Master Thesis
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Design and production of cell-like droplets using microfluidic for investigate the cell’s rheology in biological application.
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1 Introduction

The principle of biomimetism is to copy the nature. Applied to microfluidic, it allows to manipulate small amount of biomedical sample in very realistic environment. It has become a useful tool for studying the living organisms and more precisely the human diseases. It also permits to extrude a problem from its natural medium, and simplify the experiment.

The work presented here is part of a more general project combining medicine and microfluidic. The subject is the Acute Respiratory Distress Syndrome (ARDS). It is a disease which takes place in the lung and leads to the patient death because of the malfunctioning lung.

The origin of ARDS are not precisely known. One hypothesis links the disease to the neutrophils, and particularly to their behaviour in the alveoli blood irrigation vessels. Neutrophils are believed to lose their visco-elastic properties which induce the mentioned malfunction of the lung.

In our work we have tried to create a model to compare the healthy and sick neutrophils. Using microfluidic systems we have produced cell-like droplets and subjected them to the same stresses that the neutrophils sustained in the lung.

This report presents our approach to this issue, from the study of the biomedical conditions, to the final experiments.
2 biology

Our work is driven by the high mortality rate in adult intensive care units due to inflammatory disorder in the lungs. The specific lung morphology and its evolutions are highly critical during inflammatory responses for the motion of white blood cells, more than for the red blood cells (RBC).

In this part we first explain what are the specificities of the lung and the normal pathway of neutrophils in it. Then we will focus on the mechanical process of neutrophils motion in the capillaries. In the end, we will shortly talk about the cell behaviour during an inflammatory disorder.

2.1 Neutrophils description and general behaviour

2.1.1 Neutrophils

Neutrophils are one kind of blood cells which derive in the blood cell lineage from myeloblast, (figure 1). They are the most abundant blood cells, approximatively 70% in number of the total white blood cells and are parts of the innate immune system. They have a diameter around 10 µm and the normal concentration of neutrophils in the blood is approximatively $5 \cdot 10^9 \text{L}^{-1}$.

They are one of the first responders in the acute phase of inflammation. They can express cytokines and therefore amplify the immune reaction, by attract other white blood cells. They can promote phagocytosis or produce granulocytes.

Figure 1: Lineage for all the blood cells
2.1.2 Neutrophils deformation

All the cells have the ability to deform in response to a normal stress. The normal deformation of neutrophils have been studied in regards to lung syndrome, [Gabriele et al., 2009] and [Yap and Kamm, 2005]. These two papers point out the main role of cell structure during a deformation. The common value for the Young’s modulus of a neutrophil is 1kPa.

The cell is structured by a cytoskeleton made of actin filaments, and the surface is rigidified by myosin. During a deformation, the actin filament network can destruct itself, by unbinding the link between two actin filaments or depolymerising the filaments. This leads to a decrease of the Young’s modulus of the cell and this allows the cells to enter small channels or vessels. The studies
of [Gabriele et al., 2009], [Walter et al., 2011] and [B. and Khismatullin, 2009] has shown that the actin structure can unfold itself in response to the applied deformation but then reforms itself once the neutrophils has entered the vessel. Depending on the time the cell passes in the small vessel, the cytoskeleton can be rebuilt to fit the new shape of the cell. This reconstruction is responsible for the lookalike viscosity of the cell once it comes out of the small vessel. It takes again a certain time before the cell goes back to its initial shape, because once again the cell need to break its skeleton and rebuilt it.

2.2 Gaz exchange in the lung

![Figure 4: Lung Structure](image)

The lung is a complex organ where the exchange between carbon dioxide and oxygen happens. The figure 4a presents a major view of a lung showing the air channel from the trachea, to the alveoli. The same type of subdivision takes place in the blood vessel to lead the cell from the heart to the alveoli. The blood pathway around the alveoli is shown on the figure 4b. On its way from the pulmonary artery to the pulmonary vein, the blood pass near the alveoli in the capillary segments. Between one arteriole and the venule, one
blood cell goes through 40 to 100 capillary segments, and by so crosses 8 to 17 alveolar walls, [Hogg, 1987] and [Hogg and Doerschuk, 1995]. The table 1 summarizes the typical size and pathway for a neutrophil in the pulmonary bed [Doerschuk, 2001].

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Neutrophil diameters</td>
<td>6 – 8µm</td>
</tr>
<tr>
<td>Capillary segment diameters</td>
<td>2 – 15µm</td>
</tr>
<tr>
<td>Number of capillary segments in a pathway</td>
<td>40-100</td>
</tr>
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Table 1: The basic structure of pulmonary bed

By separating the blood vessel from the air with a thin membrane, the erythrocyte can capture the oxygen from the air and let the carbon dioxide and oxygen diffuse through the membrane, as seen on the figure 4c. In the figure 4c one can see that the blood capillary segment along the alveoli is approximately of the size of one blood cell, especially one neutrophils.

2.3 Acute Lung Injury

2.3.1 Inflammation

Inflammation belongs to the biological response of the immune system to any wound or pathogen in the body. It is an innate response which tries to repulse the cause of the immune response. There are two kinds of inflammation, acute or chronic. In this work we will discuss the acute one, this is to say the primary stage of the disease.

The different steps of acute inflammation are the normal immune response from the body:

- The process is initiated by cells present everywhere in the body, as they recognise the pathogen, they release agents that trigger the immune response.
- The inflammatory mediator engages the vasodilation and permits the recruitment of leukocytes, mainly neutrophils in the first moment.
- The increased permeability of the blood vessel wall results in the swelling of the surrounding tissues through the leakage of plasma proteins.
- The neutrophils are responsible for the swelling, but also mediate the recruiting of other leukocytes.
- The neutrophils are also able to pass the blood vessels wall and enter the damaged tissue.
- There, they can remove pathogens through phagocytose and degranulation.
The pain is a result from the swelling, the fact that the tissue grows and presses the surrounding nerves which are also more sensitive to pain due to some released mediators.

One major characteristic of the inflammation is that the inflammatory mediator have short half lives times. To maintain the immune response they have to be renewed frequently. It is also the reason why the inflammation stops once the stimulus is removed.

2.3.2 ARDS

ARDS description  The simpler way to describe the ARDS is to give the medical definition of this disease which falls into the following criteria:

- Acute onset,
- Pulmonary-artery blood pressure $\leq 18\text{mmHg}$,
- Acute lung injury for $\frac{PaO_2}{FiO_2} \leq 300$,
- ARDS if $\frac{PaO_2}{FiO_2} \leq 200$.

The Acute onset means that there is a rapid apparition of the symptoms. The pulmonary artery is the artery coming from the heart to the lung, and bringing the deoxygenated blood to the lung. The $PaO_2$ is the arterial oxygen partial pressure, a low $PaO_2$ is a sign that the patient is not oxygenating properly. The $FiO_2$ is the fraction of inspired oxygen. Their ratio indicates the quantity of oxygen that goes through the lung to the blood.

Physiologically speaking, ARDS is characterized by the inflammation of the lung bed that prevents the oxygen to go in the blood. The major causes of ARDS are Sepsis syndrome (disease where all the body is in inflammatory state) and severe multiple trauma.
Neutrophils role in ARDS  The figure 5 shows the process of inflammation in a Acute Lung Syndrome. As in any inflammatory response, the neutrophils pass the blood vessel wall and go into the interstitial tissue. They move along into the alveoli air space which is full of fluid. The neutrophils can then begin to fight the cause of inflammation. That normal process can not happen in acute respiratory distress syndrome because the neutrophils can not move along the capillary blood vessel.

We have said that along their pathway in the capillary blood vessel around the alveoli, most of the time neutrophils have to deform themselves to pass these small vessels. One fact, and maybe a cause of, in ARDS, is that the neutrophils ”lose” their ability to deform as much as normally. Therefore they are not able to travel any more in the alveoli vessels. The inflammatory response that begins with the action of neutrophils can not occur in that case. The trigger is not taken over by neutrophils and passed through other leukocytes. Thus, the inflammation cannot be treated by the immune system, the lung will have to partially shut down.
3 Microfluidic

As we said earlier, our aim is to produce droplets of the size of the observed cells, in our case neutrophils. Therefore we need to produce these droplets with a stable system which can provide us with highly monodisperse sample.

In this part, we describe the chosen microsystem and its fabrication.

3.1 Microsystem production

In our work and generally in microfluidic, we use small amounts of fluids and therefore very small structures. As our aim was to create a mimic of a neutrophils, we needed to manipulate fluids and create droplets approximatively of the size of one neutrophil, so below 10\(\mu\)m. To produce these systems the simpler and most common way is to use soft lithography. The main step is to make a negative mould of our system on a silicon wafer, and use it to mould our system with PDMS (Polydimethylsiloxane). The major benefit of this technique is high reproducibility. Once the mould is made, it is possible to produce similar PDMS-systems as far many as needed.

3.1.1 Lithography

The general lithography process consist of making 3D structure from a silicon wafer. One can either carve the surface and obtain structures in well. Or one can form on top of the silicon surface structure with photo-sensible resin. This last process is call Soft Lithography and it is the one we use to make our systems.

![Multi-step of Soft-lithography using a negative resist](image)

Figure 6: Multi-step of Soft-lithography using a negative resist

In the figure 6 one can see the different steps from a flat wafer of silicon to the mould with the 3D structure on it. The principle is to solidify only some parts of the photo-resist by illuminating it with a specific light. First we spin-coat the wafer uniformly with the photo-resist, figure 6b. The height of the layer we
deposit is directly dependent on the rotation speed and the resin viscosity. As the height of our future channel is equal to the height of this resin layer, the choice of rotation speed and the resin is rather critical for the process. The next step is to illuminate our resin. For that, we use masks which are represented on figure 7. There are two types of resins one can use, positive and negative. The positive one becomes soluble when illuminated, while the negative one gets insoluble to the developer. Here we use the negative one because it allows higher aspect-ratio for our system. A quasi-monochromatic light ($\lambda = 365\text{nm}$) passes through the pattern of our mask and illuminates the resin in accordance with the design of the mask, figure 6c and 6d.

(a) Mask use for the example of figure 6 (b) Mask of a flow-focusing system

Figure 7: Examples of masks for the soft-lithography

Once the photo-resist exposed, the wafer is developed in order to remove the unexposed resin and get the structure that fits our mask, figure 6e.

The figure 8 shows the result of the soft-lithography for a flow-focusing system. Here the height of the structure is 18$\mu$m.

Figure 8: Profile image of a flow-focusing device realized with an optical Profilometer. The system is 18$\mu$m high. The constriction is 15$\mu$m wide.
3.1.2 PDMS moulding

We look at our system with a microscope, so we need to make transparent systems. To achieve that, we stick together a slide of glass and a piece of polydimethylsiloxane (PDMS), an elastomer in which we mould our system.

To mould the PDMS we use our wafer with the resin structure on it. We mix PDMS with the cross-linker, here in a 1 : 10 mass ratio, pour the mixture on the wafer, (figure 9a), and wait for the complete reaction and solidification of PDMS, (figure 9b). This reaction occurs naturally at room temperature but we can accelerate the process by baking our mould at 70°C. Then the PDMS is easily removed from the wafer, leaving the structures on it so we can re-use it later, (figure 9c). To stick the PDMS on the glass slide, we place them in an oxygen plasma chamber that activates the two surfaces. Surface activation exposes silanol groups (RSI-OH) at the surface of the PDMS layers that when brought together form covalent siloxane bonds. The activated surfaces when put together bind to each other and seal the system by forming covalent bonds, figure 9d and figure 11. We have represented one entry for the connection.

![Figure 9: PDMS moulding steps](image)

(a) Pouring PDMS on the wafer  
(b) Solidification of the PDMS
(c) PDMS system removed from the wafer  
(d) Binding of the PDMS stamp on a glass slide

The figure 10 represents the same system of flow-focusing that in figure 8 but moulded into the PDMS and bound to a glass slide. We have filled it with colored water to bring out the channels.
In order to have one system with the same surface properties on each wall, we coated PDMS on the glass slide before binding it with the PDMS system. This way, all the walls, ground and ceiling of our channel are made from the same material, which will reduce the wetting problem during our experiments.

The last step of fabrication is to post-bake our system once it is bound to the glass slide. Indeed, the plasma binding activates the surfaces by creating free oxygen bonds. When the two surfaces are put in contact, the bonds link and create strong bonding, but the free bonds that remain in the channel are

Figure 11: Oxygen plasma process
leaving free. This leads to a highly hydrophilic surfaces and channel. As we want to create droplets of water in oil, our continuous phase is oil and therefore we need hydrophobic channel. Thus we have to post-bake at 90°C our system after the plasma bonding during a few hours. This eliminates the oxygen free bonds in the channels and makes them hydrophobic.

3.2 Flow-focusing

Producing mono-disperse droplets of one fluid into another immiscible fluid has been a challenge for several years. As the production of droplets is a key process in many industries (food, cosmetic), producing highly mono-disperse droplets at high speed is very critical. The most common way is to make an emulsion of one fluid into the other, but that way it is difficult to control very precisely the size and the homogeneity of the produced droplets. The other process that appeared a few years ago, is to use microfluidic. There are few different systems that can be adopted - T-junction, co-flowing streams and flow-focusing. In our work we have adopted the flow-focusing device for two reasons, the rate of production is higher and this system is well documented.

3.2.1 Physical aspect

The principle of this system have been described by [Anna et al., 2003] and [Dreyfus et al., 2003] based on the early work of [Gañán Calvo, 1998]. As one can see in the figure 12, the idea is to squeeze one fluid with a second immiscible fluid, and forcing them to pass through a narrow constriction. The second fluid will force the drops to detach. We clearly see what are the main variables in this system: firstly the flow rate of the two fluids, and secondly the size of the constriction.

![Flow-focusing principle](image)

Figure 12: Flow-focusing principle

To control the flow rate of the two fluids in the inlets, two options are possible. We can either control directly the flow-rate by using syringe pumps or control the pressure inside the fluids with a pressure source. [Ward et al., 2005] have studied the differences between this two ways of control and showed that with pressure control we can vary much faster the size of the droplets. This argument is very important, because we needed to vary the size of our droplets
as a function of the gel composition, it appears simpler not to change the design of our system for each experiment. It is also said that while controlling the fluids pressure, the breakup of the droplet is more due to surface forces rather than viscous forces. In our experiment, we have changed the composition of one of the two fluids, and perhaps its viscosity. In order to be in the same regime, controlling the breakup by the surface forces is a better option.

![Image of droplets production with a flow-focusing device]

**Figure 13:** Droplets production with a flow-focusing device. Here the constriction is 25µm wide, and the formed droplets have a diameter of 25µm.

### 3.2.2 Practical adjustment

**Scaling down the drops** One aspect of the flow-focusing is that the size of the droplets we can produce with a certain microsystem is strongly dependent of the constriction size. More precisely the common rules specify that the minimum size of the droplet we can achieve is similar to the width of the constriction, in the case of a channel with a square section. In our case we have achieved the production of smaller droplets by using high pressure for the inlet. A much simpler way of scaling down the droplets is to use smaller systems. As we are limited in width by the precision of our process (photolithography and mask resolution, 10µm), it is simpler to limit the height of our channel. This can be simply changed at the beginning of the system production process, (figure 6b). By changing the photo-resist used and the rotation speed for the coating, it is very easy to vary the thickness of the resin layer deposited on the wafer. So it is simpler and cheaper to achieve smaller channel than thinner channel. One can control the drops size by the constriction channel and easily go down to size drops of approximately 1µm.

Even if this is quite smaller than the size of the neutrophils we aim at, it is critical to produce this size of drops because the hydrogel can swell in the water. Depending on the initial concentration of reactant in our mix, the hydrogel will polymerise in a non-equilibrium state. This state is characterised by a need or excess of water inside the gel. By putting the gel freely (that means without any surfactant) into the water, it can take its equilibrium state by absorbing or releasing water. The swelling behaviour is observed in the situation of small concentration of polymer in the gel, and this will be our working conditions as
we will see later. So making only drops of 10µm is not appropriate, we need to go smaller to counter the swelling effect.

Making a 3D structure  We have already said that we achieve to get smaller drops by scaling down the height of our system rather than the width of the constriction. In the case of the lengthened system, reducing the height of the all the systems induces very high hydrodynamic resistance and therefore it requires more pressure to move the fluids. We solve this by elevating the different parts of our system at different heights. The constriction part is at the required height and the rest of the system is made bigger to reduce the resistance and slowing down the flow. The figure 15 shows a scan image of the wafer mould with this type of system printed on it. One can see the constriction part which measure only 20µm locally and the other parts measures 120µm.

![Figure 14](image.png)

Figure 14: Profile image of a flow-focusing image with a step after the constriction. Here the height is 20µm at the constriction and 120µm after the step.

Increasing the channel length  In order to increase the time the drops spend in our system, we have decided to increase the length of our outlet channel. The solution we choose is quite simple, we made a long channel with many turns. The schema of the mask we used can be seen on the figure 15.

![Figure 15](image.png)
Prevent the obstruction of the constriction  Scaling down one part of
our channel leads to the filling up of this part by some impurities coming from
the plugging or elsewhere. One way of stopping that is to add filters in our
system, an example is given in figure 16. We duplicated them and linked on a
long distance with channels smaller than the constriction. This means that the
dust that can pass through the filters can not block the constriction.
4 Biomimetic object

This project is about creating biomimetic objects and compare them with the behaviour of real neutrophils. We have presented the tool and system we have used to produce these objects. Now we are going to explain what material we use to produce them and how we actually make them.

4.1 Hydrogel

The biological experiment we want to copy imply to follow some specifications:

- The neutrophils are observed in an aqueous solution
- Our objects must have the same mechanical properties
- The objects properties cannot depend on other variables (temperature, surfactant)

For these reasons, we have chosen to use hydrogel, and more specifically hydrogel based on acrylamide. Those gels, poly-acrylamide (PAM) or poly-dimethyl-acrylamide (PDMA), are often used in biology in the DNA analysis, they are well-known and well defined. We also use Polyvinyl Acrylate (PVA), which, in the contrary of acrylamide gel, is a physical gel. The bonds in a physical gel are weaker bond, usually hydrogel bonds. This type of bonding are more fragile and are less resistant to physical deformations.

4.1.1 Acrylamide gel

Gel production To make an acrylamide hydrogel, three basic components plus one actuator are needed. The three components are:

1. Monomer, either acrylamide (AM) or N,N-dimethylacrylamide (DMA)
2. Cross-linker, methylene-bis-acrylamide (MBA)
3. Initiator, Ammonium persulfate (APS) or Potassium persulfate (KPS)

The actuator is either chemical or physical. We have used temperature and N,N,N’,N’-tetramethylenediamine (TEMED). In the first case, once the mix of components is made, putting the sample at 70°C for 4 hours initiates and
completes the reaction. Another possibility is to add TEMED in your solution and the reaction begins instantaneously.

The difference between the two gels is that the reaction for PAM is much faster than for PDMA when it is initiated with the TEMED. It means that once the TEMED is added, the reaction ends within a couple of minutes for the PAM whereas it takes more like an hour for the PDMA. This will have some importance in our droplets production.

Figure 18: Perfect hypothetical hydrogel network.

The result of the reaction is a network of polymer, as shown on figure 18. At each cross there is a cross-linker molecule. This representation of the network is idealistic. There is no reason for the polymer not to do a loop or for a cross point to gather more than four branches of polymer, or simply that for polymer branches to have the same length. But for the simplicity of the problem, we will consider that this network representation is correct on average.

In the figure 18, two variables can be stressed out. First the density of the network, and second the mean distance between two cross-points. These two variables are used to name the different gels we are making. If we name $A$ the concentration of monomer in the gel and $B$ the concentration ratio between cross-linker and monomer, one can say that $A$ is related to the density of the network and $B$ to the length between two cross-points. The bigger $A$ is, the more dense the gel is at the synthesis, and the bigger $B$ is the shorter the polymer chain will be. Therefore we name our gel $A \times B$.

4.1.2 PVA

We have briefly described the main difference between acrylamide gel and PVA. The fact that physical gels are more fragile is the reason why we have chosen to work with chemical gel. Our experiment consist to force the droplets to deform themselves largely. We also impose a cycle between their deformed state and the released one.

But at some point we observed that our gels were very elastic and not so viscous. We thought of different ways to increase the viscosity of our gel and we came up with two linked ideas.

First we thought of adding long polymer chain inside our gel. It is commonly known that longs chain, as PEO, increase visco-elastic properties of a
Fluid. The downside of this technique, is that if we add PEO in our water and push it into our microsystems, the viscosity prevents us forming droplets. It is more difficult to break the water flow with the oil if the water is too viscous [Arratia et al., 2008].

The second idea was to add another polymer network in our hydrogel, i.e. to superimpose one gel to the acrylamide one. To do so, we thought of using PVA, which is a physical gel.

**Gel production** The PVA is made of long chains of polymer, linked together by hydrogel bonds. To make the gel in bulk, one just need to follow three steps:

- Dissolve PVA into the water, this must be done at 90°C because the PVA is not soluble at room temperature.
- Freeze the sample at −20°C.
- Heat the sample at 30°C.

The freezing/ heating cycle can be done more than one time, until 60 times. Its role is to homogenize the gel. After the first freezing, some bond are created but not uniformly, by heating the sample the gel become more homogeneous and one can re-freeze it.

**4.1.3 Gel characteristic**

Our aim is to compare in the same experiment, the neutrophils and our cell-like hydrogel. This is done to compare the neutrophils behaviour with something well-know. So we need to understand and characterise our hydrogel droplets.

**Bulk measurement** At this point of our work we wanted to know the characteristic of the hydrogel we were using, and especially at the different concentration we made them. So we made bulk measurements to confirm the data we found in the literature and on which we based our work.

**Droplets measurements** We also tried to know the rheology of our droplets of hydrogel. This was done in collaboration with Olivier Theodoly from INSERM in Marseille and Atef Asnacios from Université Paris-Diderot in Paris. They have developed experiments for measuring the visco-elastic properties of living cells.

The first one consists at looking at the motion of a cell between two different deformed states. The figure 19a shows the microsystem used. The cell is pushed into a narrow channel that is smaller than the cell. At some distance in this channel there is a step, the height of the channel passes from 8µm to 4µm. At a fixed pressure, the cell passes this step and by studying the friction between the cell and the wall one can extract the Young’s modulus and the viscosity of the cell.
The second is a reproduction at the microscale of the bulk experiment of rheology. It means that the cells are placed between two plates, we imposed a fixed deformation to them and we measure the force applied by the cells on the plates. The figure 19b shows the experiment setting.

![Diagram](image)

(a) Side view of the microsystem used by Olivier Théodoly. The height of the channel is 8µm on the left and 4µm on the right. The width is 8µm. The three cells show the state before, during and after the step.

(b) Side view of the two micro plates used by Atef Asemanios. During the experiment the top plates is held still while the distance $D$ is varied with the bottom plate.

Figure 19: The two set-up used to characterise living cell and hydrogel droplets

**Swelling of hydrogel** The critical property of hydrogel in our experiment was the possible swelling or shrinking of the drops once they are put back in the water. This effect is described in [Sudre, 2011] and [Hourdet,]. In an equilibrium state, a hydrogel is subjected to two opposite forces. First it naturally swells, and secondly the elastic chains resist. To compare with the cell, we can say that the osmotic pressure inside and outside the drop are equal at the equilibrium state. But at the synthesis this equilibrium is not reached and the osmotic pressure will force the gel to swell or shrink.
We can take an example to compare the different behaviours. Let say that we have formed several droplets at the same initial volume.

First, we can look at two different drops with the same amount of monomer but different cross-linker concentrations. In the established notation, we can call the two gels $A_1 \times B_1$ and $A_2 \times B_2$, with $A_1 = A_2$ and $B_1 > B_2$. The last relation means that the first gel has shorter chains. When put into water, the second gel will tend to deploy its network and chains and therefore swell. The first one will prefer to expel water and shrink.

We can also compare two gels with different monomer concentrations, as $A_1 > A_2$ for the same cross-linker concentration. It is easy to understand that the first one will swell much more than the second one because at the synthesis it is denser. In the end the two networks will look the same but the first droplets will be bigger.

In our case, we produce very soft drops, so the concentration of monomer and cross-linker are very low. So our gels are going to swell enormously, as shown on the figure 20.

### 4.2 Droplets formation

We have described the two hydrogels we have used, their production and bulk characteristics. We are now going to describe precisely the formation of gel droplets. We have used two ways for the acrylamide gel, and we will discuss the issue of droplets of PVA.

#### 4.2.1 Diffusion

The first idea that comes to mind if we want to be able to manipulate the fluid before the reaction, and initiate the polymerisation once the droplets are formed, is to put the initiator in the oil phase. By doing so, our two fluids,
Oil + TEMED and Water + Monomer + MBA + APS, can be manipulate and do not react until they are put in contact. That means that the reaction begins at the constriction in the flow-focusing device. Because the fluid speed is quite high, approximately 10mm s$^{-1}$ in the constriction, one can say that the reaction begins after the drops are formed. As a consequence, the gel produced has a spherical shape.

This technique has one major drawback, which is the fact that the reaction is initiated at the surface of the drops. It is not fully known if the drops is therefore homogeneous. We can guess that this is the case for high concentration of reactant, figure 26, because it seems the drop breaks as a solid and homogeneous sphere. But for less dense gel this point is critical and we can imagine that the drop polymerises only at the surface and creates something like a shield.

4.2.2 Mixing

To avoid the problem of non homogeneous gel, we though of mixing all the components in our drops. We have already said that it is impossible to mix the reactants in the water phase and then to push this fluid in our system in order to form the droplets. Indeed, the water will become a gel in a few minutes and the flow will stop. The trick is to mix water + monomer + MBA + APS and water + TEMED in our system just before the drops formation.

The Microsystems used to do so are shown on figure 21. The distance between the point where the two water phases join and the flow-focusing constriction is 200µm, once again, the fluid are moving at 10mm s$^{-1}$ so the reaction does not have the time to be completed before the drops formation.

![Mixing system](image)

(a) First mixing system

(b) Second mixing system with a curve channel to mix the droplets once they are formed.

Figure 21: Microsystems used for mixing all the reactant inside the drops. There are three entries for the oil, and the two different water phase. There is also a entry at the extreme right of the system use to add surfactant in the oil after the production of the droplets.

The figure 21 shows two types of system. They differ by the mixing channel located after the flow-focusing constriction. We decided to use this design to be
sure that the two water fluids are well-mixed. One technique to mix two fluids rapidly is to impose many variations of the flux direction. Here we use a thin coil, we can see it on the figure 22 where two fluxes of water are mixed.

Figure 22: Production of water droplets with mixing two different water incoming flows.

4.2.3 PVA

One default of this production is the cold/hot cycle. If we want to get micro-droplets of PVA gel, we need to prevent them to merge for a long time even though we freeze the sample. We have solved this problem by isolating the droplets from each others in a microfluidic system containing "traps" as shown on figure 25, [Tan and Takeuchi, 2007].
This type of traps are well known in micro-fluidic, their shape allow a drop to enter the trap because the fluid can pass through, and once a drop is inside, another ones can’t expel it and are forced to make a detour. Therefore we couple those traps in our long system, figure 15, to trap a maximum of droplets.

Figure 24: Basic principle of the trap we used in our system.
4.2.4 Influence of the continuous phase

Until now, we have referred to the continuous phase as the "oil phase", without any specification. But as we have found out, the oil nature has a major importance on the reaction process.

**Fluoric oil** Because the previous article on the hydrogel and droplet of hydrogel mentioned fluoric oil [Wyss et al., 2010], we decided to reproduce their experimental conditions and use fluoric oil. This led us to many fruitless experiments. We eventually think of a simple reason for that. Fluoric oil contains many fluorine termination. It is known that fluorine can prevent the good polymerisation of hydrogel. This happens also for bulk formation of hydrogel. When using mold in Teflon, so containing fluorine, the polymerisation is not homogeneous depending on the proximity of the border in Teflon. When we found out this hypothesis, we decided to change the surrounding oil.

**Mineral oil** We then use mineral oil to form our droplets. Here again some problems have appeared. The major one we have stressed out is the internal motion of the drop and will be discuss in the next section. But we also have problems with the surfactant we used. We begin by using Span 80, a very common surfactant for water-in-mineral oil droplets. During the experiments we noticed that the outlet sample were very troubled. By looking at the droplets inside the device, we saw that along the channel, each drop was leaving behind small particles. After doing the same experiments with pure water in the same oil-surfactant mix, we deduced that these particles were small vesicles of surfactant.
We did not find a solution to this problem. We tried to change the surfactant, especially Abil EM 80, but it was not fully convincing.

4.3 Filtering and sorting the drops

In the previous section, we described how the drops were produced at the constriction point, figure 12. But once they are produced, there are still a few steps to do before being able to manipulate them.

4.3.1 Controlling the reaction process

During the production, there is no immediate way to see if the polymerisation is complete, or if it has occurred. So we need an experiment to check if the polymerisation has occurred or not.

This problem can be simply solved when using very hard hydrogel. Because they have a high Young modulus, typically of $E = 1\text{MPa}$, they are more solid and are more able to break down under an imposed deformation. As shown in the figure 26, the two pictures show four droplets of hydrogel, which are in the continuous phase, and placed under a microscope between two glass slides. The experiments consist of pressing the top slide and squeezing the droplets. The figure 26a is taken before and the figure 26b after having pressed on it. We can see that the droplets are broken in a way that leave no doubt about correct polymerisation.

![Figure 26: Break up of droplets by pressing them between two glass slides. The breakup is not exactly the same from one droplet to the others, this is due to the fact that the applied pressure was not strictly uniform.](image)

While using softer gel, the last experiment is less effective. Because that kind of gel are softer, they better resist to the imposed pressure. For that kind of gel, the best way to see if they are truly polymerised is to transfer them into the water and to see if they stay as droplets or merge into the water phase.
4.3.2 Controlling the polymerisation

From the simple design of the flow-focusing system, (figure 12), we have modified the outlet channel for two reasons. First to initiate the reaction not with TEMED but by heating the system. In order to do so, we have drawn a long channel after the constriction, as seen on the figure 15, and put this system on top of a heater made of resistive wires mould into a thin PDMS layer. This process has been put aside because even though the channel length is widely increased, the droplets travel in the system for just a few minutes at the most. The time while the droplets are heated is not enough to complete the polymerisation so we have abandoned this solution.

But the few minutes that the droplets pass in the system are enough to initiate and finish the reaction while using chemical initiation. We accentuate this phenomenon by elevating the outlet channel of the system by doing a two step lithography. After the constriction passed, the fluids and the droplets arrive in a much higher channel so they slow down. This has two major effects, one is that the droplets can take a fully spherical shape, the second is by slowing down the flow, we limit the internal movement of the water inside the drops. Indeed, when the continuous phase goes faster than the drops, a motion is induced in the droplets and can trouble the polymerisation.

4.3.3 Perturbing the polymerisation

This last effect is not well defined. But if we look at some bulk experiments we can convince us of the importance of internal motion. The simpler experiment is to take our sample with all our reactants, and mix it continuously during the polymerisation. For high concentration of reactants, the polymerisation occurs normally except that the produced gel solidifies in a complex form due to the motion. For smaller concentration, we can see the result in the figure 27.

![Figure 27: Comparison between two bulk samples of the same hydrogel (PAM 3.2x0.3). The one in the left has been stirred during the polymerisation, as the the sample on the right was not. We can see that when we tilt the erlenmeyers the gel stays still in the right one but not in the left one, which means that it is still liquid when it has been stirred.](image)

The action of stirring the solution has prevented the polymerisation to occur. We can link this result with our droplet by looking at the intern motion induced in a droplets when surrounding by a moving fluid. The figure 28 shows a still
drop surrounding by a moving fluid. The velocity field is represented and for a fluid moving at the speed $U$, the internal fluid of the drop will also move at the speed $U$. The internal fluid velocity depends on the fluid’s viscosities but it clearly appears that the drop is under a certain shear stress.

![Velocity field](image1)

Figure 28: Velocity field of the fluid inside and outside a still droplet inside a moving fluid.

Further studies have been scheduled to better understand this principle and maybe stress out a threshold of concentration that we can not pass under.

### 4.3.4 Transferring the gel into water

These parts are not well documented in the literature and in particular in the paper dealing with droplets of gel produced in a continuous phase of water, [Wyss et al., 2010]. Because the production of droplets requires surfactant, it is not trivial that just by adding water to the sample and centrifugate it, it will separate the gel from the oil phase. We have come up with a simpler and very effective way to do so.

**Removal of surfactant** During a work in collaboration with a post-doctoral student, we came up with the idea of using a device made for sorting objects depending of their size to extract our droplets from the fluid in which they are made.

![Pinched flow fractioning](image2)

Figure 29: Pinched flow fractioning used to force the drops to pass from the blue fluid to the red one.
The figure 29 shows the basic principle of the system. It consists of two entries on the left and three outlets on the right, linked by a unique channel. One inlet, here the bottom one, contains our droplets to extrude, the other one contains pure fluid. So we force the drops to pass from one fluid, here in blue, to another in red. We have tried with pure water, but the wetting issue in the system does not allow the extrusion to work. So the two fluids are oil. The one on top is pure oil whereas the bottom one is oil plus our droplets, added with surfactant. If we transfer the drops from the oil containing surfactant to pure oil, the later transfer to water will be simplified. By setting the good pressure in the inlet and outlet, one can force the fluid coming from the entry A to go in the bottom outlet while the droplets going in the middle outlet stay in a pure oil phase.

(a) Phase contrast imaging. It clearly appears that the droplets pass from one fluid to the other.

(b) Direct image.

Figure 30: Extracting of the droplet. The two entries are not shown on the right. The top one contains the oil with the droplets and the surfactant, the bottom one is pure oil. We can see that the surfactant goes in the upper outlet and the drops go in the middle one.

This process is simpler than what is commonly used in the literature. Instead of making multiple cycles of centrifugation and dilution, we just set our system and the separation occurs.

Transfer to water Once the droplets are in a pure oil phase the transfer is rather simple. As we are using mineral oil, which are lighter than water, the drops naturally sink. Adding water to your sample and centrifugate it enables the drops to pass the water/oil separation.
To achieve a purer sample, it is possible to add SDS surfactant into the water, mix it and re-centrifugate the solution. This will remove any oil remaining in the sample. Adding SDS is not a problem because its facilitates the motion of droplets along PDMS wall during later experiment. The gel droplets have a tendency to stick to the PDMS wall when they are in the water. Adding a surfactant acts against this phenomenon.

This is the last step of the production. The droplets of hydrogel are in the water phase, they can freely swell or shrink to their equilibrium state and be used in our experiments.
5 Lung experiment

We have so far described our issues and our tools. We now need to describe the experiment we made to compare the real phenomenon.

5.1 PDMS system

We have described all the parts we need to understand in order to build a biomimetic experiment. In this section we are going to give the specification of our system based on the events that occur in the lung.

5.1.1 Lung comparison

![Figure 32: Schema of an alevoli. We can see the blood capillaries, their many separations and recombinations.](image)

We have specified in the table 1 and on the figure 32 the characteristics of the blood vessels we were interested in. This gives us the general dimension for our system. We have to create a succession of short channels roughly of $5 - 10 \mu m$. We have made a microsystem based on a simple channel and put into it 2D network of pillars. These pillars create a mesh of small channels. Each end of the channel is separated in two ways and the travelling objects have to choose either the right or left path. We therefore created a alveoli-like blood irrigation system.

This is shown on the figure 33. The height we aim at for this channel is about $10 \mu m$, in the figure the pillars dimensions are $30 \times 60 \mu m$ and separated by $10 \mu m$. This is the basic model we have imagined and we can easily change the space between the pillars or their length.
This system is very interesting in comparison of the simple channel constriction because of the succession of little channels. In the simple constriction, one can study the entry time, the pressure needed to force the cell or droplet to enter it. Here we can also observe the behaviour of the objects when it is under a cycle of deformation / released state. So we can mimic all capillaries the neutrophils pass through when they travel around one alveoli.

5.1.2 Scaling

We have discussed about the problems we have encountered in making droplets at the cell-size. While we were searching for a solution, we have also made "lung-system" with a size that matches our droplets of hydrogel.

We scaled up the entire system with different factors - 2, 3 or 4 - in order to be able to make droplets of the right size.

5.2 Biological experiments

The biological experiments have been made in Marseille in the INSERM U600 with cells similar to the neutrophils, so-called THP-1. We stressed out three different behaviours of the cells along the system.
• **Entry**  The first phase is the cell’s entry in the system. It is the first deformation of the cell.

We have said that the cell needs to deform its cytoskeleton to enter a small vessel. This is what happens in the first deformation and is responsible for naturally the long entry time that we observe. This entry time can be related to the visco-elastic measurements done by [Guevorkian et al., 2010].

![Figure 34: The path of a cell shown in red. The A area corresponds to the entry, B to the transient regime and C to the stationary regime.](image)

• **Transient regime**  After the first deformation, and before reaching the stationary regime the cells have a transient regime. They accelerate, but also continue to stop for a long time at each channel entry. They also have an aleatory pathway. This means that they still have an elastic behaviour, in which they try to reform as a normal cell between two successive channels.

• **Stationary regime**  After a few deformations, meaning after a few pillars passed, the cell converges toward a stationary regime. This regime has two characteristics.

First the cell appears to go along a "line" in the system. Its motion and pathway are not aleatory. It is shown in the figure 34. One explanation is that in this regime, the cell has reached a fixed shape and does not reform between two small channels. This induces a shear stress in the cell that forces it to alternatively turn right or left when it goes off a channel.

Secondly, the cell velocity is higher. Because it has reached a stationary shape, it does not need to deform and lose time doing it.
5.3 Hydrogel experiments

5.3.1 Simple behaviour of our droplets

We have, as for the neutrophils, try to push our droplets in a single small channel. We have noted that the entry time was very long. This behaviour is comparable to the one of the neutrophils. If in the case of the cell, we can explain it by the proper elasticity of the cell but also by its internal reconfiguration, here it is purely due to elasticity of the hydrogel.

![Image](image1.png)

Figure 36: Entry of a hydrogel drops, here it is PAM 20 × 1, in a 25µm constriction. The time frequency is one image every 0.5 s.

As we have shortly noticed during our experiments, our droplets of hydrogel are very elastic. They do not behave as cells for the shape-memory. When forced into a small constriction for a long time, they do not keep the imposed shape more than a few milliseconds.

![Image](image2.png)

Figure 37: Chronophotography of a droplet coming off a constriction. The time lapse is 0.25µs
5.3.2 False cell in false lung

After all the steps we described, we have succeeded to force some droplets into our system. We will here discuss three experiments we have made.

- The first one is represented in the figure 38. The system used in this case is slightly different from the previous ones, the pillar are circular. The gel use is very stiff in comparison with a cell. The imposed deformation between two pillars is not big, the drops barely keep its spherical shape. Because the drop is very stiff, it can pass only because its size is very similar to the distance between two pillars. A bigger drop of the same gel would not move at all in this system.

Figure 38: Chronophotography of a very stiff drop, PAM 20 × 10 moving in a "lung"-like system.

- The next experiment is a very soft hydrogel moving in the same system as before. The drop is here bigger and occupies more inter-pillar space than the previous one, the distance between two pillars is 10μm and the drop is about 30μm wide. But it moves all the same. The drop deforms itself at a large rate, it embraces the form created by the pillars.

Figure 39: Chronophotography of a soft drop, PAM 3.2 × 0.2.
• The figure 40 is a chronophotography of two droplets moving between the pillars. The hydrogel used here is a PAM 3.2 × 0.2, known to have the same elasticity of the neutrophils. We can see two droplets moving from the bottom to the top. One is quite big and move by steps, stopping for a long time before entering each small channel. The second one is smaller and moves faster, more like a real cell in the stationary state we described earlier.

Figure 40: Chronophotography of a droplet of hydrogel, PAM 3.2 × 0.2, moving in a "lung"-system. The pillars are separated by 5µm, and the images are taken every 1ms.
6 Conclusion

During this master thesis in the MMN laboratory, I have joined a very promising project about the cell characterisation. The idea of imitate and compare a simple model to the living cells was very challenging. And this approach of the ARDS disease was pathbreaking.

Despite the simplicity of the issue, I have faced many technical problems and haven’t been as far as I intended. The production of the biomimetic objects was not simple. The biological context constrained us in a very narrow and specific field.

But in the end I have provided to the project a well established process to produce biomimetic droplets. I have begun to confront these droplets to the cells and this approach and the first results appears to be promising for the future.

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References


