Thesis project:

Mucosal rheology in the airways of patients with severe lung disease

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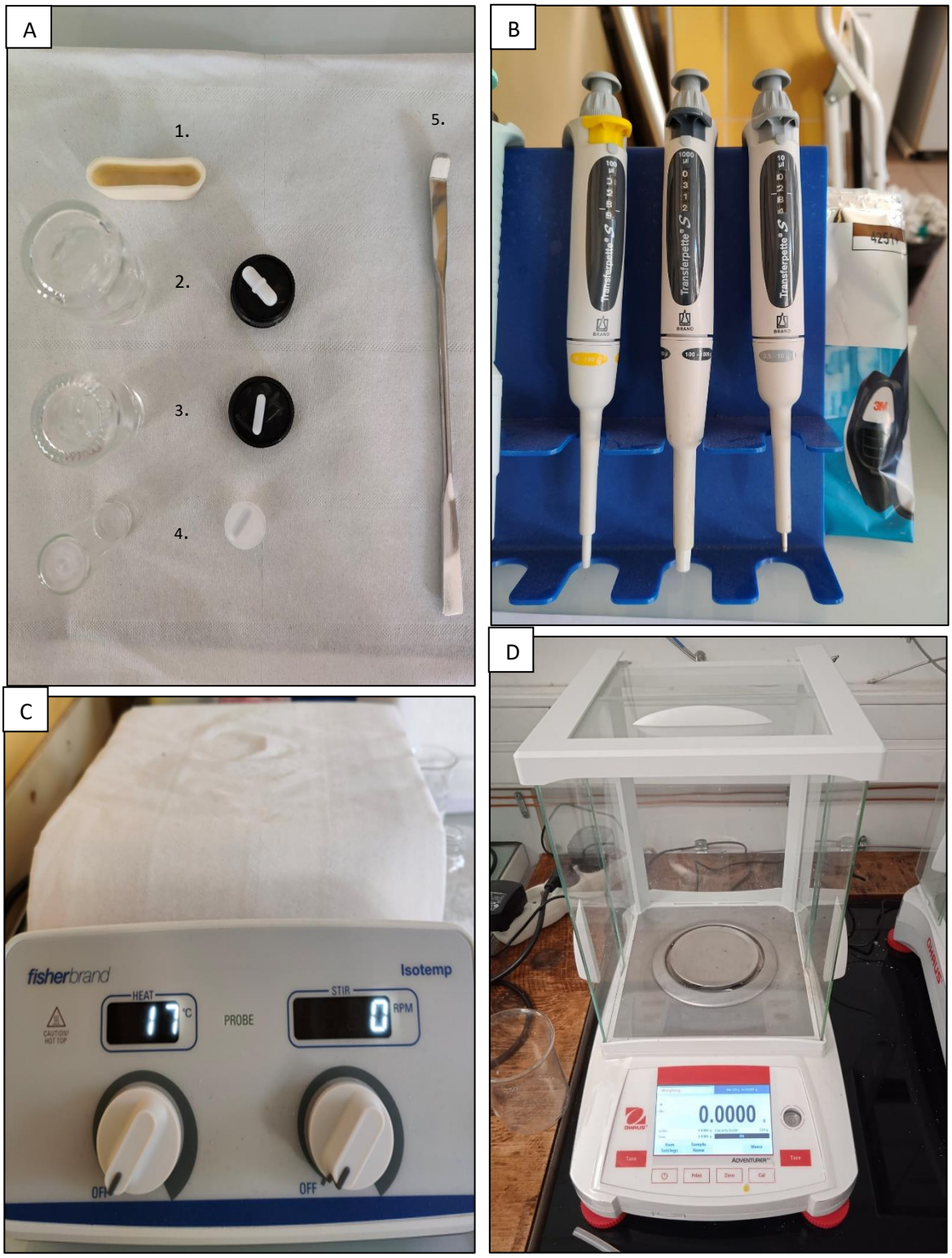
DOE1:

This annex, referred to in Chapter n. 4, aims to present the experimental procedure followed to develop DOE1, showing tools, components and basic steps for organizing the reconstruction of synthetic lung mucosa.

Contents

MATERIALS AND METHODS USED FOR THE RECONSTRUCTION

Here-below the following figures display all the tools and apparatus used during DOE 1



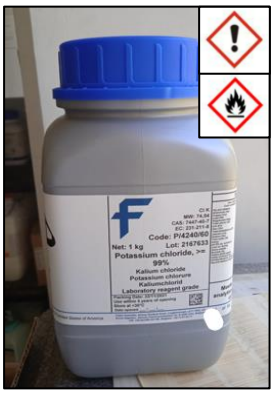
(Fig.A) 1. Ceramic pot to weight the component 2. Bottle (T) complete of stirrer and cup 3. Bottle (-) complete of stirrer and cup 4. Glass vial complete of stirrer and cup. - (Fig.B) Micropipette 1000, 100, 10 µl. (Fig.C) Magnetic Stirrer at controlled rpm and T - (Fig.D) Precision Balance

(Fig.E) Precision Rheometer "Anton Pear MCR 302".

CHEMICAL COMPONENTS of RECONSTRUCTED MUCUS

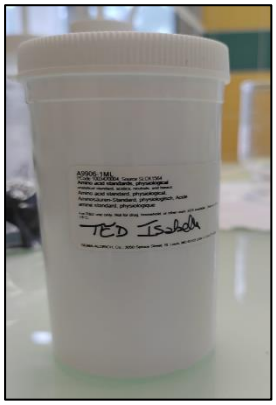
Sodium chloride chemicals.
TSCHEM
(code: 27810295)

Mucin from bovine submaxillary glands.
Sigma Aldrich
(code: M3895)



Potassium Chloride, Extra Pure, SLR, Eur. Ph.
Thermo Fisher scintific
(code: P/4240/60)

Deoxyribonucleic acid from fish sperm.
Sigma Aldrich
(code: 74782)



Amino acid standards, physiological.
Sigma Aldrich
(code: A9906-1ML)

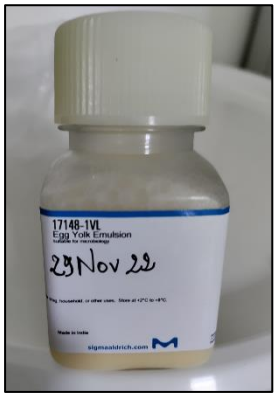
Alginic Acid.
Thermo Fisher Sienticic
(code: 177775000)

Mucin from porcine stomach.
Sigma Aldrich
(code: M2378)

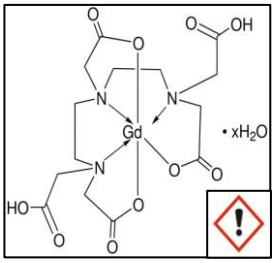
Poly(acrylic acid).
Sigma Aldrich
(code: 306231)

Tris Base (White Crystals or Crystalline Powder/Molecular Biology).
Fisher BioReagents
(code: BP152-1)

Deionised water.
(taken from the laboratory deioniser)
Demineralised water.
(taken from the tank filled by the laboratory distiller)



Egg Yolk Emulsion.
Sigma Aldrich
(code: 17148-1VL)



Diethylenetriaminepentaacetic acid gadolinium(III) dihydrogen salt hydrate
Sigma Aldrich
(code: 481667-5G)

EXPERIENCES PROCEDURE

RECONTRUCTED DIETHYsol$_{0.15M}$ – FIRST DAY

Operative condition 25 °C , 1 Atm

1. Weight a bottle with inside the stirrer and a cap (g).
2. With the help of a micropipette of 1000 µl fill the bottle at pt.1 with (ml) of DEIONIZED H2O
3. Briefly add under constant stirring at 800 rpm the so calculated grams of Diethylenetriaminepentaacetic acid (MM = 393,35 g/mol):

$$M_{DIETHY} = 0.15 \cdot MM_{DIETHY} \cdot V_{H_2O\ pt.1}$$

4. Stir the solution till it will be not homogeneous, without deposed completely Diethylenetriaminepentaacetic acid cristals.
5. Set the so reconstructed DIETHYsol$_{0.15M}$ into the fridge and wait 30 min before to use it.

RECONTRUCTED MUCUS (Part 1) – FIRST DAY

Operative condition 25 °C , 1 Atm

1. Weight a bottle with inside the stirrer and a cap (g).

2. With the help of a micropipette of 1000 µl fill the bottle at pt.1 with (ml) of DEIONIZED H_2O (Better fill with a quantity of nearly 500 µl less than how we had to add, perform a check with the balance and then add the Δ resulting from this measure and the value desired).

3. Briefly add following the order reported under constant stirring at 800 rpm :
   - NaCl (Sigma) (POWDER little spatula) (mg)
   - KCl (Sigma) (POWDER little spatula) (mg)
   - Generic amino acids (LICQUID micro piper 0.100 mL) (mL)
   - DIETHYsol$_{0.15M}$ (LICQUID micro piper 0.100 mL) (mL)
   - MUC5AC (POWDER little spatula) (mg)
   - MUC5B (POWDER little spatula) (mg)
   - DNA (POWDER little spatula) (mg)

   VIP. Use ceramics cup to weight the element with little spatula
   VIP. VIP. For the MUC5AC morphology, similar to a cotton filament (really light and volatile), it is better to weight it performing compact little balls, so pressing it a little bit, managing to better enter it into the bottle avoiding the stickiness of the mucin on the bottle wall and preventing lost in the transfer of it.

4. After 1 h of continuous stirring, perform the **first pH regulation** adding Trizma Base (POWDER little spatula) ,measuring the pH values, add the base (taking note and add on the excel file the quantity of base added and the pH value before and after the addition), stir for 5 min. at 800 rpm, measure again the pH and so on till the neutrality of the sample.

5. Weight another time the bottle at pt 1. (g).

6. Perform the Δ between the weight registered at pt.5 and the one registered at pt.1 and multiply this quantity for 0.009. This will result to be the value of PAA to add in the following pt.

   VIP. This passage is done to have approximately the 0.9% [w/V] of PAA in the reconstructed.
   VIP. VIP. By experimental evidence in this moment the reconstructed has quite the same density of the pure H_2O, for this reason the density of it is considered $\cong$ 1 kg/l.
   VIP. VIP. VIP. Entering the value obtained in the support excel file is also possible to determine the value of Alginate to add in the following pt.

7. Add under constant stirring at 800 rpm the quantity of PAA (POWDER little spatula) calculated at pt.6.

8. After 2h of stirring, perform the **second pH regulation** with the same modality and shrewdness followed at pt.4 reaching the optimum value of 6.8 with the gradual addition of Trizma Base (POWDER little spatula).

9. Weight another bottle with inside the stirrer and a cap (g).

10. Transfer the half of the sample present in the bottle at pt. 8 in the bottle at pt. 9 and perform again the weight of them.

    VIP. It is essential to take note of the actual weight of the two bottles in other to understand the quantity of all the other components to add.
    VIP. VIP. From this moment on the reconstruction of the two bottle will go on in parallel as operations and time of reconstruction but independently. For this reason, from this moment on, we will call the bottle firstly weighted at pt.1 (T) = Treated with High LV. of Alginate & the bottle firstly weighted at pt.5 (-) = Treated with Low LV. of Alginate

11. Set the bottle (-) in the fridge.

RECONTRUCTED MUCUS (Part 2) -SECOND DAY

Operative condition 25 °C , 1 Atm

BOTTLE (-)

1. Weight a vial of glass with inside the stirrer and a cap (g).
2. Take a quantity (nearly 800 µl) of (-) M.S. (LIQUID micropipette 1000 µl) performed as result of the pt.10 FIRST-DAY and set it in the glass vial of pt.1.
3. Weight again the glass vial at pt.2 , complete of stirrer and cap with inside (g).
4. Performing the Δ between the weight measured at pt.3 and pt.1, enter the value in the excel file and calculate the value of Egg yolk to add in the following pt.
5. Add under continuous stirring (800 rpm):
   - DEIONIZED H_2O (micropiper 0.100 mL+ micropipet 0.010 mL) (mL)
   - DNA (POWDER little spatula) (mg)
   - Egg yolk (LIQUID micropipette 10 µl) (µl)
   - Alginate (POWDER little spatula) (g)
6. Stir for 5 min. at 800 rpm before performing the analysis.
7. Weight a vial of glass with inside the stirrer and a cap (g).
8. Take a quantity (nearly 800 µl) of (-) M.S. (LIQUID micropipette 1000 µl) performed as result of the pt.10 FIRST-DAY and set it in the glass vial of pt.1.
9. Weight again the glass vial at pt.2 , complete of stirrer and cap with inside (g).
10. Performing the Δ between the weight measured at pt.3 and pt.1, enter the value in the excel file and calculate the value of Egg yolk to add in the following pt.
11. Add under continuous stirring (800 rpm):
    - DNA (POWDER little spatula) (mg)
    - Egg yolk (LIQUID micropipette 10 µl) (µl)
    - Missing Alginate (POWDER little spatula) (g)
12. Stir for 5 min. at 800 rpm before performing the analysis.

BOTTLE (T)

13. Weight a vial of glass with inside the stirrer and a cap (g).
14. Take a quantity (nearly 800 µl) of (T) M.S. (LIQUID micropipette 1000 µl) performed as result of the pt.10 FIRST-DAY and set it in the glass vial of pt.1.
15. Weight again the glass vial at pt.2 , complete of stirrer and cap with inside (g).
16. Performing the Δ between the weight measured at pt.3 and pt.1, enter the value in the excel file and calculate the value of Egg yolk to add in the following pt.
17. Add under continuous stirring (800 rpm):
    - DEIONIZED H_2O (micropiper 0.100 mL+ micropipet 0.010 mL) (mL)
    - DNA (POWDER little spatula) (mg)
    - Egg yolk (LIQUID micropipette 10 µl) (µl)
18. Stir for 5 min. at 800 rpm before performing the analysis.
19. Weight a vial of glass with inside the stirrer and a cap (g).
20. Take a quantity (nearly 800 µl) of (-) M.S. (LIQUID micropipette 1000 µl) performed as result of the pt.10 FIRST-DAY and set it in the glass vial of pt.1.
21. Weight again the glass vial at pt.2 , complete of stirrer and cap with inside (g).
22. Performing the Δ between the weight measured at pt.3 and pt.1, enter the value in the excel file and calculate the value of Egg yolk to add in the following pt.
23. Add under continuous stirring (800 rpm):
    - DNA (POWDER little spatula) (mg)
    - Egg yolk (LIQUID micropipette 10 µl) (µl)
24. Stir for 5 min. at 800 rpm before performing the analysis.

CONSIDERATIONS

To gain a good repeatability of the measurements and in order to maintain in an optimum working state of the Rheometer it is necessary to pay attention to the following points:

- The analysis has to be performed immediately after the end of the 5 min. at pt6.
- The analysis has to be performed at 32 °C and 1 Atm.
- The H_2O to use to maintain constant the humidity in the chamber has to be DISTILLATE.
- The humidity in the chamber of the Rheometer has to be constant.
- The two half of the chamber have not to touch the superior plate of the Rheometer. To avoid that is possible to regulate the high of the chamber wall.
- Once regulated in temperature, before performing the SAOS test, wait 5 min. so that the components into the sample will resume a stable configuration.
- Between an analysis and one other is necessary wait 15 min. so that the components into the sample will resume a stable configuration.
- Immediately after the analysis both superior and inferior plates, the two half of the cap and the glass wall of the chamber have to be cleaned and sterilised with DEIONIZED H_2O, Methanol, DEIONIZED H_2O and dried carefully with an absorbent paper.

As mentioned, this first DOE was a training ground for understanding how to perform lung mucosa reconstruction. Several improvements were made during the course of the experiments such as the change from 3 to 2 days the reconstruction time. This allowed the experiments to be performed with samples that all had the same history. This point is particularly important because from studies done on real mucosa, it was seen that storage, even in a conditioned environment, damages the rheology of the mucosa. In addition, some improvements could still have been implemented so that more control could have been had over the reconstruction. Among these perhaps the most obvious is the lack of pH regulation after the Alginate adjustment on the second day of reconstruction. It was assumed, however, that since these were only corrections and therefore only a few micrograms in magnitude, they did not decisively impact the pH of the sample. In this regard, further efficiency could have been implemented by making an overall alginate adjustment of the sample (-), monitoring and possibly adjusting its pH before proceeding to division into two vials. Such events may have conditioned, by what is said in the literature section, the structure of the reconstructed. They will therefore be taken up and evaluated in the discussion of the results of this first DOE.