.

Many improvements were also done in skin and bone tissue engineering, where tissue engineering can also be used to provide solution for tissue repair and creation<sup>[1]</sup>. This field has also a huge impact as it involves many patients with skin or bone defect, for the most common, that need external help for a good healing. Approaches in those field are mostly cell-based regeneration therapies to implement an artificial skin<sup>[21]</sup> or to improve skin<sup>[22]</sup> or bone<sup>[23]</sup> regeneration. Skin regeneration remains a key challenge in tissue engineering as it may help to regenerate skin after a huge healing or a big injury that the body is not able to regenerate naturally. Skin repair by regeneration rather than scar remains the aspirational goal<sup>[24]</sup>. With progress in biomaterials but also in regeneration process, many technics were developed to aim this skin regeneration. For this purpose, last researches aimed at the implantation of Embryonic Stem Cells (ESC) for skin regeneration but also to help the regeneration of bone<sup>[25]</sup>. One team already transplanted an engineered tissue with human

induced pluripotent stem cells (iPS) cells into rats under their skin, building up a patch of multilayer cell sheets for fabrication of myocardial like structure<sup>[26]</sup>, for heart failure applications. This opens new opportunities in the field, suggesting that implanted constructs with iPS cells, later any type of tissue would be able to be create in the body.

Despite a decrease in activity in skin, cartilage, and other structural applications by more than 50% between 2000 and 2002 (counterbalanced by a 42% increase in stem cell firms), twenty tissue-engineered products had entered Food and Drug Administration clinical trials. Four were approved but none of these are yet commercially successful<sup>[27]</sup>. There are currently about 30 clinical trials with engineered tissues (source: clinicaltrials.gov).

Despite all the improvements achieved in tissue engineering, some issues remain. Engineered tissues are mostly associated with side effects<sup>[28]</sup> and we still lack tissue models to better understand the functioning of some tissues. As an example, tissues such as heart or brain, are not fully understood yet and models to study their functioning or development are needed to improve our knowledge.

We describe below different approaches that are under development to try to solve those issues.

### **1.2. Tissue engineering approaches**

### **1.2.1 Different types of hydrogels**

Cells culture in 2 dimensions cannot provide all information about a native organ, as 2D cells patterning cannot represent cells behavior in a 3D environment with cell-cell interactions in the 3 dimensions. That is why it is important to introduce scaffolds to allow 3D cells culture that will more closely mimic the native cells patterning of a tissue.

Biomaterials were developed for tissue engineering to support either cells viability and functions<sup>[29,30,31]</sup> or the delivery of tissue induced substances<sup>[32]</sup>. Their biocompatibility is an important parameter as cells need to be viable to represents native tissue cells. Moreover, for some applications such as creating tissue models, the scaffold need to have specific mechanical properties to fit those of native tissues. Each biomaterial was developed originally more specifically for one application and was then adapted to be widely used for other type of tissues.

Here, we focused on biopolymers such as collagen, matrigel<sup>[1]</sup>, polyesters, fibrin, etc. that are commonly used in tissue engineering studies. Every biopolymer has its own properties, and is more or less degradable or biocompatible. Each biomaterial has also its own advantages and limitations such as a controllable size and organization, an easy biodegradation<sup>[33]</sup>, support more or less cells viability and are more or less rapid to crosslink. Today, composite materials<sup>[34,35,36,37,38]</sup> are mostly developed by incorporating nanomaterials within polymeric matrix to engineer bioactive scaffolds, to increase advantages without limitations. Studies are also going further to reach perfect cells viability and promote tissue function. For that, natural polymers were developed to have or help to produce extracellular matrix like in human<sup>[39, 40, 41, 42]</sup>

A quick overview of biomaterials available with their specificity is summarized in table 1.1 below.

Scaffold material	<u>Applications</u>	Principle properties and advantages	Limitations
Agarose <sup>[43]</sup>	Allograft	10 to 14 days to be colonized by surrounding cells	Cells colonization not controlled, so no control of repaired
	chondrocytes	Repaired tissue is similar to surrounding one	tissue type possible
	(cartilage)	Maintain chondrocyte phenotype	Imperfect transition graft/tissue
Fiber-based	Mostly fibroblast	Size modular	Difficult cell organization and packing within fiber
hydrogels <sup>[44, 45]</sup>	and hepatocytes,	Easy and fast crosslinking	Composite materials needed for a long term good viability
	and then extended		and cells functions
Collagen <sup>[46,36]</sup>	Cartilage	Mimic natural cartilage environment	Long and multi-step fabrication process of specific shape
		Degradability tunable for long term culture	needed (crosslinking at 37°C)
Hyaluronic	Intra-abdominal,	Lasting durability tunable	Lack of cell-specific bioactivities, such as cell adhesion,
acids <sup>[47,48,49]</sup>	skin	Directly injectable	migration and cell-mediated biodegradation
Carbon	Bone regeneration	Artificial, so no need of polymer-based component	Possible impurities and contaminates
nanotube <sup>[23]</sup>		Properties tunable	Low biological interaction if not associated to other
			material
Silk <sup>[50,51,52]</sup>	Various cell types:	Highly human cell compatible and functionalisable	Undesirable immunological problems if not purified
Fibrin <sup>[53,54,55]</sup>	epithelial, neurons,	Used in clinical translations as biodegradable	At the macroscale: small construct difficult to obtain
	endothelial, etc.	Stiffness tunable to fit native tissue properties	Optimization of mixing process with thrombin needed
Gelatin	Bone generation	Dielectrophoresis spatial patterning and for	Long patterning process for macroscale scaffold
based <sup>[56,57,58]</sup>	Vascularization	polyester gels, better viability	Crosslinking process usually via UV light and photo-
		Support cells growth with tunable gel stiffness	initiator
			Polymerization-induced porosity with diameters of microns
	Table 1.1. Non exha	ustive list of mostly used scaffold materials with their i	principle advantages and limitations

### 1.2.2. Cells sources and approaches used in tissue engineering

Cells origins for tissue engineering are either from patients or cell lines. The cell type will depend on the application, but research using stem cells is more and more developed<sup>[59,17, 25,14]</sup>. This is mostly because stem cells are able to differentiate in many cell types and, for some tissues such as corneal<sup>[17]</sup> or skin<sup>[14]</sup>, can be required for a permanent regeneration of functional tissue<sup>[17]</sup>.



Figure 1.1. Stem cells source for tissue engineering applications<sup>[60]</sup>

Three different approaches are available to obtain stem cells (see figure 1.1 above). To culture Embryonic Stem Cells, cells from the inner mass of a blastocyst are collected and placed on culture plate. When derived from skin fibroblast cells, induced pluripotent stem cells can be obtained after culture with specific growth factors. At the opposite when cells are taken from muscle, adult stem cells can be derived. Depending on the application aimed, either one or the other stem cells type can be used.

## Two different approaches to engineer complex tissues

High cell-packing density and tight regulation of cell-cell proximity are critical factors that determine the physical properties and functions of native tissues<sup>[61]</sup>. Bioengineering aspires to build biofidelic, 3Dimensional surrogates that are biologically relevant for applications including improving the function of damaged tissues or modeling native tissues and can mimic the architecture and physiology of native tissues. Bioengineering tools are thus needed to offer the ability to control the complex patterns and high cell density of native tissue, such as brain, liver or cardiac tissues<sup>[62]</sup>. In this context two different approaches can be used: "top-down" and "bottom-up" approaches (see figure 1.2. below). These two approaches have advantages and shortcomings, which prevent them yet to be widely used and commercialized.



Figure 1.2. <u>Bottom-up vs top-down approaches in tissue engineering<sup>[63]</sup></u>

For some application such as to induce a vascularization within a construct<sup>[64]</sup>, the top-down approach is mostly used. In the top-down approach, cells are directly seeded into a fully made 25

porous scaffold of predefined shape to form a tissue construct. Cells are expected to populate the scaffold and create the appropriate extracellular matrix and microarchitecture often with the aid of a bioreactor that furnish the set of stimuli required for an optimal cellular viability<sup>[65]</sup>. This approach is associated with some limitations such as the difficulty to shape scaffolds, difficulty to build large tissue constructs and low cell diffusion which result in a low cells density and non-uniform distribution within the scaffold<sup>[63]</sup>.

On the other hand, in the bottom-up approach, small cell laden modules such as spheroids or cell sheets are used as building blocks to create a larger construct<sup>[66]</sup>. In that case, the final construct organization and composition is tunable by the organization of the micro-architectural units. Usually cell seeded microbeads or small seeded hydrogels are used as functional building blocks to create the final tissue construct. With this approach, the cell distribution is more likely controlled, but an external and often complex technology is needed to assemble the building units.

New challenges in tissue engineering are to combine both approaches to enable a better cell seeding within a complex scaffold organization to create native-like tissue patterns.

#### 1.2.3. Techniques for 3D cells patterning

Novel technics were recently developed to enable 3D cells culture: hanging drop (self-assembly) or methods for bio-printing of building blocks (controlled assembly).

### 1.2.3.1. Conventional 3D culture

Hanging drop is a technology for the fabrication of cells spheroids to create cells aggregation in 3D, to mimic *in vivo* tumor models on the lab-on-chip scale, for different applications like in drug screening<sup>[67]</sup>. Techniques are based on either using specific well plates or based on the use of biomimetic super-hydrophobic flat substrates, with controlled positional adhesion and minimum contact with a solid substrate. The advantage of this technology is that it is possible to control the spheroid sizes, it is relatively easy to implement and it is low cost.

### 1.2.3.2. <u>Assembly methods in bottom-up approach</u>

The new developments to better design scaffold<sup>[68]</sup> were motivated by the need of biomaterials that integrate our understanding of cells mechanisms (cells development, attachment to surrounding microenvironment, cell-cell connections, ...) to generate native-like constructs<sup>[69]</sup>. Packing and organizing cells in a 3D microenvironment is very challenging but necessary to mimic native tissues. New technics were developed to individually create single gels and to assemble blocks together<sup>[12,30,63,70,71]</sup>. However, conventional 3D bioengineering technologies to create specific patterns, such as micro-scale hydrogels (microgel) assembly<sup>[30,72,73,74]</sup>, scaffold seeding<sup>[75]</sup> or bioprinting<sup>[76]</sup> cannot control cell-cell interactions or cell-packing density. To address these issues, new scaffold-free strategies have been developed<sup>[77, 78,79,80]</sup>, mostly using cell spheroids to build highly-packed tissue construct elements. These methods suffer from long assembly time that prohibits building large, millimeter-scale constructs, and cannot reach an assembly precision of a few hundred micrometers required to pattern constructs such as neural constructs.

These assembly methods rely on different physical methods such as magnetic<sup>[30,72]</sup>, faraday waves at liquid-air interface<sup>[81]</sup>, acoustic or 3D printer. Organ printing<sup>[76,79]</sup>, using printer, enables to build large scale scaffold with cells. This allows a precise scaffold patterns droplet by droplet but cannot go down the micro-scale size precision. In the other hand, magnetic assembly<sup>[30,72]</sup> allows spatial positioning of building blocks with cells. This technique is a contactless strategy but requires multistep procedure and a paramagnetic solution, which is not fully cell-friendly. On the other hand, acoustic strategy<sup>[81]</sup> enables a contactless, biocompatible manipulation to assemble cells following different patterns simply by tuning the acoustic frequency.

Commonly used technics generates large construct that mostly need to be cultivated in a bioreactor for proper media and  $CO_2$  diffusion within the sample<sup>[82,83]</sup>. Our hypothesis is that, with ultrasound, we will be able to easily manipulate cells, and so be able to have smaller construct to avoid the need of such a specific construct culture process.

For all those advantages, we choose to develop acoustic technics for cells manipulation and will present them in the following chapters.

#### 1.2.4. Tissue engineering applications

#### 1.2.4.1. <u>Tissue engineering approaches for tissue models applications</u>

Layered cell organization is a ubiquitous phenomenon in biological tissue in both developmental (e.g., organoids, germ) and matured stage (e.g., skin, cortical tissue, breast) of organism. Complex cell interactions in each layer and between neighboring layers as well as cell-extracellular matrix (ECM) interaction determine physical properties and function of the corresponding tissues. Hydrogels and tissue engineering methods offer several advantages to bioengineer 3D, multilayered constructs<sup>[83,84,85]</sup> resembling to native organs, such as skin, cardiac tissue and breast tissue<sup>[86]</sup>.

Different structures ranging from biopolymers films to natural hydrogels can be used for layer-by-layer tissue assembly. The structure and bioactivity of biomolecules in thin films is very important as it determines the final construct properties: adhesion of proteins, support for cells differentiation, self-degradable multilayers, mediation of cellular functions and will define the functions of biomaterials. Some of these biomaterials can be implantable or be used in applications for drug delivery, while others can be used to mimic tissues or to trap some proteins and cells<sup>[85]</sup>.

Multilayered tissue models can also be cultured in chips, to add a blood flow component to the system, and mimic the interaction between the tissue and the blood circulation. The liver, kidney, lung, intestine, fat, muscle and the blood-brain barrier have all been rendered into chip form<sup>[87]</sup>. This method allows a multilayer cell culture that contains continuously perfused chambers inhabited by living cells, to recapitulate tissue architecture and physiology, by mimicking the native surrounding environment of cells.

In this section, two applications of creating multilayered tissue models will be detailed: tubular and cerebral tissue models. I will then explain the context and challenges for these applications, and introduce the approach developed in my PhD.

#### Tubular structure

A method was developed to be able to represent tubular structure. This method uses the stress-induced rolling membrane (SIRM) technique to stepwise formation of 2D structures

through the biotin-streptavidin (SA) interaction and further construction of controlled, 3D, multilayered, tissue-like structures. In this method, the biotinylated cells connect with the SA-coated adherent cells to form a bilayer of HUVEC and Jurkat cells is transform into tubes, mimicking the tubular structure such as in blood vessel<sup>[88]</sup>. Our aim was to find a way to avoid the multistep procedure commonly used and to develop an easy and contactless protocol for spatial and temporal control of cells expression.

#### Cortex model:

Spatial organization and interconnection of neural cells in and between these layers determine functions of cerebral cortex such as memory, language and consciousness. Dysfunction of these neural connectivity is correlated with several prevalent neurodegenerative diseases such as Alzheimer's disease and Huntington disease<sup>[89,90]</sup>, which are a major burden of public healthcare system in both United States and European countries due to population aging under continuously increased life expectancy.

Bioengineering tools that offer the ability to control the complex patterns and high cell density of native tissue, such as brain, liver or cardiac tissues<sup>[62]</sup> are needed. This is particularly relevant for the brain, where the lack of preclinical models mimicking the architectural and developmental complexity of native tissue have made post-mortem functional tissues, although of limited access, essential *ex-vivo* models to study mental illnesses<sup>[91,92]</sup>, neurotransmitters mechanism of action<sup>[93]</sup>, the dynamic of proteins/ions signaling<sup>[94]</sup>, or also to screen novel therapeutics agents<sup>[92]</sup>. Studies as scanning different type of drug to choose the most efficient one or test new stimulation devices are very critical in research as they improve the quality of cares<sup>[95,96]</sup>.



Figure 1.3. <u>Cerebral cortex structure<sup>[97]</sup></u>

Cerebral cortex is a structure of about 2mm thick<sup>[98]</sup>, made of 6 layers of neurons inter connected to each other (see figure 1.3. above), giving the function of each layer and of the global cerebral cortex. Two-dimensional culture models have yielded important insights in, for instance, the molecular drivers of neural development and activity. However, the random distribution of neurons in culture dishes does not sufficiently recapitulate the 3D aspects of neural connectivity or the microenvironment of the brain.

Another alternative choice to these approaches is presented by the *in vitro* bioengineering of 3D models that recapitulate native brain tissues. Several multistep strategies have been developed to create 6-layered tissue surrogates of the cerebral cortex, either by self-assembly or bioprinting approaches.

In 2013, Lancaster and al<sup>[82]</sup> generated a cerebral cortex-like tissue by first generating spheroids self-assembly of embryonic stem cells (ESCs). Human pluripotent stem cells (hPSCs) spheroids were obtain by culture in hESC media and then in neural induction media for 11 days. Then, droplets were cultured in matrigel droplet and transferred into a spinning bioreactor (see figure 1.4. above). In each step of the process, a specific media was attributed.



Figure 1.4. <u>Description of cerebral organoid culture system<sup>[82]</sup></u>.

When cultured in a spinning bioreactor in presence of a differentiation media and retinoic acid, cells were spontaneously organized to resemble to a cortex structure (see figure 1.5. above).



Figure 1.5. <u>Sectioning and immunohistochemistry revealed complex morphology with</u> <u>heterogeneous regions containing neural progenitors (SOX2, red) and neurons (TUJ1, green)</u> <u>(arrow)<sup>[82]</sup></u>.

Neuronal differentiation of cerebral organoids reveals a heterogeneous neuron subtypes production and auto-organization, with the presence of abundant radial glial stem cells during the development. Histological analysis of the differentiated structure reveals an important degree of molecular heterogeneity and regionalization that recapitulates the different brain regions.

This method was also used to model microencephaly, a brain disorder usually difficult to recreate in mice, by introducing RNA interference and patient-induced pluripotent stem cells. In that configuration, neuronal differentiation in organoids was found to be immature that may explain the disorder cause.

In 2015, in a study using human cortical spheroids obtained from pluripotent stem cells, a laminated cerebral cortex-like structure could generated and used for the study of normal and abnormal corticogenesis<sup>[99]</sup>. Cerebral organoids were formed from deep and superficial cortical layers neurons that were characterized by immunostaining observation (see figure 1.6 above).



*Figure 1.6. <u>Cryosections of hCSs at 137 days, showing organization of layer-specific</u> <u>neurons<sup>[99]</sup></u>.* 

Different neuronal subtypes were found and located at different depth of the organoids, characteristic of both deep and superficial cerebral cortex. After complete neuronal maturation (after 180days of culture), a synaptogenesis analysis was conducted to show the visualized individual synapses activity. The developed system provides a platform where deep and superficial neurons layers are present, surrounding by astrocytes to support neurons activity.

Other alternative techniques to bioengineer 3D neuronal multilayer structures are using bioprinting approaches. For instance, primary rodent neurons were encapsulated in a layer-by-layer assembly approach in concentric 3D donut-shaped constructs, made from composite hydrogel and silk protein<sup>[52]</sup> (see figure 1.7 below). Micromolding has also been employed to

embed primary neurons in 3D networks in collagen<sup>[100]</sup> (see figure 1.8. below). The generated 3D neural surrogate tissues displayed the characteristic cell density, interlayer neurite connections and layer thickness of the cortex, as well as biochemical and electrophysiological functional responses.



*Figure 1.7. <u>Design concepts and 3D assembled tissue structures</u><sup>[52]</sup>* 

The acoustic method developed<sup>[100]</sup> was also not easily manipulable: a water tank with a 3D positioner was needed to position the samples; an amplifier was required to generate enough forces to drive cells in nodes planes. This strategy could also be too stressful for sensitive cells as collagen was chosen and it requires 15 minutes of ultrasound and 1h in incubator without media and it lacks of gel tenability to obtain native tissues properties adapted to cell types.



Figure 1.8. Process time schedule, for brain-like tissue construct fabrication<sup>[100]</sup>

Although these methods recapitulate some features of native neuronal tissues, such as 3D neuronal network design with interlayer neurite formation, neural cell density and layer thickness, and have shown biochemical and electrophysiological outcomes, some limitations hamper their wide applications in biological labs. Multistep operations required in both layer-by-layer assembly and micro-molding approaches may require special expertise and are time consuming.

To address critical challenges encountered in self-assembly and bioprinting approaches, in brain bioengineering, we developed a unique, single step bio-acoustic levitational (BAL) assembly technology that is fast and facile to implement. We used BAL technology for direct assembly of multilayer 3D geometries within one single hydrogel construct. Together, these bioengineering approaches constitute a broad toolbox for the creation of 3D architectures that recapitulate the physiological complexity of native tissues. Their applications include fundamental research, such as the elucidation of the molecules that drive cellular connectivity, and translational applications, such as regenerative medicine, therapeutic drug discovery, as well as alternative models to animal testing. Their utility includes, but is not limited to,

neuroscience, cardiovascular and cancer biology. This method will be widely developed in the following chapters 3 and 4.

### Bone and cartilage repairs

Many strategies are developed for bone or cartilage healing and regeneration.

In cartilage tissue engineering<sup>[101]</sup> the aim is to induce or provide a new efficient joint function. In the last decades new technologies were developed but some limitations still hamper their clinical use such as difficult long-term tissue regeneration because biological and mechanical properties of the new made tissue are less efficient because the neocartilage formed is very immature. Recent studies are focused on combining cells as chondrocytes and bioresorbable scaffold to produce functional cartilage<sup>[101,102]</sup>.

*In vitro* new technics are focused on engineering cartilage by *in vitro* micro-aggregate culture system, to avoid the use of any biomaterials<sup>[103]</sup>. Human nasoseptal or auricular chondrocytes cells were culture in monolayer before being seeded into a cell culture insert and culture for three weeks. Phenotypic, mechanical and molecular analyses of native and engineered elastic cartilage were found similar. This engineered tissue could be widely used as autologous cartilage graft, after were studies on long-term culture and *in vivo* tests will be done.

Some *in vivo* tests to repair large osteochondral defects were done on rabbits<sup>[104,105]</sup>. A hybrid scaffold system was made of three-dimensional porous Polycaprolactone (PCL) scaffold<sup>[105]</sup> for the cartilage and tricalcium phosphate-reinforced PCL for the bone portion seeded with allogenic bone marrow-derived mesenchymal stem cells (BMSC). Osteochondral defects were created in rabbits and repaired tissues were analyzed after three and six months. Better results in seeded construct graft than in control group were found. Mechanical properties (young modulus about 0.8MPa) were found similar to native tissues which explain that scaffolds provided support for the new cartilage regeneration. These results offer new opportunities as such a scaffold could be used as an alternative graft for cartilage regeneration.

New approach to fabricate osteochondral graft, using rat bone marrow-derived mesenchymal stem cells MSCs was also developed to regenerate bone and cartilage together in rats<sup>[25]</sup>. Cells were cultured and separately stimulated for two different differentiations: chondrogenic or
osteogenic and were loaded into two different scaffold components: sponge component (composed of a hyaluronan derivative) and ceramic component. Both component were sealed using fibrin hydrogel and implanted in rats. Both cartilage and bone were generated within the scaffold which suggests that the use MSC may be useful for bone and cartilage regeneration.

With the same scheme, progresses in bone regeneration have been achieved, first *in vitro* and then in *in vivo* models. *In vitro* bone osteoblast formation by embryonic stem cells differentiation show the feasibility of using embryonic stem cells to form bone cells<sup>[106]</sup>.

Many studies were done on stem cells embedded scaffold<sup>[107]</sup>. Silk hydrogels<sup>[108]</sup>, HEMA-lactate-dextran cryogels<sup>[109]</sup> can be used as scaffold matrix to allow long time control of molecules release such as growth factors or hydroxyapatite. This technic was, for example, used for a formation of trabecular-like bone matrix to support bone regeneration. All those approaches enable the formation of bone tissue but are limited by a difficult cells source, are time consuming and suffer from long regeneration process.

#### **1.3.** Place of Ultrasound in cells manipulation

Ultrasound have played, or have a potential to play, a role in many aspects of the process of engineering tissues. The ultrasound can be used to promote cell proliferation or to precondition cells to orient their differentiation during culture, and also to transfect cells. They can be used for the monitoring and the control of the rate of scaffold degradation, for the manufacturing of scaffolds, for the characterization of scaffold properties and for quality control, and for improving scaffold integration. Finally, ultrasound can be used to control the delivery of growth factors or the gene expression of engineered cells, but also to modulate the physical environment by heat deposition or mechanical stimulation, in order to promote regeneration. We will review some of these aspects below.

#### **1.3.1. Ultrasound cell transfection (Sonoporation)**

Ultrasound are currently used in research for cells transfection. *In vitro*<sup>[110,111,112]</sup> and *in vivo*<sup>[113,114]</sup> experiments were accomplished for sonoporation and DNA transfection, mostly

using ultrasound contrast agents, but also using inertial cavitation induced by confocal transducers.

*In vitro* cells in suspension with the transfection "reagent", such as Chinese hamster ovary cells were exposed to ultrasound at 2.25MHz and the uptake of large fluorescent dextran molecules by some cells was observed after ultrasound exposure. DNA transfection was also shown by luciferase reporter plasmid expression analysis.

Some studies combined *in vitro* and *in vivo* application to establish the transfer to *in vivo*<sup>[113]</sup>. The enhancement of gene transfection by ultrasound (US) in B16 mouse melanoma model either in suspension or implanted subcutaneously in female C57BL/6 mice were analyzed by using a reporter plasmid for a fluorescent protein (GFP). *In vitro*, 1.4% of the cells expressing GFP after two days of culture and the transfection rate was enhanced by a factor of about eight *in vivo*.

#### 1.3.2. HIFU (Heating)

Ultrasound can also be used at a high intensity. In that case High Intensity Focused Ultrasound can bring enough thermal doses to burn tumor tissue<sup>[115]</sup>. The advantage of such a technic is that side effects are limited as ultrasound are focused and only the site of interest is reached by ultrasound.

#### **1.3.3.** Physical manipulation (Acoustophoresis)

Because of their larger density and lower compressibility than the surrounding fluid, particles can be driven to the node planes of acoustic standing waves where there is minimal pressure<sup>[116,117]</sup>.

Acoustic standing waves have been explored as a non-invasive tool to manipulate cells for wide applications such as microfluidic cell sorting<sup>[118]</sup>. Although cell assembly using bulk acoustic standing waves has been demonstrated with human endothelial cells as a vascularization strategy for tissue engineering<sup>[83]</sup>, interlayers cell-cell interaction were not showed resulting in a technology not transferable to other application such as neural construct formation. We will detail these techniques in the following sections.

#### 1.3.3.1. Acoustofluidics for cells separation

Acoustic in commonly used in cells separation<sup>[119]</sup>. Due to their difference in density, size<sup>[120,121,122]</sup> and compressibility cells will be driven to different location within an ultrasound pressure field. The behavior is used to separate different cell types within a media.

Proof of concept was done with latex beads<sup>[116]</sup> of 1, 15, 25 and 45µm (1.6g/cm3 of density) using a ceramic working at 3.2MHz. A quartz reflector was positioned on top of a chamber, facing an acoustic ceramic to serve as an acoustic reflector. Incident waves, produced by the ceramic, are coherently summed with reflected waves to generate acoustic standing waves within the chamber. The chamber size was chosen to be close to the resonant frequency to generate 2 pressures nodes (minimal pressure) within the chamber. Within such parameters, beads are placed on nodes planes in 2 seconds and then formed cluster after 10 seconds (see figure 1.9. above).



Figure 1.9. <u>Temporal progression of suspended particle aggregation in an ultrasound</u> <u>standing waves (0, few seconds and after 10 seconds)</u><sup>[116]</sup>

Most biological applications are about lipid and blood cells separation as the difference in density is important between lipid and blood cells. All studies are using piezoelectric ceramic and a height chamber of half-wavelength to generate only one pressure node within the chamber. Studies shown that separation between lipid and other blood cells is possible by ultrasound combined with a flow to push the different cell type in different chamber channels exits<sup>[123,124,125,126,127]</sup>.

#### 1.3.3.2. <u>Acoustic levitation for cell patterning</u>

At the beginning of my PhD, only one application of ultrasound cell manipulation to create a tissue model had been published. The objective of this published study was to pattern endothelial cells with acoustic radiation force, in order to induced vascularization of the construct<sup>[83]</sup>. Vascularization of tissue-mimicking constructs is crucial for these constructs to be functional and more closely mimic native tissues. In this study human endothelial cells were organized in multilayer organization by applying acoustic standing waves within a 3D collagen-based hydrogel. Formed cell banding accelerated formation and elongation of capillary-like sprouts and promoted collagen fiber alignment. This technology revealed a rapid, noninvasive, formation of vascularization in lumen-like structures concentrated within layers.

Despite these progresses, some limitations still hamper on the wide application of tissue engineering. Those developments were driven by the need to bioengineer complex structure. In that context, acoustic methods could be developed to avoid current issues and enable an easy and contactless cell manipulation. The objective of the work presented in this manuscript was to develop acoustic methods to easily, rapidly and bio-compatibly manipulate cells. In the following chapters, I will present the progress made for two different tissue engineering applications: bone repair and neural tissue engineering. In the Chapter 2, I will present the strategy developed to control gene expression, with a long term objective of controlling the expression of growth factors for bone healing application. We established protocols for cells transfection and transgene activation, and also for cells encapsulation into fibrin gels, opening the way for in situ cells transfection and subsequent ultrasound activation. In Chapter 3, I will describe development of an acoustic resonator for cells manipulation. I will present the design, modeling, fabrication and the acoustic characterization of the set-up. In the following Chapter 4, I will present how we used this optimized device to create multilayered neuronal constructs, by encapsulating and differentiating Embryonic Stem Cells into neuron-like cells in 3D microenvironment. Conclusion and perspectives for this developed device will be presented in Chapter 5.

### Chapter 2 - Cell transfection with a transgene under the control of heat shock promoter: towards local control of gene expression for bone tissue repair

After a fracture, bone should be repaired naturally but, sometimes, the healing process is compromised. To solve this issue the gold standard is autograft. It is a widely used solution because there is no rejection of the graft as it is the patient own bone. However the quantity of bone graft is limited and most of the time, after such an operation, there is donor site morbidity.

An alternative to bone grafting is to fill the fracture gap with scaffolds, typically fabricated with porous structures and composed of biodegradable materials, which are frequently used in regenerative medicine as an adhesive substrate for the attachment of cells and/or the encapsulation of inductive proteins such as growth factors. But a major challenge for regenerative medicine is to reconstitute the developmental patterns of growth factors distribution that can stimulate new tissue formation.

In 2014, Wilson et al.<sup>[128]</sup> reviewed gene therapies using Bone Morphogenetic Protein (BMP)encoding vectors such as plasmids or viruses or cells engineered to express BMPs, and reported that these therapies demonstrate robust healing capacity in animal models of criticalsized bone defects<sup>[129,130,131]</sup>. Implantation of cells engineered with ligand-regulated BMP expression systems also exhibited osteogenic activity *in vivo*, and can additionally provide tight temporal control over the transgene expression<sup>[132,133]</sup>. However, methods for achieving combined spatial and temporal controls over the expression of regenerative transgenes have not been reported, yet this may be crucial for localizing the osteogenic response and reducing heterotopic ossification and other systemic toxicities that can arise from uncontrolled BMP release<sup>[134,135,136]</sup>.

Wilson et al., in collaboration with our laboratory, reported<sup>[137]</sup> a novel gene therapy approach for temporal and spatial control of two important growth factors in bone regeneration, vascular endothelial growth factor, and bone morphogenetic protein 2. The proposed method

requires the used of high intensity focused ultrasound to tightly activate cells by acousticbased heat shock and to induce a transgene expression within a cell seeded scaffolds of subvolumes of 30 mm<sup>3</sup>. In their approach, the amplitude and projected area of transgene expression was tuned by the intensity and duration of ultrasound exposure, and resulted in minimal cytotoxicity and scaffold damage. The localized regions of growth factor expression also established gradients in signaling activity. Such a system can even be further refined by using transgene that are under the control of a heat-shock promoter, and which also needs a specific chemical to be activated. This is the case for transgenes developed by our colleagues from Spain, which requires the presence of a small molecule such as rapamycin, in addition to the heat shock, to be activated. With this strategy it would be possible to introduce simultaneously different transgenes, coding for different growth factors, such as growths factors important for vascularization or for bone formation, and to specifically activate one by inducing a heat-shock and delivering its specific small molecule activator. Like that we can choose which growth factor productions we want to induce by bringing the specific chemical and heat shock to the bone healing place, where the hydrogel is.

The next step would now be to think about a way to bring this approach to the *in vivo*. Two solutions can be envisaged. The first would be to deliver to the patient cells that have been transfected *in vitro* with the novel gene switches. This would require autologous cell culture, amplification, transfection, seeding within a scaffold and ultimately implantation. The second approach would be to deliver the plasmids directly to the patient at the site of injury. We believe this solution may be more suitable as it is more straightforward and would not require cell transplantation.

In this context, we decided to collaborate with the team of Nuria Vilaboa from the CIBER de Bioingeniería, Biomateriales y Nanomedicina, CIBER-BBN, Spain, who engineered and provided us plasmids, to develop a method to deliver and transfect cells in situ, which would then be stimulated by heat-shock induced ultrasound to control growth-factor expression.

To achieve this goal, I visited the laboratory of Prof. Pandit at the Network of Excellence for Functional Biomaterials (NFB) Institute of NUI University in Galway (Ireland). The group of Prof. Pandit has worked extensively with plasmids delivery within hydrogels<sup>[138]</sup>. The 42

objective of the stay was to learn encapsulation technique in lipoplexes, to test the bioactivity of the encapsulated plasmids, to optimize a lipofectamine/plasmid formulation for *in vitro* transfection, to learn fabrication of fibrin hydrogels/lipoplexes constructs, and to test their stability and transfection potential.

In this chapter, I will present my work in Prof. Pandit's group to develop transfection technique via lipoplexes, the optimization of all the transfection parameters and the detection process. Then, I will compare the results obtained with stably transfected cells. Those stably transfected cells will serve as a model to optimize heat-shock procedures.

# 2.1. Transfection with a novel transgene: principle and plasmid characterization

#### 2.1.1. What is a heat shock protein?

Sōti et al.<sup>[139]</sup> debribed heat-shock proteins (HSP) as molecular chaperones that protect other proteins against aggregation, solubilize initial, loose protein aggregates, assist in folding of nascent proteins or in refolding of damaged proteins, target severely damaged proteins to degradation and in case of excessive damage, sequester damaged proteins to larger aggregates. These Chaperones are ubiquitous, highly conserved proteins, which utilize a cycle of ATP-driven conformational changes to refold their targets. Heat-shock proteins are called stress proteins because cellular stress, such as heat shock, leads to the expression of HSP. After stress there is an increased need for the chaperone function of HSP, which triggers their induction, to repair damaged proteins. Because HSP are expressed in response to cell stress and in particular heat shock, they are now studied as an emerging therapeutic target, and in particular as molecules that can be used to trigger subsequent events.

#### 2.1.2. Project objectives and challenges

In our approach, we are using novel synthetic gene switch activated by thermal stress to induce transgene expression, first shown by Moonen<sup>[140]</sup> and Wilson et al.<sup>[141]</sup>. By using this gene switch, they were able to control both the timing and spatial distribution of the transgene, after exposure to a thermal stress induced by ultrasound. The plasmid is composed

of a heat shock plasmid promoter which means the first part of a plasmid to be read and induce the protein translation. That promoter is only read in case of heat shock: in case of thermal stress, the plasmid will be read by the cells and will trigger transcription and subsequent translation of the protein coded in the second part of the plasmid (see figure 2.1. above).



Figure 2.1. Schematic of heat shock protein working

Temperature, time duration of heat shock and time to analyze the cells reactions after the heat shock depend on the plasmid, the cells (type and confluence), the toxicity of the transfection protocol but also of their 3D microenvironment. Plasmids used in our study, and developed by Nuria Villaboa's team, are responding to a heat-shock about 42 to 45°C of a few minutes.

The ultimate goal of our studies will be to spatially and temporally control the release of regenerative growth factors in an engineered tissue, after in situ transfection with a transgene under the control of a HSP promoter, triggering the .transgene expression by an ultrasound-induced heat-shock. To achieve this goal, we need to be able to transfect cell (integer a plasmid in the cell) with a plasmid that has a HSP promoter, so that when the cells will be submitted to a heat shock, they will express the protein for which the plasmid is coding.

To validate the approach, two steps are required: transfection in situ in hydrogels (lipoplexes) and activation of heat-dependent gene switch (see figure 2.2. above). The feasibility of the second step has been demonstrated by Wilson et al.<sup>[141]</sup>, and our main objective was to demonstrate the feasibility of the first step.



Figure 2.2. Schematic of heat activation by ultrasound, in cell-seeded hydrogel

To transfect cells within hydrogel, the final approach chosen was to bring only hydrogels with lipoplexes to the bone healing position. In the envisaged clinical scenario, patient's cells will colonized the hydrogel and will be transfected. Then, in presence of chemical such as rapamycin and of a heat shock, cells will produce growth factor to help either the vascularization development or to stimulate bone formation.

#### 2.1.3. Plasmids description and characterization

In a collaboration context, two different types of plasmids were provided to us by the laboratory of Nuria Villaboa in Madrid<sup>†</sup>: one with firefly luciferase and the other one with DsRed monomer plasmids, both under the control of a HSP promoter.

Detection of the expression of the fist firefly luciferase plasmid requires the detection and quantification of the levels of luciferase activity (bioluminescence) using a luminometer, after cell lysis. As the firefly luciferase activity is low, it is not detectable on common microscope but require the use of a CCD camera approach. The expression of the second plasmid, Red monomer, is like a fluorescent staining and should be visible by microscopy.

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Plasmid were first amplified in Madrid following a MaxiPrep procedure and then directly sent to us. To characterize our samples, the plasmid concentration and the samples purity were evaluated using a NanoDrop2000 device, for both plasmids. The NanoDrop2000 device is a spectrophotometer which measures, with just 1µl of sample, the absorbance of tested samples. The device has a wide range of wavelength which allow the detection of a variety of samples types including peptides (205nm), DNA and RNA (260nm), purified protein (280nm) or gold particles (520nm).

To operate this device and measure and estimate a plasmid concentration, a two-step procedure was required. First, a blank (reference) was obtained by measuring the absorbance of a droplet of distilled water, and the measuring unit is then cleaned with a tissue to wipe down. Then a droplet of each plasmid was put (one by one) to evaluate the samples properties. Because a plasmid sample has an absorbance peak around 260nm, the absorbance. peak measures at this wavelength gives us the concentration of plasmid in the sample (see figure 2.3. above).



Figure 2.3. Example of a nanocurve plasmid curve

The concentrations obtained were:

- HSP-70-DsRed : 1940 ng/μL
- HSP-70-fLuc : 739.2 ng/ $\mu$ L

The purity of the sample is evaluated by looking at the ratio of absorbance values at 260nm and at 230nm. If the sample is not pure, it can have a peak at 230nm which corresponds to proteins. Both samples were assessed as pure as the ratio is about 2, indicating the absence of a significant peak at 230nm.

#### 2.2. Development of Transfection parameters

To develop and optimize our transfection procedures, we decided to work with HeLa cells, which are a cancer cells line (originally from a cervix cancer) very well characterized, easy to grow and relatively easy to transfect. We developed our lipoplexes formulation using lipofectamine 2000 (ThermoFisher) and our plasmid of interest.

#### 2.2.1. Cell culture

HeLa cells were cultured in DMEM with 10% Fetal Bovine Serum FBS and 1% Penicillin/streptavidin (P/S) in 75cm<sup>3</sup> flask. At confluence, flask media was removed; cells were rinse to remove any presence of FBS. Then cells were detached with 3 ml of trypsin at 37°C for 3 minutes. After checking under the microscope that cells were detached, 10 ml of complete media (10% FBS) was added into the flask to stop the action of trypsin. Centrifugation of the cells solution was established at 300 g for 5 minutes. Media was removed and 5 ml of new media was added to the cells pellet and pipetting up and down for mixing. Cells were then counted with trypan blue and seeded back in flask. The rest of cells were seeded in well plates for transfection experiments. This cell line is growing very fast, and so was split twice a week.

#### **2.2.2. Transfection principle and protocol**

#### 2.2.2.1. Lipoplexes formulation

Liposomes are multifaceted and versatile non-viral delivery systems. They are spherical lipid bilayers of diameters in the range of 50-1000 nm that have proven useful as convenient delivery vehicles for biologically active compounds<sup>[138]</sup>. We decided to use lipoplexes to encapsulate our plasmids because of the possible liposomal gene delivery and the possible benefits of a combined liposome-scaffold approach, such as long-term expression, enhanced stability, reduction in toxicity and ability to produce spatio-temporal expression patterns.

Lipoplexes are lipids with proteins that spontaneously form a bilayered vesicle, which can contain any type of particle such as plasmid. Lipoplexes have similar properties than cells membranes, so they will naturally fused with cells membrane to deliver their content inside the cells. It is a simple, rapid and can be biocompatible at optimum concentration and time of exposure in the cells media. As transfection by lipoplexes may be toxic for cells at too high concentration, the first thing to do is to evaluate the best conditions for a transfection with reduced mortality.

Our first step was to culture the cells, and transfected them with different concentrations to evaluate the transfection rate versus cells mortality. For transfection, lipoplexes were prepared by incubating lipofectamine in 0% FBS media for 5 minutes. Concurrently plasmids were incubation for 5 minutes in 0% FBS media too. Then, both solutions were mixed and incubated for 15 to 20 minutes.

Theoretically, we would expect a higher transfection rate for the higher amount of plasmid deliverd into the wells. But, as transfection may be toxic for cells, we don't want to use too much lipofectamine. Moreover, a cell cannot expressed an unlimited amount of targeted protein, so it is important to know the DNA amount threshold above which cells are not expressing more protein of interest. To know those parameter and adapt our transfection protocol, the influence of the ratio lipofectamine:plasmid was evaluated by comparing luminescence activity for the different conditions tested.

Two distinct culture protocols had to be established to fulfill the specific requirements of the two plasmids: the Ds Red monomer has to be observed with a fluorescence microscope but

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the luciferase will be quantified with a luminometer. Cells were seeded either in 48 well plates for luciferase plasmid transfection, or in coverglass eight well plates (LabTek) for red monomer plasmid transfection.

When the cells reached about 80% confluence, a mix of lipofectamine 2000 (Invitrogen) and plasmid in media without serum was prepared and then added to the cultured cells. To prepare the lipoplexes, lipofectamine and plasmid, at different ratio, were put separately into Eppendorf tubes containing culture media with 3% of serum, for five minutes to equilibrate pH (figure 2.4. below). Then, both solutions were mixed, by pipetting, to form lipoplexes, and incubated at room temperature for five minutes. The final solution of lipoplexes was added into the wells containing cells an incubated for 48h at 37°C (within an HERA Cell incubator). Finally, a heat-shock was applied, of varying duration and at different temperature, to induce the plasmid expression, and the transfection efficiencies were analyzed using fluorescence microscopy for the Ds Red monomer plasmid, and a luciferase assay for the firefly luciferase plasmid.



Figure 2.4. Steps required for cells transfection and gene activation

#### 2.2.2.2. Evaluation of plasmid integrity

We tested the stability of plasmid after encapsulation at a concentration of 0.5µg plasmid with 1µl lipofectamine, by electrophoresis.

Electrophoresis with was made to analyze molecular weight of the plasmid to check that the encapsulation process did result in any size modification. A change of size would reveal that plasmid have been degraded during the encapsulation. Electrophoresis is performed by setting DNA upon a viscous medium, the gel, where an electric field induces the nucleic acids to migrate toward the anode, due to the net negative charge of the sugar-phosphate backbone of the nucleic acid chain. The separation of these fragments is accomplished by exploiting the mobilities with which different sized molecules are able to pass through the gels.

We prepared 1% agarose gel (agarose – A9539 – 500g Sigma) by dissolving 1g agarose with 100ml of 1X TAE (Tris, Acétate, EDTA buffer) in a microwavable flask placed a microwave for 1 to 3 minutes until complete dissolution.

Electrophoresis content was prepared using a gel tray with holes to be able to place the samples.  $10\mu 1$  SYBR safe DNA gel strain was added into 100ml of agarose (10000X concentrated in DMSO – Invitrogen S33102) and the agarose was let cooling down in the electrophoresis "tank". TAE buffer was then added to the box until it recovers the gel.

 $3\mu$ l Bromophenol blue was added in each sample of  $10\mu$ l, to be able to look at the DNA under UV light. 4 samples were loaded in the agarose gel:

- A commercially available DNA ladder of 1kB (BioLabs): serve as a standard for DNA size measurement
- 10µl of fLuc plasmid with 2µl water
- 10µl lipoplexes with 2µl water
- 10µl lipoplexes with 2µl of triton X, to dissolve the lipoplexes structure and release the plasmid

The electrophoresis tank was connected to a generator (consort E861) generating 90V for 1h30. After 1h30, the power was turned off, the electrodes were disconnected and the gel was removed from the tank. Pictures were taken under UV light (G bow, Mason) using the "GeneSnap" software (see figure 2.5. below).



Figure 2.5. <u>Electrophoresis UV picture (from left to right: DNA ladder, plasmid, Lipoplexes,</u> <u>Lipoplexes with triton X) and manufacturer data for the 1kb DNA ladder</u>

Plasmid alone and plasmid inside lipoplexes gave the same electrophoresis profile with two bands, certainly corresponding to two conformation of the plasmid. When triton X was used less plasmid was obtained resulting in a thinner band, and the apparition of bands of very large size, certainly due to opening of the DNA strands after degradation by triton X. The comparison with the DNA ladder revealed two bands, one above 10 kilobases and another at about six kilobases.

#### 2.2.2.3. Transfection of the DsRed Plasmid:

Different DNA:lipofectamine ratios (1:0.7; 1:1.7 and 1:3) were tried for different heat shock temperatures (37°C and 44°C), realized by putting well plate in a water-bath at the desired

temperatures for 30 minutes, to test the plasmid activation. Fluorescence microscopy was done after 24h (with an Olympus IX81 fluorescent microscope).

On the coverglass wells, many cells dead were observed round shape cells without attachment to the culture surface. Coverglass bottom is needed to have less signal attenuation, to be able to see the fluorescence on the microscope (see figure 2.6. below for fluorescence microscopy pictures), but it seems that cells were not enough attached on coverglass to support transfection via lipoplexes.



Figure 2.6. Microscope pictures of control (left) and transfected cells (right)

There was no difference between control and transfected cells with a heat shock for 30 minutes at 37°C. At 44°C, the fluorescence picture revealed that there were few transfected cells but the fluorescence background of the cells didn't allow to make any significant conclusion. Indeed a close look at picture of control cells (figure 2.6. above) reveals that there was a high fluorescence background which may be also due to the number of dead cells seen. So, with this protocol and this plasmid, it was not possible to detect properly the fluorescence resulting from the transfection of cells and response to a heat shock.

This is why we decided to focus on the firefly luciferase (fLuc) plasmid, which is detectable by luminometer, after cells lysis.

#### 2.2.2.4. Transfection of the luciferase plasmid:

HeLa cells were seeded in 48 well plates in low amount to enable them to reach confluence being homogenously spread in wells.

Two ratios plasmid:lipofectamine were tested to get the best transfection parameters (high transfection rate and high cell viability): 1:4 and 1:2, according to what people in Prof. Pandit's group were used to do. For the ratio 1:4, 12.675 $\mu$ L of HSP-70-fLuc was mixed with 468.75 $\mu$ L of media at 3% of serum, and then mix with 500 $\mu$ L of lipofectamine solution (937.5 $\mu$ L of media at 3% serum and 75 $\mu$ L of lipofectamine). Then 50 $\mu$ L of this mix was added into each well for a final 1:4 ratio, which correspond to a final concentration of 0.5 $\mu$ g of DNA per well. The same process was performed for the 1:2 ratio, but resulting in 1 $\mu$ g of DNA per well.

Three different plates were prepared, one for control cells (three wells cultured), and one for each of the two ratios tested (each one with six wells plated).

Lipoplexes solution was kept overnight in the cells wells and then removed and replaced by culture media with serum. Then, each culture plate transfected was submitted to a heat shock at a different temperature and luminescence analysis performed to detect the luciferase activity.

#### 2.2.3. Influence of the lipofectamine:plasmid ratio on transfection efficiency

Different heat shock temperatures were tested for each of the two lipoplexes preparation, in order to select the best conditions of plasmid activation: 37°C (control), 44°C and 49°C for 30minutes. To let time to protein to be expressed, cells were kept in the incubator 5h after heat shock. The following subsections describe the experimental procedure and the results.

#### 2.2.3.1. Assessment of transfection efficiency in 48 well plates

We first worked with 48-wells culture plate (well diameter around 11mm). To induce the heat-shock, each culture plate was placed in a water bath with controlled temperature of either 37°C (control), 44°C or 49°C, for 30 minutes. Culture plates were then placed back in the incubator for 5h after heat shock, before luciferase assay.

The luciferase assay was done according to the manufacturer protocol (Promega, luciferase assay system E1500): cells were lyzed and a luciferin solution was added to induce bioluminescence that was quantified with a luminometer. Briefly, the media was removed from wells, washed with Phosphate Buffer Saline PBS and PBS was removed. 75µl of lysis

buffer was added in each well (for 48 well plate –  $150\mu$ l in case of 24 well plate). Then, the cells were scraped, removed and pooled to be transferred to micro-tubes on ice. Tubes were vortexed for 10 to 15 seconds and centrifuged at 12000g for 15 seconds at room temperature (centrifuge, pico 17). The supernatant were transferred to new tubes. 100µl of luciferase assay reagent was added to 20µl of this cell lysate and transferred to a 96 well plate for a lecture in the luminometer (Varioskan flash, thermoscientific), measuring the light coming from each well. The results are summarized on Figure 2.7 below.



Figure 2.7. <u>Luciferase activity: percentage of cells luminescence compared to cells auto</u> <u>luminescence</u>

Three samples of each condition were mixed to have a larger amount of cells, and read on the luminometer, resulting in two values for each lipoplexes conditions. The values plotted on the graph of figure 2.7 are the mean of these two values.

At 37°C and 49°C there is no change of luciferase activity when we increase the amount of DNA (see figure 2.7. above): no luciferase activity could be detected when compared to the control cells that were not transfected. The results at 37°C are in agreement with the absence of heat-shock, and therefore the absence of activation of the transgene. For the 49°C heat-shock, the results may be explained by a high cell mortality observed (by microscopy) after the 30 minutes exposure to this high temperature.

Luciferase activity at 44°C was between three and four times larger than the control activity at 37°C, suggesting that the cells were transfected and responded actively to the heat shock. The activity is also higher with 0.5µg of DNA than for 1µg of DNA, which can be explained by a saturation effect. In presence of high concentration of DNA lipoplexes may not be well created. In fact if the DNA concentration is too high, plasmids may form complexes and so cannot fit in lipofectamine complexes. So, more DNA will stay in the supernatant and not be encapsulated in lipoplexes, which will induce a decrease in transfection rate.

One problem revealed here was the inhomogeneity of the cells spreading within the wells of the 48 well plates, which resulted in a non-uniform transfection rate over the well. To try to improve the transfection rate, we decided to conduct tests in 24 well plates; to achieve a more homogenous cell distribution throughout the well.

#### 2.2.3.2. Improving the transfection: increasing the seeding homogeneity:

#### Influence of transfection parameters on cells

To be able to compare the luminescence activity between the different conditions, we have to normalize the data by the number of cells within each well.

To assess the number of cells before luminescence analysis, the protein amount was quantified for these samples. MicroBCA protein assay kit (ThermoFisher, n°23227) was used and samples were put in 96 well plate with black borders and read with the luminometer (Varioskan) with an absorbance at 562nm (bandwidth 5nm).

Briefly, a high concentrated solution of albumin (concentration given by manufacturer) was diluted to establish a calibration curve to link fluorescence and number of protein. A working reagent for the detection was prepared following the manufacturer recommendations. Samples to analyze were put in a 96 well plate and absorbance was read with the Varioskan (see calibration curve results in figure 2.8. below).



*Figure 2.8. Calibration curve with BSA was done to link absorbance and protein concentration.* 

The absorbance and the protein amount were linked by the equation: y= 29.981x - 3.2075 so, the protein concentration is equal to (absorbance value + 3.2075)/29.981.

As, the protein amount in our samples was not known and we wanted to be within the range of the calibration curve, samples were diluted according to two different dilutions: by 30 (40 $\mu$ l in 1.2ml) and by 40 (30 $\mu$ l in 1.2ml). In each well 150 $\mu$ l of the sample was added with 150 $\mu$ l of working reagent and put in the incubator (37°C) for 1h.

As preliminary results, absorbance at 562nm was read (mean of three luminometer reading per well) and the mean of the two samples per conditions was represented in figure 2.9. below.



*Figure 2.9. <u>Protein amount according to heat shock temperature and transfection condition</u> For the concentration C2, many cells died after HS at 49°C, but the protein amount is roughly the same for all conditions tested (control, C1, C2 and C3 for the three heat shock temperature). So the difference of value with the luciferase assay was only due to the luciferase activity (does not depend on the cells amount).* 

#### Optimization of transfection efficiency for 24 well plates

To assess the influence of seeding homogeneity of the cells, 24 well plates were chosen because we observed a more homogenous distribution of the cells in these wells at the time of seeding. Cells were seeded at low concentration (10000 cells per well) and kept in culture (DMEM 10% FBS) until they reached confluence. At 70-80% confluence (about 72h after plating), medium was removed,  $500\mu$ l of fresh media at 3% FBS was put in each well, and  $100\mu$ l of lipoplexes solution was added in each well, with different DNA: lipofectamine ratios (figure 2.10. below):

- Concentration 1: 0.5µg DNA and 1µl lipofectamine per well ratio 1:2
- Concentration 2: 0.5µg DNA and 0.5µl lipofectamine per well ratio 1:1
- Concentration 3: 0.75µg DNA and 1µl lipofectamine per well ratio 0.75:1
- Control: cells in presence of media with 3% PBS (same medium as conditions 1-3, but without lipoplexes).



Figure 2.10. Schematic representation of transfection parameters tested

To obtain preliminary results, only two wells were transfected for each transfection ratio (figure 2.10). Transfection solutions were let with cells for 6h and removed to be changed for fresh complete media (10% FBS), in order to remove all lipoplexes not fused by the cells. This enabled to avoid any unnecessary toxicity effect of lipofectamine on cells.

Three plates were used to evaluate three heat shock temperatures: 37°C, 44°C and 49°C for 30 minutes. 5h after the heat shock, luciferase assay (Luciferase assay kit, Promega) was done to evaluate the luciferase activity for the different parameters tested.

Cells were observed by microscopy before and after heat shock (5h after, just before analysis). No differences in cells morphology and density were seen between before and 5h after heat shock, suggesting that heat shock did not introduce any adverse effects on cells. Pictures, taken with a 3MPixel Camera X10, just before the heat shock, are shown in figure 2.11. below:



Figure 2.11. 16h after incubation with lipoplexes, just before heat shock (C1, C2, C3, control)

Then, the luciferase activity 5h after heat shock was determined with luminometer and is showed in figure 2.12. below. Each column represents the average of the two wells studied for each condition.



Figure 2.12. <u>Evaluation of the transfection ratio by luciferase activity analysis, 5hrs after</u> <u>the heat shock.</u>

All values found were relative values of luminescence and were calculated as the ratio of the actual luminescence by the luminescence of the control cells, for a similar heat shock temperature. Luciferase activity 5h after heat shock showed (see figure 2.12. above) that:

- At 37°C: There was no difference in luminescence between transfected and nontransfected cells. This was expected as there was no heat-shock and therefore no activation of the transgene.
- At 49°C, no luciferase activity appeared. This may be due to an important number of dead cells or inactivation of luciferase expression, both due to a too intense heat shock too.
- At 44°C: A significant increase in luciferase activity was detected compared to the controls, and there were significant differences between the three formulations tested.

According to our results, the best transfection efficiency is obtained into in 24 well plates for a ratio and concentration of DNA and lipofectamine of: 0.5µg DNA and 1µl lipofectamine, as assessed after activation with a heat shock at 44°C for 30 minutes.

As the cells number was found similar for all conditions, all results are comparable. Concentration C1 was so found to be the best for cells transfection, without affecting cells viability.

## 2.2.4. Technology adapted to our laboratory - Proof of concept for luciferase detection

The first thing tried was to reproduce the transfection and to be able to detect it in our conditions, in the Pf. Dumontet laboratory. 30000 HeLa cells were seeded in 24 well plate, in DMEM 10% FBS. Three days after, cells were confluent homogenously spread within the wells and were able to be transfected. For that, media was removed by 400µL DMEM at 3% FBS with 100µL of DMEM at 0% FBS in each well of control cells. For cells to be transfected, 400µL DMEM at 3% FBS was added with 100µL of lipoplexes in media without serum, which gave a final volume in each well of 500µL.

Lipoplexes were prepared as for the previous experiments, in NFB laboratory, with 0.5µg of plasmids and 1µL de lipofectamine in 100µL of media per well. First, experiments to see the feasibility to detect luminescence with new protocol, adapted to analysis equipment available in the Pf. Dumontet laboratory in Lyon. For that, in one tube 200µL de DMEM at 0% FBS was added with 2µg of plasmids (corresponding to 2.70µl of fLuc plasmid) and incubated for 5 minutes. Concurrently, 200µL of DMEM 0% FBS with 4µL of lipofectamine were also incubated in another tube for 5 minutes too. Both solutions were mixed by pipetting and incubated for 15 to 20 minutes. Then, 100µL of newly formed lipoplexes were added in each well (3 wells per conditions).

About 6h after, media was removed and replaced by DMEM 10% FBS to avoid any lipoplexes to stay in the surrounding media and be toxic for cells.

Next day, 30min of heat shock at 44°C was done and cells were incubated for 5h before analyzing the luminescence activity, thanks to Luciferin (Promega, Luciferin-EF, 25mg, E6551), diluted at 5mg/ml.

Briefly, cells were detached with 200  $\mu$ l of trypsin, centrifuged and resuspended in 120 $\mu$ l of PBS. 20 $\mu$ l was used to evaluate the viability with trypan blue and the 100 $\mu$ l remaining were put into 96well plate (white borders) with 20 $\mu$ l de luciferin per well and luminescence was read (Chameleon V, Hidex). A mean of the luminescence readings of each well per condition was done and represented in figure 2.13. below.



Figure 2.13. Luminescence detection of transfected HeLa cells (30min heat shock at 44°C)

The luminescence was detectable even without any cells lysis, by using luciferin at 5mg/ml. Cells viability was found to be 94.3% for transfected cells and 94.8% for control cells, so these heat shock conditions were not toxic for cells and enable luminescence detection with the Chameleon V luminometer.

In conclusion, we derived an optimized lipoplexes formulation, in which the plasmid are stables, that did not affect cellular viability, and provided the maximum activation of the encapsulated transgene after incubation with cells and activation by a heat-shock at 44°C for 30 minutes.

For the next step of our project, and to be able in the future to transfect cells in situ, we wanted to embed the lipoplexes within fibrin hydrogels. I will present in the next section the

work performed to analyze the natural stability of the lipoplexes in the gel (release of plasmid) and the plasmid integrity after embedding lipoplexes in the hydrogel, and our tentative to transfect cells within gels.

#### 2.3. Hydrogel fabrication and dissolution

To bring growth factors directly in the damaged site, embedded hydrogels are needed. Analysis of their stability is also important to know all gels release properties. In our case, gels with lipoplexes were made and their supernatant were taken and analyzed. Dissolution of gels was also needed to study the lipoplexes stability after encapsulation process and to compare the lipoplexes amount with the initial amount added during the encapsulation process.

#### 2.3.1. Hydrogel fabrication

The first challenges were to find good parameters to make cohesive gels where cells and lipoplexes can then be encapsulated. Fibrin gels were chosen because it is a biocompatible and biodegradable and it is commonly used as implantable hydrogel that presents it such as a good biomaterial for our application.

Different component were used to make fibrin gels:

- Fibrinogen (Fibrinkleber SD, TIM3 US 5ml, VNK4K007, Mat Nr: 1500447)
- Fibrinogen dilution buffer (VH S/D), sterile
- CaCl<sub>2</sub>Lg (400MM 5ml, Hameln, VN125065, Mat Nr: 3500025)
- Thrombin (VH/SD, TIM5 4IE/ml 5ml, VNF4H007, Mat Nr: 1500714)

To make fibrin gels, Pandit's group was inserting, with a syringe, sterile fibrinogen dilution buffer inside the fibrinogen tube and mix, with a stirrer, for 10 minutes at 37°C (volume in respect to the fibrin tube specification -5ml). Concurrently CaCl<sub>2</sub> and thrombin were mixed and kept at 37°C (volume in respect to the manufacturer indication -5ml).

Different dilutions of gel were tested but the best ratio found was to, concurrently, pipette  $100\mu$ l of fibrinogen and  $100\mu$ l of thrombin mixing together into a 48well plate. This ratio gave a fibrinogen-thrombin solution at 2U/ml final thrombin concentration. About 10 minutes

later, the gel was cross-linked and  $200\mu$ l of media without serum (FBS) can be added into each well.

Optimization of gels fabrication protocol done, fibrin gels with embedded lipoplexes were made mixing lipoplexes with thrombin (three times more concentrated than in 2D, because we expected some lost). Lipoplexes solution was chosen to be five times more concentrated than the one used in 2D culture, not to dilute fibrinogen solution too much. 100µl of the thrombin-lipoplexes solution was mixed with 100µl fibrin into a 48 well plates. Final concentration in one single gel was 1.5µg DNA and 3µl of lipofectamine. After crosslinking time, media without serum (to preserve lipoplexes integrity) was added in each well.

To be able to evaluate a transfection rate into fibrin gels, first, the DNA release was evaluated. For that, different steps were needed:

- Take supernatant at different days (from gels with or without lipoplexes) and evaluate:
  - o DNA content
  - o Lipoplexes integrity with electrophoresis
  - Transfection with supernatant at day 0 and day 6
- Dissolve the gel and test transfection (day 0 and day 6) from gels with or without lipoplexes inside
- Put some gels on cells to test transfection in situ (6 days gels with lipoplexes, left 1.5 day in contact with cells) directly from gels made 6 days ago.

#### 2.3.2. Hydrogel dissolution and plasmid release

To dissolve fibrin gel, plasmin from human plasma was commonly used (P1867-150UG, Sigma) in Prof Pandit laboratory. In that case, aprotinin shouldn't be used as it helps the gel to be stable and not dissolved.

Tests with 100nM of plasmin into the fibrin gel and up to 10 times higher concentrations were done but no dissolution occurred, even after 24h in an incubator at 37°C. At Day 0 and day 6, no DNA released was detected with PicoGreen assay. At day 7 electrophoresis analysis revealed the same: no DNA was detected in the supernatant and we didn't manage to dissolve the gel with plasmin as we didn't see any DNA in the supernatant from the gel with plasmin.

Electrophoresis on gels dissolved with NaOH (0.1M,  $200\mu$ l) revealed that we can detect DNA but NaOH seem to degrade the DNA as the band was less clear and more spread than the one from DNA ladder.

Supernatant was also analyzed on Nanodrop to check the results obtain by electrophoresis. The peak was found at 230nm and not at 260nm which means that there was no DNA. Gels with or without lipoplexes have a peak at 260nm. The longer the gel was with the media, the more the test (PicoGreen assay) measured DNA amount values even for gel without lipoplexes, which means gels have a background at 260nm.

Even if no DNA was detected in supernatant, at day 6, I tried to see if I can transfect cells with lipoplexes from fibrin gels (after presence of plasmin or not). Different transfection parameters tested were:

- Supernatant from 7 days gels
- Supernatant from 7 days gels without lipoplexes
- Gels dissolved with plasmin
- Gels without lipoplexes dissolved with plasmin
- Cells alone
- Cells in contact with 7 days gels

Luciferase assay after transfection and heat shock didn't reveal any transfection.

# Preliminary conclusion from transfection and encapsulation processes, done in Pf Pandit's laboratory:

- Cells can be transfected with lipoplexes incorporating HSP-plasmids.
- Heat Shock at 44°C for 30 minutes seems to activate the HSP-switch
- With HeLa cells (in 2D), best luciferase expression was obtained with a ratio DNA:lipofectamine of 1:2 and 0.5µg of DNA per well in 24 well plates.
- No DNA release was observed from gels containing lipoplexes and so no transfection was obtained from supernatant.
- We encountered few dissolution issues which may be due to fibrinogen or thrombin dilution reagents used.

#### 2.3.3. Perspectives / next experiments to be conducted

According to preliminary results, different experiments can be experienced to optimize the transfection and protein detection protocol. In that context, different experiments were considered:

- Determine the ideal temperature (about 44°C) to the HS
- Determine the time necessary to transfect cells (time between the insertion of lipoplexes into wells and the time when we can remove the media)
- Determine the ideal time between HS and luciferase activity analysis
- Make new gels and try to find why we couldn't dissolve them (try different reconstitution reagents)
- Evaluate time and temperature optimal conditions for detection of luciferase activity within gels.
- Use Hela M1 (Hela cells stably transfected) to linked luciferase activity values from the luminometer to the number of cells transfected and compare reaction of stably transfected cells and the one transfected by lipoplexes.

#### 2.4. Heat shock optimization on stably transfected cells

In parallel to working on the transfection protocol, we also worked with a cell line that is stably transfected with a HSP-inducible gene switch. The cell line M1 was provided to us by Richard Voellmy from HSF Pharmaceuticals SA, who developed it in collaboration with Nuria Villaboa. The cell line has been described by Martin-Saavedra et al.<sup>[141]</sup>.We decided to work with this cell line to optimize our heat shock protocols. We also initially envisioned to develop the heat-shock induction by ultrasound with this cell line, but that will be discussed as remaining task as well as an ultrasound-induced heat shock of cells embedded within hydrogel.

#### 2.4.1. Luciferase detection on HeLa M1 cells (stably transfected cells)

#### 2.4.1.1. Calibration of luciferase detection

Luminescence activity is so detectable in our conditions. To first compare with transfected cells, a calibration curve to link luminescence and number of cells was established. In fact, HeLa M1 are stably transfected (with the fLuc plasmid) cells so detection of one luciferase activity corresponds to one cell.

Different cells concentrations were seeded (from 10000 to 500000 cells) in a 24 well plate and a heat shock of 43°C and 1h was chosen, according to commonly used heat shock for this cell line<sup>[141]</sup>.

Luminescence was read with the luminometer Chameleon V (Hidex). Luciferine (Promega, Luciferin-EF, 25mg, E6551) was diluted at 5mg/ml and 20 $\mu$ l was added with 100 $\mu$ l of cells solution, in 96 well plates. Each well was read 10 times and luminometer is giving the mean. Then, the mean and the standard deviation of the three wells per condition were established and reported in table 2.1. below.

<u>Cells number</u>	Mean according to cells number	<u>Standard deviation</u> according to cells number
500000	10638	2814
100000	9443	1520
80000	6143	689
60000	2925	1331
40000	1117	520
20000	397	66
10000	116	35
No cells	35	9

Table 2.1. <u>HeLa M1 luminescence, after 1h heat shock at 43°C</u>



The calibration curve (figure 2.14 above) reveals that it seems that either there is a maximum luminescence for which we cannot detect properly with the luminometer or there is a cells concentration threshold from which the curve is not anymore following a "simple" equation (which may be due to the cells growing conditions – too much concentrated for the well). If we delete the highest cells number in the curve, we can see that the luminescence and the number of cells are linkedby an exponential equation until 80000 cells (see figure 2.15. below).



Figure 2.15. Calibration curve for HeLa M1 luminescence, after 1h heat shock at 43°C

After knowing the link between cells number and luminescence, a study of the effect of the heat shock temperature was done, with each time three wells of cells (at confluency) submitted to 1h heat shock (water bath), with temperature check with thermometer.

#### 2.4.1.2. Heat shock optimization

**Optimale temperature** 



Figure 2.16. Effect of heat shock temperature on luminescence activity (1h heat shock)

Figure 2.16. reveals that the maximum luminescence value is about 30000, so the change in the calibration curve after 80000 cells is not due to a luminescence maximum of the device. Luminescence seems much higher for 43°C than for 45 and 41°C. shouldn't be transcripted into number of cells to keep being consistant.

To better have a look of luminescence activity (except at 43°C), the curve below (figure 2.17) is showing all the other conditions tested.



Figure 2.17. Effect of heat shock temperature on luminescence activity (1h heat shock)

In figure 2.17. luminescence activity is increasing until 41°C and then decreasing again.

To be sure that 43°C is the optimal heat shock temperature, new heat shock were done for temperature around 43°C and were representing in the same graph (figure 2.18. below). A control condition with just PBS and one with PBS and luciferase reagent for detection were also experiment to see if there is any luminescence threshold of those components.



*Figure 2.18. Effect of heat shock temperature on luminescence activity (1h heat shock)* 43°C is the optimal heat shock temperature as the luminescence is more than twice the one at 42°C and 44°C. Luminescence at 42, 43 and 44°C is about 10 times more important than for other temperatures. The difference between those 3 temperatures and the others is so high that we can wonder if this difference is linked to cells viability. So, a viability analysis on those wells was performed (see figure 2.19. below) to see this difference is due to a change in cells death.



Figure 2.19. Effect of heat shock temperature on cells viability (1h heat shock)

The cells viability is about the same for all temperatures until 43°C. Then, from 44°C the cells viability seems to decrease which may explain the luminescence activity decrease. So, to keep good cell viability and detect the maximum luminescence activity, 43°C is the temperature chosen for the heat shock.

#### Optimal heat shock duration

The optimal temperature found, a time analysis was done to evaluate, at 43°C the luciferase activity depending on heat shock duration.



Figure 2.20. Effect of heat shock duration on luminescence activity (at 43°C)

Cell viability was 90% for 10 minutes and 30 minutes and 75% for 1h, 1h30 and 2h. Luminescence is more important for 30 minutes heat shock than for 1h and 1h30. Also, luminescence activity is higher for 2h than for other time duration but as the viability is less important (see figure 2.20. above), 30minutes was chosen to be the best compromise between keeping cells alive and detecting the luminescence.
## 2.5. Conclusions and perspectives:

The best conditions to transfect HeLa cells, including seeding protocol, transfection protocol, heat shock duration and temperature intensity to be able to detect the transfection were established for 2D culture. Our optimized protocol is a heat-shock of 43°C, for 30 minutes, resulting in the largest luminescence detection associated with a high cell viability (similar for control and transfected cells). Luminescence was also detectable without any cells lysis.

We also reported the fabrication of stable fibrin gels with embedded lipoplexes. Because these hydrogels do not degrade the lipoplexes, we believe that this approach would be suitable for in situ (within the hydrogel) transfection.

These preliminary results open many perspectives in tissue engineering and especially for bone repair applications. The proposed approach is envisioned as an alternative to growth factor delivery by hydrogels, where the release kinetics is very difficult to control and which are often associated with a burst release in the first days and sustained delivery. The proposed growth factor delivery would rely on in situ transfection within fibrin gels embedded with lipoplexes loaded with HSP-plasmids. Similarly to Martin-Saavedra et al.<sup>[141]</sup>, ultrasound would be used to trigger and temporally and spatially control the activation of the plasmid expression by locally inducing a heat-shock.

To go toward an *in vivo* application, different steps are now required: *in vitro* ultrasound heat shock production validation, cells encapsulation with lipoplexes in gel and with an acoustic heat shock generation<sup>[142]</sup>, for a final specific activation proof of concept with plasmid activated by concomitant presence of heat shock and specific chemical. Then, *in vivo* first tests would be able to be done.



<u>Hydrogel in situ</u> (lipoplexes inside) <u>Activation in situ:</u> Schematic representation of transfected cells in situ

## Figure 2.21. In vivo schematic representation of ultrasound controlled release of growth factors

The idea is to insert in an animal model hydrogel full of lipoplexes, let some time for cells colonization of the hydrogel (see figure 2.21.). Then, ultrasound would be used to activate the plasmid expression by the transfected animal cells.

A spatially and temporally control production of growth factors by the own patient cells open many perspectives for bone healing, especially as it will avoid any side effect such as a too much concentrated growth factor release or an non-well localized release.

# Chapter 3 – Design and characterization of an acoustic device for cells levitation

In tissue engineering, a new research field that is extensively explored nowadays is to understand how the cells react in three-dimensions, in order to more precisely understand how an organ could work. Layered cell organization is a ubiquitous phenomenon in biological tissue in both developmental and matured stage of organism such as for skin, breast or cerebral cortex. As of now, it is difficult and time consuming to assemble cells in a 3D multilayer construct. Several technologies are being developed to control cell patterning in scaffolds, such as micro-scale hydrogels (microgel) assembly, scaffold seeding or bioprinting, but suffer from a difficult control of cells organization, multi-steps time consuming procedures, adverse effects and an impossibility to control cell-cell interactions or cellpacking density and require experimental apparatus and expertise. For wide applications in biological laboratories, such a patterning technology method should be simple, easy to implement and affordable. Here, an acoustic device was developed, in order to mimic such multilayered cell assembly, aiming a neural tissue engineering application. With an ultrasonic set-up, cells can be patterned contactless in a very fast way. Multilayers are formed in a fibrin 3D microenvironment by the effect of bulk acoustic radiation pressure on mammalian cells. Because of their larger density and lower compressibility than the surrounding fluid, cells are driven to the node planes of acoustic standing waves where there is minimal pressure. The interlayer distance is proportional to the frequency according to the equation  $f=c/\lambda$ , where c is the sound celerity and  $\lambda$  the wavelength. This technique will avoid the processing of multiple very thin gels layers to be stacked together.

Particles and cells suspended in the bulk acoustic standing waves are driven by acoustic radiation force to the regions with the lowest force potential, which corresponds to node planes of the standing waves (see figure 3.1. below<sup>[116]</sup>). The radiation force is the force generating by the ultrasound and acting on a compressible spherical particle. This force depends on the acoustic frequency, on the particles size, their compressibility and density and on the fluid properties. Commonly, ultrasound are combined with microfluidics devices for cells sorting according to their size and density, for example to separate lipids from blood cells.



*Figure 3.1. <u>Temporal progression of suspended particle aggregation in an ultrasound</u> <u>standing wave (0, few seconds and after 10 seconds)</u><sup>[116]</sup>* 

To develop this project, I spent almost one year in the laboratory of professor Demirci at the Canary Cancer Center of Stanford School of Medicine. The aim of this collaboration was to develop a device for easy and fast cell manipulation to trap cells in a multilayer sheet in fibrin hydrogels.

This project will be described in the two following chapters.

In the current one, the ultrasound device is described. We first describe the design of the acoustic resonant chamber and its acoustical characterization. We then discuss the formulation of our fibrin hydrogels and their rheological characterization. Finally, we present the experimental validation of the technique by forming 3D multilayer using polystyrene beads with geometrical (size) and physical (density) properties close to the ones of biological cells.

In the next chapter, the use of the bulk acoustic levitation assembly method to pattern embryonic stem cells derived neuro-progenitor cells will be presented.

#### **3.1. Design of the acoustic chamber**

The idea was to have a petri-dish based device for easy and reproducible experiments. If the resonant chamber is a petri dish stick by acoustic gel on the ceramic, it should easy to remove and change for sterile applications.

In order to trap cells at particular locations, acoustic standing waves have to be produced inside the acoustic chamber. Inside the chamber, incident and reflected waves are coherently summed (see figure 3.2.c. below) to produce ultrasound acoustic standing waves: a succession of minimum and maximum pressure nodes or antinodes planes, respectively. This will produce an acoustic radiation force acting on the particles that will trap them on nodes or antinodes, depending on the contrast of acoustical properties between the particles and the surrounding fluid. To generate this resonant system, a plane piezoelectric transducer (working on thickness mode, where ultrasound are produced in the direction of the ceramic thickness) is facing a reflector with high acoustic impedance (to have a large difference of impedance, between liquid in the chamber and the reflector, to allow the reflection), and the thickness of the chamber is proportional to an odd number of quarter-wavelength. The size of the resonant chamber has to be proportional to a half-wavelength of the acoustical wave within the chamber ( $\lambda$ =c/f, with f the frequency and c the sound celerity in water c = 1520 m/sec at 37°C).



Figure 3.2. <u>Schematic representation of experimental setup (a) and principle of acoustic</u> standing waves generation (b) Schematic of ceramic housing

The design of the different resonant chambers we fabricated in our studies is now presented. A piezoelectric ceramic was mounted on a poly(methyl methacrylate) (PMMA) housing, with an air backup, to force the ceramic vibrations to the ceramic center frequency (see figure 3.2.a. above). The air backup has to be at least half-wavelength height to be efficient. The

PMMA housing was designed with CorelDraw software, the different plates composing it were cut with a laser cutter (Versa Laser), and then stacked with doubled side adhesive of 80µm thickness. The piezoelectric ceramic was soldered with conductive epoxy (Atom adhesive, AA-DUCT 902). Its electrical impedance was measured with an impedance analyzer (Agilent, 4294A) and impedance matching circuit was built when needed.

The acoustic resonant chamber, which will hold the cell-fibrin prepolymer solution, was made of a poly(methyl methacrylate) ring (PMMA; inner diameter of 16mm; outer diameter: 18mm) with one side sealed with a polystyrene film of 0.2mm thickness (see figure 3.2.b. above). To mount the final system, the resonant chamber was squeezed in between the ceramic transducer and the acoustic reflector. The acoustic coupling between the polystyrene film and the ceramic transducer was realized via ultrasound gel (Aquasonic clear, Parker).

The ceramic was driven by a waveform generator (Agilent, 33522A) on Continuous Waves mode. The transmitted power set and the driving frequency were adjusted for the different piezo-ceramics used.

The first experiments were conducted to select the most efficient reflector material and to evaluate the influences of the reflector, of the chamber size and shape and of ceramic frequency.

#### 3.1.1. Selection of the reflector

In our preliminary experiments, we tested different types of reflectors to optimize the formation of standing waves within a resonant chamber of 24mm height (see figure 3.3. below). Four conditions were tested : no reflector (fluid/air interface), PMMA 6mm thick, glass 6mm thick or a second transducer facing the first one (see figure 3.3 and 3.4. below). When we started, we did not use cells or particles, but we increased the power delivered to the ceramic so that bubbles were created inside the chamber by acoustic cavitation. The different reflectors were then tested on their efficiency to create and trap bubbles. The working frequency was chosen at 680kHz, leading to a 2mm wavelength standing wave in the chamber.



Figure 3.3. Example of reflectors tested: no reflector, plastic 6mm, glass 5 mm, second ultrasound device



Figure 3.4. Zoom on the experimental setup with 2 transducers in parallel

Without reflector, no bubbles were formed. This means that, even if acoustic standing waves were generated, they were not powerful enough to create new bubbles within the chamber. Moreover the absence of reflector at the top of the chamber can lead to unexpected problems: meniscus at the borders of the chamber and vibrating water-air interface, which were visible when first tests with water were done.

With glass or PMMA reflector we observed similar bubbles patterns: bubbles were aligned every millimiter, which correspond to a half-wavelength, suggesting that the standing waves were indeed trapping bubbles at pressure nodes. With two ceramics facing each other, bubbles were also formed but merged together very quickly to form bubbles bigger than halfwavelength diameter.

Based on these data, either glass or PMMA seemed the most suitable as material for our reflector. We decided to select glass for the following experiments because the glass reflector is heavier, which make it easier to manipulate and more stable. Moreover, it has higher

impedance so acoustic standing waves generated should be better (even if it was not eyevisible).

The next step was to try to levitate and evaluate the influence of the size of the resonant chamber on the generation of a uniform acoustic pressure field (leading to uniform beads or cells layers). We also wanted to test for heterogeneous distributions of cells within a node plane, as radial patterns may be inadvertently caused by secondary acoustic forces and the associated radial acoustic pressure gradients, generated by resonance within the levitation chamber.

## 3.1.2. Influence of the resonant chamber properties

Generated bubbles were very useful to easily evaluate, by eye, the generation of acoustic standing waves. However, generating bubbles needs high power and can damage cells. So, to avoid the creation of bubbles, on a timescale corresponding to the one necessary to aggregate cells, different setups were tested.

The first hypothesis was that having a bigger chamber would avoid edge effects, allowing uniform particles trapping along the lateral dimension of the chamber. In all the setup tried here, the reflectors were stacked on the chamber. The chamber was filled with fluorescent microbeads<sup>‡</sup>, of properties similar to cells (size, compressibility and density) using the tubes located on one side of the chamber. Bead sizes of  $8\mu m$ ,  $49\mu m$  and  $202\mu m$  were tested to evaluate the versatility of the device in levitating a range of objects, ranging from single cells up to cell spheroids. All chamber borders were stacked with glue and sealed on the ceramic with silicon.

Thermo Scientific, Fluoro-Max 36-3 (8μm diameter) and 36-5 (24μm diameter) and Chromospheres BK050, RD100, RD 200 (8μm, 49μm and 202μm diameter)



Chamber 2 Chamber 3 Figure 3.5. <u>Example of chamber design tested</u>

Four different home-made square chambers were tested (see figures 3.5. above): chamber 1 (L 15mm x 1 15mm x H 24mm), chamber 2 (L 35mm x 1 35mm x H 24mm), chamber 3 (L 15mm x 1 15mm x H 12mm) and chamber 4 (see figure 3.6 below) (L 15mm x 1 15mm x H 20mm).

With the chambers 1 and chamber 2, bubbles were nucleated and we could not achieve beads levitation. For chambers 1 to 3, levitation was tested with and without any chamber bottom. Without bottom, chambers were directly stacked on the ceramic by using silicon. For all the chambers tested without bottom (1 to 3), levitation was achieved when the chamber was directly stacked on the ceramic. Levitation was also achieved with chamber 4 (see figure 3.6. below) which had a thinner chamber bottom (plastic height of about 0.5mm compared to a minimum of 1mm for other chambers).



Figure 3.6. Chamber 4: commercially available square chamber (24um beads in levitation)

The shape of petri dish seems to have an influence on layers formation. In the commercially available chamber the levitated layers of beads were not flat (see above). In fact, a square petri dish was used to generate layers and flow was detected probably due to convection. Moreover, with a square petri dish with a plastic bottom, the layers were curved. That may be due to the bottom of the petri dish that does not have the same height than its larger borders. Such patterns were never found with a round petri dish or a square chamber without bottom

(directly stacked on the ceramic). Within chamber 3 and 4 levitation occurs but in chamber 4 the layers formed are curved.

Therefore, for the proof-of-concept experiments, we used a chamber with a design similar to the chamber 3 to characterize the system. The design had to be adjusted in order to reduce the volume as it is considerably high for the experiments with cells, so a home-made tiny round chamber was designed (see below section "3.2.2.2. Final encapsulation protocol") for the applicative experiments. It has been checked that, in this chamber, the particle layers were flat (but difficultly eye-visible).

The next step was so to try to levitated beads and see the influence of the frequency to demonstrate the possibility of particle levitation with acoustic standing waves. All following experiments were done with the chamber 3.

## 3.1.3. Influence of the ceramic working frequency on particles trapping process

The frequency at which the standing waves are generated inside the resonant chamber will determine the interlayer thickness, which is equal to half-wavelength.  $24\mu m$  beads were mixed in Optiprep solution of  $1.049g/cm^3$ , to balance the gravity force (see figure 3.7.).



680kHz

340kHz

*Figure 3.7. Levitation tests for different ultrasound parameters (340 and 680 kHz) – scale* <u>bars indicate 250µm</u>

Three frequencies were tested: 340 kHz, 550 kHz (not shown here) and 680 kHz. At 340 kHz and 550 kHz the layers are not completely flat, there are curved. This may be due to the working frequency of the ceramic which is 680 kHz. So, working at another frequency may induce a non-uniform pressure field. This may also just be due to the frequency. In fact, if the frequency is lower, the radiation force will be changed and so the beads may be submitted to a smaller pressure gradient, which results in wavy layers.

The inter-layer distance is equal to half wavelength corresponding to 1.13mm at 680 kHz and 2.26mm at 340 kHz. Depending on the frequency the interlayers distance is different, so the number of layers seen in a resonant chamber will be different. As layers are more homogenously horizontally spread at central frequency and to obtain an inter-layer distance of about 250um, a ceramic working at 2.78MHz was chosen for cells experiments. To illustrate this influence of frequency on the number of layers in the two appropriate levitation setups, figure 3.8. below presents results for chamber 3 and for the round home-made chamber used for cells experiments.



Figure 3.8. Theoretical number of layers VS frequency for different chamber height

The higher the frequency is, the higher the wavelength is, so the distance between layers is smaller.

In a 12mm height chamber, at 680kHz (as used for validation with beads), 11 layers can be seen whereas in a 2.2mm height chamber at 2.78MHz (used for cells experiments) the number of layers present is 8.

## **3.2. Influence of particles properties on assembly process**

It is of great importance to know the system limitations, to better understand the levitation process and feasibility. To evaluate the setup limits, beads of different sizes and density were levitated in different conditions, within the chamber 3 (see 3.1.2.).



Figure 3.9. <u>Photo of the experimental setup for beads levitation tests and zoom in of</u> <u>the chamber for 24µm beads levitation at 680kHz</u>

First setup designed was using 2 tubes stacked at two edges of the chamber (see figure 3.9. above), to be able to fill the chamber.

## 3.2.1. Influence of beads size and density

Optiprep media was used and diluted with PBS following manufacturer recommendation to obtain a solution with precisely known density. Polystyrene beads of 1.06g/cm<sup>3</sup> and different sizes were levitated in Optiprep 1.049g/cm<sup>3</sup> as proof of concept, to show the feasibility of levitating particles. Beads size were enough important not to be transparent to ultrasound

waves and their density not too high to allow the radiation force to be higher than the gravity. First, beads were tested all separately to validate the levitation feasibility.

Beads were mixed homogenously in the chamber before applying acoustic pressure field at 680kHz. Tests were performed at this frequency to test the biggest beads possible. If interlayers distance is too small compare to beads size, no levitation can be seen.

To visualize the chamber before and after applying acoustic standing waves, pictures were taken just before turning on the power and 30 seconds after (see figure 3.10. below).



Figure 3.10. <u>49um (in optiprep 1.049g/cm<sup>3</sup>)before (left) and during (right) levitation</u>

Different beads diameters were submitted to ultrasound to evaluate the size range of particles that could be levitated with the acoustic device (see figure 3.11. below).



Beads diameter D=8µm

D=49µm

D=202µm

Figure 3.11. Levitation of different beads size in optiprep 1.049g/cm<sup>3</sup> (scale bars 250µm)

All beads from a range of 8µm to 202µm diameter were able to levitate within the chamber. For the 202µm beads, there were less beads on top chamber as they are going down by gravity before applying ultrasound, suggesting that those beads are at the higher size limit possible to levitate as the gravity start to have effects on them (see figure 3.11. above).

Then, to observe if the beads are all levitating at the same level or not, a mix with 2 different beads sizes were put in the device and power was turned on to check the levitation (see figures 3.12., 3.13. and 3.14. below).



Figure 3.12. Beads of same density but different size (pink: 24µm / red: 200µm) together



Figure 3.13. <u>8 (pink) and 49µm (black) beads in optiprep 1.049g/cm<sup>3</sup>: before and during</u> <u>levitation</u>



Figure 3.14. <u>8 (pink) and 49µm (black) beads in optiprep 1.049g/cm<sup>3</sup>: chamber zoom in,</u> <u>during levitation</u>

The contrast between white and the pink beads (figure 3.14.) is not large enough to see those beads with our camera. There are flows at the border of the chamber (round shape).

Beads of different sizes are levitating at the same level, so sizes (in the 8-202µm range) do not affect their behavior within acoustic standing waves.

A test with beads of higher density was done: beads are levitating but it was very complicated to mix those beads with media as they were in media and not dry powder.

To summarize, bead sizes of  $8\mu$ m,  $49\mu$ m and  $202\mu$ m were tested to evaluate the versatility of the BAL device in levitating a range of objects, ranging from single cells up to cell spheroids. For all the tested microsize beads, the acoustic levitation system was able to generate multilayers with identical interlayer distances equal to half-wavelength of the ceramic transducer working frequency, confirming the theoretical calculations. All beads densities and sizes are levitated at the same level, as they are seen at the same height within the chamber. Furthermore, we analyzed the effect of acoustic frequency on the tunability of the interlayer distance. Increasing the acoustic frequency from 340kHz to 680kHz decreased the interlayer distance from 2.2mm to 1.1mm, and increased the number of layers in the levitation chamber. The number of layers was also tunable by changing the chamber height.

#### 3.2.2. Establishment of an appropriate encapsulation protocol

After validating the layers formation, we needed to find a way to trap beads and later cells in that specific shape. Similarly to what has been proposed by Garvin et al<sup>[83]</sup>. We decided to trap the cells in a hydrogel that would polymerize while the cells are levitated inside the resonant chamber. Different hydrogel are available: collagen, silk, gelMa, etc. Chemical crosslinking was preferred to UV or thermal crosslinking so that we would be able to crosslink the entire volume. Fibrin was chosen as extracellular matrix of the 3D constructs because its unique, tunable gelation properties and viscoelasticity make it a very suitable substrate for 3D tissue engineering purposes, as well as clinical applications. Different tests for different prepolymer concentrations were done to evaluate the assembly feasibility.

#### 3.2.2.1. Influence of thrombin concentrations

Optimization of fibrinogen-thrombin concentrations is required to be able to encapsulate beads in horizontal sheets. A careful tuning of the prepolymer concentrations (and their ratio) of fibrinogen and thrombin will influence the gelation time and the mechanical properties of the resulting hydrogel. The crosslinking time must be carefully evaluated and should stay within a specific time window. If it is too slow, cells will remain out of their media and incubator for a long time. If it is too fast, cells will not have sufficient time to be pushed to pressure nodes before the gelation, and consequently won't be aligned. Another issue of manipulation with gels that polymerize too rapidly is that polymerization may occur before we even can turn on the power to the ultrasound source.

10mg/ml fibrinogen concentration in PBS is commonly used for cells culture, and we used this concentration to study the influence of the thrombin concentration. Three thrombin concentrations were used: 0.1U/ml, 0.7U/ml and 1U/ml (see figure 3.15. below).



*Figure 3.15. <u>Pictures of gels obtained with three thrombin concentrations: 0.1U/ml (left), 0.7U/ml</u> (center) and 1U/ml (right)* 

At the lowest thrombin concentration, layers were first seen and then clusters appear but the polymerization never happened. The solution never becomes a gel even after 1h. This thrombin concentration is so too low to allow the polymerization process in a time line acceptable. At the thrombin concentration of 0.7U/ml, the polymerization process occurred but beads were trapped after they made clusters. At 1U/ml, beads were aligned in layers and trapped in that sheets organization. This concentration was therefore selected.

### 3.2.2.2. Final encapsulation protocol

As the final aim is to encapsulate organized cells in fibrin gel, a new device was made. A circular ceramic (modified PZT 4, SMD20T08F2500R, Steminc, working frequency of 2.78MHz) was mounted with 3mm air backup. A resonant chamber of 2.2 mm height (see figure 3.16. below), made of Plexiglass stacked with a 0.2mm thick plastic film (McMaster-Carr) was used as resonant chamber. Acoustic gel was used in between ceramic and chamber, as coupling.



Figure 3.16. Plastic border for the resonant chamber: 2.2mm height

The chamber volume is about  $540\mu$ l. To be able to fill it entirely without making any bubbles when the reflector is on, a volume of  $580\mu$ l is decided.

Thrombin and fibrinogen solution were prepared separately. Fibrinogen was dissolved in PBS and put in a water bath at 37°C to help the dissolution and keep the fibrinogen active. 10U/ml solution of thrombin (stock solution at 600U/ml in distilled water) was diluted in PBS and put on ice.

When fibrinogen was well dissolved, it was filtered to make it sterile. Beads were mixed with fibrinogen.

 $540\mu$ l of fibrinogen-beads solution was pipetted into the resonant chamber.  $81\mu$ l of thrombin at 10U/ml was then added and well mixed. The reflector was directly put on top of the resonant chamber and the power turned on to generate acoustic standing waves for 10min (see figure 3.17. for experimental setup pictures). After this time the gel is stiff enough to remove the reflector and to be removed from the chamber. Gels can then be cut to be observed under a microscope.



*Figure 3.17. <u>Experimental setup</u>* (a) Piezoelectric ceramic connected to arbitrary signal generator. (b) Acoustic levitation chamber filled with a cell-fibrinogen-thrombin solution, covered by a glass reflector allowing formation of acoustic standing waves

A pressure threshold for banding can be predicted from an analysis of the forces acting on the cells, including acoustic radiation force and acoustic streaming, gravity, buoyancy and drag forces. Working acoustic pressure within the chamber has been set-up to values slightly above the banding threshold, found here to be 46kPa for  $10\mu m$  diameter cells (see annexe 1).

Experimentally we also observed that there is a threshold to levitate and trap particles. To be able to find this one, different gels were made with different power intensity for 10 minutes. Gels were then cut and observed by microscopy (figure 3.18.).



Figure 3.18. Electrical power effect for levitation of 24µm beads: 1mW, 8mW and 22mW. 8mW is the power threshold for beads levitation.

The threshold to levitate the beads is about 8mW (transmitted power), as before no layers were seen and for higher power we can see the layers, all spaced by 250µm.

## 3.3. Rheometry measurement for gel characterization

#### 3.3.1. Rheometry principles

Concentration of fibrinogen influences the final fibrin gel stiffness. 10mg/ml fibrinogen dilution is suitable for cells growth. Analyzing viscosity and elastic modulus of such a hydrogel can help to predict material behavior and so improve its performance and properties. In fact, viscosity and elasticity parameters give information about how deformable the gel is. A fluid with much higher elastic modulus than viscous one is considered as a gel because a small deformation is reversible as soon as it is stopped. Here we wanted to optimize the gel properties for neural tissue engineering applications to have gel with similar properties than native cortex tissue. Rheometry is the best technic to evaluate elasticity and viscosity of fluid as it allows to know G' and G'' (storage and loss modulus, respectively) according to the applied strain or frequency. Loss modulus is an "indirect" measurement of the gel viscosity (G''/w) and the storage modulus defines one elastic property of the hydrogel.

Here the methodology to characterize the gel will be presented following by measurements of gel properties after optimization of gel pre-polymer concentration. Then, as values were found similar to cerebral cortex tissue, this gel fabrication process was kept for all following experiments.

Figure 3.19. below shows the rheometer device used to characterize the gel properties.



Figure 3.19. Photo of the rheometer (Anton-Paar, MCR 301)

According to the application, different rheometers exist with particular geometry (see figure 3.20.):

- Couette (concentric cylinders): suitable for low viscosity liquid (less hook line or bubbles effect on measurement), need large volume
- Plane-plane: suitable for other liquids, small volume



Figure 3.20. <u>Plane-plane (left) and couette (right) geometries</u>

The lower part of the geometry is fixed and the top part (the head) is the one inducing the constraints. The principle is that the rheometer is calculating the torque G' / G'' from all movements and the forces applied to the head. G' gives the elastic modulus, G'' gives the viscosity in Pa.s.

First to remind some stress-strain relationship for different classes of fluids:

For visco-elastic fluid: Constraint  $\sigma = G^*(\omega)\gamma$  where  $\gamma$  is the shear rate.

With  $G^* = G' + iG'' = G e^{i\varphi} = G cos(\varphi) + iG sin(\varphi)$ 

G' elastic modulus (in phase) and G'' viscous modulus.

For viscous fluid:  $\sigma = \eta \gamma = \frac{\eta d\gamma}{dt} = i \,\omega \eta \gamma = G'' \gamma$ 

For elastic gel:  $\sigma = E\gamma \implies G' = E$  (E deformation modulus, here shear modulus – there is one modulus for each deformation).

Deformation percentages are calculated on the gap between the two plates. To stay in the elastic regime, low frequencies (typically 1Hz) are required. The first measurement done consisted in evaluating the fibrinogen viscosity and elasticity modulus.

#### 3.3.2. Fibrinogen characteristization:

After calibrating the rheometer, 1ml of fibrinogen was put in a plane(plane geometry. Different strains from 300s<sup>-1</sup> to 3s<sup>-1</sup> were applied. Loss modulus was found to be 0.2Pa and storage modulus was 0.8Pa. It appears that fibrinogen is just twice more viscous than water but, these data cannot be trusted totally as being very small quantities and because the plane-plane geometry is not suitable to viscosity measurement of such solution. Viscosity analysis was so then done with a couette geometry, in a shear stress condition. 25ml of fibrinogen 10mg/ml were put in the couette geometry and viscosity was analyzed (see figure 3.21. below).



Figure 3.21. Fibrinogen viscosity for different shear stress applied

For all the shear stress conditions tested, fibrinogen viscosity was found to be around 0.002Pa/s, which indicates that fibrinogen has a low viscosity, close to the one of water.

#### 3.3.3. Fibrin gel characterization:

Initially, while the gel polymerized, we applied a constant strain. Then we wanted to test the gel properties after polymerization. We choose a 3% strain, 1Hz frequency. Each point is acquired every 5 seconds during polymerization.

A gap height of 0.8mm was chosen for the plane-plane configuration. 1.16 ml solution prepared, containing 1ml of fibrinogen and  $162\mu l$  of thrombin at 10U/ml, in order to get the same proportions than in resonant chamber.

The principle for setting-up the gel on the rheometer was the following. Fibrinogen and thrombin were mixed on the measuring plate glass. Then, we applied some strain to better mix the gel and positioned the top plate (gel spreads over the entire surface of the geometry)

Slope between stress (ordinate) and shear rate (x-axis) gives the viscosity (see figure 3.22.).



Figure 3.22. Evaluation of storage and loss modulus for the first 1200seconds of polymerization: gel mixed on top of plate

Storage and loss modulus are increasing slowing all along the 1500sec of analysis, which suggest that the gel is still polymerizing. In fact, even if the solution is already more elastic than viscous it seems that the gel continues to polymerize for a longer time than 1500sec.

G' (storage modulus, gives elastic modulus). From the started point, the elasticity (given by G', went from 5 to 130Pa in 20min) is higher than the viscosity (G' $\approx$ 10 \* G''). The solution has consequently, from the beginning, the properties of a gel.

To manage to catch the first time point, fibrinogen and thrombin were mixed in a Falcon and then placed on the rheometry plate.

We tried to catch the first seconds of the polymerization process as since the beginning we are higher in elasticity than in viscosity (figure 3.23. below).



Figure 3.23. Evaluation of storage and loss modulus for the first 2000seconds of polymerization: pre-mixed gel

G' and G'' are evolving for the whole timescale, otherwise during the first 600 seconds the elasticity changes with stronger slope. The polymerization always occurs although it is much faster during the first 10 minutes. From the beginning the fluid seems to be more elastic than viscous, the first polymerization effects are seen during the first minute.



Figure 3.24. Photo of the gel between the two plates of the rheometer

After 1h of polymerization on the rheometer, when the gel is well formed (see figure 3.24. above), we tested its responses to different constraints to get its viscosity and elastic properties. First, we studied gel behavior depending on frequency, with a strain at 1% and frequencies ranging from 0.1 to 10 Hz (figure 3.25.).



From 0.1Hz to 10Hz we are in linear regime as G' and G'' are about to be constant. The increase in G' (storage modulus) may be due to the gel which is still polymerizing.

Secondly, for a constant frequency of 1Hz, the deformation varies between 0.5 and 5% (see figure 3.26.).



Figure 3.26. Storage and loss modulus Vs deformation

Both modulus meet a steady-state for the entire range of tested strains. We are therefore always in linear mode for 10% deformation. 3 % strain was therefore a good choice for measurements of elasticity and viscosity of the gel during the polymerization time.

G' (474Pa) and G'' (27.8Pa) values are stable for that strain range which show that the gel is tolerant to those constraints. G' is more than 10 times higher than G'' that shows that we have a stable elastic gel. The viscosity and elasticity modulus of the gels are respectively 4.42Pa.s (loss modulus/ $\omega$ ) at 1Hz and 474Pa.s. At a fibrinogen/thrombin ratio of 1/0.162 (fibrinogen concentration of 10mg/ml and a thrombin concentration of 10U/ml), the storage modulus (*G*'=474Pa) was found to be 10 times larger than the loss modulus (*G*'' =27.8Pa) (**Figure 3e**), similar to those of the native brain<sup>[54,143]</sup>. So, these concentrations were kept for all the following experiments in order to get a tissue with cortex-like properties.

#### 3.4. Acoustical and physical device characterization

**3.4.1.** Characterization of the levitation process: ceramic displacement, acoustic pressure field generated, trapping time involved

#### 3.3.2.1. Interferometry measurement of ceramic displacements

Theoretical modeling of the net forces acting on the cells - acoustic radiation force, viscous drag force arising from acoustic streaming, and vertical force due to gravity and buoyancy - can predict the minimal pressure to levitate and maintain cells in parallel horizontal layers. This pressure leads to a null vertical velocity of cells in the bulk acoustic field. Accordingly, the pressure threshold to levitate 10µm diameter cells, modeled as spherical objects, in homogeneous, multilayer node planes was estimated at 46kPa (using Matlab software, see annexe 1). Laser interferometry (interferometric probe SH-140, Thales laser SA –  $\lambda$ =532nm) was used to determine if it is possible to experimentally achieve this pressure threshold. Vibrations at the surface of the ceramic were measured in air and in the full device (filled with water). The set-up is composed of an optical head to send the laser light, and an electronic probe for signal processing (response proportional to object displacement). The reflected light from the ceramic surface has a phase shift with the incident light. This shift is proportional to the ceramic displacement and converted into amplitude. Surface of the ceramic was scanned

using a 3D motorized stage while ceramic was driven by a generator (Agilent). At each scan point, the amplitude of ceramic displacement was recorded by a Picoscope (Pico Technology) and processed into a home-made LabVIEW (National Instruments) program to display the values on each point of the ceramic.

The ceramic vibration was also simulated with Comsol software to calibrate future simulation of the pressure field inside the chamber.

The ceramic was driven at the electrical power 8mW, to fit with experimental data for particles levitation.



Figure 3.27. Simulated (a) and experimental (b) ceramic displacements

Interferometry measurements (figure 3.27.b.) reveal that ceramic is vibrating following concentric rings. Ceramic is vibrating from center to the edges. This is typical of piezo ceramic and depends on the frequency<sup>[144]</sup>.

## 3.3.2.2. <u>Acoustic characterization of the device: pressure field measurement</u> <u>and simulations</u>

## Acoustic pressure field

The ceramic was driven by a generator (Agilent) with a transmitted power of 8mW. Acoustic pressure field generated inside the resonating chamber was measured with a fiber optic hydrophone (FOPH, Precision Acoustics LTD). To measure pressure within a ZY plan, the fiber optic was moved inside a petri dish of 8mm height (see figure 3.28.) from 0.1mm to 2.1mm in the Z direction with a step of  $50\mu$ m and along the entire ceramic diameter with a step of  $500\mu$ m.



Figure 3.28. Picture of the resonant chamber used for ZY plane pressure field measurement

Due to the small volume and the time required for a plan acquisition, the recording was divided into 3 planes (each one of 5mm length) and last 30 minutes. Between each plane acquisition water was added to the chamber to fully fill it and have a flat top.

The 3 planes obtained were combined using Matlab and the obtained acoustic pressure field is shown in figure 3.29. below:



Figure 3.29. Experimental pressure field on the resonant chamber: 2mm height were scanned

The incident waves, generated by the ceramic and the reflected ones are coherently summed inside the petri dish or custom designed container made from PMMA to produce acoustic standing waves. This results in a succession of pressure node planes where the pressure is 100

equal to zero and antinode planes, where pressure is maximum. We can also see radial patterns.

To measure the pressure along the Z axis (vertical direction), a hole was drilled through a reflector and the fiber optic was moved along the Z axis with a 50 $\mu$ m step (figure 3.30.). A 2.2mm height chamber was used for these measurements.



Figure 3.30. Schematic representation of Z axis measurement with fiber optic

Two different positions were chosen to measure Z axis: in the center of the resonant chamber and 1.3mm away from the center (see dotted lines in figure 3.31.).



Figure 3.31. Zoom on the pressure field, showing the position of Z axis recording

The resulting pressures along Z axis are showed in figure 3.32. below.



*Figure 3.32. Experimental pressure along Z axis, for 2 different positions from the center* 

There are variations of pressure along vertical direction (Z axis), resulting in horizontal planes of pressure nodes. Seven pressure nodes where found in 2mm height. The pressure maximum in the center of the chamber is 140kPa whereas the maximum one at 1.3mm away from the center is 100kPa.

There is an asymmetry between the center of the chamber and when you moved from the center. This is in correlation with the plane pressure field we observed as it has radial mode effects.

Further, the performance of the complete system, comprised of the air-backed ceramic coupled to the levitation chamber closed with the plexiglass lid, was then analyzed using Comsol Multiphysics<sup>TM</sup> software (Comsol 5.0, Burlington, MA). We characterized the acoustic levitational assembly with numerical modeling to correlate the findings with actual experimental results. We analyzed the acoustic pressure fields and numerical setup characteristics using COMSOL software and matched with experimental findings, obtained with the fiber optic hydrophone.

## **Comsol simulation**

Comsol multiphysics software with MEMS and acoustic modules were used to simulate the pressure field.

An axial symmetry was chosen to be faster for calculation and as the setup is axisymmetric. Three different rectangles were defined in the geometry to represent the ceramic, the chamber and the reflector.

The components were defined with their dimensions and material:

- 2.2 mm height for the chamber with 8mm radius of water,
- 0.8 mm height for the ceramic with a working frequency at 2.78MHz, PZT5 material
- 6 mm height for the reflector, with 12mm of radius, glass window or steel (to get an ideal model, with higher impedance so higher reflection created).

A PZT 5 material was chosen to represent the ceramic as it is the one closer to the properties given by the manufacturer to characterize our ceramic (PZT modified).

All the calculations to get acoustic pressure were in frequency domain. We first identified the input optimal voltage (5V) needed to obtain the above derived minimal pressure of 46kPa across the entire antinode planes surface. The chamber borders were defined as hard wall boundary whereas the boundaries were free between ceramic and chamber and between chamber and reflector. Like that, all reflections were calculated by the software based on impedance of each material.

3D planes and axes were previously defined to get the graphical representation we want to.

The mesh was chosen to be of 0.99mm maximum distance between two points and the resulted acoustic pressure field is shown in figure 3.33.below.



Figure 3.33. Comsol simulation of pressure field inside the chamber

Simulation shows that there is a succession of pressure node planes and antinode planes. We can also see radial patterns. Mostly all pressure is localized in the center of the chamber. There are also rings patterns on the top of the chamber which are with low pressure gradients: from 0 to 0.06MPa.

To compare simulated pressure field with experimental one, pressures along Z axis was done at the same position than for the experimental data. Pressures along the radial axis were also calculated for three positions corresponding to node, antinode and a middle position (see figure 3.34. below).



*Figure 3.34. Zoom on the pressure field, showing the position of Z and R axis simulation* 

The resulting pressures along Z and R axis are showed in the figure 3.35. below.



Figure 3.35. Simulated acoustic pressure field along axial and radial axis

By simulating the acoustic field generated by the piezoelectric ceramic, we confirmed the formation of pressure node layers in the levitation chamber with an interlayer spacing of  $250\mu$ m.

Simulated pressures along the Z axis show the same patterns than the experimental ones: 7 pressure nodes in 2mm height with higher pressure variation in the center of the chamber. Also, same pressures along center Z axis are found: about 140kPa meanwhile the pressure at

1.3mm away show lower pressure values than experimental data: 50kPa compared to a maximum of 100kPa.

The presence of acoustic pressure gradient in the radial direction will result in secondary lateral radiation force that will act within the pressure node planes. However, because the pressure gradients measured in the radial direction are lower than the vertical direction, the time to reach equilibrium within a node planes is much longer than the time to reach equilibrium position in the vertical direction.

#### 3.3.2.3. Levitation time characterization

To evaluate the time schedule for levitation of microparticles in water, a CMOS-based highspeed camera (Vision Research, Phantom V12.1) was used with a 0.5 zoom lens and a cadence of 100 images per seconds. To synchronize the video and the levitation process, the video beginning was trigged by the signal generator. Four beads sizes were levitated (8, 24, 49 and 96µm) in an 8mm thickness resonant chamber (figure 3.36).



Figure 3.36. High-speed camera pictures of levitating beads (after layers formation)

The time to reach equilibrium at pressure nodes is different according to beads sizes. The bigger the beads are the faster they are aligned on nodes (see figure below). Beads are going to pressures nodes in about 1.70sec for 8um, 1.30sec for 24um, 0.90sec for 49um and 0.4sec for 96um beads (figure 3.37.).



Figure 3.37. <u>Time schedule of formation of layers for different beads sizes.</u>

The bigger the beads are the less time they are staying in the nodes, they are making clusters and so become bigger and need more acoustic power to be maintained at their position.

This can be explained by forces applied to particles. In fact the radiation force is dependent on the particles diameter, increasing as a power of 3 of the radius. By the same time gravity will have higher influence but in less proportion.

There is a pressure threshold to make beads levitating, which is of 40kPa for 15µm diameter beads but higher for bigger beads.
As the acoustic levitation occurs in less than 2 seconds for  $24\mu m$  beads and as the gel is already more elastic after about 1 minute (time to mix everything and positioned on the rheometer), we can wonder if the beads are trapped in the gel in less than 10 minutes.

Different ultrasound exposure time were performed to evaluate the time for the gel to become enough elastic and stiff (fibers already made) to trap the beads. Ultrasound was turn on from 1 minute to 5 minutes (test every minute) and the chamber was stayed untouchable for the rest of the usual 10 minutes. The gels were then cut and observed on the microscope to evaluate how fast the gel is enough stiff to trap the beads inside (figure 3.38.).



Figure 3.38. <u>Microscope pictures of gel exposed respectively to 1min, 2min and 5min of</u> <u>ultrasound</u>

After 1 minute, beads are already aligned in layers but not all of them. There are still few not completely in the alignment. After 2 minutes all beads are aligned and so it is the same after 5 minutes.

The gel is enough viscous and elastic, the fibers are already enough formed after 2 minutes to trapped the beads even if the gel at that time is not enough stiff to be manipulable.

## 3.4. Conclusion

An acoustic platform was developed for an easy and contactless particles manipulation. This system, composed of circular piezo ceramic mounted face to a glass reflector, with a resonant chamber of 2.2mm height, allows organizing particles in parallel layers. Layered organization 108

is a ubiquitous phenomenon in few biological tissues such as skin, cerebral cortex or breast. Being able to reproduce such an organization *in vitro* may help to get models for different tissues.

The device allows the production of acoustic standing waves inside a resonant chamber. The pressure field was simulated and experimentally measured with fiber optic hydrophone. It shows that it has radial modes but, thanks to the dynamic of polymerization process, beads can be trapped in parallel planes. After 1 minute, beads are nearly all trapped in layers either as the gel may be too visco-elastic for beads to move or fibrin fibers are already too much close to let beads going out of their place. This opens perspectives for cells manipulation, if we can culture the cells in 3D inside the gels. If we increase the crosslinking time we may be able to create other patterns, as the radial modes will have effects on the pressure field. We could also play with frequency to tune layers thickness.

# Chapter 4 – Bio-acoustic levitational (BAL) assembly method for engineering of multilayered, threedimensional brain-like constructs

The acoustic platform developed has to be transferable to cell manipulation and particularly in neural construct formation. In fact, generating a 3D scaffold with neural network would have a huge interest for many applications from understanding neural connection formation, neural development, drug testing to brain stimulation devices test.

For now, technologies to produce a neural construct are time consuming and mostly used primary rats neurons which result to a use of many animals. Trying to find a way to avoid the use of animal and generating a neural network in an easy way is very challenging but of huge interest from scientific to ethical. Our technology allows cells alignment and encapsulation in a single step. We wanted to see if our system is cell-friendly, if in use in a sterile environment such as under a culture hood. The neocortex is a multilayer structure, so the chosen approach was to use ESC to differentiate into neurons. The advantages of such a strategy is that ESC can be cultured so no need of alive tissue is requires and as the system is easy, it should allow to manipulate cells fast and so not causing too much stress to cells.

In this chapter, I will develop the optimization of the protocol to keep and culture cells in such 3D environment, the staining protocol to be able to see cells in the construct for an easy cell line. Acoustic effect on viability was also done to detect if the process has any viability effect on cells. Then, I will show how we transfer that to ESC to differentiate them in 3D and their characterization (differentiation step and the type of cells present after differentiation) and will quickly go over the perspectives.

## 4.1. Evaluation of the levitation and encapsulation feasibility on cells

The first thing to do was to validate if this technology is transferable to cells manipulation. First tests were done at low frequency, for different concentration to get the right parameters to see if we can levitated and encapsulate cells. The second step was to use the proper device and see ultrasound toxicity to then find a way to observe layers in the microscope.

## 4.1.1. First proof of concept, using uncultured cell lines

First test was done in a commercially round petri dish with ceramic at 680 kHz; to be sure than by eyes we will be able to see the layers.

Different cells concentrations were tested. If the concentration is too low, the layers are not visible because not enough compact but if the concentration is too high, there are too much cells, so the layers are too thick and cannot be seen.



Figure 4.1. <u>Photo of the gel, after polymerization: top (left) and side (right) views after 10</u> <u>minutes of ultrasound and 15 more minutes of crosslinking to remove reflector.</u>

For this first test, hepatocytes were used at the concentration of 1 million per ml of fibrinogen (figure 4.1. above). A ratio 1:0.16 fibrinogen-thrombin was chosen, based on experiments with beads with prepared solution of thrombin at 10U/ml to get a final concentration inside the gel of about 1U/ml.

As hepatocytes are big cells compare to most of common cell line, from 1 million cells per ml layers are visible.

It is difficult to remove the reflector, without cutting of piece of gel which stay stacked on the reflector. So, I developed a protocol/technique to be able to remove it from the top of the chamber without any gel breaking.

Then, when the reflector removal protocol was established I needed to find a way to cut the gel to look at a piece on the microscope. The second thing to do then is to stain the gel to be able to easily see the cells.

With smaller cells, it is more difficult to see layers from the side of the chamber. A colon cancer cell line HCT116 was used to test the gel cutting and staining feasibility. A vial of 8 million cells was thawed, centrifuged, the media was removed and the cells were re-suspend in 5ml of fibrinogen at 10mg/ml. The final concentration of thrombin in the chamber was 1U/ml.

After gel polymerization the gel was sliced in small pieces and those slices were incubated into Live/dead kit staining for mammalian cells ( $2\mu$ M Calcein AM and  $4\mu$ M ethidium-homodimer) for 30 minutes in the incubator and kept away from light. After 30minutes, the slices were washed 3 times with PBS and visualized on an epifluorescence microscope (using 2.5X and 10X objectives with respectively 0.06 and 0.25 Numerical aperture, Zeiss).

At that time, the smaller magnification was X10 and the field of view of camera was so small than we cannot see 2 layers in a camera field of view. A picture was so taking from the eyepath, with a camera to get the 2 layers (figure 4.2. below).



Figure 4.2. Live/dead staining on a gel slice: photo (left) and microscope fluorescence (right)

By eyes and with the fluorescence microscope we can distinguish 2 layers of cells. There is no cell between two layers, which shows that the dispositive is efficient.

Live/dead stain also fibrin gel; so the contrast between gel and cell is not very good. So, the next step was to find a way to stain cells with specific markers.

3T3 cell line was used for those experiments as it is a good cell model, growing fast with elongated shape.

## 4.1.2. Evaluation of the process toxicity and visualization feasibility on fibroblast cells

## 4.1.2.1. Expansion and encapsulation of fibroblasts

NIH 3T3 mouse fibroblast cells (ATCC CRL-1658) were cultured in DMEM media with 10% FBS, 1% penicillin/streptomycin, 1% GlutaMax Supplement (Life technologies) and 1% HEPES (Life technologies). Cells were seeded in 75cm<sup>3</sup> flask with 13ml of media and put in a 5% CO2 humid incubator at 37°C.

After cells reached about 80% of confluence (in about 3 days), they were trypsinized with 3ml of trypsin at 37°C and put in the incubator for 3 minutes, until cells were detached from the flask. 10ml of media was then added to stop the action of tryspin which is not good for cells. The solution was then transferred to a flacon tube to be centrifuged at 1300rpm for 3 minutes. Media was removed, and 5ml of new culture media added in the falcon tube.

 $50\mu$ l of this solution with  $50\mu$ l of trypan blue were mixed in a 96well plate well and  $10\mu$ l was put in a counting chamber. Cells were then counted on the microscope distinguishing dead and alive cells.

1 million cells were seeded back in a culture 75cm<sup>3</sup> flask with 13ml of media and the rest of cells were used for experiments.



Figure 4.3. <u>3T3 NIH fibroblast cell line in 2D culture (photo from ATCC)</u>

## 4.1.2.2. Effect of ultrasound on cells viability

PrestoBlue viability test is a test based on fluorochrome color changes by viable cells. To analyze the effect of ultrasound a series of tests were done to compare ultrasound exposed and non-exposed cells.

To link the fluorescence values with the number of viable cells, a calibration curve was done. For that, six different cells concentrations were tested. Cells were counted, and after 4h cells were attached. Media was removed to be replaced by a PrestoBlue solution composed of a ratio of 1:9 PrestoBlue:Media (1ml in each well) for 1h at 37°C. After 1h, top read fluorescence was then performed using a fluorimeter (Infinite M1000, Tecan – Ex 560nm, Em 590nm, bandwidth 10nm). Three values for each well were taken by the fluorimeter and the mean was calculated.

Mean for each concentration is represented in the graph below (figure 4.4.):



Figure 4.4. Calibration curve for 1h of PrestoBlue with fibroblasts

The fluorescence of PrestoBlue-Media solution alone, without cells is 2700. A linear curve fits to the values with a correlation coefficient of 0.9871. Fluoresence and number of cells is linked by the equation: fluorescence=0.0042\*number of cells + 2700.

To determine the effect of ultrasound on cells, fibroblasts were suspended in media at 4 million cells per ml. 580µl of the solution was placed inside the resonant chamber and ultrasound were applied or not for 10 minutes. Then 100000 cells were seeded in each of 3wells of a 6 well plate. Three samples for each condition (ultrasound d and no ultrasound) were seeded for PrestoBlue viability tests for different days (figure 4.5.).



Figure 4.5. Effect of ultrasound on cells viability

At day 1 and 3 there is not any significant difference between ultrasound exposed cells and control ones. Whatever the cell concentration at day 0, fibroblasts are growing very fast and after 3 days they are too much confluent to do another PrestoBlue test. In fact at day 5, cells are already 100% confluent and so are detached easily when the media is change by the PrestoBlue solution. Anyway, PrestoBlue test is sensitive to confluence. In fact, at confluence cells are not reacting the same and so any viability test won't give significant results.

Ultrasound do not have effect on cells for 3 days. To look at the effect for longer time and analyze the effect of the entire encapsulation process, encapsulated cells were culture and PrestoBlue viability test was done over 5 days.

## 4.1.2.3. Effect of encapsulation protocol with or without ultrasound

After the cells were counted, they were separated into falcon tubes with 1 million cells each, and centrifuged just prior encapsulation. Medium was removed, and cells were mixed into 500 $\mu$ l of fibrinogen (Sigma) at 10mg/ml (reconstituted in PBS and filtered at 0.22 $\mu$ m), and kept at 37°C. The fibrinogen-cell solution was transferred into the petri dish, with 81 $\mu$ l of thrombin 10U/ml.

After gel crosslinking, the hydrogel was removed from the petri-dish (figure 4.6.), transferred into 12-wells culture plate and culture in presence of culture medium.



Figure 4.6. Photo of a gel after polymerization

Effects of the encapsulation process on cells viability were tested using PrestoBlue. Five gels with 3T3 were incubated 2h with PrestoBlue-media. In 1h solution were not enough color changed.

The fluorescence values were read on the fluorimeter and convert to number of viable cells, thanks to the calibration curve.



Figure 4.7. <u>Number of viable cells in fibrin gel over time: comparison ultrasound and no</u> <u>ultrasound</u>

The number of viable cells increase over time (figure 4.7.), that suggested fibrin is supporting fibroblast growth. The first two days, it seems that there is no increase in number of cells, but from day 4 this number increases.

#### 4.1.2.4. Gel histology and immunostaining

Gels with 1 million cells encapsulated after acoustic levitation were cultured for 3 days. Then, gels were fixed in 4% paraformaldehyde for 30 minutes at room temperature, followed by permeabilization with blocking buffer containing PBS, 1% BSA (Sigma) and 0.5% saponin (Sigma) for 1h30 at room temperature.

Slices of 180µm were cut and incubated in blocking buffer in the presence of Phalloidin (ratio dilution 1:500). The samples were shake away from light for 1h30 (Shaker), and then rinsed with PBS. DAPI (Sigma) staining was added for 5 minutes at 1:1000 ratio, and the sampled were rinsed before imaging with epifluorescence microscopy using 2.5X and 10X objectives with respectively 0.06 and 0.25 Numerical aperture, or confocal microscopy used 10X, 20X, 40X and 63X objectives with numerical apertures of 0.45, 0.8, 1.3 and 1.4, respectively (LSM

710, Zeiss). Image analysis was performed with FIJI ImageJ (NIH, Bethesda) software and IMARIS and are shown is figure 4.8. below.



## Blue: DAPI / Green: Phalloidin

## Figure 4.8. 3T3 fluorescence microscopy pictures: X2.5 (left) and X20 (right)

Phalloidin alexaFluor 847 is a membrane marker with a fluorochrome, so it stains all the cells contours; DAPI is a nucleus marker of dead cells. As the cells are permeabilized with the blocking buffer, the membrane's pores are opened and so, DAPI can enter in the nucleus and will stain them in blue.

Fluorescence microscopy reveals that at low magnification, we can see parallel layers of cells. Microscopy pictures also allow checking that the distance achieved is the one predicted. In fact we expected 270µm theoretically and in microscopy picture we see that this distance is 250µm. The small difference between calculation and experiments is due to the number of cells. In fact, fibroblasts are 10µm diameter cells and in one layer of cells soma we can see multiple cells layer height.

X20 magnification pictures reveals that many elongations were made to establish connections between layers. This shows that fibroblasts in 3D are extending elongations, which is not a 2D behavior. In fact in 2D fibroblast culture, cells have a fusiform shape but are expanding allow the flask surface and they are not developing such elongations.

To better see those connections, and being able to completely see the 3D behavior and construct patterns, confocal microscopy was done (figure 4.9.).



Figure 4.9. <u>3T3 connection between layers, after 3 days of culture, confocal images (DAPI purple,</u> phalloidin blue): scale bars 250µm.

Those pictures are slices of the gel slice. We can well see the layers and few connections between layers.

To see all the connections in 3D it is requires to stack all the slice view taken. 3D confocal pictures are very heavy in terms of memory, so we used an image analysis platform to process the 3D reconstruction of the fibrin gel slice with encapsulated 3T3. We used Imaris to be able to open all the pictures and reconstitute the 3D view. Images were compressed meaning four points of a picture to make one. Then a 3D reconstruction was done with ImageJ (figure 4.10.).



Figure 4.10. <u>3D reconstruction of confocal microscopy (scale bar 250µm)</u>

This picture is a 3D reconstruction of a gel slice which is rotated a bit to see a part of layers. Here, we can see 2 layers with inter-layers connections but also a sheet fully filled of cells.



To post-processed layer zoom in was done to see precisely one layer (figure 4.11.).

Figure 4.11. Zoom in on a layer, to see intra-layers connections (scale bar 250µm)

We see cells everywhere in one layer (purple point for nucleus) and connections made in all the layers. One layer is fully filled of fibroblasts. In fact there is phalloidin everywhere which means we have cells membranes everywhere.

Cutting and staining protocol was so validated. We can fix and permeabilize the gel to then cut it and stain the slices to observe them on the fluorescence or confocal microscopy.

So, the next step was to it this protocol with other cell types such as encapsulated ESC that we can differentiate into neurons.

## 4.2. Embryonic stem cells approach for a neural tissue construct application

#### 4.2.1. Cell differentiation into neuron application



## 4.2.1.1. <u>Principle</u>

## Figure 4.12. <u>Schematics of the application principle</u>

The idea (principle schematic in figure 4.12.): embryonic stem cell derived neuro-progenitors in a fibrinogen solution are placed in an assembly levitation chamber. Thrombin is then added to slowly (< 10min) initiate the fibrin hydrogel gelation process. An acoustic ceramic is used, to generate incident waves, which coherently interfere with the waves reflected from the glass reflector placed on the top of the levitation chamber. The resultant standing waves induce cells to levitate, within seconds, in 3D multilayer constructs. By placing the 3D constructs in differentiation medium in conventional culture dishes, the neuro-progenitor cells are differentiated in neural cells, resulting in a 3D neuronal construct with inter and intra-layer neurite elongations.

#### 4.2.1.2. <u>Cells types</u>

Stem cells are a unique type of cells

a. They are not specialized and they can proliferate for long periods of cellular division.

b. They are capable, under specific physiologic or experimental conditions, to differentiate into different cell types.

They are two types of stem cells: embryonic stem cells and adult stem cells.

Embryonic stem are derived from Blastocyst Inner Cell Mass (structure of 16 to 40 cells from fecundated oocyte). They can induce all tissues. They are totipotent and have infinite division at undifferentiated step. Embryonic stem cells keep a normal genotype and karyotype whatever the number of proliferations as they are not mutating.

Embryonic stem cells can be pre-selected selected a specific membrane marker to get cells predisposed to a type of differentiated tissues. Human embryonic stem cells HUES 64 (Harvard University) (Stanford SCRO protocol number 506) were FACS sorted for neural progenitor cell surface marker p75. Those cells were named as neural progenitor cells (NPC).

#### 4.2.1.3. Embryonic stem cells culture in 2D

When thrawing a vial of about 4 million cells, the cells solution has to be slowly (droplet by droplet) transfer into 40ml of induction media, composed of DMEM F12, GlutaMax, 1% penicillin-streptomycin, N2 supplement, B27 (all from Life Technologies), 20ng/ml FGF (R&D Systems) and 20ng/ml EGF (Sigma). NPCs were then cultured on Matrigel (BD Biosciences) coated culture plate with neural induction media.

A vial of  $300\mu$ l of matrigel (REF) was put on ice for about 1h to let it become liquid. Matrigel was then mixed with 25ml of empty DMEM and kept on ice. 1ml of this solution was added in each well and the plate was shake slowly by hand to be sure that matrigel with be homogenously spread on the wells. The plates were then put in the incubator for at least 1h.

Just before seeding the cells, the matrigel-DMEM solution was removed from the wells and cells were seeded at a concentration of about 300000 cells per well in induction media. At confluency, to detach the cells, media is removed from the wells. PBS is used to wash the wells and replaced by 800µl of accutase for 3minutes in the incubator at 37°C. Plates were used two by two, not to let cells to much in presence of accutase. Accutase-cells solution was then transfer into 30ml induction media (with FBS) to stop the action of accutase.

Cells were then centrifuged, media was removed and change by 15ml of new induction media. Cells were then counted and seeded back in pre-coated plates or used for experiments.

Those cells in induction media just proliferate for about 2 weeks to become confluent and can be differentiated with the use of differentiation media.

Embryonic stem cells are very sensitive cells, so an ulrasound toxicity analysis was done to see if ultrasound has an effect on those cells or not.

## 4.2.2. Ultrasound toxicity

To quantify the potential impact of acoustic levitation on cell viability, NPC were put in the resonant chamber in the presence of ultrasound acoustic waves or not. Cells were then seeded back in pre-coated 24 well plates.

The viability of NPCs after exposure to ultrasound, compare to ones without ultrasound exposure, was assessed with Live-Dead assay (Life Technologies) for 14 days and with PrestoBlue at day 7. 1 million cells in  $540\mu$ l of media were placed into the resonant chamber with or without ultrasound and appropriate amount of cells were plated back in matrigel precoated wells with  $500\mu$ l of induction media.

## 4.2.2.1. Qualitative analysis of ultrasound effect on cells

For live/dead, cells were plated in 24 well plate at a concentration of 100000 cells per well. At the analysis day, samples were incubated in  $2\mu$ M Calcein AM and  $4\mu$ M ethidium-homodimer (total volume of 200 $\mu$ l per well) for 20 minutes at 37°C and washed with PBS prior the imaging with fluorescent microscope (AxioView, Zeiss). This test colors live cells in green and dead cells in red (figures 4.13., 4.14. and 4.15. below).

## No ultrasound



<u>Ultrasound</u>



<u>Day 1</u>

<u>Day 3</u>

<u>Day 5</u>

<u>Day 7</u>

Figure 4.13. <u>Live/dead staining on ESC after exposition or not to ultrasound (scale bars: 100um)</u>



No ultrasound

<u>Day 7, 20X</u>

<u>Ultrasound</u>

Figure 4.14. Zoom in, at day 7(scale bars: 50µm)



No ultrasound

<u>Ultrasound</u>

Figure 4.15. Zoom in, at day 14(scale bars: 50µm)

Day 14, 20X

Live/dead pictures reveal that, even after 14 days of culture after ultrasound exposition, there is no difference between exposed and no cells. We clearly see that the concentration of cells per well increased from day 7 to day 14, as at day 14 cells are confluent rather than at day 7 there was still placed for cells to grow.

To better see that, quantification of number of dead and live cells were done thanks to a home-made Matlab software to count every structure. The percentage of live cells was calculated by divided number of live cells per the total number of cells (dead+live) (figure 4.16.).



Figure 4.16. Percentage of live cells over time

#### 4.2.2.2. Quantification of percentage of live cells over time

Percentage of live cells remains stable over 14 days, in case of ultrasound exposure or not.

The difference from exposed to ultrasound or not cells suggests that ultrasound doesn't have effect on cells death, it doesn't induce apoptosis. In fact apoptosis is about 7 days after stress. As after 14 days the percentage of live cells remains the same than at day 7 cells were not induce to an apoptosis process.

To see if ultrasound also doesn't have an effect on viability, PrestoBlue test was assessed after 7 days of culture (with a 1:9 PrestoBlue:Media ratio, according to manufacturer protocol). As for live/dead, 1 million cells in 540µl of media were placed into the resonant chamber with or without ultrasound and 250000 cells were plated back in matrigel pre-coated wells (6 well plate) with 2ml of induction media.



Figure 4.17. PrestoBlue viability test on ESC after 7 days of culture in induction media

At day 7, PrestoBlue viability test reveals that there is no significant difference between nonexposed and exposed to ultrasound cells (figure 4.17.).

This suggests that the levitation process has no effect on cells viability. So, the next step was to encapsulate them.

#### 4.2.3. Optimization of encapsulation and differentiation process

#### 4.2.3.1. Optimization of encapsulation process

At 80% confluence, cells were harvested with accutase (Stem Cell Technologies), cells between passage 4 and 8 were used in the experiments. 2 million NPCs were mixed with 500 $\mu$ l of 10mg/ml fibrinogen solution supplemented with 10 $\mu$ g/ml BDNF (R&D systems) and kept at 37°C. The fibrinogen-cell suspension was transferred into the resonant assembly chamber, on top of the acoustic ceramic. 81 $\mu$ l of 10U/ml thrombin was then added and mixed by pipetting. At that time the reflector was positioned on top of the petri dish, avoiding the presence of air bubbles. Ultrasounds were then applied for 10 minutes, corresponding to the duration of the crosslinking fibrin gel. After the crosslinking, hydrogel was retrieved from the assembly chamber and cultured in neuronal differentiation media. Differentiation medium is composed of neurobasal media, 1% Glutamax, 0.2% Dextrose, 0.5% MEM NEAA (Life Technologies), 20  $\mu$ l/ml B27 (Life Technologies), 10  $\mu$ g/ml BDNF (Sigma), 10  $\mu$ l/ml acid ascorbic (R&D systems), 2 $\mu$ l/ml cAMP (R&D systems).

Gels are degraded in few hours and cells are making clusters (figure 4.18.). The cells are degrading the fibrin gels as they are differentiate.



Figure 4.18. Fibrin gels after about 7h (scale bar: 100um)

To be able to keep the gel longer 2U/ml of aprotinin and 10mM of  $CaCl_2$  (Sigma) were added in the culture media. After that, gels were stable for up to one month. As aprotinin is a chemical which is slowing done the cells mechanism, an affinity test was done to get the most cells proliferation. BDNF and/or Matrigel were added in fibrin droplets containing cells and the droplets were observed on the microscope for up to 7 days. All the conditions were tested with and without aprotinin in the neuronal differentiation media (table 4.1.).

Fibrin	Matrigel	BDNF
10mg/ml	$12\mu l/ml \Rightarrow 1.8\mu l$	/
10mg/ml	$6\mu l/ml \Rightarrow 0.9\mu l$	/
10mg/ml	/	20µl/ml
10mg/ml	/	10µl/ml
10mg/ml	$12\mu$ l/ml => 1.8 $\mu$ l	20µl/ml
10mg/ml	/	/

Table 4.1. Conditions tested for ESC affinity in fibrin droplets

In all conditions, if there is no aprotinin in the media, the droplets were degraded in few hours.



Figure 4.19. <u>Matrigel 9µl/ml (left); BDNF 20µl/ml (middle); BDNF20µl/ml with Matrigel 18µl/ml</u>

<u>(right)</u>

After one day cells already started to spread. After 3 days we clearly see differences between conditions (figure 4.19.).

Fibrin droplets with either BDNF or matrigel cells are spreading much faster than within control fibrin droplets. In presence of matrigel we can see that the fibrin gel is making clusters of cells. This may be due to matrigel which become hard at 37°C, so the mixing process with fibrinogen, which is used at 37°C, may induce cluster when matrigel warm at 37°C. In presence of BDNF in the droplet, cells are spreading much better than in fibrin droplet. So, from now, it was decided to include BDNF at 10µl/ml inside the fibrinogen solution.

## 4.2.3.2. Encapsulation effect on cells

To quantify the potential impact of 3D hydrogel encapsulation and acoustic levitation on cell viability and proliferation, NPC encapsulating fibrin gels were prepared inside the resonant chamber in the presence of ultrasound acoustic waves or not (figure 4.20.).



Figure 4.20. Gels with levitated ESC after crosslinking, inside resonant chamber

The gel was removed from the resonant chamber and cultured in differentiation media for up to 7 days. The viability of fibrin encapsulated and acoustic levitated NPCs was assessed with Live-Dead assay (Life Technologies). Samples were incubated in  $2\mu$ M Calcein AM (membrane marker of live cells) and  $4\mu$ M ethidium-homodimer (bound DNA of dead cells) for 20-40 minutes at 37°C and washed with PBS prior the imaging with fluorescent microscope (AxioView, Zeiss).



Figure 4.21. Live/dead kit on levitated and encapsulated ESC at day 0 (left) and day 7 (left) after encapsulation

After levitation and encapsulation, fluorescence pictures (figure 4.21. above) show that at day 0, there are few dead cells (in red). This number increase at day 7 but the number of live cells too. It is normal that in the differentiation process few cells are dying.

To compare with encapsulated cells not submitted to ultrasound, the proliferation potential of NPCs in fibrin gels were determined using PrestoBlue (Life Technologies) assay at different culture periods up to 7 days. Samples were incubated in PrestoBlue solution, diluted with cell culture media in 1:9 ratio according to the manufacturer instructions and incubated for 1h at 37°C. The supernatant was analyzed using a fluorescent plate reader (Infinite M1000, Tecan) with an excitation 560nm and an emission 590nm wavelength and 10nm bandwidth.



Figure 4.22. PrestoBlue viability test on ESC other days

PrestoBlue results (figure 4.22.) show that the number of viable cells increase over time, without any difference between control cell in gel and the ones exposed to ultrasound to stabilized them in a layer sheets structure. Ultrasound and encapsulation process does not affect cells viability. The next step will be to visualize and characterize the type of cells we have over time in the gel in differentiation media.

#### 4.2.3.3. Protocol for gel slicing and immunostaining

After Different days of culture in differentiation medium, gels were prepared to be visualized on fluorescence microscopy and/or confocal microscopy.

First thing to see was to characterize the stage of differentiation we are at day 0 (control) and day 7 (day around which first connections are seen on the microscope). At day 0 and day 7, gels were fixed in 4% paraformaldehyde for 30 minutes at room temperature and permeabilized with 0.5% saponin (Sigma) and blocked with 1% BSA (Sigma) for 1.5h at room temperature. Gels were sliced in 150-200 µm stripes and stained for primary antibodies: DCX (MABN707, EMD Millipore) or NeuN (MAB377, EMD Millipore). Samples were incubated overnight at 4°C. Antibodies conjugated with goat anti-mouse AlexaFluor 488 (for DCX) and donkey anti-chicken AlexaFluor 647 (for NeuN) all from Jackson ImmunoResearch Laboratories were used for secondary staining and cell nuclei were stained with DAPI for 5min (Sigma). Samples were incubated for 2h at room temperature in dark and excessively rinsed with PBS. 3D hydrogels were kept wet and imaged with confocal microscopy (LSM 710, Zeiss). Image analysis was performed with FIJI ImageJ (NIH, Bethesda) software.

DCX is a microtubule associated protein that is required for the initial steps of neuronal dispersion and lamination during cortex brain development. Expressed by neuronal precursor cells and immature neurons, DCX expression is decreased by the time neurons are mature and express the mature neuronal marker NeuN (NEUronal Nuclei). NeuN antibody (NEUronal Nuclei; clone A60) specifically recognizes the DNA-binding, neuron-specific protein NeuN, which is present in most CNS and PNS neuronal cell types of all vertebrates tested.

DCX expression is localized in the cytoplasm and along microtubule tracks while NeuN protein distributions are apparently restricted to neuronal nuclei.



Figure 4.23. Cells stained with DCX (top) and NeuN (bottom) at day 0 (left) and day 7 (right)

At day 7, NeuN is much more present than DCX (figure 4.23. and 4.24.). A fluorescence intensity of both DCX and NeuN was done at day 0 and day 7 to quantify the evolution of the associated protein expression.



Figure 4.24. Quantification of DCX and NeuN intensity

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At day 0 NPCs are positive both to neural precursor protein DCX and NeuN. After one week of 3D differentiation of acoustic levitated NPC we observed 3-fold decrease in DCX expression. There was no significant change in the expression levels of NeuN. The decrease in DCX expression suggests that the progenitor cells respond to the neural differentiation in 3D microenvironment and maintain their neural phenotype.

Further we evaluated other early neuronal markers such as Tuj-1 and Nestin on multilayer constructs.

#### 4.2.4. Multilayered structure analysis over time

#### 4.2.4.1. Differentiation process and analysis

To have an overview of the construct over time gels with levitated ESC were cultured for different timeline: day 7/8, day 14 and day 30.

NPC encapsulating fibrin gels were fixed in 4% paraformaldehyde for 30 minutes at room temperature and permeabilized with 0.5% saponin (Sigma) and blocked with 1% BSA (Sigma) for 1.5h at room temperature. Gels were sliced in 150-200 µm stripes under dissecting microscope and stained for primary antibodies: Tuj1 (MAB1195, R&D Systems), Nestin (N5413, Sigma), MAP2 (PA1-10005, Thermo Scientific), GFAP (ab50738, Abcam), CaMKII alpha (ab111890, Abcam), DCX (MABN707, EMD Millipore) or NeuN (MAB377, EMD Millipore). Samples were incubated overnight at 4°C. Antibodies conjugated with goat anti-mouse AlexaFluor 488, goat anti-rabbit AlexaFluor 568, donkey anti-chicken AlexaFluor 647 all from Jackson ImmunoResearch Laboratories were used for secondary staining and cell nuclei were stained with DAPI for 5min (Sigma). Samples were incubated for 2h at room temperature in dark and excessively rinsed with PBS. 3D hydrogels were kept wet and imaged with confocal microscopy (LSM 710, Zeiss). Image analysis was performed with FIJI ImageJ (NIH, Bethesda) software.



Figure 4.25. Zoom in on a connection at day 7: Tuj1 (green), Nestin (cyan), DAPI (blue), MAP2 (red) staining (scale bars 100µm)

First results showed few connections between layers and revealed that the staining protocol is fine to detect the cells in the fibrin gel (figure 4.25.).

TUJ 1 (betaIII Tubulin) is present in human neural progenitors (see annexe 2 for markers specificities). Tubulin is the major component of microtubules, essential cell cytoskeleton proteins involved in mitosis, cytokinesis and transport of vesicles and organelles. There are several isoforms of beta tubulin which are expressed in a tissue-specific manner. Beta-III Tubulin, also known as tubulin beta-4, is regarded as a neuron-specific marker. The expression of beta-III Tubulin has been suggested to be one of the earliest markers to signal neuronal commitment in primitive neuroepithelium. These antibodies label neuronal cell bodies, dendrites, axons, and axonal terminations and are commonly used for the identification of newly committed neurons. Nestin is an intermediate filament protein which is abundantly expressed in neuroepithelial stem cells early in embryogenesis, but is absent from nearly all mature central nervous system (CNS) cells. Microtubules are associated with a family of proteins called microtubule associated proteins (MAPs), which includes the protein  $\tau$  (tau) and a group of proteins referred to as MAP1, MAP2, MAP3, MAP4 and MAP5. MAP2 isoforms are expressed only in neuronal cells and specifically in the perikarya and dendrites of these cells. Antibodies to MAP2 are therefore excellent markers on neuronal cells, their perikarya and dendrites.



Figure 4.26. <u>Superposition of confocal pictures for a gel slices: day 7 (scale bar 100µm)</u>

After 7 days, NPCs started their differentiation as they were Tuj1 positive neuronal cells in their 3D multilayer assembled structure. The cells are still positive to Nestin, which is also an early neural marker. We demonstrate neural differentiated cells start to form network-like organizations in each single layer (figure 4.26.).

Other gels were then cultivated for different time to confirm differentiation and keep the gel longer to possibly get more connections. Gels fixation, permeabilization and staining were done.



*Figure 4.27. <u>Gel slice staining with Tuj1 (cyan), Nestin (green), DAPI (blue), MAP2 (red) at</u> <u>day 5</u>* 

After 5 days (figure 4.27.) we can see that cells are already in differentiation as they are positive to neuronal markers such as MAP2. They are also already making axons which suggest that they are in good process to make connections.

## 4.2.4.2. <u>Neural differentiation over time</u>



*Figure 4.28. <u>Gel slice staining with Tuj1 (green)</u>, <u>Nestin (cyan)</u>, <u>DAPI (blue): different gel</u> <u>magnification at day 8</u>* 

After 8 days, NPCs are Tuj1 and Nestin positive in their 3D multilayer assembled structure. We can well see the different layers stabilized in the fibrin gel. Then, at higher magnification, we can see a neural construct within layers and also few interlayer connections (figure 4.28.). A zoom in on the interlayer space shows that the connection is really established from one layer to the other, but different cells somas are involved. We can also better see the network formation within a layer.



Figure 4.29.<u>Gel slice staining with Tuj1 (red)</u>, Nestin (green), MAP2 (cyan) and DAPI (blue) <u>at day 14</u>

At day 14 (figure 4.29.), much more connections are established between but also within layers. Many cells are MAP2 positive suggesting that NPC are already well differentiated.

To characterize the type of cells we have in the neural construct after 30 days (late differentiation), gel staining with GFAP and CaMKII was done. CaM-kinase II (CAMK2) is a prominent kinase in the central nervous system that may function in long-term potentiation and neurotransmitter release while GFAP is an intermediate filament, is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells. So, CaMKII suggests that cells can be "active" and produce neurotransmitters, and GFAP expression reveals a presence of glial cell like astrocytes. Both markers are well-known in mice but not yet fully characterize in primates.



Figure 4.30. <u>CaMKII (red)</u>, <u>GFAP (cyan)</u> and <u>DAPI (blue)</u> staining at day 30 (10X, 20X and <u>40X scales = 250um</u>, 100um and 50um)

After 30 days in 3D differentiation cells are positive for excitatory neuron marker CaMKII and glial cell marker GFAP (figure 4.30.). Magnified zone shows a neuro-differentiated cell and its connections.

A neuronal network is established, as one cell is connecting with different cells via many connections.

After 30 days, ESCs are differentiated into neurons and are making many connections within layers but also inter-layers which create a neuronal network. The cerebral cortex is made of different cell types. Each layer is composed of a specific cell type. The layers are also of different thickness. To be closer to cerebral cortex different steps has to be done: being able to have layers of different cell types and being able to modulate the layer thickness.

## 4.3. Improvement for generation of a cortex-like construct

#### 4.3.1. Layer by layer modeling: beads validation

#### 4.3.1.1. <u>Two step gel</u>

The first feasibility test to be done is to know if we can encapsulated 2 types of cells: one in bottom layers and one in the top layers. First, beads validations were done.

After the gel is crosslinked it is stiffer. So, the question to solved was to know if we can levitate beads after a crosslinked gel. In fact, ultrasound may not go through an already made gel as the properties of the gel are different to water.

Usually an entire gel requires  $500\mu$ l of fibrinogen and  $81\mu$ l of thrombin at 10U/ml. So, two different gels were done: first one without beads, second one with beads encapsulated.  $250\mu$ l of fibrinogen with  $40\mu$ l of thrombin were mixed in the resonant chamber and after 10 minutes the gel is crosslinked. At that a mix of fibrinogen-beads ( $250\mu$ l) was add on top of the first gel. Thrombin is then added ( $40\mu$ l) and mixed, reflector is positioned on top of the chamber and power is turned on (has to be careful to mix on all the gel surface).

First test at 8mW revealed that it all beads were in between the two gels. There isn't levitation process. To solve that issue, different approaches were tested:

- Different crosslinking time for 1 gel
- Increase power to 22mW transmitted

#### Different crosslinking time

Different samples were done with different crosslinking time for the first gel. Fibrinogen and thrombin were mixed for 1min, 2min, 5min and 10minutes. Then solution with beads is added on the top, mixed with thrombin and ultrasound were applied for levitation.

For fast crosslinking time (1 and 2 minutes), when the fibrinogen-beads solution is added, it is directly mixed with the first gel. So, that time is not enough as the first gel is not enough stiff

to support a new volume on the top. For 5min and 10 minutes (control), same patterns is seen than on the previous experiments: beads are all aligned in between the two gels.

## Increase power to 22mW transmitted

Same protocol than previous experiments:  $250\mu$ l of fibrinogen mix with  $40\mu$ l of thrombin. After 10minutes, fibrinogen-beads solution was added on top of the first gel with thrombin. After the reflector is positioned 22mW transmitted power is sent to the ceramic (correspond to 10V on the generator, ceramic not adapted).



Figure 4.31. <u>Picture of the gel after 10 minutes of ultrasound for the top gel part: X4</u> <u>magnification</u>

Pictures in figure 4.31. are taking from the ocular as the camera field of view was too small to see the entire gel, on the fluorescence microscope (Nikon, Eclipse Ti with CoolLED pE-300-W fluorescence light). We can clearly see that layers were formed on the top gel. So, ultrasound can go through a piece of fibrin gel already crosslinked. The two gels were stacked together but the limit between them was clearly visible and did not seem "stable". This may be due to the first node which corresponds to this interface. So, many beads are at this interface.

As, it is possible to levitate beads on top of a first gel, it can be imagined to later, levitate two type of cells both parts of the gel.

## 4.3.1.2. Without reflector

To be able to create a gel, layer-by-layer, few tests of  $8\mu$ m beads levitation process was performed with the interface air-liquid as reflector, for different acoustic parameters (at a 2million beads/ml concentration).

Gel analysis showed that the formation of layers can be achieved using the same parameters than in presence of a glass reflector: 8mW transmitted power at 2.78MHz resonant frequency.

## 4.3.1.3. Tunability of layer thickness

In the cerebral cortex layers are of different thickness, so improving the process by being able to tune the layer thickness is of huge interest.

Different beads concentration (2million beads/mL, 3million beads/mL and 8million beads/mL) were tested.  $250\mu l$  of fibrinogen with beads was mixed with  $81\mu l$  of thrombin and ultrasound (8mW transmitted) were applied for 10minutes (figure 4.32.).



Figure 4.32. Pictures of gel slices for different 8µm beads concentration: 2,000,000 beads/mL, 3,000,000 beads/mL, 8,000,000 beads/mL

The more the concentration of beads (up to 8millions per ml was tested) is important, the more the layers are thick. This shows that layers thickness is tunable by playing with the cells density.

## 4.3.1.3. Three step gel

A multilayer assembly would allow the fabrication of gels of different cell type in each generated layer.

167ul of fibrinogen-beads solution were transferred to the levitation chamber.  $27\mu$ l of 10U/ml freshly dissolved thrombin was added to start the gelation process, and mixed by pipetting.

Different acoustic parameters from 8mW to 22mW were tested. The best parameters found were to send 8mW during the first gelation process, 15mW during the second one and 22mW during the last gelation process (schematic in figure 4.33.).



Figure 4.33. Schematic process of the layer-by-layer assembly

#### 4.3.1. Layer by layer modeling: cells validation

Three types of cells were used: HeLa cells, HeLa-eGFP and HeLa-mCherry. HeLa cells were cultured in 75cm<sup>2</sup> flask at 1 million in DMEM media, 10% Fetal Bovine Serum, 1% penicillin-streptomycin and 1% L-Glutamate. HeLa-eGFP and HeLa-cherry were cultured in 75cm<sup>2</sup> flask at 1 million in DMEM media, 10% Fetal Bovine Serum, 1% penicillin-streptomycin and 1% geneticin (G418, final concentration at 500µg/ml). Cells were confluent every 3 days, harvested with trypsin and used for acoustic levitation experiments solution.

167µl of the solution transferred to the levitation chamber. 27µl of 10U/ml freshly dissolved thrombin was added to start the gelation process, and mixed by pipetting. Ultrasound waves were applied for 10 minutes through the gelation process, with a power set at 8mW. After 10 minutes the same process was repeated on top of the first gel, with another cell type. The power was set at 15mW for 10 minutes. The last gel part was made with the last cell type available at with the power set at 22mW.
After those 3 gelation process on top of each other, the hydrogel was retrieved from the assembly chamber and either prepared for microscopy or culture in HeLa culture media.

For analysis gels were placed in formol 4% for 1h and then in blocking buffer for 1.5h before being cut and analyses on the fluorescence microscope (Nikon).



Figure 4.34. <u>Cells multilayer assembly: microscope pictures for cherry (G filter), GFP (B</u> <u>filter) and common HeLa cells levitation (scale bars 250µm)</u>

The last results (figure 4.34.) showed that cells multilayer assembly is possible by tuning the acoustic power sent for each gel layer formed. Composition of each layer is also tunable by choosing the cell type to mix with the prepolymer solution.

# 4.4. Conclusion

In sterile conditions, the system is able to generate multilayer constructs to be cultured. Particularly, the culture of ESCs levitated by ultrasound and stabilized within the fibrin gel is possible for at least 30 days. The culture within a differentiation media induce them to neural cells which are connecting to each other via axons elongations inter and intra-layers.

This single-step and easy method could be used for many applications such as drug screening, validation of brain stimulation device, or even studying the neural differentiation and connections establishment in a 3D environment.

The system is biocompatible as it doesn't affect cell viability, even for ESCs, which are very sensitive cells. Also, the gel properties are similar to the brain stiffness, so gel parameters are well defined to get a model close to human cortex.

The use here of human ESC-derived neural progenitor cells sets the stage for the bioengineering of 3D models of human brain disease that are based on human induced pluripotent stem cells (iPSCs)<sup>[145]</sup>. For instance, defects in iPSC derived neurons from patients with psychiatric illness are well documented<sup>[146,147]</sup>, but it is still difficult to recapitulate cerebral cortex to study neuronal differentiation and cerebral cortex organization.

Our approach is currently limited to levitate homogenous cell populations or heterogeneous cell populations without spatial difference. As it is possible to levitate through a piece of gel, the next steps possible are to generate a neural network composed of different cell types. For example, having bottom layers with one neuron type and top layers full of another cell type is feasible. This will results to a neural construct a bit closer to human cortex. Also, the cortex layers are more or less thick. The interlayer distance and thickness is tunable by playing with acoustic frequency.

It would also be possible to use radial mode to get different patterns. In fact, increasing power and/or decreasing the crosslinking time will results on the formation of radial patterns. Using different resonant chamber shapes is also a possibility to achieve different cells patterning.

# Chapter 5 – Conclusions and perspectives – Ultrasound for cell patterning

Some human tissues are constituted of several cells spheroids. For instance, hepatocytes formed 3D spheroids with lumen in middle. That kind of patterns (shape with lumen) is not easily achievable in tissue engineering. Being able to manipulate and pattern spheroids would be of huge interest as it will allow many tissues model fabrication. For that different approaches can be feasible: playing with different ceramics or using a different first approache to make spheroids and then encapsulated them in gel thanks to acoustic standing waves, in the same way than for neural tissue engineering application.

In the idea to use different ceramic to directly fabricate spheroids within a gel, Comsol simulations will be useful to predict the cells patterning but many possibilities can be seen that will results in a high optimization time.

To solve those issues, using the levitational assembly method used for neural tissue engineering is feasible. The bioacoustics levitation technique I have developed has been applied to fabricate 3D multilayer structures mimicking the microarchitecture and part of the physiology of the primate cortex. But the technology is not limited to neural tissue engineering. Before concluding, I would like to present two possible future research directions that I explored during my PhD: creating cell spheroids in 3D to recreate an environment similar to the liver, and the use of acoustic levitation for cell sorting in large sample volume.

# 5.1. Cells spheroids in 3D

Human liver is made of cells spheroids. Recreate a 3D culture of spheroids would be of great interest for research on liver. In fact, hepatocytes in 2D culture do not show the same shape as in human body. In 3D they are making spheroids. Finding a way to reproduce this shape would be of great interest and useful for many applications such as for studies of hepatitis and to analyze cells behavior in the presence of the virus in a microenvironment that could mimic a natural liver.

#### 5.1.1 Methods to assemble in vitro spheroids of hepatocytes

A new strategy was studied to recreate spheroids inside a 3D microenvironment, using alginate beads. This preliminary work was performed in collaboration with Ophélie Berthuy and Léa Pourchet from the Institut de Chimie et Biochimie Moléculaires et Supramoléculaires (ICBMS, équipe GEMBAS) UMR CNRS 5246– Université Lyon 1, who develop 3D printing of cells encapsulated in alginate beads or fibers. Our idea was to encapsulate cells in alginate beads, levitate the beads, stabilize them in a fibrin matrix and then culture the constructs in order to obtain spheroids micro-patterns.

Encapsulation of cells within alginate beads (figure 5.1.), was achieved by extruding drops of cells in alginate solution in a calcium chloride (CaCl<sub>2</sub>) solution at 10mM. Calcium polymerizes alginate beads to be able to keep them intact and usable for levitation.



Figure 5.1.<u>Alginate beads of approximately 100µm diameter, encapsulating cells (yellowish</u> smaller dots). Picture courtesy of Ophelie Berthuy, ICBMS)

#### 5.1.2. Development protocol for HeLa cells

We had to optimize our protocols to be able to encapsulate alginate beads in fibrin hydrogel. Fibrinogen is usually diluted in PBS, but the calcium precipitate in PBS and we observed that our alginate beads were unstable. The best protocol found for this application was to dissolve fibrinogen in DMEM media without any supplement and thrombin in water. Until now, we were able to see beads levitating in media but not yet to encapsulate them in layers. In fact, to be able to have nice layers we need a high concentration of beads but for now the method to create beads (3D printer) makes them close to each other in the chamber and it make them difficult to mix within the media. So, then when ultrasound are on, all the beads are floating on top of the chamber, because of the surface tension.

So, next step is to develop a good mixing protocol to be able to have alginate beads homogenously mixed within the chamber.

# 5.2. Standing waves: perspectives for cells sorting

Another application of the device good would be for cell sorting such as circulating tumor cells sorting from whole blood. For now most technics for cells sorting are using magnetic antibody to target cells or acoustic standing waves in small microchannel. The idea here is that with such a device we could be able to sort cells within a big volume. In fact if within a chosen volume chamber we apply acoustic we may be able to make different cells types levitating at different level (nodes or antinodes).

#### 5.2.1. Blood cell sorting

Red blood cells behave to ultrasound like embryonic stem cells as they are making layers (figure 5.2.).



Figure 5.2. After 1 minute of ultrasound, 680kHz

After 1 minute red blood cells are still forming homogenous layers but after 15minutes (figure 5.3.) cells are making clusters.



Figure 5.3. After 15minutes of US, at 680kHz (left), 340kHz (right)

Depending on the frequency applied, radial patterns are different.

Whatever the frequency used, it did not seem that other cells than red blood cells have enough contrast to levitate on antinodes plans. The solution thought was to use acoustic contrast agent targeted on cancer cells to enable a contrast enough high to make those cancer cells levitated on antinodes plans when blood cells will levitate in nodes plans.

# 5.2.2. Cells sorting with ultrasonic contrast agents

Blood cells are able to be levitated by acoustic standing waves. To distinguish and sort blood cells from circulating tumor cells, the next step was to first try the feasibility of levitating ultrasound contrast agent and see if contrast agents are able to go to pressure nodes of the acoustic standing waves field.



# Figure 5.4. UCA after 5 minutes, US (3mVpp compared to 20mVpp for beads), 680kHz

Figure 5.4. shows that it is possible to make UCA levitating and it needs less power than beads.

To distinguish if UCA and cells levitate at the same level, it is necessary to improve the microbubbles fixation protocol on cells.

We used ultrasound contrast agent (UCA) previously specialized with biotin antibodies  $(6\mu g/100 ul of UCA)$ , fixed on streptavidin agent coating. UCA and cells were mixed and incubated for 30 minutes to let UCA go on the top of the Eppendorf tubes.

MS1 cells, Thy1 stably transfected (expressed in inflammation) were used and mixed with UCA at different concentration (see table 5.1). Thy1 is presented at a concentration of 30 millions per cell.

For each condition, the top and the bottom part of the mixing tubes were taken, with a 27G syringe, to be analyzed on by microscopy.

100µl cells + 20µl UCA	100µl cells + 30µl UCA
50µl cells + 20µl UCA	50µl cells + 30µl UCA

Table 5.1. Number of bubbles per cells (UCA initial concentration was 500millions per ml)



Figure 5.5. Condition 1: bottom part of tube, after 1h of incubation

Even after 1h of incubation most cells are still in the bottom of the tube which suggest that the number of bubbles fixed on them was not enough to enough increase the contrast.

Around 5 bubbles were attached to one cell in this condition.

To be able to detect the levitation level UCA, beads and UCA with beads were levitated and pictures were put all close together to analyze if there is a difference.



Figure 5.6. Levitation of UCAQ (left), beads (middle) and UCA+beads (right)

It was difficult to mix UCA and beads as UCA directly sticked to beads. So, for next step to prove the feasibility of cells sorting, we need to find to protocol to mix UCA and beads together.

This cell sorting could also then be combined with a specific transfection.

### 5.3. Discussion and conclusions

In this PhD we presented a new device for easy, contactless, cells manipulation which was applicable for neural tissue engineering to which opens opportunities for other applications such as: hepatic tissue engineering or cells sorting.

Using a plan transducer interlayer distance can be tuned by tuning the acoustic frequency but also different patterns can be used playing with acoustic parameters or combining with other technics such as alginate beads, to obtain model closer to native tissue structures.

By introducing the BAL technology for direct assembly of multilayer 3D geometries within one single hydrogel construct, we have significantly improved on the assembly technologies for building 3D tissue constructs, in which individually created single gels are combined together to create a macro-scale organization. Together, these bioengineering approaches constitute a broad toolbox for the creation of 3D architectures that recapitulate the physiological complexity of native tissues. Their applications include fundamental research, such as the elucidation of the molecules that drive cellular connectivity, and translational applications, such as regenerative medicine, therapeutic drug discovery, as well as alternative models to animal testing. Their utility includes, but is not limited to, neuroscience, cardiovascular and cancer biology.

Annexes

#### Annexe 1: Theoretical modeling of microbead levitation

Levitation of a single cell was modeled (Matlab 2007b, The Math Works Inc., Natick, Massachusetts, United States) as the combined net force of acoustic radiation force, a viscous drag force arising from acoustic streaming, and a vertical force due to gravity and buoyancy. The minimum pressure (P<sub>0</sub>) for levitation is calculated under the assumption that the vertical velocity of the particle  $\dot{z}$  is zero. The radiation pressure force acting on a compressible spherical particle can be expressed by equation (1), if the particle size is at least 10 times smaller than the acoustic wavelength.

$$F_{RP} = \left(\frac{-\omega P_0^2 V}{4\rho_f c_f^3} - \right) \Phi \sin(2kz) \tag{1}$$

with  $\Phi$  is the acoustic contrast factor:

$$\Phi = \frac{5\rho_p - 2\rho_f}{2\rho_p + \rho_f} - \frac{k_p}{k_0}$$

in which V,  $\rho_p$  and  $k_p$  are the volume, the density and the compressibility of the particle respectively.  $\rho_f$ ,  $k_f$  and  $c_f$  are the density, the compressibility, and the speed of sound of the fluid, respectively.  $P_0$  is the amplitude of the pressure field,  $\omega$  the angular frequency and k the wavenumber.

Non-linear effects of ultrasound give rise to acoustic streaming, and the resulting force arising from viscous drag on the particle is expressed by

$$F_{ST} = -6\pi\eta a (\dot{z} - U_z)$$

in which  $\eta$  is the dynamic viscosity of the liquid, and  $U_z$  the vertical component of the streaming velocity in the vertical direction given by:

$$U_z = \frac{-3P_0^2}{8\rho_f^2 c_f^3} \left(1 - 2\frac{r^2}{R^2}\right) \sin(2kz)$$

in which R is the radius of the cylindrical levitation chamber ,and r the radial position from the axis of the cylinder.

Finally the force due to buoyancy and gravitational force is expressed by

$$F_{GB} = V(\rho_p - \rho_f)g$$

in which g is the gravitational constant and V the particle volume.

Species	Human	Mouse	Mouse	Human	Human	Human	Human	Human	Human	Human	Human
<u>Observed specificty</u>	Late neural stem/progenitor cell marker <sup>[148]</sup>	$GFAP + nestin = astrocytes^{[149]}$	Nestin and MAP2 expression to check for an early differentiation stage / intermediate filament protein and a well-known marker of NSC / associated with GFAP expression = indicate that astrocytes might dedifferentiate and be a source of $NSC^{[150]}$	Neural stem/progenitor differentiation associated marker whose expression is necessary for the generation of neural stem cells / Nestin expression is considered as a marker for neural stem/progenitor cells and is necessary for neural differentiation <sup>[151]</sup>	Early neuronal maker <sup>[152]</sup>	Neuronal specific marker <sup>[151]</sup>	Involved specifically during differentiation of neuronal cell types <sup>[153]</sup>	neuronal marker <sup>[154, 155]</sup>	Neuron-specific cytoskeletal protein that is used as a marker of neuronal phenotype / expression weak in neuronal precursors but becomes pronounced later ( about one day after Tij1) <sup>[153]</sup>	Neuronal specific marker <sup>[151]</sup>	In most neuronal cell types throughout the central and peripheral nervous systems of adult mice / detectable in both embryonic and adult neurons with the exception of cerebellar Purkinje cells, olfactory bulb mitral cells, retinal photoreceptor cells, and dopaminergic neurons in the substantia nigra <sup>[153]</sup>
Staining			Nestin				Tuj1	MAP2	(microtubule associated protein 2)		NeuN

Annexe 2: Neuronal marker for different species

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	Astrocytic marker <sup>[155]</sup>	
GFAP	Most of the GFAP positive cells failed to express neuronal markers; therefore, we concluded that these cells were in fact astrocytes <sup>[155]</sup>	Human
nomalous glial	Astrocyte specific marker / intermediate filament protein normally expressed by astrocytes and schwann $cells^{[151]}$	Human
fibrillary cid protein gene)	One of the most widely used markers of astroglial cells / intermediate filament that forms a network to provide support and strength to cells / More recently, a number of GFAP isoforms have been identified, including GFAPô, which is expressed in proliferative radial glia during development as well as neural stem cells in adulthood <sup>[153]</sup>	Human

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