ANNEX 3204
Technical Description

Preparation and mechanical characterization of Bionate membranes

Table of Contents

1 Scope of the Annex:

This Annex was developed to describe the fabrication of the DALI membrane, which works as support for the alveolar barrier. Moreover, the characterization of the material is here presented.

2 Abbreviations:

dH_2O	Distilled Water
EtOH	Ethanol
GPTMS	γ-glycidoxypropyltrimethoxysilane
HFP	1,1,1,3,3,3 Hexafluoro-2-Propanol
NM	Nanomaterial
PBS	Phosphate-Buffered Saline
UV	Ultraviolet

3 Principle of the Method:

These methods aim at describing the fabrication of the DALI membrane and its mechanical characterisation. Firstly, a description of the membrane materials is presented, as well as an overview about the electrospinning technique, which was selected to obtain the porous support. Then, structural and mechanical properties are investigated in order to ensure the feasibility of this material as stretchable support. The contact angle was measured to investigate the hydrophilicity of the material, therefore its cell adhesion. Then, the Young's modulus was evaluated considering three different testing conditions: *dry conditions*, to evaluate the material stiffness; *wet conditions*, to evaluate the effect of an aggressive environment such as the cell culture environment; and *cyclic conditions*, to evaluate the structural properties after fatigue tests, studying material changes after cyclic stretching.

4 Description of the Method:

4.1 Chemicals and reagents used:

Membrane fabrication:

- Bionate® II 80 A (Koninklijke DSM N.V., Heerlen, the Netherlands)
- Gelatin Type A from porcine skin (300 bloom, Sigma Aldrich, Milan, Italy)
- 1,1,1,3,3,3 Hexafluoro-2-Propanol (HFP, from Sigma)

- γ-glycidoxypropyltrimethoxysilane (GPTMS, from Sigma)

Membrane characterization:

- 70% ethanol (70% EtOH)
- Distilled water (dH_2O)
- Phosphate-Buffered Saline (PBS)

4.2 Apparatus and equipment used:

Membrane fabrication:

- Electrospinning setup: syringe equipped with a blunt needle (extruder), a pump, a high voltage power source and a collector

Membrane characterization:

- A laboratory-made setup for permeability evaluation (detailed description in section 4.4.2.1)
- Twin column-testing machine (Z005, Zwick-Roell) equipped with a high precision load cell (max loading 100N, resolution 0.1 N; Instron, Massachusetts, USA)10 µL, 200 µL and 1000 µL pipette tips

4.3 Procedure:

4.3.1 Membrane fabrication: from the requirements to the adopted solution

The main actor of the DALI system is the membrane that works as support for the alveolar barrier. The membrane must be porous, to allow the communication between the two compartments divided by the physiological barrier, recreating a barrier-like system. Its biocompatibility and cell adhesion are essential requirements to allow cells growth in a functional device; therefore, the membrane must be sterilizable without losing its characteristic properties. Moreover, the support must be highly elastic and flexible, to replicate the cyclic mechanical strain of the alveolar barrier that occurs during the breathing, allowing for physiological levels of stretch on the cultured epithelium. For this reason, it is important to select a membrane material that maintains its mechanical properties also after long term cyclic stretches. Finally, since the membrane is used in an aggressive environment (cell culture media), it must not degrade during cell culture experiments.

A commercial poly(carbonate)urethane copolymer (Bionate® II 80A) was used as a support of the physiological barrier, thanks to its combination of elastic characteristics from the urethane and the biostability from the carbonate segment. Additionally, in order to increase the material cell adhesion, gelatin was used in combination with Bionate® to obtain the final formulation for the membrane. Gelatin is a natural biopolymer, which is obtained by hydrolyzing the collagen of skin, tendons, cartilage, and bone [1]. Because of its biodegradability, biocompatibility, and the presence of arginine-glycine-aspartate motifs in its structure, it provides an appropriate platform for cell adhesion, proliferation, and migration [2].

The electrospinning technique was selected to obtain the porous support due to its important advantages. In this regard, electrospinning generates supports with high porosity and high surface area, which can mimic extracellular matrix structure, making itself an excellent candidate for cell culture applications [3]. It allows to fabricate membranes composed by very thin fibers to the order of few nanometers with large surface areas and superior mechanical properties. Moreover, membrane characteristics (thickness and pore and fiber dimensions) can be controlled by varying the electrospinning parameters. Finally, the possibility of large scale productions combined with the simplicity of the process makes this technique very attractive [3].

Bionate® and gelatin were dissolved at 10% (w/v) in HFP. In order to stabilize the gelatin, 368 µL of GPTMS per gram of gelatin were added to the Gelatin/HFP solution. Different percentages of Bionate® conjugated with gelatin were investigated (Bionate®: Gelatin at 50:50, 70:30, 80:20, 90:10, 100:0) to obtain a membrane with different hydrophilic and mechanical characteristics. Table 1 shows the electrospinning parameters.

Table 1: Electrospinning parameters.

Voltage	**D extruder-collector**	**Needle diameter**	**Solution flow**
30 KV	15 cm	0.41 mm	1 mL/h

4.3.2 Membrane characterization

4.3.2.1 Methods

Once fabricated the membranes, images were obtained with the Scanning Electron Microscope (SEM), and geometrical characteristics (fibers and pores dimensions) were derived via image analysis (ImageJ).

Then, the wettability of the material was evaluated, since it is believed to be one of the most important parameters that influences biological response of biomaterials [4]. Wettability was evaluated measuring the contact angle and analysing how different percentages of gelatin in the composition vary hydrophilic properties. Moreover, to further increase the wettability, the test was repeated after dipping the membranes in 70% ethanol/distilled water (70% EtOH/dH_2O) for 15 minutes.

Membrane permeability was evaluated using the method described by Karande *et al*. [5]. Briefly, a constant hydrostatic pressure is applied to a constrained sample and the flow rate of water through it was measured. Darcy's Law (Equation 1) is then used to determine the value of permeability K [6].

$$K = \mu_F \frac{L}{S} \frac{Q}{\Delta P} \qquad (1)$$

In Equation 1, μ_F is the viscosity of the water, L and S are, respectively, the height and the cross-section of the membrane; Q is the measured flow rate and ΔP is the pressure applied using a constant head of water. Membranes were tested with and without a preliminary dipping into 70% Ethanol for 15 minutes. The experimental setup is shown in Figure 1. Briefly, the membrane is fixed on a bottleneck using a cap. The cap is cut in correspondence of its central region, allowing fluid flow through the membrane. The end of the bottle is cut, and a tube is fixed in correspondence of the bottleneck, in order to maintain a diameter continuity passing from the cap to the tube. The tube is then filled with distilled water, maintaining a constant fluid level during the test.

Figure 1: Experimental setup of the permeability test.

Then, tensional stress-strain tests were performed on porous membranes using a twin column-testing machine (Z005, Zwick-Roell) equipped with a high precision load cell (max loading 100N, resolution 0.1 N; Instron, Massachusetts, USA). The samples were prepared cutting a 10x30 mm piece from a sheet of material. Firstly, the Young's modulus was evaluated for the porous membranes in dry conditions at room temperature (25°C), in order to understand how gelatin can influence the stiffness (setup shown in Figure 2 A). Mechanical tests were performed with a constant strain rate of 0.1%/s and the resulting stress was evaluated until a maximum strain of 20%. Moreover, since the membranes will be used in a rather aggressive environment and application, the mechanical characterization was repeated for long-term tests. To do so, their structural properties were evaluated performing the tensile test at 37°C, after several days of incubation (0, 1, 3 and 7 days) in Phosphate Buffered Saline (PBS 1X) at 37°C. we refer to this test condition as *wet condition*, whose set up is shown in Figure 2 B. Moreover, a cyclic pre-conditioning was performed, stretching the material for 1, 2, and 4 hours in PBS, and studying its changes after the cyclic actuation (*cyclic condition*). The membranes were stretched at a maximum strain of 5% and a frequency of 0.4 Hz, in order to simulate the normal breathing cycle. After this preconditioning phase, the Young's modulus calculated within the linear region was evaluated with a traditional tension stress-strain test to determine the occurrence of plastic behaviour (set up shown in Figure 2 B).

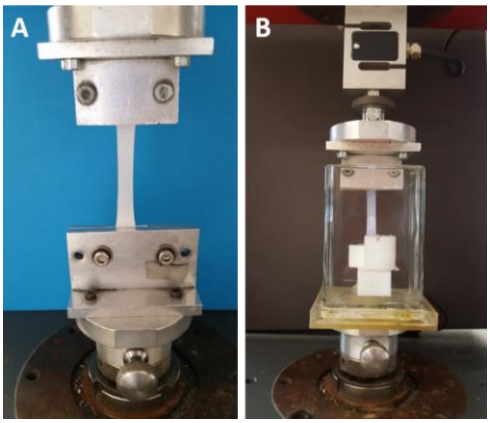

Figure 2: Pictures of the setup during mechanical tests with the column-testing machine: A) dry conditions setup, B) wet and cyclic conditions setup.

Mechanical and structural tests data are reported as mean ± standard deviation for at least three recorded values (n > 3). Statistical analysis was performed using the One-way ANOVA-test, setting the significance at $p < 0.05$ for each test.

4.3.2.2 Results and discussion

Table 2 summarizes the geometrical characteristics of the electrospun membranes. They have a thickness in a range between 50-85 µm, resulting thick enough to be handle by an operator during laboratory procedures. The fiber dimension is almost 2-3 µm, while the pore dimension 4-5 µm, similar to traditional cell culture Transwell inserts.

Table 2: Geometrical characteristics of the electrospun membranes.

Bionate®:Gelatin	Thickness [µm]	Fiber Diameter [µm]	Pore Diameter [µm]
50:50	74.9±10.6	2.7±0.8	4.9±2.1
70:30	82.3±16.9	3.6±1.0	5.8±1.3
80:20	48.8±6.8	2.4±0.5	3.9±1.7
90:10	85.0±17.7	2.9±0.9	5.0±1.1
100:0	54.1±10.7	2.4±0.6	4.3±1.9

Hydrophilicity was evaluated analysing water droplet contact angle on control membranes and on membranes treated with 70% EtOH/dH2O (Table 3).

Table 3: Contact angle (θ) of a water droplet on electrospun membranes. The contact angle was measured on control membranes and on membranes dipped in 70% Eth for 15 minutes.

Bionate®:Gelatin	θ (°) - Control	θ (°) - 70%EtOH treated
50:50	-	-
70:30	-	-
80:20	117.2±1.9	70.9±10.1
90:10	119.3±4.8	86.2±3.0
100:0	136.9±4.6	120.8±12.4

Considering the control membranes (Table 3), a low wettability was found in 80:20, 90:10 and 100:0, with a decrease of the contact angle by increasing the amount of gelatin within the material composition. The 50:50 and 70:30 membranes showed high wettability, so that the contact angle could not be measured since the membranes quickly absorbed the drops. Drops on 70:30 membranes were absorbed slower than drops on 50:50 membranes. However, the treatment with 70% EtOH/dH2O improved the wettability. In fact, 80:20 and 90:10 membranes became hydrophilic, while the 100:0 membranes remained hydrophobic, but they decreased the contact angle. Since the enhancement in the wettability can improve the cell attachment, cell proliferation and cell-support interactions, after the treatment with 70% EtOH all the membranes are potentially adapted for cell culture application, except for the 100:0 ones that remain hydrophobic, and for the 90:10 that probably are not enough hydrophyilic.

Then, membrane permeability (K) was evaluated before and after the treatment with 70% EtOH/dH2O (Table 4).

Table 4: Membrane permeability before and after the treatment with 70% EtOH/dH2O.

Bionate®:Gelatin	**$K_{\text{untreated membranes}}$ [m^2]**	**$K_{\text{treated membranes}}$ [m^2]**
50:50	6.89 ± 0.97 E-14	6.31 ± 0.99 E-14
70:30	1.64 ± 0.62 E-16	4.02 ± 0.72 E-14
80:20	1.88 ± 0.89 E-16	3.17 ± 0.44 E-14
90:10	2.60 ± 0.63 E-17	6.67 ± 0.63 E-14
100:0	2.84 ± 1.67E-15	2.71 ± 0.74 E-13

Considering the untreated membranes, Table 4 shows that the permeability decreases with the increase of Bionate® within the composition, except for the pure Bionate® (100:0). This behaviour could be due to an interaction between the cross-linker used to stabilize the gelatin (GPTMS) and the Bionate®. In this regard, membrane permeability decreases when increasing the amount of Bionate®, apart from the 100:0 membranes, in which the gelatin and, therefore, the GPTMS are not present. For this reason, it is reasonable to conclude the decrease of the permeability is not due to the presence of the Bionate®, but probably to its interaction with the GPTMS. Moreover, permeability does not change significantly for the 50:50 membrane after the treatment with 70% EtOH/dH2O, while it increases of two order of magnitude for the 70:30, 80:20 and 100:0 membranes. Concerning the 90:10

membrane, permeability increases of three orders of magnitude. This difference in the behaviour after the treatment with ethanol can be attributed to the lower surface tension of ethanol with respect to water, which facilitates the substitution of air in the copolymer network with liquid and afterwards by water. Furthermore, after the treatment with ethanol, all the membranes with different composition are characterized by a permeability with the same order of magnitude, except for the 100:0 membranes that present a higher permeability. This behaviour is probably due to the absence of GPTMS in the composition of the 100:0 membranes. To conclude, the most relevant aspect concerning permeability tests is that the membranes were effectively porous, allowing for material translocation between the apical and basolateral compartments of the bioreactor.

Mechanical tests in dry conditions (Figure 3) show that increasing the amount of gelatin within the material composition causes a decrease of the linear elastic region of the electrospun membrane.

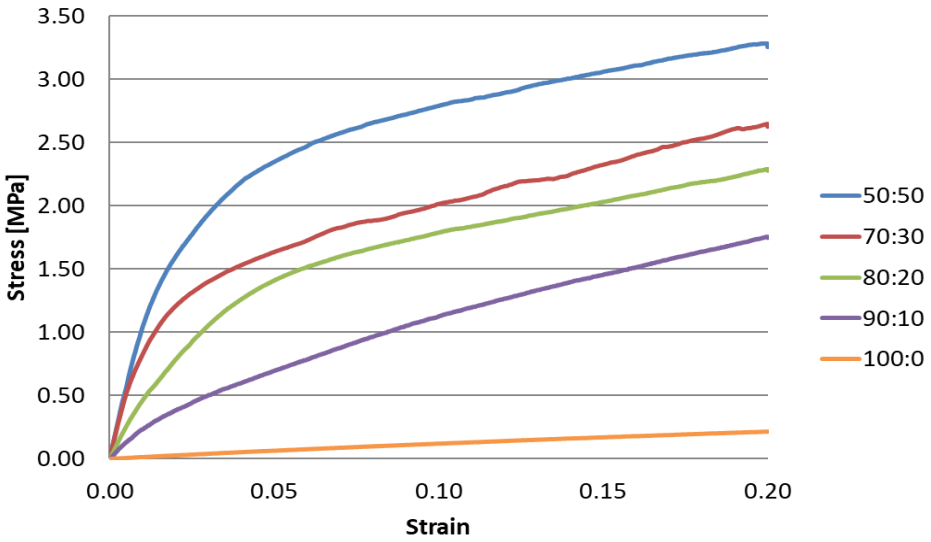

Figure 3: Stress-Strain curves of the electrospun membranes tested in dry conditions.

Table 5 shows that the Young's Moduli calculated within the linear region increased with the increase of the amount of gelatin in the membrane composition: it is almost 1 MPa for the pure Bionate®, reaching ≈ 90 MPa in 50:50 membranes.

Table 5: Young's Modulus within the linear region (1% strain for 50:50, 70:30 and 80:20 membranes; 3% strain for 90:10 membranes and 5% strain for 100:0 membranes).

Bionate®:Gelatin	Young's Modulus [MPa]	Linear Region (@strain)
50:50	89.25±14.46	0.01
70:30	74.72±16.50	0.01
80:20	43.35±3.60	0.01
90:10	21.76±6.42	0.03
100:0	1.10±0.26	0.05

Mechanical tests were repeated in wet conditions. Stress-strain curves referred to the wet condition test (Figure 4) show that the samples had a linear elastic behaviour within the entire deformation range, as opposed to the dry condition test, suggesting that all the membranes are suitable for the applications where a linear-elastic behaviour is necessary (i.e. cyclic stretching of the membrane). As an example, Figure 4 shows the curves referred to the 7th day of incubation, as the ones obtained at different incubation times had the same linear elastic behaviour.

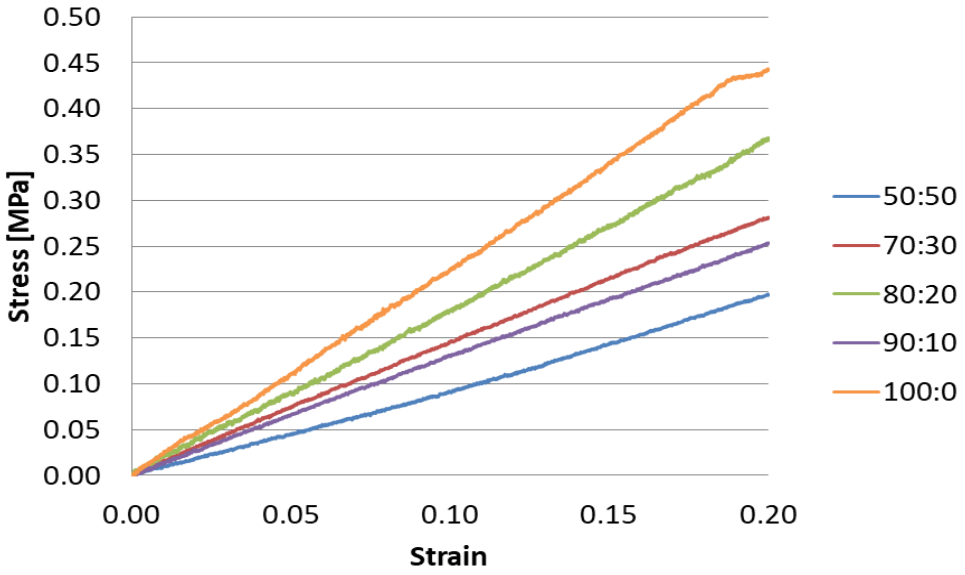

Figure 4: Stress-Strain curves of the electrospun membranes tested in wet conditions after 7 days of incubation in PBS.

The histogram in Figure 5 shows the Young's Moduli of the membranes grouped according to their composition, after 0, 1, 3 and 7 days of incubation at 37°C.

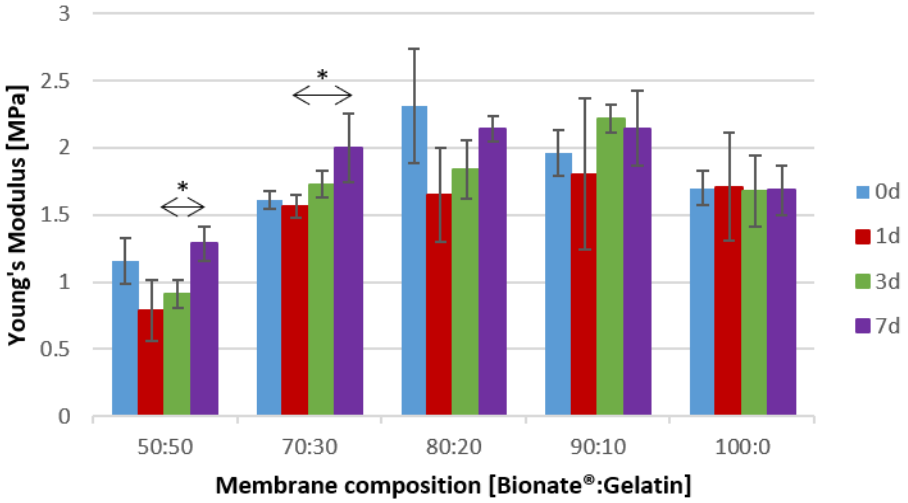

*Figure 5: Young's Moduli of the membranes with different Bionate®:gelatin composition (50:50, 70:30, 80:20, 90:10, 100:0) with respect the incubation time (0, 1, 3 and 7 days). *p < 0.05.*

The analysis of the histogram revealed that:

- 80:20, 90:10 and 100:0 samples did not show a statistically significant variation of the Yong's Modulus with respect to the different incubation times (one-way ANOVA test, $p > 0.05$).
- 50:50 and 70:30 samples showed a statistically significant variation between the samples incubated for 1 and 7 days (one-way ANOVA test, $p < 0.05$).

These results suggest that all the membranes are suitable for long-term cell culture applications, since the Young's Modulus did not change significantly during the analysed incubation time. Considering also 50:50 and 70:30 membranes, which showed a statistically significant difference in the Young's modulus at day 1 and 7, did not degrade: the Young's Modulus is higher at the 7th day of incubation. Probably this difference is due to heterogeneity of the electrospun membranes (fibers deposit randomly on the collector during electrospinning).

Table 6 shows the calculated Young's Moduli of the membranes with respect to the incubation time.

Table 6: Young's Moduli of the membranes with different composition with respect the incubation time.

Bionate®:Gelatin	E_{0d} [MPa]	E_{1d} [MPa]	E_{3d} [MPa]	E_{7d} [MPa]
50:50	1.16±0.17	0.79±0.22	0.91±0.10	1.28±0.13
70:30	1.61±0.07	1.56±0.09	1.72±0.10	2.00±0.25
80:20	2.31±0.43	1.65±0.35	1.83±0.21	2.14±0.10
90:10	1.96±0.17	1.80±0.56	2.21±0.10	2.15±0.28
100:0	1.70±0.13	1.71±0.40	1.67±0.26	1.68±0.19

Finally, cyclic pre-conditioning of the material was performed to verify if the membranes were suitable for applications in which a cyclic stretch is applied. Figure 6 shows the Young's Moduli of the membranes grouped according to their composition after 0, 1, 2, and 4 hours of cyclic stretching (with 0 hours we refer to the samples tested in wet conditions without performing a cyclic stimulation, 0h wet cond in Figure 6).

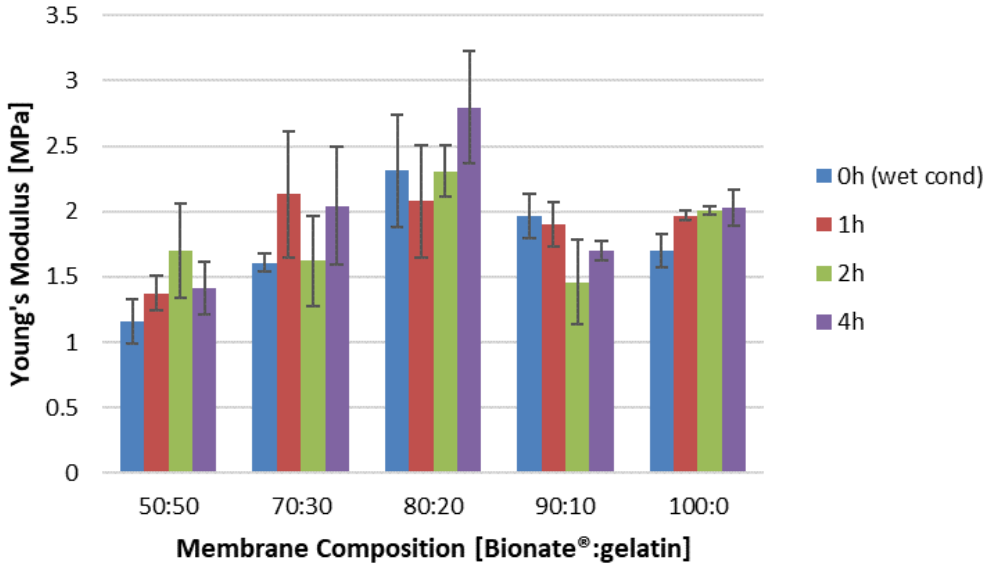

Figure 6: Young's Moduli of the membranes with different Bionate®:gelatin composition (50:50, 70:30, 80:20, 90:10, 100:0) with respect to the cyclic stretching time (0, 1, 2 and 4 hours).

No significant difference in elastic modulus was observed (one-way ANOVA test, $p > 0.05$), suggesting the tests were performed into the linear range of the material, without any residual plastic deformations. Table 7 shows the calculated Young's Moduli of the different samples with respect to the cyclic stretching time.

Table 7: Young's Moduli of the membranes with different composition with respect to the stretching time.

Bionate®:Gelatin	E_{0h} [MPa]	E_{1h} [MPa]	E_{2h} [MPa]	E_{4h} [MPa]
50:50	1.16±0.17	1.38±0.13	1.70±0.36	1.41±0.20
70:30	1.61±0.07	2.13±0.49	1.62±0.35	2.04±0.45
80:20	2.31±0.42	2.07±0.43	2.31±0.20	2.80±0.43
90:10	1.96±0.17	1.90±0.17	1.46±0.32	1.70±0.07
100:0	1.70±0.12	1.97±0.04	2.01±0.03	2.03±0.14

4.3.2.3 Conclusion

Measuring the contact angle, the membranes made of pure Bionate (100:0) showed a low wettability also after the treatment in EtOH/dH2O, and so they are not

recommended for cell culture. Mechanical tests in dry condition showed a viscoelastic behaviour of all the membranes with different composition, and the decrease of the linear elastic region with the increase of the amount of gelatin in the membrane composition. However, repeating mechanical tests in a wet environment at 37°C, the membranes showed a linear-elastic behaviour within the entire range of deformation, suggesting their suitability also for applications where a linear-elastic behaviour is mandatory (i.e. in vitro models of the lung, where the alveolar barrier is cyclically stretched). After fatigue tests, the Young's Modulus of the membranes did not change appreciably. Moreover, after performing the tensile tests in wet condition, none of the membranes showed any mechanical degradation over 7 days of incubation in PBS at 37°C. These results indicate that all the different membranes are suitable for long-term cyclic stretching applications. Since the 50:50 membrane showed the highest wettability, which suggests that it could be a better support in applications where cell adhesion is crucial, it was selected for performing the preliminary biological studies involving cell stretching.

4.3.3 Membrane handling and sample preparation for experiments using the DALI System

The procedure to obtain a sample from a sheet of membrane material is shown in Figure 7 and schematize as follow:

1. Turn upside down the aluminium sheet with the electrospun membrane
2. With the help of the holder and a pencil, draw a circle (try to obtain the circle from the centre of the aluminium sheet, where the membrane it is thicker). From a membrane it is possible to obtain approximatively 5 membranes, depending from the sheet
3. Cut the circle and then remove the membrane from the aluminium sheet
4. Place the membrane in its holder.

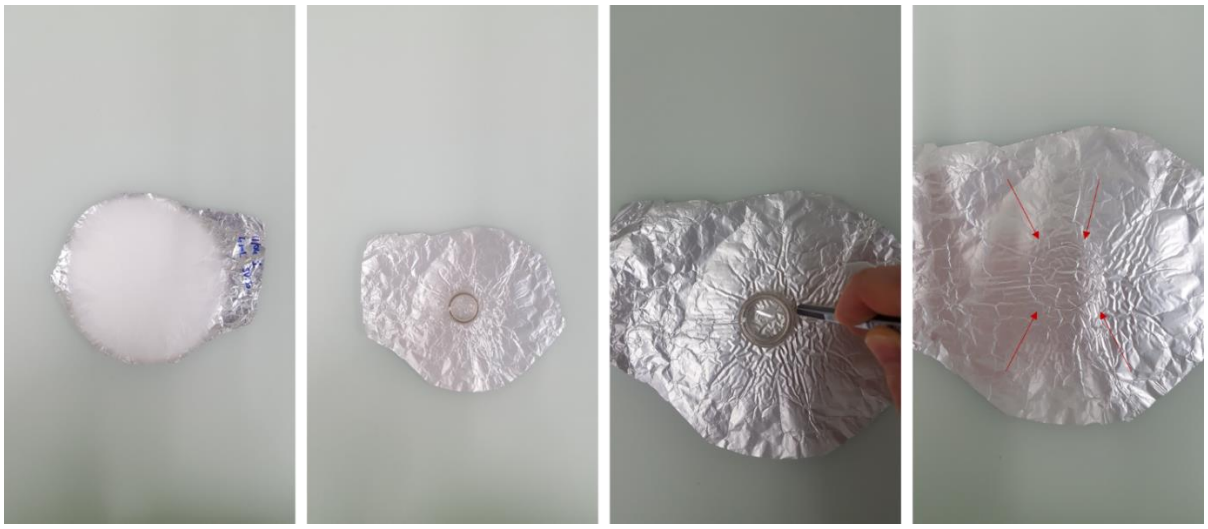

Figure 7: Picture showing the procedure to obtain a sample from a sheet of membrane material.

4.3.4 Membrane sterilization

1. Fix the membrane in its holder
2. Dip the membrane in a 70% EtOH in deionized water solution (V/V) for 15 minutes;
3. Wash in PBS twice and let it dry under a laminar ventilation hood;
4. Expose to UV light for 15 minutes, each side.

4.3.5 Conclusions

Biohybrid membranes made of a commercial polycarbonate urethane (Bionate®) combined with gelatin were fabricated and characterized in terms of mechanical properties, fatigue strength, permeability and wettability. Bionate® was selected as an excellent candidate for cell culture systems due to its biostability, flexibility, electrical properties and tensile strength [7]. However, because of its low wettability, we used it in combination with gelatin. The 50:50 Bionate®:gelatin solution was electrospun, allowing to obtain a porous support with the suitable geometrical characteristics necessary for cell cultivation (i.e. membrane thickness, pore size, fiber size).

4.4 Quality control & acceptance criteria:

Measuring the contact angle, the membranes showed a high wettability. Mechanical tests in dry condition showed a viscoelastic behaviour of the membranes. However, repeating mechanical tests in a wet environment at 37°C, the membranes showed a

linear-elastic behaviour within the entire range of deformation, suggesting their suitability also for applications where a linear-elastic behaviour is mandatory (i.e. *in vitro* models of the lung, where the alveolar barrier is cyclically stretched). Moreover, after performing the tensile test in wet conditions, the membrane did not show a degradation during the 7 day incubation in PBS at 37°C. After fatigue tests, the Young's Modulus did not change significantly.

5 References

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