MASTER THESIS

Non-invasive Integration of electrodes for time-resolved monitoring of 3D engineered organs

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1 Abstract

Organs on chip have arised as a new possible generation of devices for screening drugs in preclinical studies and as research tool for tissue engineering studies. Actual research is directly targeted to the increase of the complexity of these organs to achieve a most reliable simulation of different organs and the coupling of various micro-organs systems at the same time. This increase of complexity makes necessary to use new materials and different microfluidic approaches in order to reconstitute organs activity. Despite widely possible biomymetic application, attention was directed to the simulation of proximal tubule structure of the human kidney. In this study, the use of different carbon based inks were used and an optimization of the cell culture condition over electrospun (e-spun) membranes was performed in order to make the first steps into the creation of a foldable-organ on a chip (origami-organ) system which could recapitulate human proximal tubule. The creation of a foldable system, would make imaging over the micro-organs impossible. Due to this problem, trans-epithelial electrical resistance (TEER) was used as a possible solution for cellular characterization. Various carbon based inks were created upon obtainment of interesting mechanical and electrical characteristic that could gave us as reliable results as obtained using metal electrodes for cellular characterization. These inks were used to simulate TEER in a home-made system and compared with well-established systems using different salt concentrations. Furthermore, proximal tubule (PT) epithelial cells culture conditions over e-spun were optimized through experiments with different purposes: biocompatibility of cells with the e-spun, cell monolayer comparison and characterization, membrane selection and collagen coating optimization. Results show good properties regarding conductivity and wettability of certain ink carbon-chitosan ink mixes but lack of sensitvity when the ink is applied for TEER simulation. Cell culture results helped to find the proper conditions for a monolayer formation of proximal tubule cells over a e-spun membrane showed and ensure presence of basal lamina (laminin presence) and functionality of the cell on the e-spun (albumin uptake assay). Both discoveries, will help to make the first steps into a better physiological recapitulation of the kidney using an origami-organ approach and left the door open to a future scaling-up system which could completely reconstitute the whole organ.

2 Introduction

2.1 Drug development

Medical innovations require great investments from governments, pharmaceutical companies and other entities. Opposite to other technological developments, pharmaceutical industry is highly regulated in order to prevent possible safety and security issues of the patients. Furthermore, these regulations seek to ensure the medical success of the pharmaceutical drugs without the creation of biases that could not really bring improvements to the targeted disease. Regulations about ethical treatment of human experiments were not fully formulated until the end of World war II. The code of Nuremberg of 1949 englobed 10 basic principles that were posteriorly developed through the years in different parts of the world, for e.g.: the declaration of Helsinki in 1964 and the Belmont Report create by the US government in 1979. Clinical trials are designed to observes the results of an experimental treatment on humans, controlled by scientists. In order to assess all the possible parameters within a clinical trial, there should be a good control of all the variables and a number of well stablished steps in order to reduce the possible biases. The complete process of a single drug commercialization can take between 10-12 years as the are several steps and procedures to be made [1]–[3]. The steps internationally followed for the approval and sell of a drug into the market are the next:

- **Research and development.** Process of investigation and acquiring of knowledge about a specific disease. Theorizing and making in vitro experiments showing the possible application of certain treatments to a specific pathology. The research does not always have to rely in the study of several molecular compounds against a single disease, but the study of these compounds against a large number of diseases. Once an interesting compound is identified, there are experiments conducted to study how the drug is processed by the body (absorbed, distributed, metabolized and excreted), the best dosage, the best way to give the drug (mouth or injection), possible in vitro effectiveness of the compound [1]–[3].
- **Preclinical phase.** Use of *in vitro* and *in vivo* (animal) models to simulate the human disease. It is used to assess the safety and biological activity of the drug studied. Furthermore, in the late years, it is arising the use of *in silico* (computer models) to study the possible behaviour of the compound in the body using computer simulations. models to study This process can be long if the disease develops different in animal models compared to humans. Furthermore, if the studied disease needs the use of higher mammals as animal models (e.g.: apes), the development of the research can be subjected to several problems related with both time and ethics. The selection of an animal model to be used in the research depends on the criteria necessary to understand the physiopatology of the studied disease [1]–[3].
- **Clinical phase I.** First human trial of the drug, it is used to determine the safety and tolerability of a drug in humans. Usually implies between 20 and 100 healthy volunteers

which will take either the studied drug or a placebo. Further than the safety and the tolerability, the clinical phase I is used to assess the proper dosage for human body after comparing the with model animals or other relevant data available form preclinical phase. The dosage management is usually studied following two methodologies: a single ascending dose (SAD) studies where every subject receives a different concentration of the studied drug (or a placebo) but only a single dose of it, and the multiple ascending dose (MAD) where the individual subjects also receive different concentrations of the drug or a regular dose of placebo, but multiple times instead of only one. The process takes an average of 1.5 years and approximately 70% of all presented drugs pass to the next phase [1]–[3].

- **Clinical phase II.** It is intended to evaluate the efficacy and dose response of the drug. Opposite to clinical phase I, this phase uses real patients instead of healthy volunteers. It uses several hundreds of patients to study the disease. This phase can be divided at the same time into phase IIA and IIB. The clinical phase IIA is intended to study dosing requirement while the phase IIB puts attention into the drug efficacy. The success rate on this phase is between a 30-33% of all presented drugs [1]–[3].
- **Clinical phase III.** There are performed on large numbers of patients to assess the efficacy of the drug and to continue the study of drug safety which was already studied previously in clinical phase I and II. It uses between 300 to 3000 patients and usually It has a length of 1 to 4 years. This is the most time consuming and difficult phase to be performed, especially if the drug is intended for chronic diseases. As occurred with clinical phase II, the success percentage of this phase is very low, being in this case between 25-30% [1]–[3].
- **Marketing approval.** Once clinical trial phase III is done, if the results are successful, the company can apply for approval by a specific official organism. In the USA the organism designated is the food and drug administration (FDA). In the European union (EU) the process is carried by different national competent authorities or by the European medicines agency (EMA). Within the EU, the decision of which authority is in charge of the process, depends on the type of application pursued. If the drug is purposed to be commercialized in the whole EU (centralised authorisation procedure), the EMA will carry the assessment of the application and review if the drug should be released to the market or not. The other possible paths followed are the mutual-recognition procedure and the decentralised procedure [1]–[3].
- **Clinical phase IV.** Post-marketing surveillance. Once the drug has been approved into the market, there are still studies dedicated to see possible effect derived from the drug that could not be firstly observed previous to approval [1]–[3].

The most common approach used during the different clinical phases are the single-blind and the double-blind studies. This terms mean that neither the participants (single-blind) or both participants and doctors (double-blind) have the knowledge of which medication is

being used in each subject. This methodology is used to prevent the alteration of the possible results by both groups, reducing the possible bias of the results as for e.g.: placebo effect, observer bias and experimenter bias. As explained before, one group of participant will take a placebo (control group) and other will take the studied drug [4]. Even if the process of blind studies is based on the randomization of the different groups, the participants are always free to choose if they want to take part of the study and they are allowed to leave the process at any moment if they are not comfortable or if they feel that they are having many unexpected and undesired symptoms derived from the treatment. In the case of children or disabled people which could not consciously agree to take a novel treatment, the family or legal tutors have the decision if the patients should go into the clinical treatment.

The patents ensure the right of the exclusivity of the drug for several years, which allow the companies to have benefits after all the expenses made during the drug development. Only once It expires, other manufactures have the right to make a drug with similar characteristics. Generic drugs do not need to be subjected to all the new drug application as their characteristics (dosage form, strength, safety, quality, intended use) is the same as the previously released drug.

All these clinical phases and possible issues that can be found, make the process of drug discovery long and economically exhausting for pharmaceutical companies. Furthermore, the low success rate, decrease the confidence of investors into pharmaceutical industry. There have been steps made in order to try to optimize this process in order to make a quicker way to release a drug if their safety and efficacy can be proved. But there is still a great level of optimization necessary in order to reduce the time and price of the pharmaceutical research without exposing the patients' health. Further than legal changes and adjustments that can allow the introduction of bias within the process, there are emerging new biomimetic technologies that could reduce the time of research and preclinical phase developments thank to organ simulations using different biomaterials and human cells.

2.2 **Tissue engineering**

Tissue engineering is a multidisciplinary field which englobes specialist of different disciplines (biologists, engineers, pharmacologists, computer science specialists, etc). Its subject of study goes from the micro- nanotechnologies to the organ regeneration/simulation for research or direct clinical application. This interdisciplinary field uses different new materials and technologies in order to have advancements in the study of disease or studying the regenerative capacities of the different parts of the body and so on to try to solve the shortage of organ donors. First successful organ transplant was achieved by Joseph Murray in 1954, transplanting a healthy kidney from between twin brothers. Nevertheless, Organ shortage became a problem as compatibility is hard to find out of the

patient's family, leading to an increasing waiting list. Around 22 people die each day in USA while waiting for a transplant. This makes this nowadays problem a great challenge that can be solved by the means of TE thanks to the new technological advances [5]. The first tissue engineering techniques were used for the creation of skin tissues and allografts. These first synthetic skins developed between 1962 and the early 1980s was were tissue engineering is actually considered to have started, despite the term ''tissue engineering'' was not introduced until 1987. During the development of tissue engineering through the last decades, different technologies have been developed based on the advances in molecular biology and different micro- and nano-engineering techniques. These advances had led to a development of molecular diagnosis techniques thanks to the tissue engineering cross work [5], [6] .

Opposite to classic cell culture and regular biochemistry assays, tissue engineering tries to recapitulate the mechanical cues of the cells further than only putting attention to the biochemical cues of the cells. In order to fulfil this purpose, there are used different approaches with a variety of cells and scaffolds combinations. Scaffolds are intended to simulate the Extracellular matrix (ECM) of the body, in order to recapitulate better the three-dimensional (3D) living environment of the cells. The complexity of organs and Its functions make necessary also the use of other clinically relevant technologies for the reconstruction and/or research purpose as: genetic engineering, material engineering, chemical functionalisation of molecules. For instance, the use of genetic engineering could be used to induce a certain genetic disease in cells and simulate their behaviour on a biomimetic system as organs on chip or into the creation of induced pluripotent stem cells (IpSCs) that can be reprogrammed to differentiate into different cell types [7]. Functionalisation can be used to chemically link different growth factors and adhesion molecules to different scaffolds as happens in the ECM [8]. The use of material engineering can join forces with biology for the development of biocompatible scaffolds is a relevant issue to avoid the immune response to by the body, to achieve a controlled degradation inside or outside patients body and to control cell growth due to tailored biochemical and mechanical properties. Some scaffolds are made of natural materials (chitosan, silk, etc) while other are completely artificial [5], [6].

The most developed technologies derived or that are directly working with tissue engineering are bio-printing and the micro and nanotechnologies: 3D printing allows a controlled deposition of different materials on various substrates, which makes possible the future construction of personalized organs that could replace a whole organ. 3D printing is being used by tissue engineers in order to obtain new materials and scaffold embedded with cells. Bio-printing has been used for instance to create a heart valve [9], which could replace previous obsolete mechanical models. Furthermore, the advances in precision to the level of the microscale in 3D printing, has been used to substitute the use of soft lithography in the creation of microfluidic devices or even to create full devices using only 3D printing. While classic lithography uses several steps into the patterning of chambers and channels over

PDMS, 3D printing allows a quick prototyping of patterns for casting PDMS [10]. There are still steps to be made in order to fully substitute classic lithography techniques with 3D printing approaches, as the creation of 3D printers which can precisely create molds of just few micrometers and the development of new biocompatible materials which can be used to print cells without causing major stress to them [5], [6]. The Wyss institute has already use bio-printing for the creation of biomimetic Organ chip system for heart [11].

Some trends and advances in TE are leading to new future direction in regenerative medicine as: personalized medicine due to the advances in bio-printing and microengineering technologies. But there are still several economic and technological issues to solve until TE could be easily applied to daily medical care in order to fully substitute organs, tissues or be used for drug discovery and molecular diagnosis. There is still necessary a great understanding of the mechanical and biochemical cues of the cells over different materials in order to obtain a full organ/tissue regeneration *in vitro* or *in vivo*.

2.3 **Organs on chip**

Organ chips are biocompatible microengineered systems that have been perfused with biomaterials and/or cells, in order to create a dynamic culture device. They are based in the creation of chambers and channels applying templated molds over polydimethylsiloxane (PDMS), a transparent biocompatible silicon. Organs Chips are used to recall the structure and function tissues and organs, obtaining a tissue- and organ- physiological simulation that could not be mimicked with conventional two-dimensional (2D) culture or 3D statics culture [12] [13].

This technology has also been depicted as a possible substitute of animal models in the future, and as the microfluidic systems become more and more complex also arises the advantages over the long breeding periods and the ethical issues of animal models [14]. Several organs as the liver, the heart, the bone marrow and even the brain [11], [15]–[17] have been simulated in Organ chips, being possible to use different engineering approaches to manufacture them (soft lithography, stereolitography, inkjet 3D printing, extrusion printing, etc) [18]. Further than the facility to handle and the possibility of using human cells and biomaterials for a more accurate physiological approach, Organ Chips have the advantage of being a culture system that enables a high-resolution, real-time imaging analysis of the simulated organs and the integration of sensor systems.

Following this trand, organs chips can be a remarkable tool to face the challenges in tissue engineering and drug development as it makes the use of different cells lines and materials that can mimick better the physiological structure of human body possible. The high control of cellular microenviroment combined with a high spatiotemporal precision,

can help to get new cues about the behavior of cells under different situation and/or substances without the necessity of animal models and so on improving the effectiveness of developmetn and preclicinal phase predictions to drug studies. As an example, Biomimetic 3D organs on chip, can be used to mimick the interface between organ and the blood vessels connected to it. These experimental approaches cannot be assesed with a regular classic 2D culture approach. The Wyss institute has previously studied the interaction of air-blood lung interfaces, using a human-lung on chip (fig. 1). In this study, alveolar epithelial cells and pulmonary microvascular endothelial cells were co-cultured in opposite parts of a porous membrane. The membrane was stretched using a group of sealed side chambers which make the control of pressure in the chip possible. This system was used to reconstitute inflammatory responses and neutrophil migration to intra-alveolar compartment after addition of *Escherichia Coli* [14].

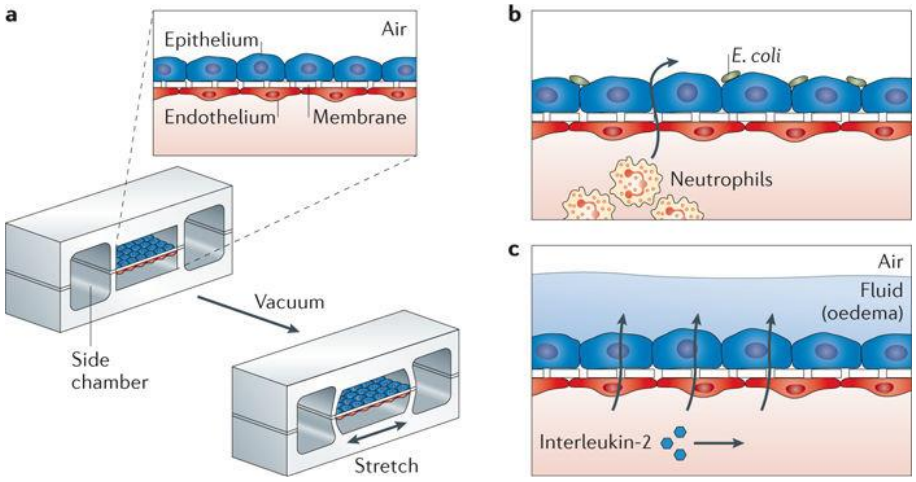

Figure 1. Human-lung organ on chip used for the study of immune response into alveolar compartments.The image, was taken from [3].

Toxic compounds and drug effects are often not constrained to just one organ, but mostly characterized by an intricate cascade of interconnected inter-organ events. The human-lung on a chip is an example of how Organ on chips cannot only studied as single organs, but also the interaction of these ones with other tissues within the body, as the alveolar layer with the endothelial tissue and part of the immune system in this example. These multi-organ platforms present a promising tool in toxicity screening of pharmaceutical compounds. By the coupling of different tissues, these devices enable a recapitulation of human tissue-tissue interactions related to the drugs and Its associated passage throughout the human body. The overall process of creation of Multi-Organ Chips has not undergone a revolutionary change yet, although Its potential necessity in pharmaceutical industry [19] (fig. 2).

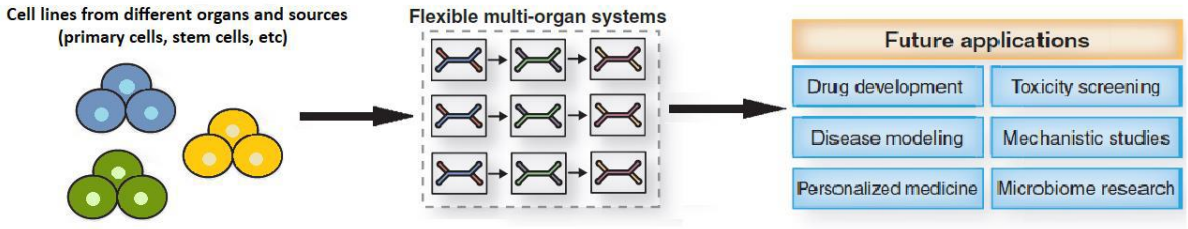

Figure 2. Flexible Multi-organ systems and future applications. The combination of different cell lines and assembly of the these multi-organ systems could have several uses in the medical field. The image was modified from [19].

2.4 **Kidney and its pathology**

Kidneys are big bean-shaped organs situated above the waist, between the peritoneum and the posterior wall of the abdomen. They are posteriorly located between the last thoracic and third lumbar vertebrae, where they are protected by the eleventh and the twelfth pair of ribs. Every adult human kidney is around 12cm long,7 cm wide and 3cm thick. The concave border of each kidney faces the vertebral column. In the upper part of the kidney. in the middle of the concavous part of the kidney, we can find the renal hilium which is the main entrance for of vascular channels and nerves [20], [21].

The external part of the kidney possesses three the layers of tissue. The most external one, also called renal fascia, is a capsule of dense irregular connective tissue, which include two cell layers: an external layer of collagen fibres and fibroblasts and an internal layer of miofibroblasts. The middle layer, the adipose capsule, is a mass of fatty tissue tha surrounds the internal part of the kidney, protecting it from traumas and holding it to the abdominal cavity. The inner layer, the renal capsule, is a transparent sheet of dense irregular connective tissue that serves as barrier against traumas and helps to maintain the shape of the kidney [20], [21].

The internal structure of kidneys is divided in two main regions: the outer layer, called the renal cortex and the inner region called renal medulla. These two parts are easily differentiated by their colour, being the renal cortex dark red coloured while the renal medulla is a pale red color. This color differs in the different structures of the kidney due to the distribution of the blood within the organ, being around 90% of the blood in the cortex and just a 10% in the in medulla. The renal medulla consists on cone-shaped structures called renal pyramids. The base of these renal pyramids, face to the renal

cortex, while the upper part of the pyramid structure faces to the inner part of the kidney [20], [21].

Parenchymal (functional) proportion of the kidney, is formed by the renal cortex and the renal pyramids. Inside this parenchymal structure, is where the essential structural unit of the kidney is found, the nephron. Each human kidney, possesses between 600.000 and 2 million of nephrons. The nephrons are in charge of filtering the blood, reabsorbing filtered electrolytes and solutes. Furthermore, it is also in charge of excrete the excess of electrolytes and water. The nephron is composed by a renal corpuscle and a renal tubule. The two main components of the renal corpuscle are the glomerulus and the Bowmans capsule. Blood and plasma is filtered in the Bowmans capsule and then it passes to the renal tubule. Renal tubule itself also has separated parts depending of its distance from the Bowman's capsule and the histology of the cells found on it. Next to Bowmans capsule we find the proximal convoluted tubule, characterized by simple cuboidal epithelial cells with prominent microvilli. The term convoluted comes from the fact that its coiled tubule instead of a straight structure like in the rest of the renal tubule. Its role is the. Next structure found in the renal tubule is the loop of Henle, which makes a hairpin connecting the proximal and distal tubule. The loop of Henle is the main structure that differs between the different types of nephrons. Between 80-85% of nephrons have a short loop of Henle which lies mainly in the cortex and slightly penetrates into the external region of the renal medulla, these are denominated cortical nephrons. Within the rest of the nephrons, the Bowmans capsule is nearer the medulla than cortical nephrons and the loop of Henle is much longer and It extends into the deepest regions of the medulla. The Longer loops of Henle makes possible the kidney to excrete very concentrated or very dilute urine. The loop of Henle epithelial cells is mainly composed by simple squamous (descending and partially ascending limb) or cuboidal (ascending zone) epithelial cells. Last structure within the renal tubule are the distal convoluted tubules. Distal convoluted tubules usually empty in a single collecting duct which is connected with the rest of the urinary system [20], [21].

Regarding Functional activity in the nephrons, there are three basic process in urine production: Glomerular filtration, tubular reabsorption and tubular secretion. Through glomerular filtration a great quantity of water and solutes from blood plasma passes from blood to the renal corpuscle (fig 3A). The pressure forces the liquids and solutes to pass through a membrane. Around 99% of these liquids and solutes will be returned to blood through the step of tubular reabsorption. Reabsorption (fig 3B) function is carried by the epithelial cell all along the renal tubule, especially the ones in the proximal convoluted tubule which make the largest contribution to the process. Last third function is tubular secretion, which transfer substances from blood into renal tubules (fig 3C). This

secretion includes several molecules as water, drugs, ions, etc. This secretion function, helps to control blood pH and to eliminate substances from the body [20], [21].

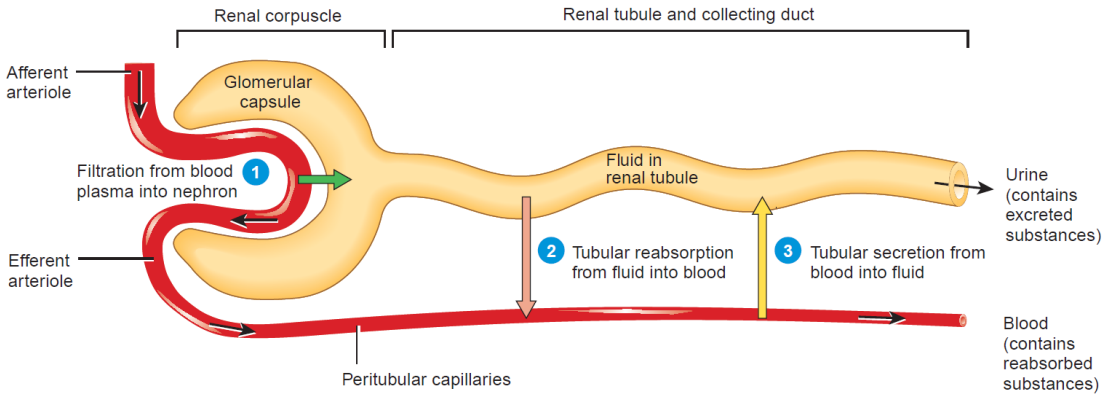

Figure 3. Relation between blood vessels and nephron structure for the three basic renal functions: Glomerular filtration (A), tubular reabsorption (B) and tubular secretion (C). The image was taken from [16].

The numbers of nephrons do not change through life, remaining constant from birth. This means that the body cannot create new nephrons if the regular ones are destroyed. This physiological structure lead to a great quantity of possible abnormalities within the urinary system function. Chronic kidney disease (CKD) is the denomination given to the pathologies that create a slow and progressive loss of kidney function over the years. Further than kidney problems, CKD patients have a higher risk of quick development of cardiovascular diseases. CKD have a global prevalence rate between 11 and 13%. The epidemiology is quite variable from one country to another, having the highest prevalence countries like Australia (5.8%), EEUU (6.7%), and England (5.2%). There are several CKD risk factors: age, diabetes, low number of nephrons at birth, obesity etc. Other endemic factors are much more difficult to define but might be related with differences in climate, nutrition and behaviour [22].

The number of patients in waiting list for kidney transplant at july 2016 was near 100.000 people in USA. Only about a 17% of those patients could get a transplant. Due to this lack of available transplant organs, the management of CKD patients is highly concentrated in the early detection and prevention of the disease. Moreover, due to the lack of available transplants, dialysis is the most common treatment used as kidney replacement therapy [22]. Prevalence of renal replacement therapy world-wide can be visualized in figure 4.

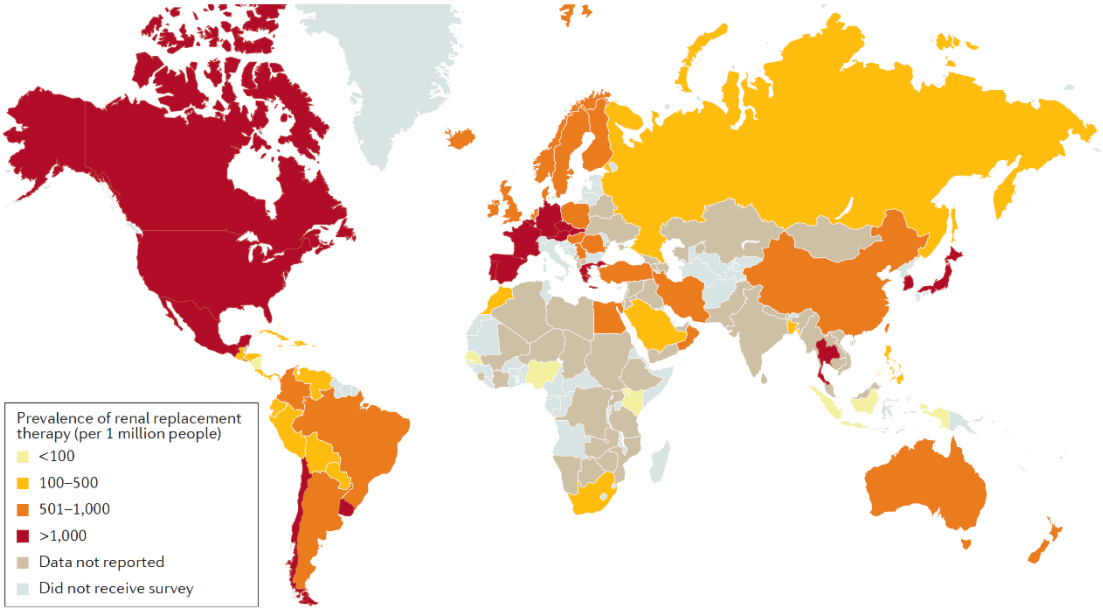

Figure 4. world-wide prevalence of renal replacement therapy. Due to lack of transplant donor available, continous dylaisis is the most common treatment available.

The high percentage of CKD world-wide and the long time needed for the development of a single pharmacological drug, make necessary the creation of a model which could simulate the structure of the kidney in order to reduce the time necessary through preclinical studies. Due to the complexity of the nephron, a simple channel on a microfluidic chip, might not be the most accurate approach to obtain reliable model within an organ chip device. The complexity of the nephron makes necessary a device that could be used to study all the functional activities of the nephron (glomerular filtration, tubular reabsorption and tubular secretion). The increase of surface ratio given by a foldable device could help to solve these issues.

2.5 Biosensors and Trans epithelial electrical resistance (TEER)

Biosensors are analytical devices used for the measurement of different relevant chemical or biological parameters that could be used to obtain information about the health of an individual, a population, an environment or a consume product (meat, eggs, vegetables, etc). There is not a single category for biosensors as they are differenced depending of which biological tissue/molecule is used, how the information is transduced and how is the output obtained. The most common biosensors are the enzyme-based, tissue-based, immunosensors, DNA biosensors. All biosensors take advantage of the changes in mass, pH, electrical current or any other parameter to transduce a signal which can be displayed and analysed [23].

Trans-Epithelial Electrical Resistance (TEER) is a non-invasive assay that is usually applied to conventional static cultures in order to evaluate the level of integrity and differentiation of *in vitro* epithelial monolayers as the electrical impedance across an epithelium is directly related to the formation of robust tight junctions between neighbouring cells. TEER can also be used to evaluate the transport of drugs through a membrane without damaging the cell integrity. The concept is relatively simple, electrodes are placed on both sides of a cellular monolayer and current between both sided is measured in order to calculate the resistance of the cellular barrier. The electrical resistance of a monolayer of cells in a transwell is a classical setup measurement of TEER. The most common tool for measurement of TEER are stx/chopstick electrodes and the EndOhm electrodes. Stx/chopsticks are as their name says, there are a couple of chopstick electrodes which different length, allows the measurement of the electrical resistance through a transwell. EndOhm electrodes are a more robust type of electrodes, with two fixed electrodes. These EndOhm electrode system is based in a bottom electrode that also works as container for the transwell and an upper electrode which works as cap for the container. The calculation of the resistance of a cell monolayer includes the measuring of a semipermeable membrane with membrane only (without cells) and other measurement of the monolayer with cells, with the objective of calculating the resistance given by the membrane itself and subtracting it from the final measurement with seeded cells [24]. obtaining the following equation:

$$Rtissue(\Omega) = \mathrm{Rtotal} - \mathrm{Rblank}$$

Variations in cells value can arise due to different factors as temperature, the medium and the passage number of cells. Ideally, TEER should be measured at a temperature of 37ºC, to ensure that there is not an alteration on the mechanical behaviour of the cell monolayer [24].

The use of static stx/chopstick and EndOhm electrodes is mostly confined to non-dynamic cell culture system, but due to different engineering techniques, it is easy to pattern different type of electrodes that can adapt to organ chip systems. The use of new materials and different engineering techniques to introduce the electrodes nearer to the cellular monolayer, can also reduce the contribution of electrical resistance from the cell culture medium and the signal noise generated in the systems [25]. On the other hand, it is important to ensure the proper conductivity and uniformity of the electrodes when used within organ on chip systems, as the non-uniformity of the electrodes is one of the main reasons for erroneous measurement.

2.6 Project aims. Origami organ chip

Given the good results obtained in the creation of these Organ chips, the next logical step is to try to scale-up and improve the complexity of system without the loose of measurement accuracy. A quite promising approach to obtain a relevant cell biomass, which can be translated into preclinical data in Organ Chips, it is the insertion of foldable biomaterials into the microstructures, and the interconnection of single-organ chip platforms to create Multi-Organ chips.

Our approach seeks to use Fibrous electrospun (e-spun) engineered scaffolds, applying a combination of folding and cutting (origami approach) to increment the surface-area of the material and adapting it to a culture device. Origami protocol is already a common objective for the construction of foldable lithium batteries in the electronic consume technology [26]. Besides, It has a remarkable potential for the creation of foldable architectures applied to the reconstruction complex human tissue scaffolds for transplantations and drug testing in Organ Chips [27] [28].

In contrast, the assembly of two single Organ channels embedded with origami foldable scaffold and posteriorly seeded with cells, will make impossible the optical characterization of the Organ Chip, particularly due the non-transparent nature of the scaffold [29]. The use of TEER into Organ Chips thanks to Patterning gold and platinum electrodes in polycarbonate (PC), has already been a subject of research in the Wyss institute. Patterned electrodes using different materials will make possible the creation of real-time sensors that could be used not only for monitoring the formation and disruption of epithelial monolayers without the necessity of optical techniques or the destruction and posterior analysis of the cells in the devices, but could also the measurement of other values based on electrical impedance as ion channel activity, tissue conductivity, cell proliferation, cell migration and many other cell behaviors [30]. This research is search to make the first steps into the creation of a fully foldable organ chip device which could be used to recapitulate the kidney function. The research process will include: First, the development of a proper foldable engineering device that can contain differentiated chambers and be used for cell culture. Second, the optimization of the culture conditions of primary kidney cells over e-spun membrane. Finally, the development of a conductive and biocompatible ink in order to construct a reliable TEER electrode system which could be easily deposited over an e-spun membrane and also give feedback of the characteristics of the cell monolayer without the necessity of destroying the device.

3 Materials and methods

3.1 Ink development

Chitin is the second most abundant polymer found in nature. It can be extracted from the exoskeleton of crustaceans and different molluscs. Chitin is a copolymer of N-Acetyl-D-glucosamine units linked by an α (1-4) glyosidic bond. Chitosan, is the deacetylated form on chitin. Both chitin and chitosan are very abundant polymers and have excellent properties such as biodegradability, biocompatibility, non-toxicity and adsorption. Chitosan shows certain advantages over chitin, as its higher solubility and its reactive free amino group (-NH2) [31]. These characteristics, added to the fact that e-spun membrane used during all the research was partially made of chitosan, lead to the use of this material for our first experimental approaches on biocompatible ink creation.

Carbon nanotubes (CNTs) have recently appeared as a useful material for a wide spectrum of uses. They were first discovered by Sumio Ijima in 1991 while studying carbon sphere structures called fullerenes. CNT are cylindrical molecules with a diameter ranging from 1nm to a few nanometres and length up to a few micrometres. Its electronic structure is based on carbon covalently linked to three neighbouring C-atoms via sp2 molecular orbits. This structure is what gives carbon nanotubes their remarkable mechanical, electronic and thermal properties [32] [33]. It was expected that the characteristics of this material were interesting for a conductive ink development.

Chitosan powder (Sigma-Aldrich®) and carbon nanofibers powder (Sigma-Aldrich®) were weighted until obtainment of desired calculated amount and added to a specific volume of acetic acid 1N in order to obtain a conductive ink. The protocol was partially extracted and modified from M.Martins *et al* [34]. Chitosan was weighted and added to a specific volume of acetic acid 1N and left to stir overnight to ensure the total dissolution of the organic polymer. On the Next day, the carbon nanofibers were weighted and added to the chitosan acetic acid mix at the desired concentration. The order of addition of the components was not significant the final mix properties, but a sequential procedure was preferred in order to avoid the agglomeration of molecules due to the insolubility of the chitosan. This process was repeated several times, leading to the creation of inks from 1-5% W/V of chitosan and 1-5% W/V of carbon nanofiber concentration.

3.2 CAD design for electrodes

SolidWorks® is a computer-aided design (CAD) software which can be used to make an easy recreation of 2D and/or 3D structures. The software makes the exportation to several file types possible. This versatility makes the software useful to be used with other devices as paper cutting plotter and 3D printers [35].

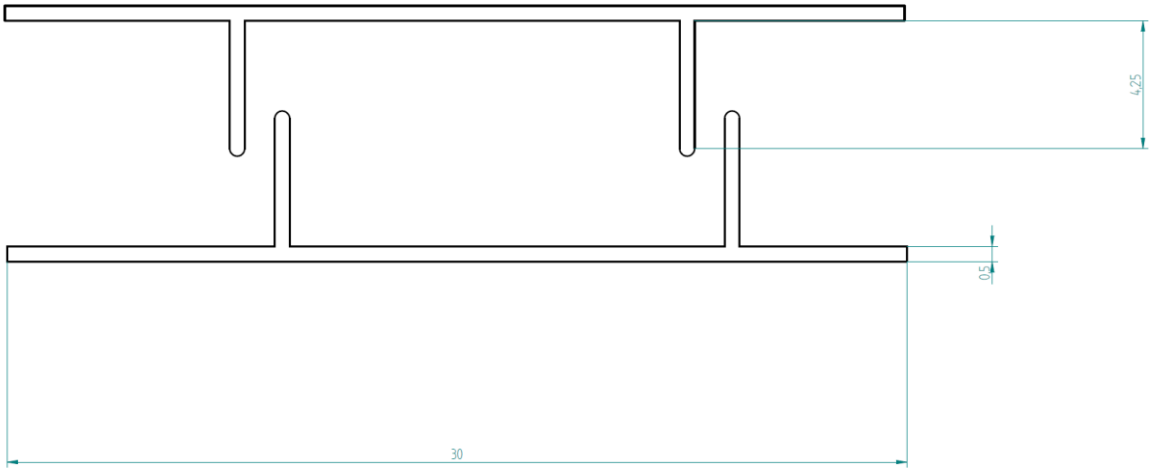

Figure 5. Electrodes technical draw. The main body of the electrodes is constructed by Parallel lines separated 82 mm with two perpendicular lines of 4.25 cm of length

SolidWorks was used in order to design electrodes and other structures for the different experimental approaches (e.g.: external windows for screen printing and plastic cap for the TEER carbon-chitosan simulation). The structure of the electrodes had the following dimensions: 300 mm length, 5 mm width and two perpendicular lines of the same width and 42.5 mm length as explained in figure 5. This design could have had a more complex shape and different width, but main information and application could be better extracted from a simple design.

3.3 **Ink deposition**

Screen printing was the selected technique to perform the first experiments related with carbon-chitosan ink. Screen printing is a technique which has been widely used in clothing business to create various pattern into different materials. The principle of screen-printing is based on the deposition of a paint or ink over a porous mesh which is strongly attached to a support frame. This makes the selective pass of molecules of ink and/or paint through the holes of the mess possible when a mask or blocking material with a specific design is applied over it. Once the patterned mask is applied, ink or paint is spread using a squeegee, allowing a homogeneous deposition of material over the selected surface. The experimental approach followed made necessary an external frame that could maintain the screen-printing frame itself as static as possible to avoid the anomalies on printed electrodes. Despite the existence of commercially available supports, the necessities of our design made the creation of a home-made one necessary. Home-made support for screen-printing frame was designed with the CAD software SolidWorks, as it was done previously with the electrodes. Acrylics sheets of 1 cm width were cut using laser.

Previously designed masks for electrodes were printed in vinyl paper using a Graphtec® cutting plotter CE5000-60. The mask was then applied and stuck to the mesh of the screen printer. PC membranes were aligned and deposited between the screen-printer and the external frame. The substrate selected for the screen printing application were polycarbonate (PC) films. PC films were chosen as material due to its common use in different cell culture technique applications, which would ensure biocompatibility of the support material if cell culture assays were needed to be performed on them. Prior to their application for screen printing, films were treated with corona discharger plasma activator under fume hood to make their surface hydrophilic. Surface activation is a common task performed on microfluidics which is used to change the surface properties of different materials thanks to the chemical modifications created by plasma. Corona discharger has the same principle of actuation of a plasma chamber but allows a simpler hand-controlled protocol, which was enough for our PC membranes.

3.4 Electrode characterization

The conductivity of the screen printed electrodes was measured with a multimeter at different distance through their length in order to ensure the obtainment on average value of conductivity per cm. Furthermore, to ensure it mechanical resistance, the electrodes were repeatedly folded 90 degrees and the conductivity was measured while applying mechanical deformation to ensure the consistence of the electrodes. For solubility testing, the electrodes were submerged on Phosphate buffer (PBS) for 3 hours and posteriorly dried carefully at room temperature (RT).

3.5 Transwell creation (injection molding)

Transwells are quite commonly used for cell culture experiments. Their advantage over 2D systems is that they can be used to mimic internal body membranes, thanks to their top and bottom membrane-separated parts that allow several interesting experiments as molecule permeability and cell migration. It has been used to simulate biological barriers such as skin to study the physiopathology of the organ [36]. These previously proven experimental characteristics makes them perfect for experimental purposes preparatory for an origami foldable system. Transwells can be purchased directly from several companies, but their price is high and the necessity of performing several experiments with a relatively high amount of transwells lead to the creation of home-made transwells using injection molding.

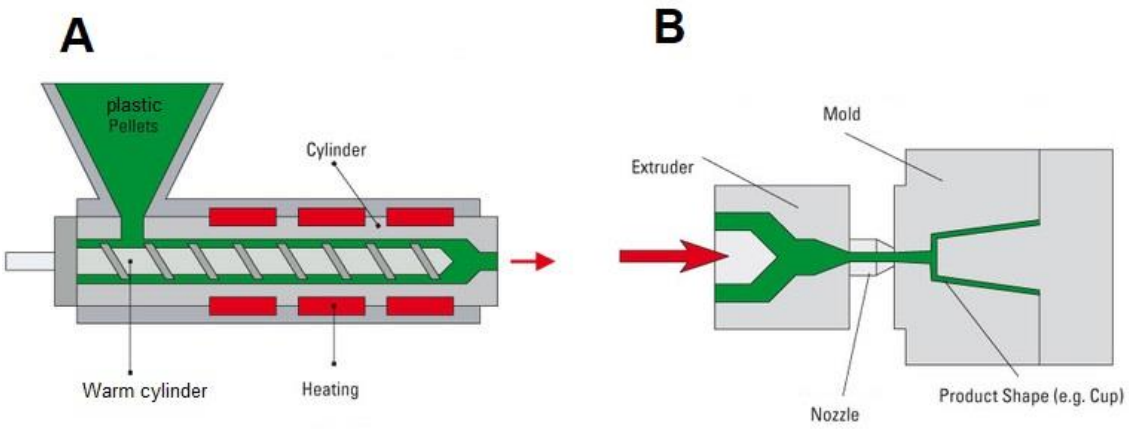

Figure 6. Injection molding process. Plastic pellets pass through a heating cylinder (A) and are posteriorly extruded through a nozzle and injected in a mold (B). Modified from [37].

Injection molding is based on the melting and molding of plastic polymer at high temperatures. Beads made of plastic cyclo-olefin polymer (COC) ZEONEX® 1500 were inserted into conical heating cylinder and pressed down through it (fig. 6A). Melted polymer goes out through an extruder and falls into an aligned mold, making the creation of a desired pattern possible (fig. 6B). In our case, two-pieces of transwell mold were designed using SolidWorks and milled using a computer numerical control (CNC) milling machine. Plastic was extruded into the previously heated and aligned mold and the resulted piece was posteriorly separated from the mold using pliers.

3.6 **TEER simulation with carbon electrodes.**

TEER was the selected method to use in a future Origami organ on chip system. As previously explained, TEER can be used to characterize cell monolayer formation, further than other different biologically relevant parameters as for e.g.: degradation on a scaffold.

EndOhm electrodes (Fig7) were used as standard to have a reliable comparison of the capabilities of carbon-chitosan electrodes. EndOhm for transwells is a commercially available electrode system which can be used to measure the resistance through the membrane of a transwell based on a top and a bottom metallic electrodes. They can be coupled to measurement devices as EVOM^2 or IVIUM, which allow the obtainment of a value for the resistance between the electrodes and/or a complete graphic representation under different frequencies using alternative current (AC). In order to make possible a comparison with a homemade system, EndOhm was connected to the IVIUM system through an adapter. The IVIUM potentiostat allows the use of different frequencies to measure the resistance through a membrane or a liquid, further than other application as changing the applied amperage. The IVIUM system was connected to EndOhm and different KCl concentrations (0.1M, 0.25, 0.5M, 0.75M and 1M KCl) were tested. Posteriorly, in order to simulate the top

electrode of EndOhm system, a cap was 3D printed using a Fortus® 400mc printer and glued to a screen printed electrode over a PC membrane. For the bottom electrode simulation, home-made transwells were also glued to PC membrane with screen printed electrodes. The transwell was deposited over a 12 well-plate to have a stable support and connected to IVIUM by an alligator cable.

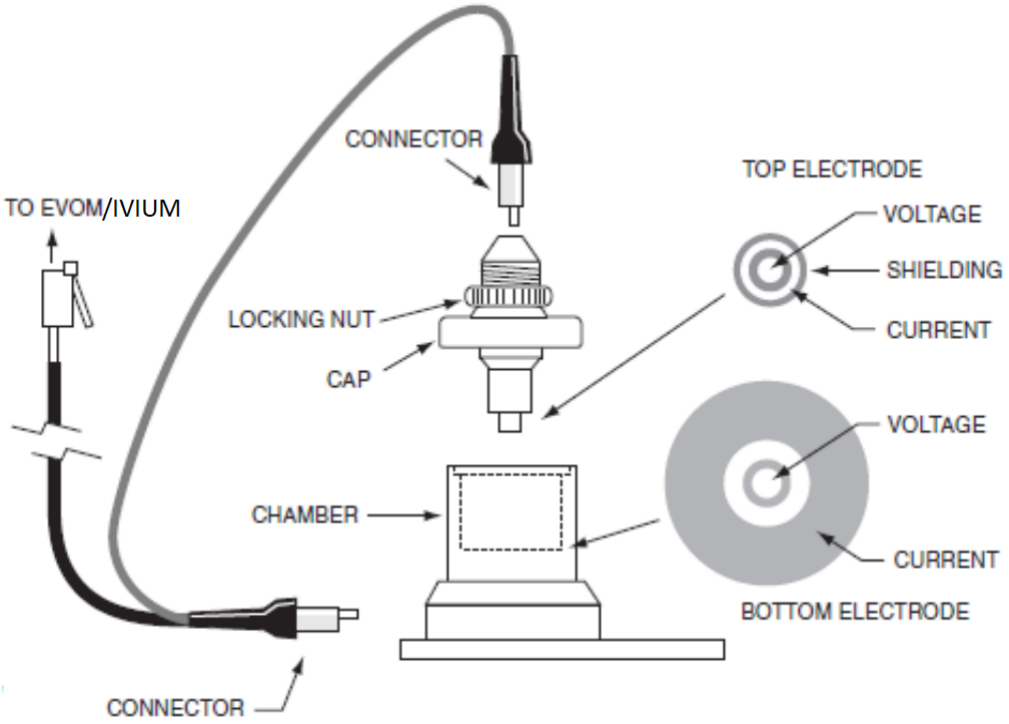

Figure 7. Structure of the EndOhm electrode system. The transwell is deposited on the chamber after medium is added. Bottom electrode is found at the end of the chamber and the top electrode is attached to a cap. Both electrodes are wired directly to the EVOM² or the IVIUM system depending on the experimental necessities. Modified from [38]

3.7 **E-spun membranes**

Electrospinning is a simple technique that relies electrostatic repulsion of liquid polymer within a syringe when an electric field is applied on it. This allows the expulsion of controlled polymer fibres through the syringe. The resulted e-spun membranes have different characteristics depending of the material and settings used in the process. E-spun membranes of different characteristics were purchased from Stellenbosch nanofiber company (SNC®). Membranes selected for these studies were made of different concentration ratios of poly ε-caprolactone (PCL) and chitosan. This ratio was selected regarding previous experimental attempts carried by the research group, where e-spun fibres were home-made in the laboratory at the same ratio. Other parameter that differenced one material from another was the speed collection of the e-spun, which was performed at medium or high speed for both 40:1 and 20:1 ratio concentrations. This lead to the use of a

total of 4 different e-spun types during the experiments: 20:1 medium speed (20:1m), 40:1 medium speed (40:1m), 20:1 high speed (20:1H) and 40:1 high speed (40:1H). Numerical data of speed recollection is confidential information from SNC®. Polyethylene terephthalate (PET) was used in some experiments as standard for comparison with e-spun.

E-spun or PET membranes were glued to home-made transwells using Loctite® 651, a biocompatible and UV-curable glue. A small amount of glue was deposited on a plate and transwells were carefully dip into the glue until the bottom surface was completely covered with a thin film of glue. A small square of material (e-spun or PET) was cut with a scalp and attached to glued surface of the transwell. Once attached, the transwells were flipped to avoid direct contact of the glued bottom with any other surface. Glued transwells were then cured for 8 minutes using an UV curing chamber. Finally, the leftovers of the square of PET or e-spun were trimmed with a scalp or scissors.

3.8 Cell culture

3.8.1 Cell lines

Cells selected perform the first experiments were epithelial proximal tubule cells. As previously explained in the introduction, epithelial proximal tubule cells are the most populous and carry several endocrine, regulatory and filtering functions. Progression of many CKD first manifest in the proximal tubules [39]. These characteristics made these cells perfect for their use in an organ on chip system.

3.8.2 Collagen coating on transwells

Collagen IV was used to coat transwells previous to cell seeding. Collagen IV is used as mechanical support of the cells as it is one of the most abundant proteins of the extracellular matrix (ECM). Vials of collagen IV 1mg/ml stock were unfrozen and diluted into PBS enriched with calcium and magnesium (also called PBS++) in order to obtain appropriate concentration for the experiment. PBS++ (800 μl) was added to the bottom of the 12 well-plate to avoid the diffusion of the collagen mix deposited on top. Finally, 200 μl of collagen IV coating mix were added to the top of the transwells and incubated for 2 hours at room temperature (RT) to ensure total gelification of collagen IV. Prior to cell culture over prepared transwells, the collagen coated membranes were washed two times with PBS.

3.8.3 Cell preparation and seeding

Primary PT epithelial cells were obtained from primary source of Beth Israel hospital in Longwood medical area. Cells were seeded on T75 flasks using home-mixed proximal tubule medium (table 1)**,** to ensure the proper cell growth. Cells were incubated at 37ºC during several days until obtainment an appropriate number of cells were obtained.

Table 1. Components of PT medium		
Component	Volume	Final concentration
Fetal Bovine serum (FBS)	2.5 ml	0.5%
Triiodothryonine	0.5 ml	10 nM
Rh EGF	1.0 ml	10ng/ml
Hydrocortisone Hemisuccinate	0.5 ml	100 ng/ml
rh Insulin	0.5 ml	5 µg/ml
Epinephrine	0.5 ml	1 µM
Transferrin	0.5 ml	5 µg/ml
L-Alanyl-L-Glutamine	6.0 ml	2.4 mM

Once the cells were ready for their culture on transwells, the flasks were washed with PBS and incubated with trypsin for several minutes to separate the cells from the bottom of the flask. Once cells were resuspended, they were counted using a Neubauer® cell chamber to calculate the total number of cells available from the solution and the final concentration needed for the experimental purpose. While counting was being performed, cells were centrifuged at 650g for 5 minutes in order to eliminate the rest of trypsin and adjusted with new medium until obtainment of desired concentration. To all the transwell used during the experiments, 200 µl of a suspension of 500.000 cells/ml were pipetted to the wells, in order to obtain 100.000 cells/transwell. As performed during collagen IV coating, 800 µl of PT medium was added to the bottom of the transwells to avoid diffusion of medium from top to the bottom and so on preventing the drying and death of the seeded cells.

3.8.4 Cell freezing

To avoid the continuous passage of cells and therefore the change of certain properties (spreading, migration, accumulative mutations), a pool of frozen cells was created.

The main reagents needed for cryopreservation of cells were fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO). The approach followed for the creation of a pool of frozen cells was as follows: Resuspended cells were counted and on Neubauer cell counter chamber after the trypsin elimination using centrifugation at the same settings as when cells were used for experimental purposes (650g and 5 minutes). The mix of cells ($1*10^6$ cells per ml) and cryopreservants reagents (10% DMSO and 20% FBS) were aliquoted to cryotubes and deposited on the Thermofisher® freezing container ‘’Frosty’’. Freezing containers allow an optimal cooling rate of -1ºC/minute, making the freezing process easy and automatic. Finally, the freezing container with the aliquots were stored at -80ºC.

3.8.5 Immunostaining protocol

Immunostaining is a technique based on the selectiveness of antibodies (abs), to stain different selected parts of the cell for experimental purposes. Immunostaining can be divided in different subtypes, depending of the number of abs used. Primary immunostaining (fig. 8A use a single ab covalently attached to a fluorophore. Secondary (indirect) immunostaining use 2 Ab in order to obtain a signal. The first Ab is directed against the target tissue, lacking of any modification on its structure of any type of fluorophore. The secondary ab is modified with a chemically linked fluorophore is directed against the primary Ab, making a complex of two Abs and one fluorophore. The use of secondary immunostaining (Fig. 8B) is more common due to the obtainment of a good signal amplification compared to the direct staining. In contrast, primary staining is still widely used if a quicker protocol than indirect staining is needed. Furthermore, non-specific binding may be reduced by the use of a single Ab [40].

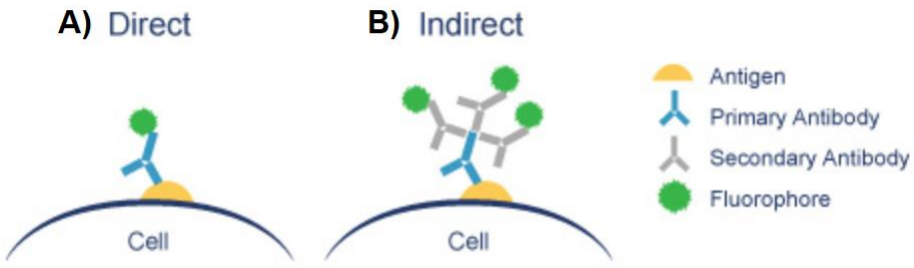

Figure 8. Summary of the two main immunostaining methods. Direct immunostaining (a) uses one single Ab chemically linked to a fluorophore dye. Secondary immunostaining (B) uses a second Ab chemically linked to fluorophore dye, allowing the amplification of the signal due to multiple reactions with primary Ab. Modified from [29]

Cells seeded on transwells were fixed using 4% paraformaldehyde solution. After 3 times washing with PBS, transwells were permeabilized using a permeabilization buffer (0.125% Triton X-100 in 1x PBS) and incubated for 5-10 minutes. In order to reduce the number of unspecific reaction of antibodies, incubation of transwells with blocking solution (2% BSA in permeabilization buffer) was perform for a minimum of 30 minutes at RT followed by 3 times washing using permeabilization buffer. To avoid the use of a high volume of ab for the staining, from this step on, transwells were taken out of the well plate and covered with parafilm. Due to this extra step, the use of extra volume of buffer (800 µl) at the bottom of the transwell could be avoided. The primary ab was diluted into antibody buffer (1:5 blocking buffer into PBS) and pipetted into transwells covered with Parafilm (about 200 µl/transwell). The samples were incubated at 4ºC until the next day. On the next day, after 3 washes with permeabilization buffer, the secondary antibody solution (ab on ab buffer again) were

pipetted into the top of the transwells and incubated for 1 hour at RT while covered with aluminum foil. Dilutions of both, primary and secondary antibodies were selected depending on the ab used, but ranged between 1:200 to 1:500 for primary Ab and 1:1000 to 1:5000 for secondary ab. The final wash with permeabilization buffer (3 times) and PBS (3 times) was carried out before preparation for imaging or storage at 4ºC.

3.8.6 Hoechst staining

The Bisbenzimide, also called Hoechst, is a cell permeable compound that permits an easy observation of the nucleus of the cells using fluorescence microscopy. The structure of Hoechst allows it to easily bind to AT-rich zones in the DNA [41]. Its convenience and popularity relies in is easy-to-use protocol, that only needs few steps to complete bright nucleus staining. Its use can be combined with several other fluorophores. For the different experimental approaches, several counterstainings during the different assays were performed. Hoechst staining was combined with immunostaining (both direct and indirect) and phalloidin staining.

The Hoechst stock solution was diluted 1:10000 in PBS. This protocol was always performed directly after immunostaining, so the samples were washed 3 times with permeabilization buffer before adding Hoechst, to ensure the permeability of the cell. Hoechst solution (200 µl) was added to the top of transwell and 800 µl to the bottom of them. They were covered with aluminium foil and incubated for 10-20 minutes at RT. Finally, they were washed 3 times with PBS to ensure the elimination of residual of Hoechst solution.

3.8.7 Phalloidin staining.

Phalloidin is a toxic peptide synthesized by *Amanita Phalloides*. It interests in cell biology relies on Its specific binding to actin, which makes it a useful reagent for staining the cytoskeleton of the cell. Phalloidins can be chemically modified to be covalently attached different type of fluorophore dyes [42] [43]. As happens with Hoechst, the most important advantage of phalloidin over other immunostaining is the necessity of 1 single step to have a good image of the actin skeleton, further than the cheaper purchase price when compare to monoclonal Abs for immunostaining.

The phalloidin (covalently modified with 647) stock solution was diluted 1:40 in PBS. After the removal of PBS, the phalloidin solution was added to the top and bottom channels and incubated 20 minutes at RT. The transwells were then washed 3 times with PBS to eliminate the rest of non-attached phalloidin molecules.

3.8.8 Albumin uptake

Albumin is one of the most common proteins found in the human blood. It is produced in the liver and it has a main role in transporting hormones, fatty acids, and other compounds. The albumin re-uptake can be performed receptor-mediated by both PT cells and podocytes in the proximal tubule. The uncapability of this cells of processing albumin is a marker of structural or functional alterations into these cells [44]. For these reasons, albumin linked to fluorescent dye (emission wavelength 488 nm) fluorescein isothiocyanate (FITC) was used to characterize the function of our proximal tubule cells.

The FITC-albumin stock solution was diluted in DMEM-F2 medium to obtain a 10 µg/ml solution. Transwells were transferred to a new plate and medium of both top and bottom channels was removed. DMEM-F2 medium was added to every transwell. The samples were then incubated for 15 minutes at 37ºC. DMEM-F2 was then removed and 200 µl of FITC-albumin solution were added on top and 800 µl on the bottom of the transwells as in previous experimental approaches. After 15 minutes' incubation at 37ºC, transwells were washed with medium several times and fixed using the previously explained fixation protocol.

3.8.9 Live-dead staining

Live-dead staining allows the observation of living and dead cells on membranes (PET and e-spun in this case) due to a strong fluorescent signal given by calcein (495nm) and ethidium bromide (EthD-1). The internal esterase activity of living cells can be used to convert nonfluorescent calcein intro fluorescent calcein, producing a visible signal on the microscope. In contrast, EthD-1 can pass through damaged cells membranes but it is totally excluded from intact plasma membrane of live cells. EthD-1 produces a strong red fluorescent signal within dead cells [45].

This staining protocol which was performed without previous fixation of the cells. Samples were washed with PBS in order to eliminate possible membrane or cell residues. Reagents were diluted with PBS and added to both the top and the bottom of the transwells until total submerging of the samples. Transwells were incubated at 37ºC at 5% CO_2 for 20 minutes and posteriorly observed using epifluorescence microscope.

3.8.10 Inulin-FITC and TEER (permeability assay)

Permeability assays can be used to study how different substance affect the cell-cell adhesion and/or the cell-substrate adhesion. Transwells are especially useful for this purpose as they have differentiated top and bottom chambers, where liquids can diffuse depending of the integrity of the cell membrane culture and the porosity of the substrate itself. In the experiment, certain known permeable molecule linked to specific dye were used,

in this case: the permeable molecule inulin, bonded to the fluorophore FITC. Calculations of permeability are made upon the difference on molecules left on top and bottom after the same time. TEER has a similar procedure to Inulin-FITC assay, but it uses the potential between two electrodes to calculate the integrity of a membrane [46] [47]. Values obtained by measuring the TEER and permeability must be inversed as the resistance of a membrane is higher when its permeability is low and vice versa. In this experiment, instead of studying how a substance affects the membrane integrity, the growth and monolayer formation of PT cells on different materials was studied.

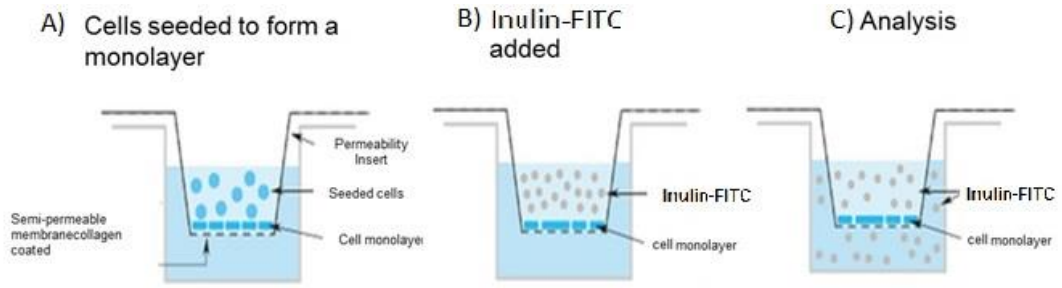

Figure 9. Inulin-FITC workflow. Cell are cultured over e-spun membrane (A) in order to obtain a monolayer. The inulin-FITC conjugate is added on top of the transwell (B) and incubated for a certain time until diffusion occurs in the cell monolayer. Medium on top and bottom of the transwells is posteriorly reaspirated and analyzed for quantification (C). Modified from [48].

The inulin-FITC stock solution was diluted in PT medium. Previously seeded cells on transwells (fig. 9A) were washed with PBS prior to addition of the regular medium on bottom, to avoid quick diffusion from the top as usual and as essential part of this quantification experiment. The Inulin-FITC medium mix was added on top of the transwells (fig. 9B) and samples were incubated for 30 minutes at incubation chamber. After 30 minutes both the media on top and bottom was recollected (fig. 9C) and the fluorescence level measured using a synergy NEO (BIOTEK®) microplate reader on a 96 Costar® well plate.

3.8.11 Microscopy preparation and image processing

Confocal and epifluorescence microscopy were used in order to image the different stained samples. The chemical structure of the fluorogenic dye molecules allows an excitation of their electrons under certain wavelength. Due to their chemical structure, the electron relaxation results into the emission of light instead of the loss of energy in form of heat. These both microscopy techniques use the excitation of fluorogenic dyes, which were previously linked to biological samples, in order to obtain an emission by these fluorophores at a higher wavelength (emission wavelength). This excitation can be produced by a lamp or a laser.

Fixed samples from e-spun or PET membranes were separated from the transwells using a scalp or scissor and deposited over goldprobe mounting media on a glasslide. A coverslide was applied on top and nail was polisher applied to the edges of the coverslide to seal both parts. The mounted samples were incubated in the dark at RT overnight and posteriorly kept at -4ºC until imaging was performed. The samples were transferred to microscope and cells imaged using Zen black® software. The settings were adapted for every samples to ensure the most efficient imaging results in every experiment.

4 Results.

4.1 Screen printer assembly.

The montage of screen printer for homogeneous ink deposition, was simple: the paper printed mask was applied over the mesh and mounted over the external laser cut frame. First attempt of application the mas was only applied the top of the screen printer, between the squeegee and the mess. After first attempt, it was noticed that this approach was not the most reliable one, as the inks tended to attach to the glues side of the mask. In order to solve this issue, another mask was printed and carefully stuck at the opposite side of the mesh after hand-made aligning. Neither of the components was described as toxic in their safety data sheet (SDS) but carbon nanofibers stain all objects around them and solvent (acetic acid 1N) can be corrosive, so all the experiments were performed under chemical fume hood.

The size of the mesh seemed to be adequate for this application. A previously homemade screen printer was available in the laboratory but its small pore size made it impossible to use it with carbon nanofibers as they seem to be too big to pass through it (10 nm x 20-200 µm).

4.2 Ink optimization

The development of a foldable system based on a e-spun membrane which could be characterized using TEER, makes it necessary that a conductive material which can be easily deposited over a sensitive and thin material as e-spun. Gold electrodes are commonly used for biological applications due to their high conductivity and biocompatibility, and give a good sensitivity when cellular characterization with TEER is used [30]. In contrast, the price of these materials is high and they cannot be deposited easily on a surface without aggressive techniques that could deteriorate the structure of the e-spun. Carbon based materials, have previously been used to make conductive scaffolds, biocompatible electrodes and other variety of applications [34], [49]–[52]. The first step for the development of a TEER based foldable system, was the creation of an ink that could maintain a good level of conductivity but could also be used in the future with different deposition techniques as screen-printing and 3D printing.

These inks were created to study how the percentage of each component affected the conductivity and viscosity of the system. Solvent was selected regarding the work of M. Martins *et al*. [34] where they used a similar mix of components but applying a different protocol, as their objective was scaffold formation instead of an ink creation. Because chitosan is an insoluble polymer, it was expected that it increases the viscosity of the ink but also that it interrupts the connection between carbon nanofibers. Due to their structure chitosan can easily agglomerate in insoluble clusters that causes a rough ink. Due to this

issue, several inks were prepared in a first moment without achieving a proper dissolution of both components. This problem was first attempted to be solved using detergents as Triton Pluronic F-127 (Sigma Aldrich®), to try to avoid the aggregation of molecules on the mix due to the change of surface tension by the added surfactant. Subsequently optimization showed that the only step necessary to avoid agglomerations was the sonication of the ink during 10 minutes shortly before using it.

Both components were studied in a concentration of 1-5% (W/V). As expected, the increase of carbon nanofibers on the mix, lead to an increase in the conductivity of these inks. As shown in figure 10A, there is a big change in the conductivity of the inks when the percentage of carbon nanofibers is increased from 1% to 2% W/V. From that point, inks seem to only increase their conductivity slightly, even if more nanofibers are added to the mix. In contrast, we obtained exactly the opposite result when the concentration of chitosan was increased on a mix, as is shown in figure 10B. This made us thing that there is a point of carbon nanofiber concentration where every fibre is in contact with other neighbour fibres, allowing a good conductivity through the electrodes. But when chitosan concentration is increased, these molecules interrupt the contact between carbon nanofibers, and consequently reducing the conductivity of the system.

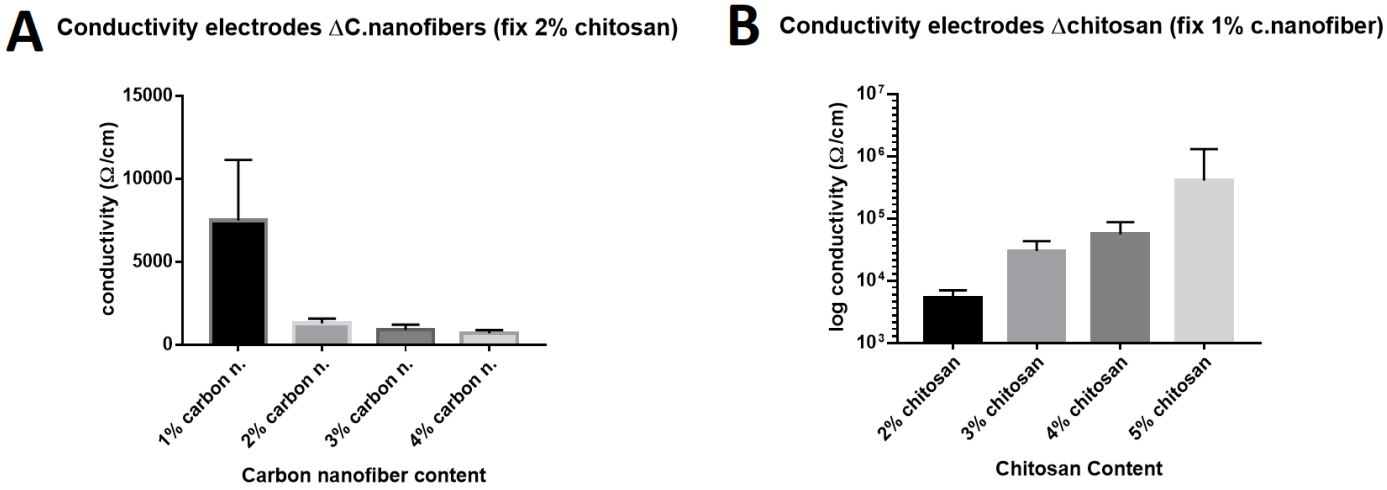

Figure 10. Measurement of conductivity of electrodes made with (A) different percentange of carbon nanofibers and (B) different percentage of chitosan.

At first sight, this could make think that a low chitosan concentration will be the best for the ink, but the concentration of this component had an essential role on the rheological behaviour of the final ink, being impossible under certain concentrations to make complete electrodes using screen printing, as the inks where too liquid to be deposited using any technique.

At the same time that different mixes were created in order to find the best solutions for conductivity and rheology, wettability of the electrodes was tested. Once the influence of the carbon and chitosan components in the inks, it was necessary to determine their resistance in water. Several electrodes were created with different concentrations of XXX and submerged for 3 hours in PBS. Figure 11 shows the comparison of these electrodes before (fig. 11A) and after being washed (fig.11B).

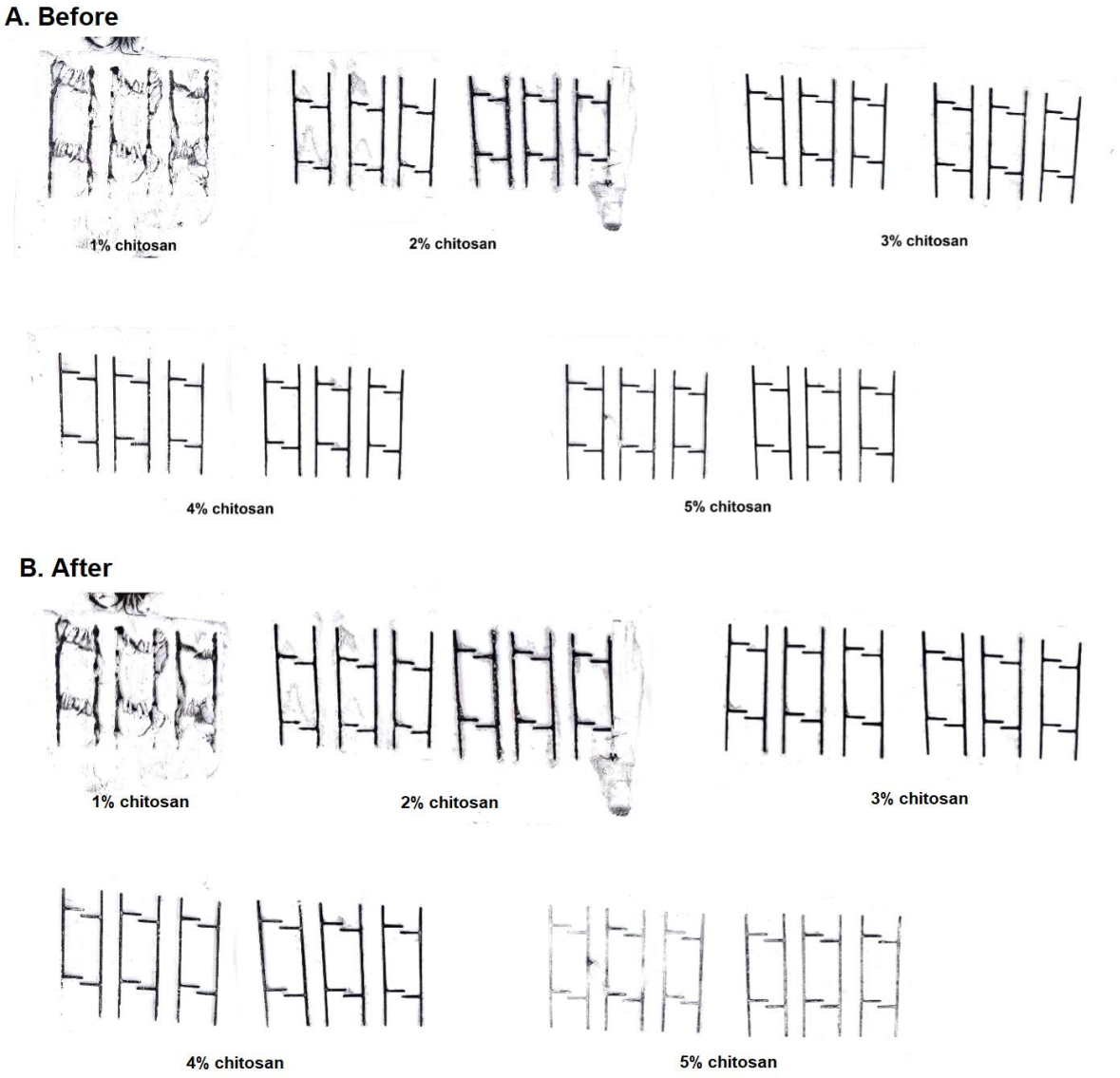

Figure 11. Screen-printed electrodes before (A) and after (B) soaking them in PBS for several hours.

The image of the before and after washing of the electrodes showed a clear pattern of how the concentrations affect the mixes regarding its deposition. When the percentage of chitosan is too low (1%), the ink is too liquid to be deposited using the screen printing

technique. As chitosan increases, the patterns are more defined and clear. In contrast, after submerging them, the electrodes started peeling off when they achieve a high chitosan concentration (4-5%). At 4%, it is possible to observe a certain amount of peeling before and after washing the samples, but the most remarkable results could be observed at 5% chitosan. At 5% chitosan, the loss of electrodes after washing was very clear, making them totally useless for sensing purposes.

After obtaining these results, I was thought that washing in PBS was probably affecting the conductivity of the electrodes. From this point the experiments were constrained to variations of both components from 2 to 3%, as they were the range of concentrations that showed to be more stable as result of the soaking experiment.

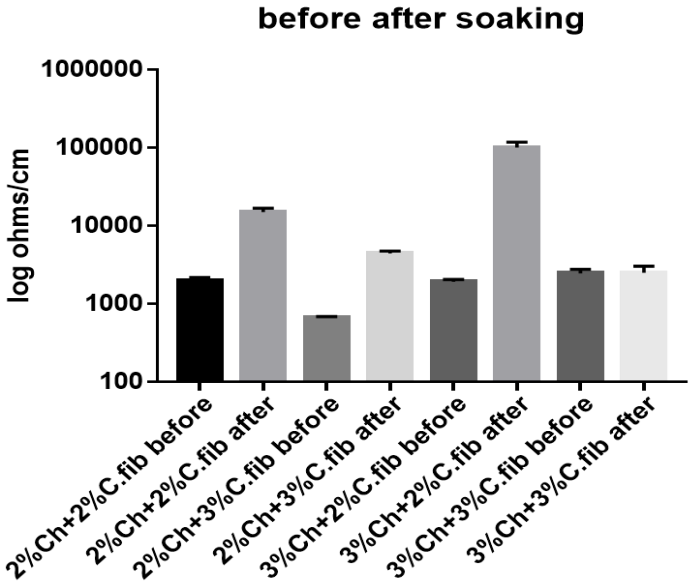

Figure 12. Conductivity of screen printed electrodes before and after soaking. All ink mixes lose conductivity after being soaked in PBS, except the mix of 3% chitosan and 3% carbon nanofibers, which seems to remain at the same level of conductivity.

The conductivity measurement and the soaking experiment was repeated in order to obtain information of the maintenance of electrical properties of carbon-chitosan electrodes. Figure 12 shows the result of measuring the conductivity before and after soaking the electrodes in PBS. The data shows interesting results: Most of the mixes tend to highly decrease their conductivity once they are submerged in PBS. This is a significant information to have, as the decrease of conductivity could lead to obtainment of unreliable results when applied for TEER. In contrast, the mix of 3% carbon nanofibers and 3% chitosan, seems to maintain Its electrical properties after being submerged and dried. The most likely explanation is that this chitosan concentration is enough to allow the maintenance of the structure of the electrode

(as previously shown in the fig. 11) and at the same time, the carbon nanofibers are in a concentration high enough to allow the contact between the fibres without any loss of conductivity after soaking.

4.3 TEER simulation using carbon-chitosan electrodes

The first success acquiring an ink with interesting properties, lead us to the attempt of simulating TEER using these electrodes. Before using them in a complex setup as cell culture monolayer formation, it was decided to make a simulation using only different concentration of potassium chloride (KCl). A simple salt dilution would give exact information of how the electrodes behave when compared to a well-established system as the EndOhm. To make the setup as similar as possible to EndOhm system, a cap was printed using the Fortus® 400mc 3D printer. This 3D printed cap was used as top electrode once glued to a screen printed electrode on a PC membrane. A PC membrane glued to the bottom part of a transwell was used as bottom electrode, making a complete EndOhm simulation after wiring it to IVIUM potentiostat device.

Figure 13A depicts the results obtained with EndOhm when different concentration of KCl were tested. If attention is put on the black line (which represent the resistance found in the sample), It is possible to observe a clear pattern whereas the concentration of the salt increases, the resistance decreases, showing a slightly smaller value in the graphic. This results shows how a proper TEER system should work in regards of data output. Figure 13 A also shows the data obtained when performing the same experiment using the home-made simulation system. As it was expected, conductivity is much lower compared to well established gold electrodes of the EndOhm but seems to show some degree of conductivity. On the other hand, the differences between different KCl concentrations is practically indistinguishable and even results do not correlate with the concentration used as they did with the EndOhm.

At the beginning, it looked as the system was very unstable compared to the EndOhm, as the cap was not totally fixed in vertical position, leading to changes in the measurement results due to the instability. To solve this problem and still ensure the good capabilities of the electrodes, it was decided to make a more stable system based on a simple microfluidic channel. This microfluidic channel was based on microfluidic channel patterned on double sided tape and covered on the top and the bottom by an PC membranes embedded by carbon-chitosan ink through screen-printing technique. This system was more stable than the previous one as the distance between electrodes was fixed and shorter than before. With these advantages, it was expected that the differences between different concentration of KCl were clearer.

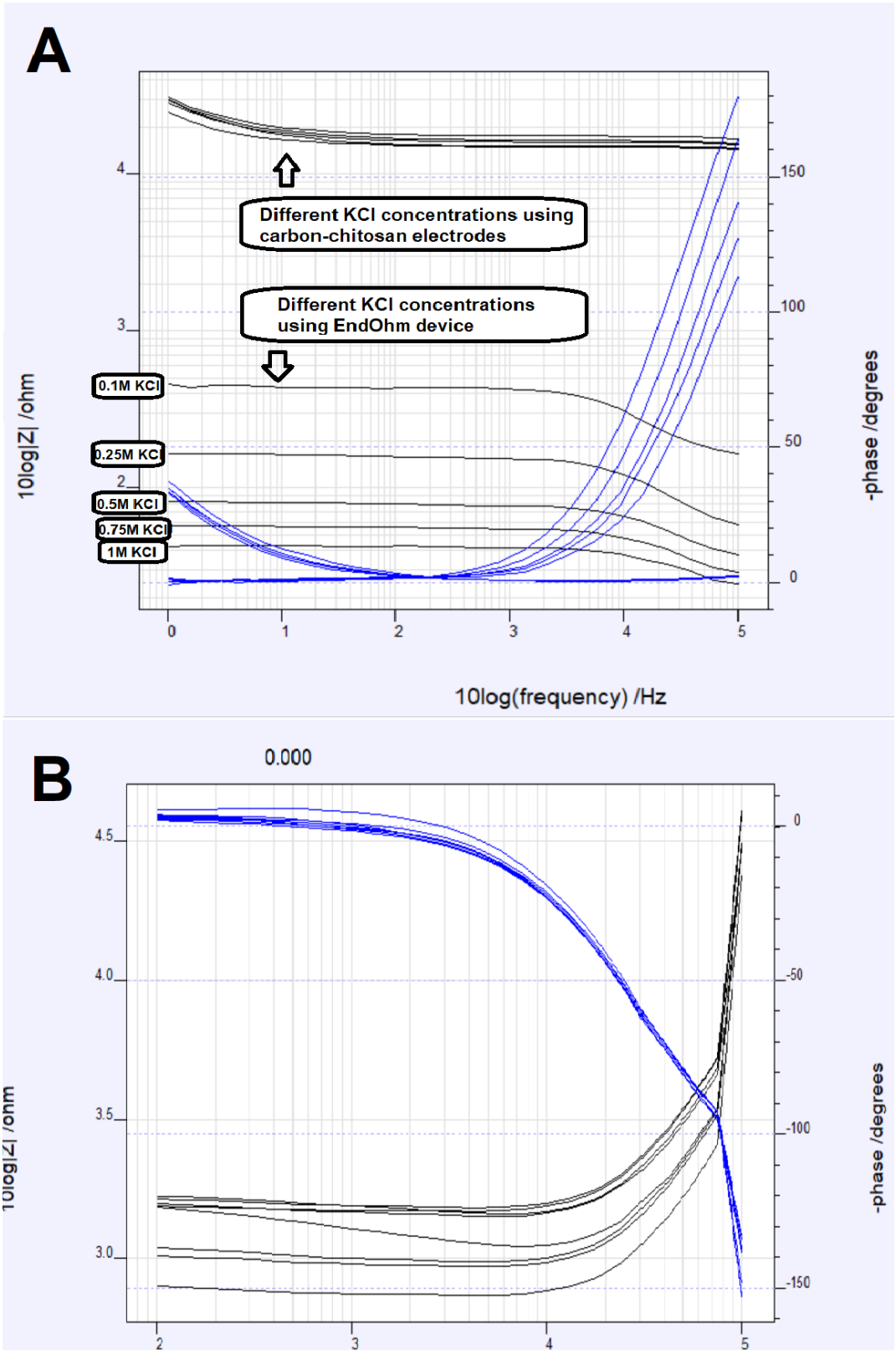

Figure 13. Electrical resistant measurement using different KCl concentrations. The samples were measured using EndOhm system, carbon-chitosan ink simulation (A) and carbon-chitosan microfluidic channel (B) .

This approach made possible to have a higher conductivity (figure 13B) as the electrodes were nearer to each other when compared with the previous simulation, but the problem of the precision and correlation with EndOhm remained unclear. This result may have an easy explanation: Carbon nanofibers show good conductivity due to the fact that they are in contact with each other, this makes it easy to obtain conductivity within a screen printed electrode but makes it very hard to obtain a result with two electrodes that are not in contact. This problem does not occur in metal electrodes as they are much more conductive and current can easily travel within an electrode and even through medium from one electrode to another without direct physical contact. Regarding this issue, next steps have to be directed to improve the conductivity of the system adding or changing materials to the ink. Regarding carbon nanofibers, their use can still be interesting even if another conductive material is added. While studying their mechanical stability (data not shown), It was discovered that the conductivity changed when folded. This fact can be explained by the separation of the carbon nanofibers when the membranes were folded. conductive properties instability when folded could seem at first sight as a disadvantage but It can be used as an interesting advantage as strain tensor. The differences in resistance given by the folding of carbon nanofiber electrodes can give information of the degree of pressure and folding in an origami organ chip system.

4.4 Cell culture experiments

The future obtainment of origami organ chip, makes the optimization of cell culture conditions for PT kidney cells over e-spun material and carbon-chitosan ink necessary. The first experiment was directed to ensure the biocompatibility of the e-spun material. For this purpose, a live-dead staining was used. Results ended up in a 2nd experiment to ensure the biocompatibility regarding the imaging of the cells and the cell monolayer formation. As there were not specific known differences between different e-spun (20:1m, 20:1h, 40:1m and 40:1h) membranes, all of them were subjected for these two first experiments. Posterior approaches (3rd experiment) were attempted to elucidate which was the best membrane to continue working with and so on reduce the number of samples necessary. Final (4th) experiment was performed in order to observe different parts of the cells into selected e-spun membrane to try to analyse the function and behaviour of cell within the e-spun membranes.

4.4.1 Cell viability on e-spun (1st experiment)

The first experiment regarding the use of cells was directed to ensure the biocompatibility of the e-spun membranes available. All membranes, including a PET membrane were coated with 50 µg/ml collagen IV and incubated for 3 days. Fig x shows the number of living cells in green and dead cells in red.

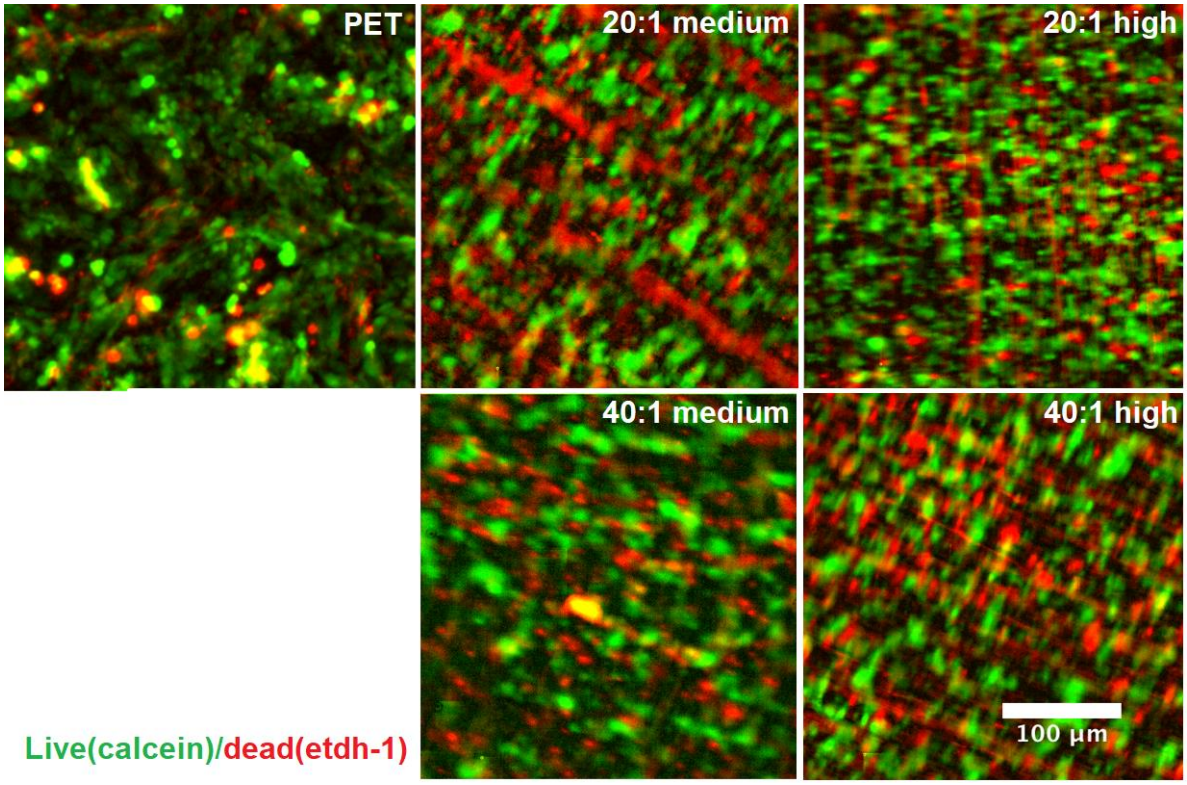

Figure 14. Live/dead staining over different PET and e-spun membranes. Cells seeded on PET membrane, clearly show the differences between alive and dead cells but int the rest of the images (e-spun membranes) the result is not clear.

Results (fig. 14) are clear on the PET membrane were cells can be clearly observed, but the case is different for the e-spun membranes. All e-spun membrane seem to show both live and dead cells but It also seems that ethd-1 is getting absorbed by the e-spun.

4.4.2 Cell viability (2nd experiment)

As the results obtained from the live-dead staining showed a good quantity of living cells, it was performed a second experiment to observe better the cell-cell contact interactions of PT cells over the different membranes. We performed Immunostaining for the zona occludens-1 (ZO-1) protein, which is found in the tight junction of epithelial cells, followed by a Hoechst staining. Support materials used were PET and different e-spun membranes (20:1m, 20:1h, 40:1m and 40:1h) again. Tight junctions also called occluding junctions are complexes of proteins that create intercellular boundaries between plasma membrane domains, and have

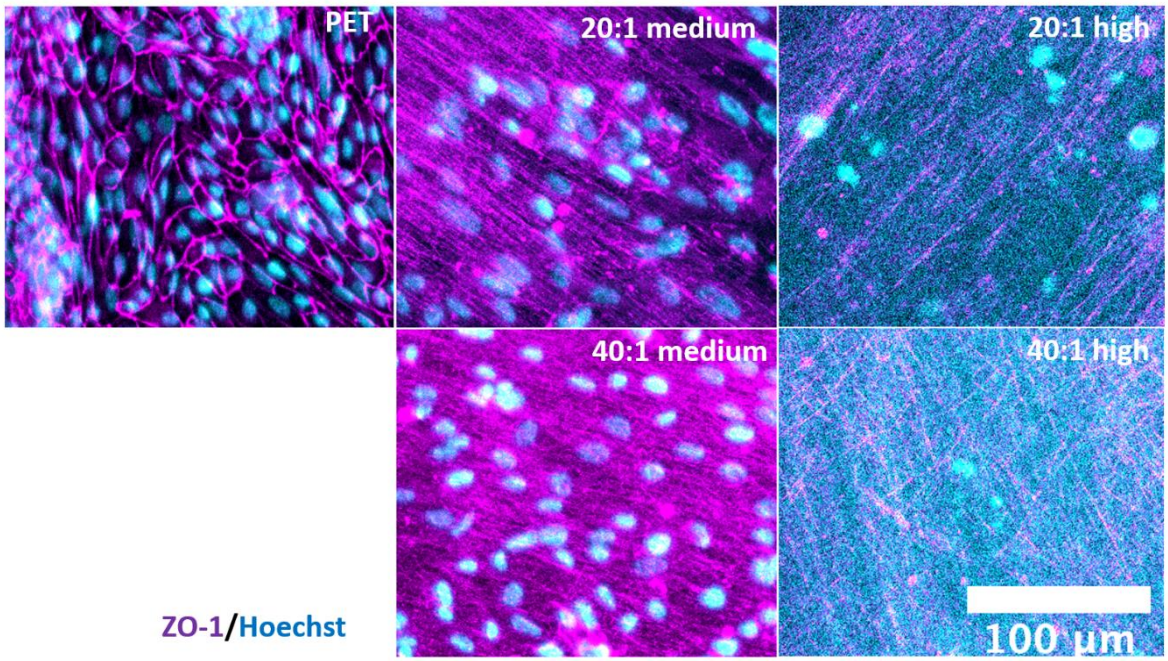

Figure 15. PT cells seeded on different materials and stained with Hoechst and immunostaining for ZO-1.

an essential role into barrier function, polarity and cell-cell contact. Several membrane proteins compose tight junctions, being the most commonly known the claudins and occludins. Further than the main protein structure of tight junctions, there are many other peripheral membrane proteins that are associated with these structure, being one of the most known is the ZO-1 protein [53]. The imaging of this protein using immunostaining will help to characterize possible formation of a monolayer of epithelial cells on different membranes.

The PET membrane (fig. 15) shows a clear monolayer of cells with a well define ZO-1 bright signal. Having a look at the e-spun membranes (fig. 15), the image is not that clear. Both 20:1m and 40:1m membranes have a clear presence of cells but there is a high background noise signal which avoid the actual visualization of tight junctions. In order to solve this issue, the imaging process was repeated using a 3rd channel to allow the extraction of the background signal from the actual signal given by the immunostaining. The fluorogenic dye used was Alexa-488, which has a peak of emission at 488 nm. The 3rd channel used for background signal was entitled to a slighter lower wavelength than 488 nm. 20:1h and 40:1h did not show a good number of cells over it, the reason was that due to the difficult handling of the e-spun, it was easy to break the membranes at any given moment.

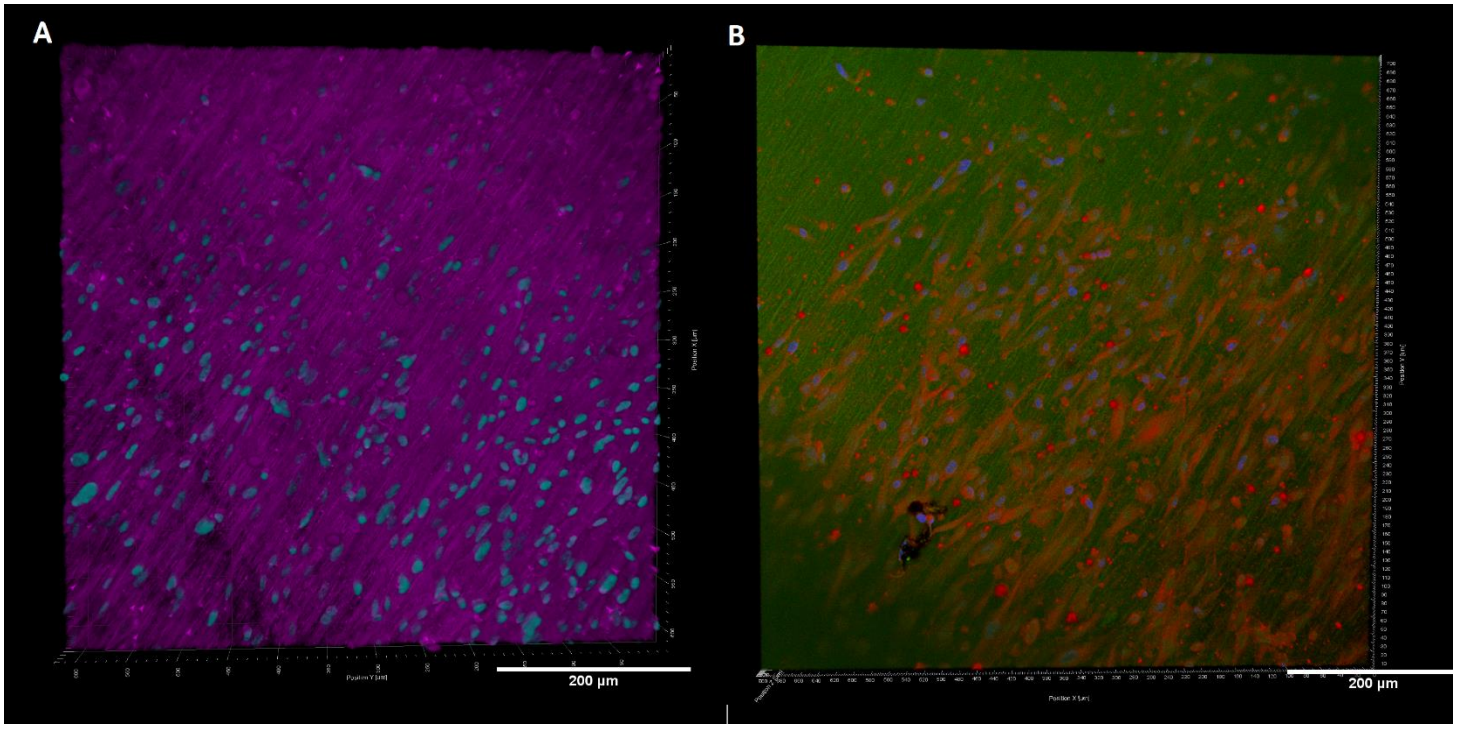

Figure 16. PT cells over 20:1M e-spun. Before background treatment (A) and after background extraction treatment using a third signal (B).

Background signal extraction resulted in a clearer image as illustrated in Figure 16. Before the image treatment, only the nucleus of the cells could be observed (fig. 16A), which also had to be overexposed in order to have a bright signal. After background extraction (fig. 16B), individual profile of the cells can be easily observed but opposite to the result obtained in the PET membrane, the staining was totally found on the cytoplasm of the cell. The fact that tight junctions cannot be observed means that the cell-cell interaction is not remarkable and so on there is no monolayer formation in the sample. The fact that tight junction cannot be observed even after background subtraction mean there is not a cell-cell interaction and so on there is not monolayer formation in the sample Furthermore, we found unspecific dye stains all over the samples. This can be explained due to the properties of the e-spun, which facilitates the absorption of substances thanks to its porous structure, giving a realistic explanation of what happened in the previous live/dead assays.

The results of the experiment showed us what we should expect from a staining of monolayer of PT cells and the possibility of seeding the cells on the e-spun. Furthermore, we noticed posteriorly that the collagen IV coating used for the transwells was less in the first experiment (50 µg/ml vs 100 µg/ml). This coating difference made us thought about the fact of collagen IV being a limiting factor for cell culture optimization as results were good enough in 2nd experiment but not in the 1st experiment (live-dead staining). Collagen IV concentration might be the second reason of the unclear results obtained in the first live/dead staining and in

other experiment within the same conditions performed by the research supervisor (data not shown).

4.4.3 E-spun selection (3rd experiment)

Once the questions about the biocompatibility of the membranes and the monolayer characterization were solved, it was decided to reduce the number of e-spun membranes used (20:1m, 40:1m, 20:1h and 40:1h) to concentrate the efforts in just one. Furthermore, it was necessary to investigate how time affected the cells in the e-spun, regarding monolayer formation. The experimental approach followed was the seeding of cells at same concentrations on the studied e-spun membranes with 4 different samples per membrane. One sample of each group of e-spun membranes would be fixed at a different time point (4 hours, 24 hours, 96 hours and 120 hours) in order to study how the cells evolved in time when seeded on the membranes.

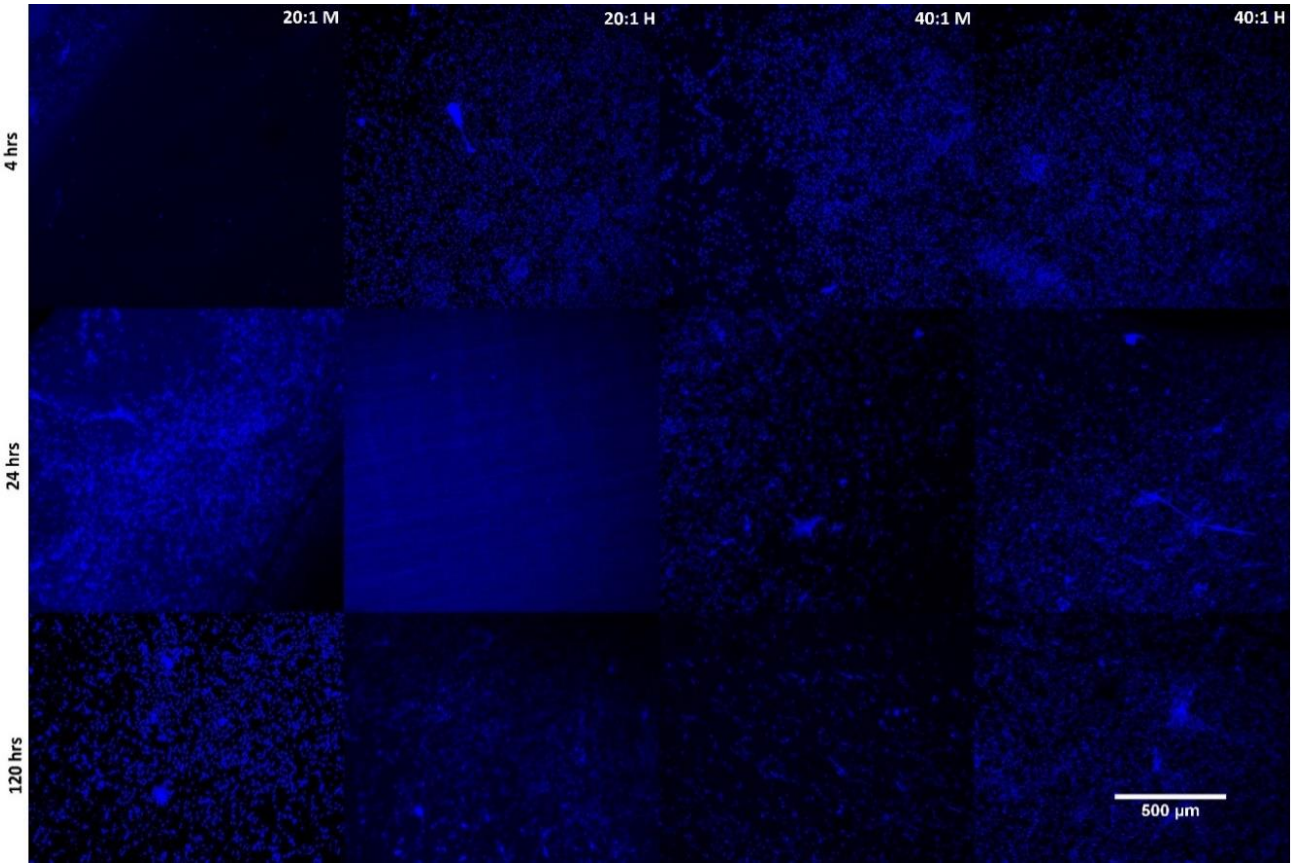

Figure 17. Hoechst staining of different membranes (20:1M, 20:1H, 40:1M, and 40:1H) at different time points: 4, 24 and 120 hours (hrs).

Results depicted in Figure 17, show a clear nucleus staining thanks to Hoechst but no signal at all from ZO-1 staining. This could be explained with problems with the ab, which was changed despite the good result obtained in the previously performed immunostaining.

Analysing the data obtained in figure 17, we can see that the quantity of cells in all membranes seems to be enough to consider that cells properly attach to the e-spun. Results obtained after 96 hours are not shown at 3 out of 4 samples were broken in the process due to the fragility of the e-spun. Only sample that seems to don't have cells is 20:1m after 24 hrs, and as the 96 hrs samples, this can be also justified by the fragility of the e-spun on transwells, which probably made us oversee a hole in the membrane that avoided a proper collagen coating or cell seeding as happened in previous experiment.

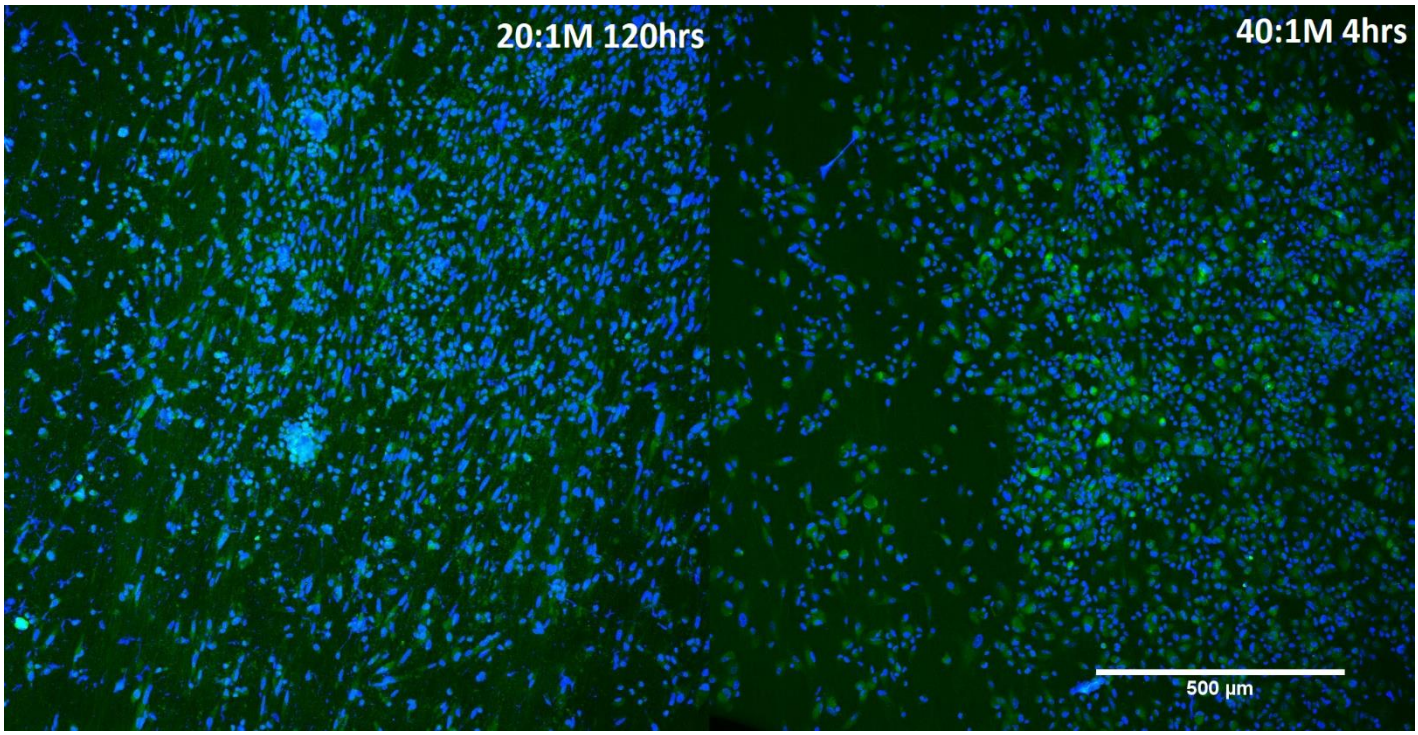

Figure 18. Different e-spun membranes (20:1M and 40:1M) at different time points (120hrs and 4 hrs respectively). Further than the Hoescht staining, a signal can be observed at 488nm. This signal does not come from the immunostaining against ZO-1 as this was performed with an Ab coupled to Alexa 647.

Interestingly, despite the unsuccessful immunostaining, we found a cell-shaped signal at 488 nm in several samples. As previous immunostaining had the issue of an overlap between the e-spun membrane signal and the ZO-1 immunostaining at 488 nm, the new immunostaining was performed using a primary Ab coupled to Alexa 647. This difference is enough to make us think that the green (488nm) signal depicted in figure 18 is given by the e-spun membrane and not the immunostaining. This cell-shaped green fluorescent signal could be given by a degradation of the e-spun by the cells. The possibility of degradation of the e-spun by the cells is supported by the fact that both chitosan and PCL are biodegradable polymers [31] [54] . The control and/or the knowledge of the degradation rate of the e-spun and the optimization of the conditions for the formation of a monolayer, could give us an important tool for the formation of a 3D origami organ on chip. Finally, the 20:1m membrane was selected, as standard for future experiments as it showed good results in all previously

assays performed. This decision was open to change the membrane in the future if necessary.

4.4.4 Collagen coating optimization (4th experiment)

Previous experiments lead to the obtainment of information about the biocompatibility, imaging optimization and possible endocytosis of e-spun. But the question about the collagen IV coating optimization was still open for clarification. As previously commented, various experiments were performed with a collagen IV concentration lower than 100 µg/ml and the results showed unclear data about the cell behaviour on the e-spun membrane. After

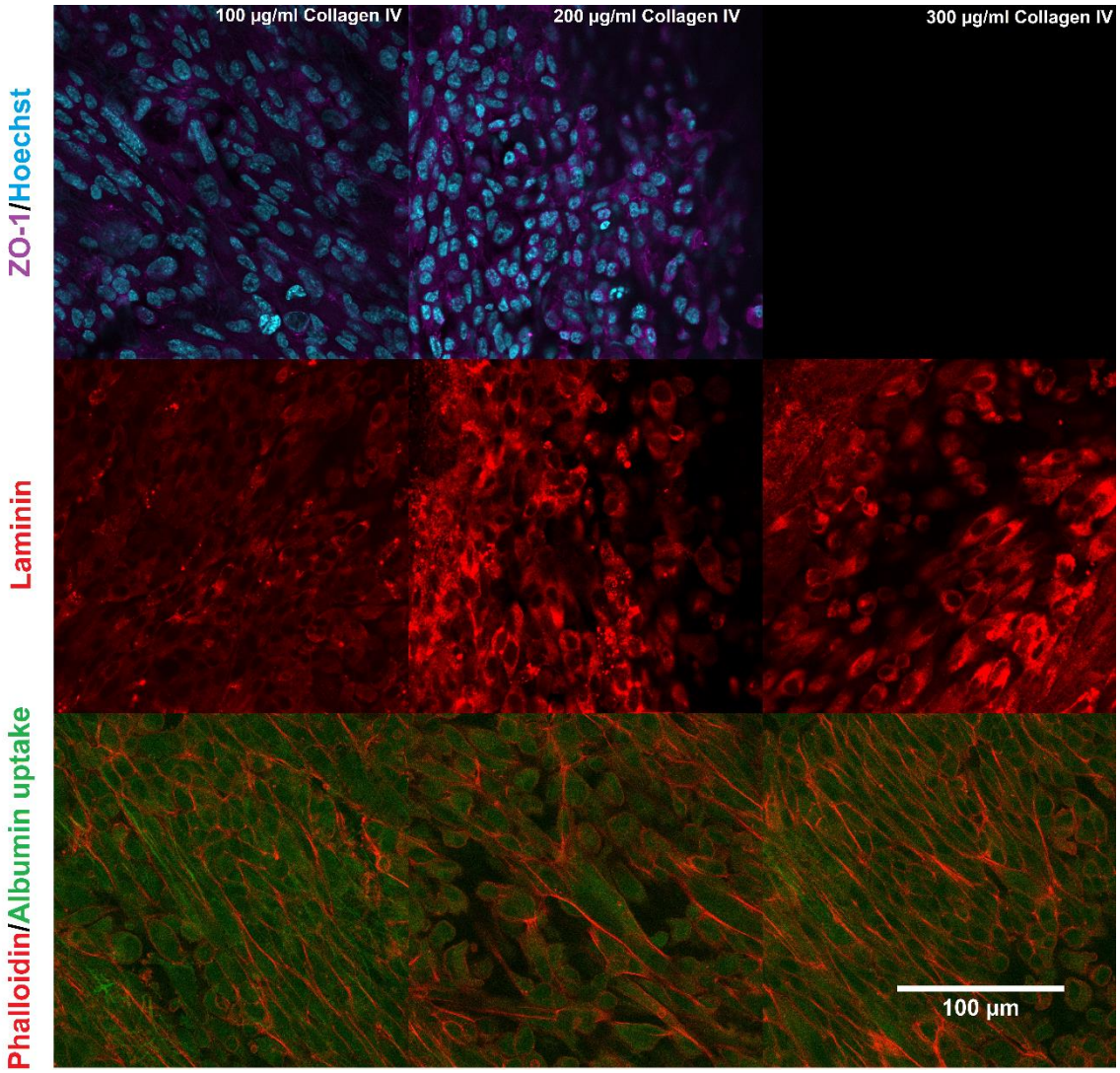

Figure 19. Collagen Iv Coating experiment. Different coating and stainings were applied over 20:1m e-spun membrane and incubated in order to study different characteristics an parameters.

selection of one membrane (20:1m), it was decided to study what is the collagen IV concentration necessary for a proper cell culture for PT cells on the studied membrane

(20:1m). Further than the study of collagen, this experiment also intended perform different staining of cells that could give feedback of how the cells are evolving in the e-spun.

I was decided to perform 3 different conditions and 3 different types of staining's as shown in figure 19. The coating concentrations selected were 100, 200 and 300 µg/ml of collagen IV. Staining selected were: Hoechst + ZO-1, laminin and phalloidin + albumin. As in previous experiments, the presence of ZO-1 presence will be useful to characterize the cell-cell interaction and monolayer formation. The monolayer formation over e-spun remained unclear through the 2nd experiment, so we decided to incubate the cells for several days more than before. Albumin uptake is an important factor regarding the proper activity of epithelial cells on glomerular filtration, so Its observation within the cells may indicate a proper functioning of the PT cells. Laminins are proteins synthesized by the epithelial cells in the basal lamina of the ECM. The basal lamina is a layer of the ECM which separates two sheet of cells as for e.g.: epithelial and endothelial cells. In the nephron, the basal lamina acts as a molecular filter for the pass of water and small molecules [55]. The presence of laminin in the studied culture, would help to identify different characteristics (as permeability if compared with other results) and give more cues about the proper function of the cells on the e-spun.

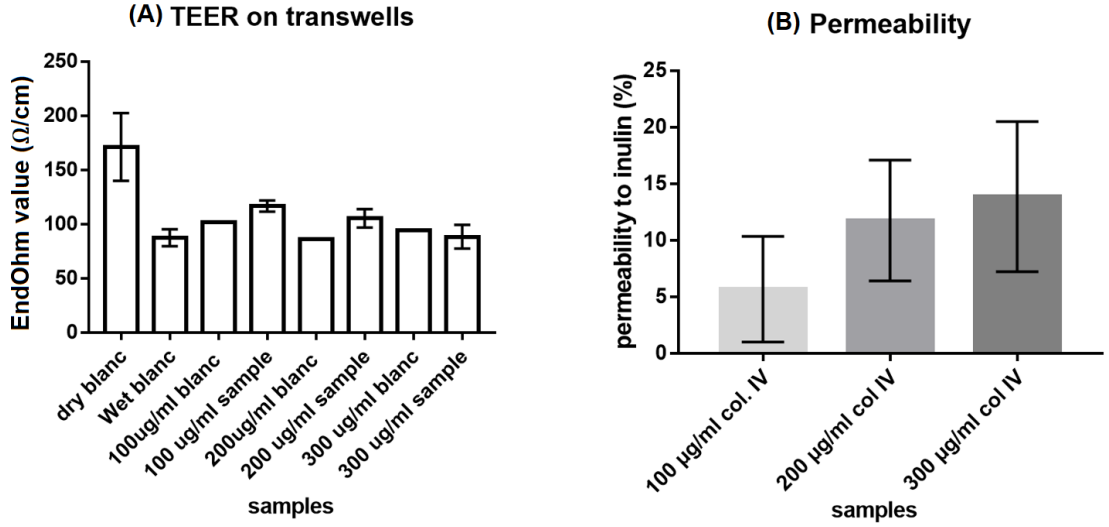

Figure 20. (A) TEER performed over transwell with and without cells in different conditions. (B) Inulin-FITC assay to study the permeability of cell-seeded membranes under different collagen IV coating conditions.

Further than imaging, TEER using EndOhm chamber and Inulin-FITC permeability assay were performed in an attempt of characterizing the formation of monolayer and permeability on different collagen coating conditions. These experiments were performed previous to fixation and staining of cells. Figure 20A shows the results given by TEER measurement. As

it can be observed there is a big difference if the e-spun scaffolds have previously been submerged in medium than if they were dry previous to TEER measurement. The changes found between the coated blanks and cell seeded samples is not remarkable and the error can be given. When attention is given to the permeability data (fig. 20B), it is possible observe that there is not a significant different from one collagen coating concentration to another. This result correlate with the good images obtained in all the samples of the experiment (Fig. 19). These results suggest that all concentrations of collagen over 100 µg/ml are enough for the formation of a cell monolayer and cellular activity.

5 Discussion

Organs on chip are still in a quite early development and there are many issues to be solved regarding a proper simulation of complete human organs in the microscale as the inclusion of microbiota and immune system on the chips. During this 6 months' research, the first steps were performed into the creation of a foldable kidney organ on chip which could recapitulate the physiological cues of the human kidney. The origami approach is a promising novel solution to preclinical screening of kidney treatments which might accelerate the development of pharmacological treatment for different diseases. The intricate structure of the organ, which made it impossible to develop a fully functioning prototype in only 6 months of research. But to our knowledge, this is the first research that uses the classic Origami technique approach to study the possible engineering of a human-kidney on a chip. This report does not directly address the design of a foldable membrane but puts attention to the sensing technology and biological cues. Membrane folding design was carried by supervisor in the group and highly supported by the representatives of the Cooper-Hewitt New York museum. This opens a great collaboration that might be interesting for both scientific and artistic worlds. The Wyss institute has already had art expositions in the modern art museum of New York and expect to increase it collaboration in future expositions of art.

It was shown that carbon-chitosan inks gave a relatively good level of conductivity and wettability resistance, but the inks were fabricated in a way that still show several issues regarding their use to measure the TEER for PT cell monolayer characterization. Here, the situation was left open to the inclusion of new materials to the fabricated inks in order to obtain a reliable measurement. Poly(3,4-ethylenedioxythiophene) also known as PEDOT is a conductive polymer with various chemical and biomedical applications due to their biocompatibility and conductivity. PEDOT can be doped with polystyrene sulfonate (PSS), another ionomer polymer, to increase its conductivity [56]. The use of PEDOT:PSS as an extra step in the engineered ink, could be helpful for increasing the electron transfer properties of the ink. In contrast, as previously commented in the results, the carbon nanofibers can still be interesting for the sensing electrode system of the organ on chip as the separation from one carbon nanofiber from another can be used as strain tensor to get feedback when pressure is applied over the foldable system. Another remarkable issue left to optimize within the sensing system was the electrode deposition. Screen-printing is a simple technique which is very useful for a quick homogenous deposition of ink over different materials, but it lacks of the precision which would be expected for a microengineering electrodes. Regarding these issues, two possible approaches were started to be studied: first, the use of a sputtering system that could deliver a thin controllable thin layer of material over an e-spun membrane without deforming or breaking it. Second, the use of 3D printing techniques or 2D robotic controlled deposition. This 2D/3D printing was the option most likely valued during the research as a conductive printable ink could be used in other interesting applications for the Wyss institute. Due to lack of time, we could not perform any experiment

that used this 3D printing for conductive ink deposition. This process would take time regarding the optimization of the rheology of the ink, which needs to be viscous enough to pass through a printing extruder without losing efficiency on electrode size deposition. This leaves the door open to the inclusion of other non-conductive materials that can substitute the chitosan as for instance: cellulose, Arabic gum, etc. Furthermore, other parameters as control of the mechanical stability of the e-spun needs to be optimized to avoid possible membrane disruption commented during different cell culture experiments.

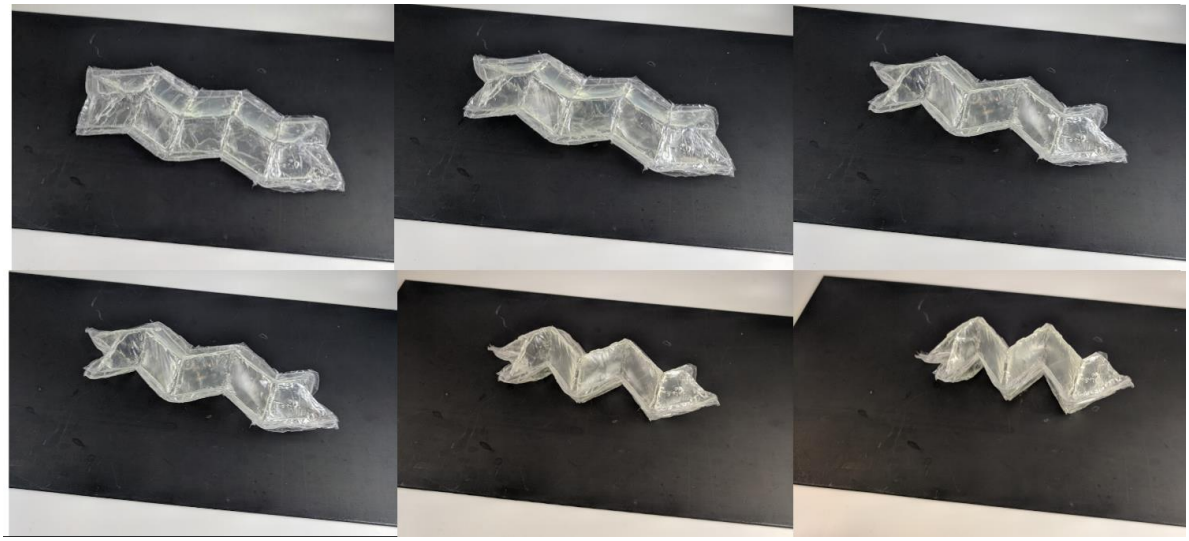

Figure 21. Examples of first prototypes of foldable membrane for origami organ on chip. Membrane used is PET as the material is quite resistant to temperature and easy to fill with gels and resins in order to oberserve mechanical folding.

Cell culture experiments were also helpful for optimization of future organ on chip conditions. We obtained information about the biocompatibility of the different e-spun membranes, and made a first comparison of how a proximal tubule cells monolayer should be. In the 3rd experiment, more than helping with the selection of the best membrane for our future experiments, gave us a new challenging but interesting question: are the cells degrading and phagocytising the e-spun? The 4th experiment was the most successful one in acquirement of data as it showed the wanted monolayer formation over the e-spun membrane due to the staining of ZO-1. The laminin presence within the culture did not show many clues about the possible differences between the different collagen IV coating concentrations, but its presence shows the possible formation of a basal lamina. The basal lamina could lead to future experiments related with changes in the permeability of the cell monolayer. Moreover, the 4th experiment ensures the functional activity of the cells due to the results obtained in the albumin uptake assay. In any case, despite of these interesting results, there were some experiments that were left to do: The measurement of a possible e-spun degradation, as results observed in 3rd experiment indicated that might be occurring, and the quantification of cytotoxicity of inks on PT cells. For both experiments, were tried to

be successfully performed on time, however due to a new pool of frozen primary there were several problems on these two last experiments, so results were not good enough to be analysed and studied. The fact that the e-spun might be degrading is supported by the individual biodegradability of both of its components [31] [54] and its measurement may be interesting in order to control the time needed and quality of cell monolayers formed on it. The characterization of this degradation phenomena could be measured using TEER and an Inulin-FITC assay as well as with 3D imaging using confocal microscopy images and treated using Imaris® software. The biocompatibility of the carbon nanofiber based scaffolds is highly bibliographically supported [25], [40]– [43] but it was necessary to obtain an experimental result on cytotoxicity to ensure the biocompatibility of our manufactured inks. The importance of the failure of this last experiment is minor if it is considered that there are still many changes left in the composition and characterisation of a carbon based conductive ink if it has to be adapted for new deposition methods.

Summing up the results this research, there has been a good advance in the creation of conductive inks that can be used over different materials and showed the monolayer formation and proper glomerular activity of PT cells over the studied e-spun membranes. The findings are still not in directly practical relevance as there still many steps have to be done in the creation of the organ chip system. The next step of this research is the confirmation of the questions left open during the cell culture experiments and the optimization of ink biocompatibility and rheology. Looking to a further future, it is still necessary to adapt the foldable membrane shown in figure 21 to cell culture conditions and be able to condense a high quantity of cellular material in both layers of the designed structure. Furthermore, several test and simulation will be necessary regarding the flow rate within the micro-organ system, to avoid the dead or destruction of cells or coating gel. Finally, but not less important, we have to put attention into the challenge of inclusion of more cell types into the Origami organ. All our cell culture experiments, have been performed with a single type of cell, ignoring the crosstalk between different types of cells within the kidney and the implication of this within the glomerular filtration. The following attempts will be directed to the addition of endothelial blood vessel cells on the other side of the membrane, increasing the complexity of the cell culture system. Moreover, the complete electrode integration for TEER will be quite relevant for the characterization of both cell types. Independent electrodes on the different parts of membranes can facilitate the independent characterization of all the types of cell added onto the different membrane compartments.

As the project is still in their first steps, the possibility of inclusion of new materials and new cells types is still open. Many challenges remain until the complete creation of a fully functional origami organ chip but the experiments performed in this research give the first clues of what is the path to follow. In addition to the kidney chip, the origami organ chip system may also open the door to its use in different project that could use one or more organ in the microscale. For instance: origami vascular system on a chip which could be

used to study the interface between the endothelial cells and different organs. This vascular organ could also be used for screening drugs in a relatively long folded vascular system, which could not be simulated using a simpler organ chip.

6 Bibliography

[1] "Food and Drug Administration," 2003. [Online]. Available: https://www.fda.gov/ForPatients/Approvals/default.htm.

[2] "Trial stages of clinical research." [Online]. Available: https://www.profil.com/knowledge-center/trial-stages.

[3] "European medicines agency." [Online]. Available: https://www.ema.europa.eu/human-regulatory/research-development/clinical-trials-human-medicines.

[4] "NIH clinical center." [Online]. Available: https://www.nih.gov/health-information/nih-clinical-research-trials-you/basics.

[5] A. Shafiee and A. Atala, "Tissue Engineering: Toward a New Era of Medicine," *Annu. Rev. Med.*, vol. 68, no. 1, pp. 29–40, 2017.

[6] F. Berthiaume, T. J. Maguire, and M. L. Yarmush, "Tissue Engineering and Regenerative Medicine: History, Progress, and Challenges," *Annu. Rev. Chem. Biomol. Eng.*, vol. 2, no. 1, pp. 403–430, 2011.

[7] S. Yamanaka, "Induced pluripotent stem cells: Past, present, and future," *Cell Stem Cell*, vol. 10, no. 6, pp. 678–684, 2012.

[8] B. Guo, B. Lei, P. Li, and P. X. Ma, "Functionalized scaffolds to enhance tissue regeneration," *Regen. Biomater.*, vol. 2, no. 1, pp. 47–57, 2015.

[9] S. Jana and A. Lerman, "Bioprinting a cardiac valve," *Biotechnol. Adv.*, vol. 33, no. 8, pp. 1503–1521, 2015.

[10] S. Waheed *et al.*, "3D printed microfluidic devices: enablers and barriers," *Lab Chip*, vol. 16, no. 11, pp. 1993–2013, 2016.

[11] J. U. Lind *et al.*, "Instrumented cardiac microphysiological devices via multimaterial three-dimensional printing," *Nat. Mater.*, vol. 16, no. 3, pp. 303–308, 2016.

[12] S. N. Bhatia and D. E. Ingber, "Microfluidic organs-on-chips," *Nat. Biotechnol.*, vol. 32, no. 8, pp. 760–772, 2014.

[13] D. Huh, B. D. Matthews, A. Mammoto, M. Montoya-Zavala, H. Y. Hsin, and D. E. Ingber, "Reconstituting Organ-Level Lung Functions on a Chip," *Science (80-.).*, vol. 328, no. 5986, pp. 1662–1668, 2010.

[14] E. W. Esch, A. Bahinski, and D. Huh, "Organs-on-chips at the frontiers of drug discovery," *Nat. Rev. Drug Discov.*, vol. 14, no. 4, pp. 248–260, 2015.

[15] N. S. Bhise *et al.*, "A liver-on-a-chip platform with bioprinted hepatic spheroids," *Biofabrication*, vol. 8, no. 1, p. 014101, 2016.

[16] S. Dauth *et al.*, "Neurons derived from different brain regions are inherently different in vitro: a novel multiregional brain-on-a-chip," *J. Neurophysiol.*, vol. 117, no. 3, pp. 1320–1341, 2017.

[17] Y. Torisawa *et al.*, "Bone marrow–on–a–chip replicates hematopoietic niche physiology in vitro," *Nat. Methods*, vol. 11, no. 6, pp. 663–669, 2014.

[18] S. Waheed *et al.*, "3D printed microfluidic devices: enablers and barriers," *Lab Chip*, vol. 16, no. 11, pp. 1993–2013, 2016.

[19] J. Rogal, C. Probst, and P. Loskill, "Integration concepts for multi-organ chips: how to maintain flexibility?!," *Futur. Sci. OA*, vol. 3, no. 2, p. FSO180, 2017.

[20] M. H. R. Wocjkej Pawlina, *Histology: A Text and Atlas*. .

[21] G. J. Tortora and B. Derrickson, *Principles of Anatomy & Physiology 14th Edition*. 2014.

[22] V. B. Kute, N. Prasad, P. R. Shah, and P. R. Modi, "Kidney exchange transplantation current status, an update and future perspectives," *World J. Transplant.*, vol. 8, no. 3, pp. 52–60, 2018.

[23] P. Mehrotra, "Biosensors and their applications - A review," *J. Oral Biol. Craniofacial Res.*, vol. 6, no. 2, pp. 153–159, 2016.

[24] L. Shuler and J. J. Hickman, “TEER measurement techniques for in vitro barrier model systems,” vol. 20, no. 2, pp. 107–126, 2016.
[25] S. Choi, H. Lee, R. Ghaffari, T. Hyeon, and D. H. Kim, “Recent Advances in Flexible and Stretchable Bio-Electronic Devices Integrated with Nanomaterials,” *Adv. Mater.*, vol. 28, no. 22, pp. 4203–4218, 2016.
[26] Z. Song *et al.*, “Kirigami-based stretchable lithium-ion batteries,” *Sci. Rep.*, vol. 5, no. 1, p. 10988, 2015.
[27] P. J. Mehner *et al.*, “Toward Engineering Biological Tissues by Directed Assembly and Origami Folding,” 2015.
[28] S. Kamrava, D. Mousanezhad, H. Ebrahimi, R. Ghosh, and A. Vaziri, “Origami-based cellular metamaterial with auxetic, bistable, and self-locking properties,” *Sci. Rep.*, vol. 7, p. 46046, 2017.
[29] A. G. Guex, G. Fortunato, D. Hegemann, H. T. Tevaearai, and M. N. Giraud, “General protocol for the culture of cells on plasma-coated electrospun scaffolds,” *Methods Mol. Biol.*, vol. 1058, pp. 119–131, 2013.
[30] O. Y. F. Henry, R. Villenave, M. J. Cronce, W. D. Leineweber, M. A. Benz, and D. E. Ingber, “Organs-on-chips with integrated electrodes for trans-epithelial electrical resistance (TEER) measurements of human epithelial barrier function,” *Lab Chip*, vol. 17, no. 13, pp. 2264–2271, 2017.
[31] S. Pokhrel, P. N. Yadav, and R. Adhikari, “Applications of Chitin and Chitosan in Industry and Medical Science : A Review Demineralised powder,” *Nepal J. Sci. Technol.*, vol. 16, no. 1, pp. 99–104, 2015.
[32] P. R. Bandaru, “Electrical Properties and Applications of Carbon Nanotube Structures,” *J. Nanosci. Nanotechnol.*, vol. 7, no. 4, pp. 1239–1267, 2007.
[33] N. Saifuddin, A. Z. Raziah, and A. R. Junizah, “Carbon nanotubes: A review on structure and their interaction with proteins,” *J. Chem.*, vol. 2013, 2013.
[34] A. M. Martins, G. Eng, G. Caridade, F. Mano, and R. L. Reis, “Electrically Conductive Chitosan/Carbon Sca ff olds for Cardiac Tissue Engineering,” 2014.
[35] “SOLIDWORKS TUTORIALS.” [Online]. Available: https://www.solidworks.com/sw/resources/solidworks-tutorials.htm.
[36] B. De Wever, S. Kurdykowski, and P. Descargues, “Human Skin Models for Research Applications in Pharmacology and Toxicology: Introducing NativeSkin ® , the ‘Missing Link’ Bridging Cell Culture and/or Reconstructed Skin Models and Human Clinical Testing,” *Appl. Vitr. Toxicol.*, vol. 1, no. 1, pp. 26–32, 2015.
[37] “Moldeo por inyección.” [Online]. Available: http://www.rose-plastic.es/22258.8.html.
[38] L. and medical supplies (LMS), “No Title.” [Online]. Available: https://www.lms.co.jp/products/docs/ENDOHM.pdf.
[39] N. Nakhoul and V. Batuman, “Role of proximal tubules in the pathogenesis of kidney disease,” *Exp. Model. Ren. Dis. Pathog. Diagnosis*, vol. 169, no. 1, pp. 37–50, 2011.
[40] “Direct vs indirect immunofluorescence.” [Online]. Available: https://www.abcam.com/secondary-antibodies/direct-vs-indirect-immunofluorescence.
[41] A. F. Excitation, “Hoechst Stains,” *J. Histochem. Cytochem.*, no. 3, pp. 9–12, 2005.
[42] Abcam, “Phalloidin staining protocol.” [Online]. Available: https://www.abcam.com/protocols/phalloidin-staining-protocol.
[43] J. A. Spudich, “Effects of Cytochalasin B on Actin Filaments,” *Cold Spring Harb. Symp. Quant. Biol.*, vol. 37, no. 0, pp. 585–593, 1973.
[44] L. Gianesello *et al.*, “Albumin uptake in human podocytes: A possible role for the cubilin-amnionless (CUBAM) complex,” *Sci. Rep.*, vol. 7, no. 1, pp. 1–10, 2017.
[45] Invitrogen Molecular Probes, “LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells,” *Prod. Information, Cat. number MP 03224*, pp. 1–7, 2005.
[46] A. N. N. V Rowlands, R. G. Eston, D. K. Ingledew, V. Ann, R. G. Eston, and K. David, “A simple, nonradioactive method for evaluating single-nephron filtration rate using

FITC-inulin," *Am. Physiol. Soc.*, pp. 1843–1849, 1999.
[47] T. Duff *et al.*, "Transepithelial resistance and inulin permeability as endpoints in in vitro nephrotoxicity testing," *ATLA Altern. to Lab. Anim.*, vol. 30, no. SUPPL. 2, pp. 53–59, 2002.
[48] "Best Cellular Assays." [Online]. Available: http://cellomaticsbio.com/molecule-testing.php.
[49] P. By, O. O. Omolade, S. Kanta, B. Plangklang, and W. Subsingha, "Implantable electrodes with carbon nanotube coatings," *Energy Procedia*, vol. 56, no. C, pp. 604–609, 2017.
[50] S. H. Lee, H. Y. Fang, and W. C. Chen, "Electrochemical characterization of screen-printed carbon electrodes by modification with chitosan oligomers," *Electroanalysis*, vol. 17, no. 23, pp. 2170–2174, 2005.
[51] E. Eksin and A. Erdem, "Chitosan-carbon Nanofiber Modified Single-use Graphite Electrodes Developed for Electrochemical Detection of DNA Hybridization Related to Hepatitis B Virus," *Electroanalysis*, vol. 28, no. 10, pp. 2514–2521, 2016.
[52] S. Cinti, V. Mazzaracchio, I. Cacciotti, D. Moscone, and F. Arduini, "Carbon black-modified electrodes screen-printed onto paper towel, waxed paper and parafilm m®," *Sensors (Switzerland)*, vol. 17, no. 10, pp. 1–12, 2017.
[53] C. Zihni, C. Mills, K. Matter, and M. S. Balda, "Tight junctions: From simple barriers to multifunctional molecular gates," *Nat. Rev. Mol. Cell Biol.*, vol. 17, no. 9, pp. 564–580, 2016.
[54] S. J. Garcı, J. C. Monllau, J. Suay, and M. Monleo, "Biodegradable PCL scaffolds with an interconnected spherical pore network for tissue engineering," pp. 17–22.
[55] V. Iorio, L. D. Troughton, and K. J. Hamill, "Laminins: Roles and Utility in Wound Repair," *Adv. Wound Care*, vol. 4, no. 4, pp. 250–263, 2015.
[56] J. Ouyang, "" Secondary doping "' methods to significantly enhance the conductivity of PEDOT : PSS for its application as transparent electrode of optoelectronic devices," *Displays*, no. September, pp. 1–14, 2013.