OPTIMIZATION OF BIOINK FOR 3D PRINTING OF HUMAN FEMALE REPRODUCTIVE TRACT

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Professional practice

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1. INTRODUCTION

Today the manufacturing industry is in the process of developing a new concept that is revolutionizing it in different sectors such as: electronics, automotive, fashion, and even medicine [1]. This new technology is 3D printing (also called additive manufacturing), which has been in development for the last 4 decades. But what is 3D printing? In general, it is defined as a process in which an object is manufactured in 3 dimensions from a 3D model using additive processes, where successive layers of a desired material are placed with computer control [1].

Along with the development of 3D printing, another new technology, 3D bioprinting, has emerged. This represents one of the latest technological advances widely used in regenerative medicine and tissue engineering. Its main goal in these areas is to replicate complex tissue structures like native organs and tissues [2]. Like 3D printing, it consists of depositing layer after layer of a material, with the particularity that now, these layers are made of biomaterials loaded with cells, with which a predetermined architecture is sought to recreate functional tissues or organs, the latter being created from tissue engineering scaffolds, with controlled permeability, porosity and mechanical properties [3]. It is important to highlight that this technology offers reduced manufacturing costs and a compromising production speed [4].

Among the many applications that 3D bioprinting has today in medicine, applications in obstetrics and gynecology stand out, the following are some examples: firstly, uterine fibroids, this technology is used for preoperative simulations, intraoperative guidance, and teaching, making use of 3D printed tumor models. Secondly, applications focused on cervical, endometrial and ovarian cancer, its application lies as in the previous case, in preoperative simulations, intraoperative guidance, teaching, radiotherapy and chemotherapy; all this thanks to 3D printed tumor models, models of surrounding tissue, in vitro cultures and in vivo cultured animal models, which seek to identify more quickly the invasion of lesions and surroundings, in addition, facilitate guidance and surgical planning to minimize physical injury and the development of personalized chemotherapy regimens. Thirdly, there is Premature Ovarian Failure or (POF) [5], this condition usually causes infertility and difficulty in conception, as well as other comorbidities; in this case the application focuses on 3D printing a model of the ovarian tissue in order to be able to perform their basic research, which can facilitate the search for an effective treatment [6]. And so, many more applications.

Now, going deeper into the last application mentioned (premature ovarian failure or POF), it is known that it occurs in 1% of women, where 1 out of every 100 women is under 40 years old, and 1 out of every 1000 women is under 30 years old [7]. Currently, research is underway to understand and treat the different causes of infertility in women, such as POF.

For the development of these studies and new technologies, it is important and very useful to be able to recreate the tissues and the native female reproductive organ to simulate the behavior of new treatments, and this is where 3D bioprinting comes into play.

Considering the above, for the creation of new technologies focused on the study and development of treatments for the female reproductive organ, it is important to start by getting to know it. As shown in Figure 1, it is mainly composed of the cervix, vagina, uterus, and ovaries, which, in turn, are supported by ligaments, fasciae and muscles. This organ has surprising mechanical properties, as it has the capacity to undergo great deformations without breaking, which makes it incredibly strong [8].

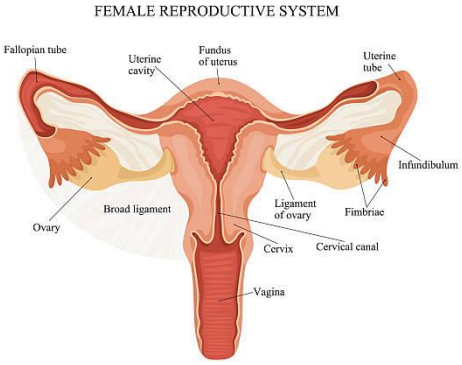


Figure 1. Female reproductive tract. Taken from [9]

Some studies such as [8], [10] y [11] have been able to characterize some of their tissues, yielding results such as those shown in Table I.

Table I
ELASTIC MODULE OF THE FEMALE REPRODUCTIVE SYSTEM

Female reproductive tract area	Elastic modulus
Uterine tissue	5 KPa
Uterus	2,5 - 30 MPa
Uterine neck	2,17 – 243 KPa
Associated ligaments	2,17-243 kPa
Cardinal ligament	0,5-5,4 MPa
Round ligament	9,1-14,0 MPa
Uterosacral ligament	0,75-29,8 MPa
Cervix	2.17–243 kPa
Oviduct	11,5 kPa

Once the characteristics of the real tissue are known, it is possible to proceed to the development of a bioink that achieves a result like these characteristics.

Having said that, to print a 3D model of the female reproductive organ with biomaterials, many printers that facilitate these processes can be found on the market. This is the case of the Lumen X printer of the company CELLINK, this printer works through digital light processing (DLP), offering high resolution, high performance, and fidelity in the

prints, being useful for applications in microfluidics, hydrogels, microporous structures, among others [12]. This printer can be seen in Figure 2.

Figure 2. Lumen X™ printer.

In [12] the supplier indicates the most commonly used bioinks in this printer: Gelatin methacrylate (GelMa) and Polyethylene glycol diacrylate (PEGDA), but it should be noted that this printer works with a wavelength of light projected at 405 nm, which indicates that it can be functional with other biomaterials as long as the bioinks created react to this wavelength and achieve the necessary polymerization to successfully obtain the 3D structure.

However, to obtain good results at the time of printing, there are certain printing parameters that are essential to achieve a 3D structure with good characteristics, resolution, and definition. These parameters vary depending on the bioink being used and its composition [13]. These parameters are:

- Power (mW/cm^2): It is the power of the laser or light source used in the printing process. In this case where the Lumen X printer uses the photopolymerization process, the ultraviolet light source is used to solidify layers of the photosensitive bioink. As for the unit of measurement "mW/cm^2" it refers to milliwatts per square centimeter and expresses the intensity of light power per unit area. It is set to control the speed of solidification of the bioink and thus affects the accuracy and quality of the print. [14].

- Layer Height (µmIt indicates the height that each layer of the print will have or the vertical distance between two consecutive layers of deposited material. It is measured in micrometers (µm). This value influences the resolution of the resulting structure. The smaller this value is, the thinner the layers will be and the higher the resolution will be, in turn, the longer the printing time will be [15]. Two options are available for the Lumen XTM printer, 50 µm and 100 µm.

- Exposure Time (s): Refers to the time during which each layer of the print is exposed to the light source (ultraviolet light) to solidify the photosensitive material.

When this is set, you are controlling how long the light hits the material to harden the current layer. This parameter is vital to achieve proper curing and solidification of the material in each layer, ensuring that the layers are solid enough to support the upper layers and form a coherent three-dimensional object [16].

- 1st Layer Time Scale Factor: It is a time scale factor applied specifically to the first layer of the print. This parameter adjusts the duration of the exposure time or printing time for the first layer compared to the subsequent layers. It allows adjusting the exposure time or print time to ensure better adhesion of the object to the build surface (usually the print bed). The first layer is crucial to establish a solid and uniform base [17].

Each of the above plays a fundamental role in the result of the printed object. That is why it is important to have knowledge of the functions of each parameter to identify the best values at the time of printing.

On the other hand, thanks to a preliminary work based on [18], we had the recipe for a PEGDA-based bioink, which was given the name PEGDA-Homemade, which will be shown and explained later. This bioink was the starting point, i.e., this bioink composed of PEGDA, Orange G, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) and water, is the basis on which the optimization is sought.

In that sense, it is necessary to be clear first what PEGDA is, it is a derivative of polyethylene glycol which has several applications in drug delivery and tissue engineering. It is also used as a prepolymer solution in the formation process of cross-linked polymeric systems [19]. PEGDA has been a biomaterial with an important role in the manufacture of hydrogels due to its excellent biocompatibility, high water content and its great ability to promote cell growth and proliferation [20]. This is why it is a great candidate for the formulation of bioinks.

Having said the above, and bearing in mind the concept of what PEGDA is, a literature search is carried out on the development of new PEGDA-based bioinks that have presented characteristics in their mechanical properties like the desired ones, i.e., similar to the values shown in Table I.

To this end, a study developed in China proposes the realization of a bioink for printing hydrogels composed mainly of Alginate, PEGDA and PVA (Polyvinyl alcohol), with the addition of these components they managed to obtain elasticity moduli between 3.08 MPa and 6.77 MPa [21], values that may be related to the objective of this study. They also found another research where they propose a bioink composed of GelMa (Gelatin methacrylate) and PEGDA, this was characterized by being soft, having low stiffness and good elasticity, because by varying the concentration of these, they obtained hydrogels with a tensile modulus of 10kPa and a compression modulus of 0.8 kPa, besides being able to find that it has excellent biocompatibility and that it greatly favors cell growth [22].

With the information found in the previous articles, a bioink was proposed to obtain 3D prints with the mechanical properties previously mentioned. For this purpose, in the present study the bioink made with only PEGDA is reformulated, working now additionally

with PVA, Alginate and GelMa. For its characterization, pH tests were performed, to know the resistance of the materials to different environments and compression tests to obtain the modulus of elasticity of each material.

This report is divided into 3 main parts, the first section is a brief introduction, where theoretical and necessary information for the execution of the study is shown, as well as the objectives and the methodology used. The second section shows the results obtained, and finally, in the third section, an analysis of what was achieved, obtaining some conclusions and ideas for future work.

2. OBJECTIVES

2.1. General

1. Optimize a PEGDA-based bioink for 3D printing of the human female reproductive organ, to achieve a print with mechanical properties that more accurately simulates real tissue.

2.2. Specific

1. Find the best printing parameters for PEGDA-Homemade bioink.
2. Search for new components to formulate a new bioink that achieves more elastic and less rigid prints.
3. Optimize the proportions of each biomaterial that makes up the formula of the proposed new bioink.
4. Perform compression tests on the printings achieved with the proposed new bioinks.
5. Perform pH test on the printings.
6. Analyze and compare the information obtained from the new bioinks with the characteristics of the female reproductive organ tissue.

3. METHODOLOGY

3.1. Problem to solve.

3D printing today has several applications in medicine, and many of them are still under development. In the present study, the optimization of a PEGDA-based bioink is sought to achieve a 3D printing with mechanical properties as similar as possible to the real tissue of the human female reproductive organ, with the aim of printing this organ and being able to replicate it to use this model as a simulation and test other technologies under development.

3.2. Project phases

To start this project, the following Gantt chart was proposed to have a better control of time and a good distribution of activities to achieve each of the objectives.

Table II
GANTT CHART

Activity ↘ / **Week →**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1																
2																
3																
4																
5																

Activity

1. Literature study
   - Study on the female reproductive system.
   - Mechanical properties of human tissue.
   - Reading about Lumen X.
   - Study on PEGDA, commercial and others.
   - What has been done so far to improve these features?
2. Proposal of the new recipe.
3. Printing of material samples.
4. testing of material characteristics (compression test and pH test).
5. Preparation of the final document

Once the time and activities for each week were organized, the phase-by-phase development of the research was as follows.

First, a literature and state of the art study was carried out to learn more about fundamental concepts that would be useful throughout the project, and to get an idea of the current state of research on bioinks for 3D bioprinting with a focus on applications in medicine. This was done by searching in repositories such as PubMed, in information analysis pages such as Elsevier, in the academic Google search engine and in the center of resources for learning and research (CRAI) of the Universidad del Rosario. Searches

were performed using commands such as 'AND' and 'OR', accompanied by keywords such as 'bioprinting', 'PEGDA', 'bioink', 'female reproductive tract', among others.

Having enough theoretical information to start the project, the following stages of the study were carried out:

3.2.1 General analysis of PEGDA-Homemade bioink, varying concentration of PEGDA in the recipe and varying printing parameters.

As mentioned above, thanks to preliminary work, the laboratory had the recipe for a PEGDA-based bioink (PEGDA-Homemade). The first thing that was done was to prepare this bioink with the protocol and recipe shown in Table III.

Table III
PEGDA-HOMEMADE (RECIPE AND PROTOCOL)

RECIPE (PEGDA-Homemade)	PROTOCOL
• Water 10 ml • PEGDA 30% = 3g • Orange G 0.03% = 0.003 g • LAP 0.03% = 0.003 g	• Add all components in a test tube and mix until a homogeneous ink is obtained.

After the bioink was prepared, small cubes were printed as shown in Figure 3, this was done at different printing parameters to identify which of these parameters yielded a print with better precision, definition, and good quality.

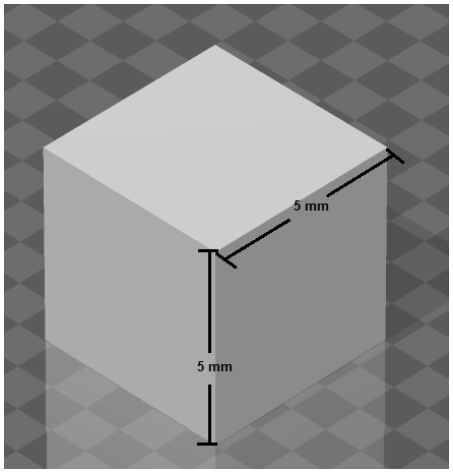


Figure 3. Cube design for printing with different parameters

The parameters to be tested are shown in Table IV.

Table IV
VALUES OF THE TESTED PRINTING PARAMETERS FOR PEGDA-HOMEMADE BIOINK

Power (%)	Exposure time (s)	1st Layer time	Resolution (µm)
65	12	3X	100

45	10	3X	100
35	12	3X	100
50	6	3X	100
35	6	3X	100

After identifying the best printing parameters, we tested varying the concentration of PEGDA in the recipe, maintaining the original concentrations of Orange G and LAP, to see what changes the prints presented. It is important to remember that the concentration of PEGDA in the original recipe is 30%. The variations in the recipe are shown in Table V.

Table V
VARIATIONS IN PEGDA CONCENTRATIONS IN THE ORIGINAL RECIPE

Water mL	PEGDA	ORANGE G	LAP
5	40% = 2g	0.03% = 0.0015g	0.03% = 0.0015g
5	20% = 1g	0.03% = 0.0015g	0.03% = 0.0015g
5	15% = 0.75g	0.03% = 0.0015g	0.03% = 0.0015g
5	10% = 0.5g	0.03% = 0.0015g	0.03% = 0.0015g

3.2.2 Reformulation of bioink by adding GelMa to the recipe

The next step was to add GelMa to the original formula with different concentrations, the proposed recipes are shown in Table VI:

Table VI
3 PROPOSALS FOR NEW BIOINKS WITH GELMA AND PEGDA

Bioink	Recipe	Printing parameters
GelMa-PEGDA 1:1	- Water: 10 mL - LAP: 0,03% = 0,003g - Orange G: 0,03% = 0,003% - GelMa: 10% = 1g - PEGDA: 10% = 1g	Power : 70% Exposure time (s): 20s 1st Layer time: 3X
GelMa-PEGDA 4:1	- Water: 10 mL - LAP: 0,03% = 0,003g - Orange G: 0,03% = 0,003% - GelMa:12% = 1,2g - PEGDA: 3% = 0,3g	Power : 70% Exposure time (s): 20s 1st Layer time: 3X
GelMa-PEGDA 1:4	- Water: 10 mL - LAP: 0,03% = 0,003g - Orange G: 0,03% = 0,003% - GelMa: 3% = 0,3g - PEGDA: 12% = 1,2g	Power : 70% Exposure time (s): 20s 1st Layer time: 3X

For the protocol of the formula with GelMa, initially the latter should be put on the plate at 57°C for approximately 20 minutes while mixing at the same time, to achieve a liquid consistency. After that, put all the components into a test tube and mix the solution until a homogeneous consistency is achieved.

Once the bioinks were made, samples were printed in the form of a cube, as shown in Figure 3, to observe the consistency and behavior of the prints with these bioinks.

3.2.3 Reformulation of the ink with new components

Based on studies found in the literature, the PEGDA-based bioink recipe was reformulated and a bioink with PVA and additional alginate was proposed. After several tests were carried out on the bioink and the appropriate viscosity to be able to work it in the Lumen X printer, the formulas shown in Table VII were studied.

Table VII
NEW FORMULATIONS OF BIOINKS WITH PVA AND ALGINATE. PROTOCOL, RECIPE AND PRINTING PARAMETERS. FOR PEGDA CONCENTRATION ≤10%

BIOINK	RECIPE	PROTOCOL
PEGDA+PVA	• Water 10 mL • LAP 0.03 % = 0.003 g • ORANGE G 0.03% = 0.003 g • PEGDA 10% = 1g • PVA 3% = 0.3 g Recommended printing parameters: 65% 12s 2X	1. Heat the water in the iron to 130°C, this will bring the water to 90°C, when it is hot adding the PVA and mix at the same time for 2 hours. 2. After, wait until the water is at room temperature and add the LAP, Orange G and PEGDA 3. Mix P.S.: If you observe lumps, put the ink for about 20 min in the incubator at 37°C, then mix again.
PEGDA + ALGINATE	• Water 10 mL • LAP 0.03 % = 0.003 g • ORANGE G 0.03% = 0.003 g • PEGDA 10% = 1g • ALGINATE 1% = 0.1 g Recommended printing parameters: 60% 12s 2X	• Add all components in a test tube and mix until a homogeneous ink is obtained. P.S.: If you observe lumps, put the ink for about 20 min in the incubator at 37°C, then mix again.
PEGDA + PVA + ALGINATE	• Water 10 mL • LAP 0.03 % = 0.003 g • ORANGE G 0.03% = 0.003 g • PEGDA 8% = 0.8 g • PVA 3% = 0.3 g • ALGINATE 1% = 0.1 g Recommended printing parameters: 65% 15s 3X	1. Heat the water in the iron to 130°C, this will bring the water to 90°C, when it is hot adding the PVA and mix at the same time for 2 hours. 2. After, wait until the water is at room temperature and add the LAP, Orange G, PEGDA and Alginate. 3. Mix P.S.: If you observe lumps, put the ink for about 20 min in the incubator at 37°C, then mix again.

The above table shows recipes for inks with a concentration of PEGDA ≤10%, 3 other inks with a higher concentration of PEGDA were also proposed, these are listed in Table VIII.

Table VIII
NEW FORMULATIONS OF BIOINKS WITH PVA AND ALGINATE. PROTOCOL, RECIPE AND PRINTING PARAMETERS. FOR PEGDA CONCENTRATION 18%

BIOINK	RECIPE	PROTOCOL
PEGDA+PVA	• Water 10 mL • LAP 0.03 % = 0.003 g • ORANGE G 0.03% = 0.003 g • PEGDA 18% = 1,8g • PVA 3% = 0.3 g Recommended printing parameters: 50% 10s 3X	1. Heat the water in the iron to 130°C, this will bring the water to 90°C, when it is hot adding the PVA and mix at the same time for 2 hours. 2. After, wait until the water is at room temperature and add the LAP, Orange G and PEGDA 3. Mix P.S.: If you observe lumps, put the ink for about 20 min in the incubator at 37°C, then mix again.
PEGDA + ALGINATE	• Water 10 mL • LAP 0.03 % = 0.003 g • ORANGE G 0.03% = 0.003 g • PEGDA 18% = 1,8g • ALGINATE 1% = 0.1 g Recommended printing parameters: 50% 10s 3X	• Add all components in a test tube and mix until a homogeneous ink is obtained. P.S.: If you observe lumps, put the ink for about 20 min in the incubator at 37°C, then mix again.
PEGDA + PVA + ALGINATE	• Water 10 mL • LAP 0.03 % = 0.003 g • ORANGE G 0.03% = 0.003 g • PEGDA 18% = 1,8g • PVA 3% = 0.3 g • ALGINATE 1% = 0.1 g Recommended printing parameters: 50% 8s 3X	1. Heat the water in the iron to 130°C, this will bring the water to 90°C, when it is hot adding the PVA and mix at the same time for 2 hours. 2. After, wait until the water is at room temperature and add the LAP, Orange G, PEGDA and Alginate. 3. Mix P.S.: If you observe lumps, put the ink for about 20 min in the incubator at 37°C, then mix again.

3.2.4 pH test

Samples were printed with the bioinks in Table VII using the design in Figure 3, while these prints were being made, solutions with different pH levels were prepared; these buffer solutions had the following values:

- Very acid pH = 1.035
- Moderately acid pH = 3.540
- Slightly acid pH = 6.820
- Neutral pH = 7.082
- Slightly alkaline pH = 8.580
- Moderately alkaline pH = 10.105
- Alkaline pH = 12.442

The protocol to be followed was as follows:

Print samples of each material and put the samples in the above solutions for 24 h in the incubator at 37°C. Observe the reactions and differences after that.

3.2.5 Compression test

After the bioinks were prepared, samples of each material were printed to perform their respective compression tests. For this purpose, a cylinder was designed (Figure 4), since according to the literature, the cylinder shape is the most recommended for performing such tests due to the distribution of the force applied during the test along the figure [22].

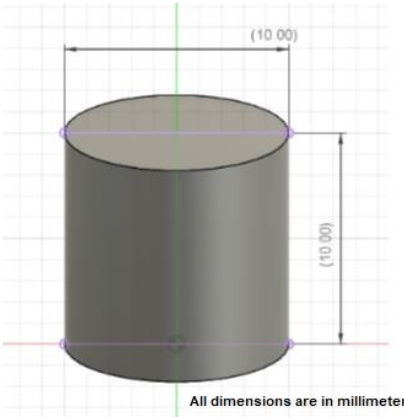


Figure 4. Print design for compression testing.

The Univert 1Kn machine from CellScale was used, as shown in Figure 5.

Figure 5. Compression testing machine, Univert 1Kn

For the execution of the compression tests, each of the samples was tested in an aqueous medium due to its natural and application, since we are working with hydrogels

and in addition to the fact that these must remain in an aqueous medium, their application is also intended for the impression to be in a humid medium, this is shown in Figure 6.

Figure 6. Arrangement of the samples in the compression test machine.

It should be noted that two types of tests were performed, the first was to subject the samples to compression for 10 cycles and the second was to apply a pressure of 10 N to the samples until the sample reached the fracture point. The machine parameters used for each of these tests are shown in Table IX.

Table IX
PARAMETERS FOR THE PERFORMANCE OF THE TWO TYPES OF COMPRESSION TESTS

Cyclic compression	Application of force up to the fracture point
- Control mode: displacement - Stretch magnitude: 3 mm = 30% - Timeout (s): 5 - Preload magnitude (N): 0.1 - Stretch Duration (s): 10 - Repetitions: 10 - Recovery duration (s):10	- Control mode: Force - Cell loan: 10N - Timeout (s): 5 - Stretch Duration (s): 10

After the data were obtained, they were processed in Excel. For each of the samples, the following procedure was carried out:

a. First, the value of the cross-sectional area of the design used was obtained, as shown in Figure 4, a cylinder of height 10mm and diameter 10mm was used, that is, a radius of 5mm. Knowing this, the cross-sectional area was found as shown in equation 1.

$$(1)\ A = (\pi \times 0{,}005^2) = 0{,}0000785\ (\text{m}^2)$$

b. Then, the value of Strain (ε) was obtained for each sample, using the equation 2.

$$(2)\ \varepsilon = \frac{(\Delta L)}{L_0} * 100\ (\%)\ \ [23]$$

Where ΔL is the change in length and L_0 is the initial length.

c. The next step was to find the value for Stress (σ), for this we made use of equation 3.

$$(3)\ \sigma = \frac{\frac{F}{A}}{1000}\ (\text{kPa})$$

Where A corresponds to the value of the cross-sectional area in m^2 and F is the applied force in N. It is divided by 1000 so that the units are in kPa [24].

d. With the values of Strain (ε) and Stress (σ) the behavior of the sample in the compression test is plotted to calculate the value corresponding to the modulus of elasticity (E), with equation 4, seeking to have a graph similar to the one shown in figure 7.

$$(4)\ E = \frac{\sigma}{\varepsilon}$$

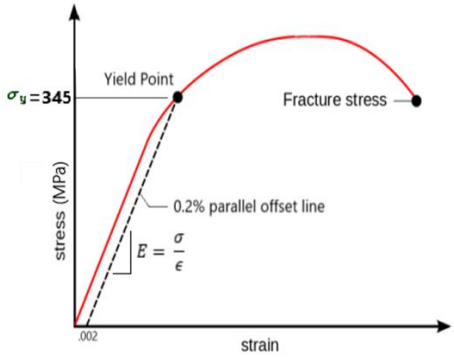
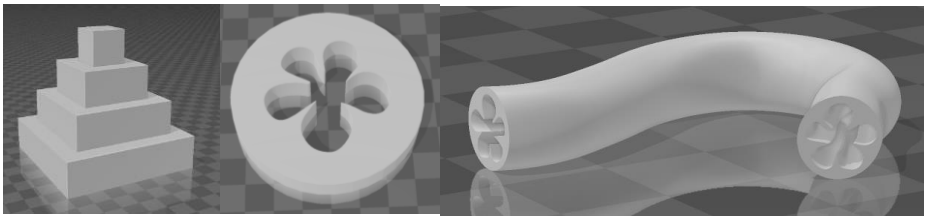


Figure 7. Strain vs Stress graph. Taken from [25]

a. Finally, with the values of the modulus of elasticity of each sample of each material, an average was made between them to arrive at a final value that could characterize the modulus of elasticity of each bioink tested.

3.2.6 Printing of more elaborate structures

Finally, some prints with more elaborate structures were made with the bioinks in Table VII to observe the performance of the new material proposal when printing more complex shapes, some of the printed designs were the following:



A *B* *C*

Figure 8. A) pyramid with stairs, B) cross section of the oviduct, C) oviduct of the human female reproductive organ.

We worked with the methodology proposed, and the results are shown below.

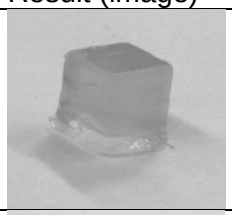
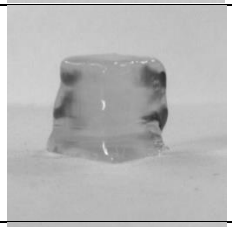
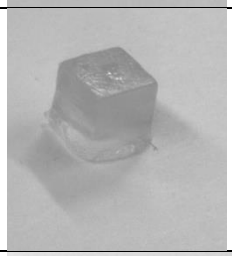
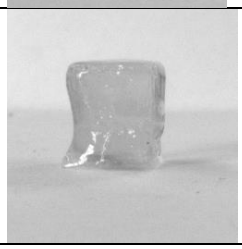
4. RESULTS

Once the protocols indicated above in the methodology had been carried out, the results obtained for each of the tests were as follows:

4.1 General analysis of PEGDA-Homemade bioink, varying PEGDA concentration in the recipe and varying printing parameters.

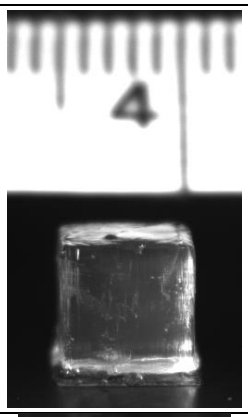
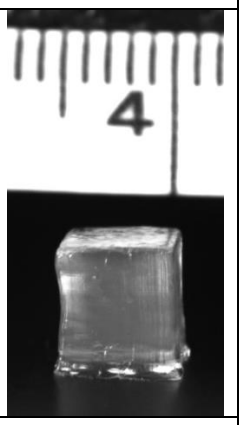
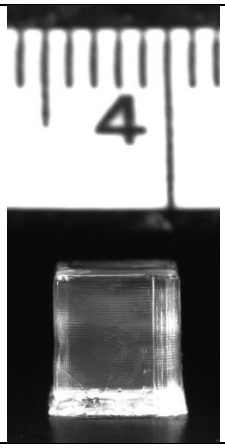
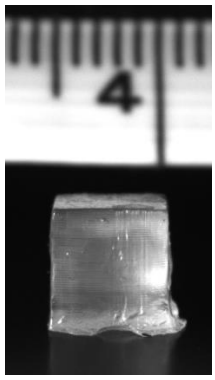
After preparing the PEGDA-Homemade bioink using the materials and the protocol shown in Table III, cubic samples were printed with the printing parameters shown in Table IV, the results are shown in Table X.

Table X
PEGDA-HOMEMADE PRINTS WITH DIFFERENT PRINTING PARAMETERS

Parameters	Result (image)
65% 12s 3X	
45% 10s 3X	
35% 12s 3X	
50% 6s 3X	
35% 6s 3X	

Once the best printing parameters were identified, the next step was to print cubic samples using the bioinks in Table V. The results are as follows:

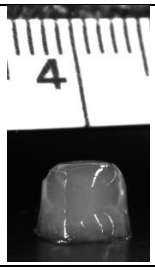
Table XI
RESULTS, PRINTS USING BIOINKS WITH DIFFERENT CONCENTRATIONS OF PEGDA

PEGDA Concentration	Result	PEGDA Concentration	Result
10%		20%	
15%		40%	

4.2 Reformulation of bioink by adding GelMa to the recipe

PEGDA and GelMa are two materials very commonly used in bioprinting as discussed above, so 3 bioinks were proposed (Table VI) where both materials were included. The results are shown in Table XII.

Table XII
GELMA-PEGDA BIOINK PRINT RESULTS

GelMa-PEGDA 1:1	GelMa-PEGDA 4:1	GelMa-PEGDA 1:4
		

4.3 Reformulation of the ink with new components

Now, as shown in Tables VII and VIII, a composite bioink with PEGDA, PVA and Alginate in different concentrations was proposed, the observations of the prints are shown in Table XIII.

Table XIII
GENERAL REMARKS ON PRINTING WITH PEGDA-PVA-ALGINATE BIOINKS

Recipe	Observations
PEGDA-Alginate	Works well It looks very good, is transparent, that is good, it is soft.
PEGDA-PVA	Works well Looks good too, it is not so transparent like just with alginate, but is soft and it has good resistance
PEGDA-PVA-Alginate	Works well Looks good too, but it breaks easily, then I need to try with different concentrations of the chemicals
PEGDA-PVA-Alginate Changing the LAP to I-2959	Didn't work, it is possible that the problem is about the photo initiator

It is important to highlight that, at this stage of the research, the bioink was tested with the Irgacure 2959 photo initiator (as shown in the previous table), trying to follow the recipe indicated in the base article [21] and in addition to this, the Orange G component was removed to achieve more transparency in the prints. For this last purpose, the test was carried out by eliminating the bioink component, adding only 0.01% concentration and adding 0.02% concentration.

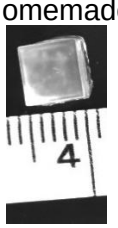
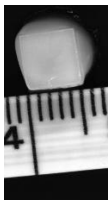
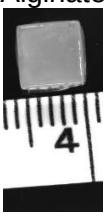
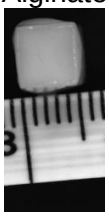
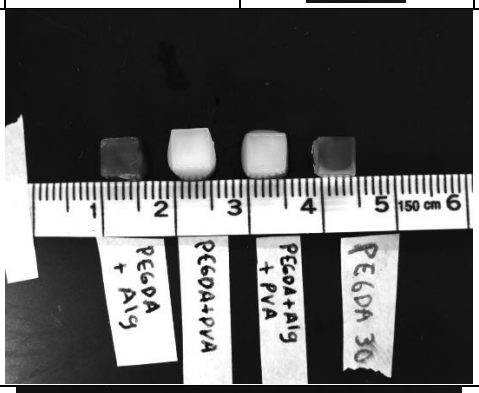
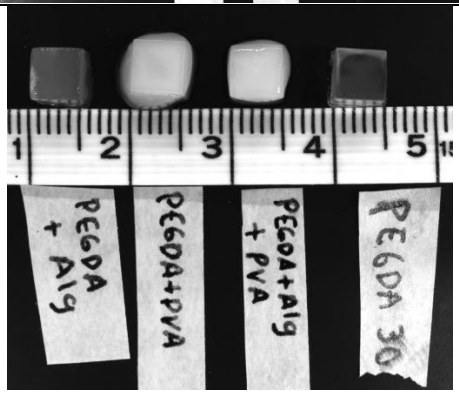
For the cases where Orange G was completely annulled and where only 0.01% concentration was added, the results for both cases are as shown in Figure 9.

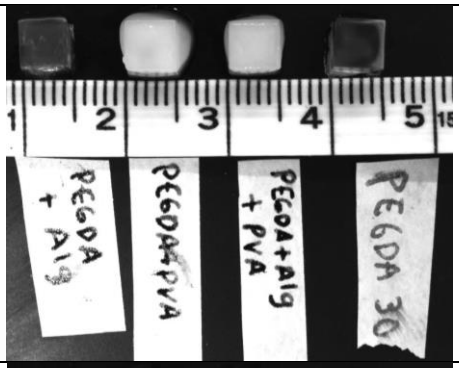
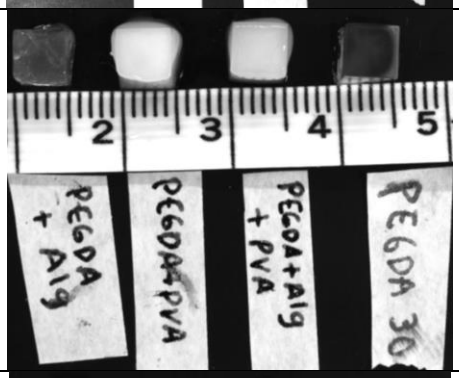
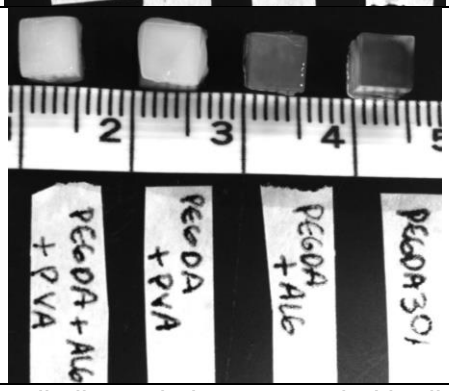
Figure 9. Printing without Orange G

4.4 pH Test

Having identified the best printing parameters and having defined the new bioink proposal, a pH test was carried out with the process indicated previously in the methodology. After having the samples for 24 hours in the different solutions, the results are shown in Table XIV.

Table XIV.
SAMPLES OF THE 4 BIOINKS AFTER 24 HOURS IN SOLUTIONS AT DIFFERENT PH VALUES

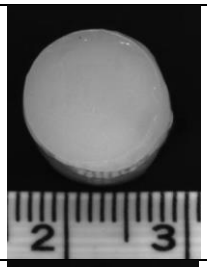
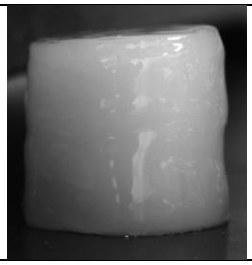
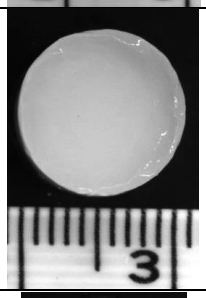
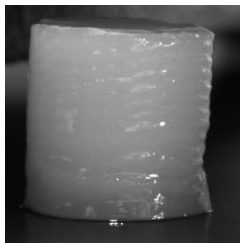
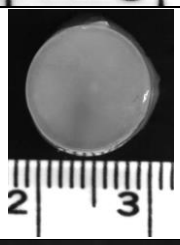
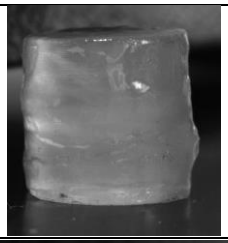
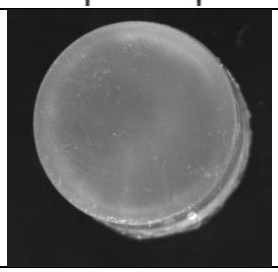
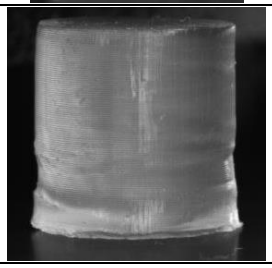
pH ↓	Control →	PEGDA-Homemade	PEGDA-PVA	PEGDA-Alginate	PEGDA-PVA-Alginate
					
Very acid					
Moderately acid					

Slightly acid		
neutral		
Slightly alkaline		
Moderately Alkaline		
Alkaline	After 24 hours, the alkaline solution succeeded in disintegrating the 4 prints of different bioinks.	

4.5 Compression test

First, the design used was a cylinder as mentioned above, Table XV shows some of the models printed for the present test.

Table XV.
SOME CYLINDER-SHAPED IMPRESSIONS FOR COMPRESSION TESTS

PEGDA 8% +PVA+Alginate		
PEGDA 10% +PVA		
PEGDA 10% +Alginate		
PEGDA-Homemade		

After printing the samples of each bioink, the values of the elastic modulus found by the compression test for each of the materials in Tables III, VI, VII and VIII are shown in Table XVI.

Table XVI.
VALUES OF THE ELASTIC MODULUS FOR THE 9 STUDIED BIOINKS

Bioink	Elastic modulus
GELMA-PEGDA 1:1	0,14 kPa ± 5%
GELMA-PEGDA 1:4	0,26 kPa ± 2,3%
PEGDA18%+ALG	0,90 kPa ± 0,6%

PEGDA18%+PVA	0,77 kPa ± 1,18%
PEGDA18%+PVA+ALG	0,62 kPa ± 0,5%
PEGDA10%+ALG	0,320 kPa ± 0,48%
PEGDA10%+PVA	0,369 kPa ± 0,09%
PEGDA8%+PVA+ALG	0,039 kPa ± 6,15%
PEGDA 30% - Homemade	4,432 kPa ± 0,99%

For each of the materials the Strain vs Stress graph was made to determine the elastic modulus, these graphs were compared grouping them in 3 large figures, the first one which is figure 10, shows the behavior of the impressions with GelMa-PEGDA. Figure 11 shows the behavior of the impressions with a concentration of 18% of PEGDA, using both Alginate and PVA. Figure 12 shows the behavior of the impressions with PEGDA concentrations of 10%, with Alginate and PVA, as well as the behavior of the impression made with the PEGDA-Homemade bioink.

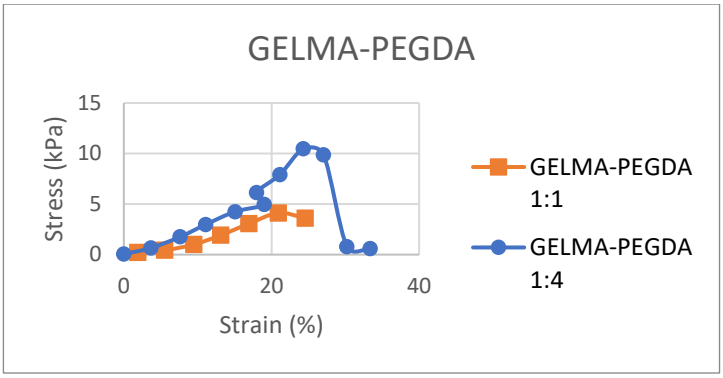


Figure 10. Stress vs Strain graph of the impressions made with different concentrations of GelMa-PEGDA.

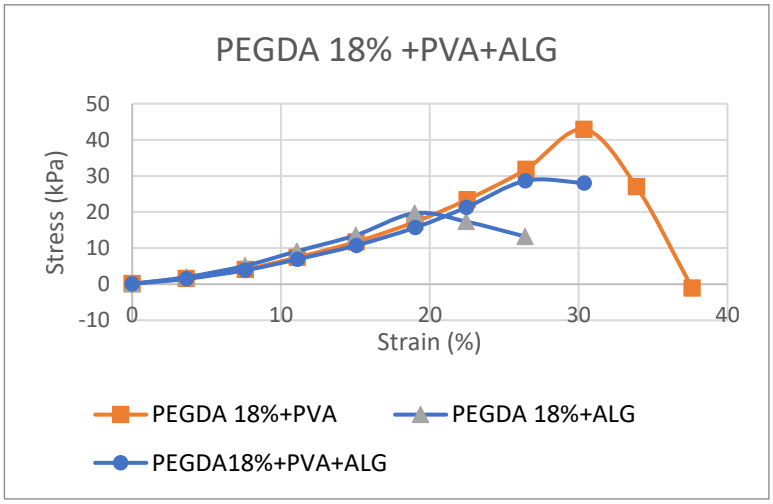


Figure 11. Behavior of impressions with PEGDA 18%, Alginate and PVA bioinks.

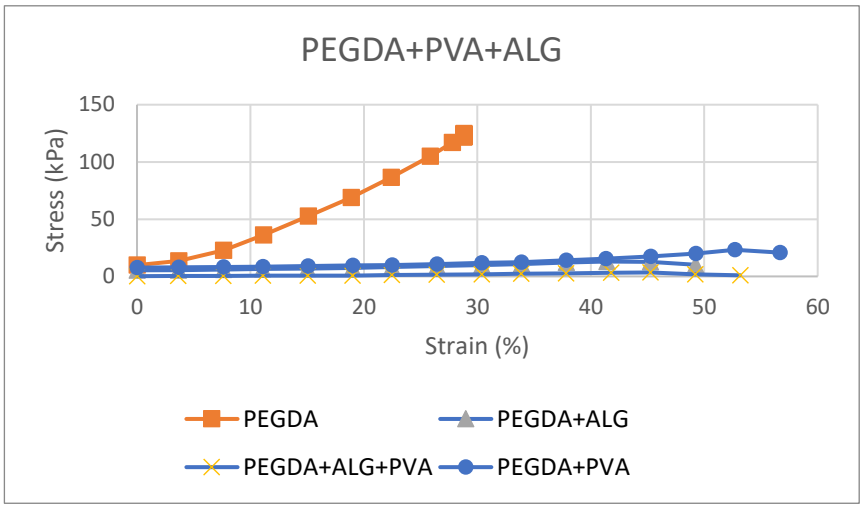


Figure 12. Performance of impressions with PEGDA 10%, Alginate, PVA and PEGDA-Homemade bioinks.

In addition, it was possible to determine the value of the maximum force resisted by each material before rupture and the maximum percentage of deformation presented by each material. These values are shown in Table XVII.

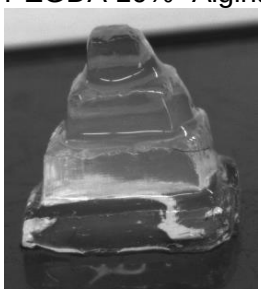
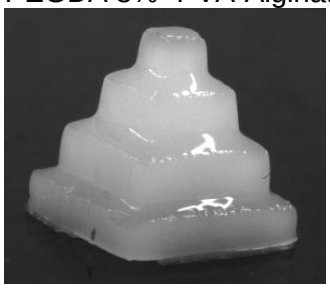
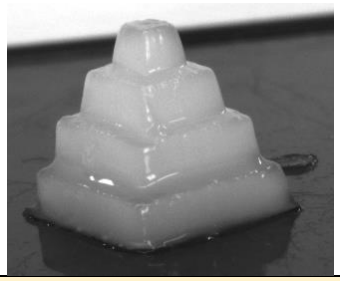
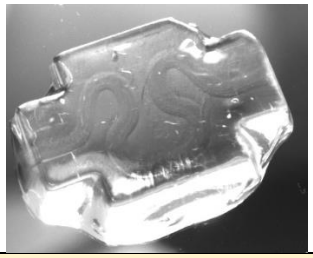
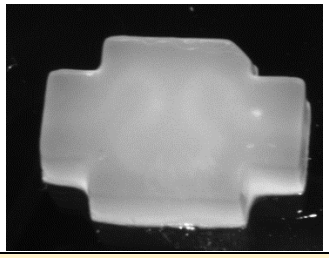
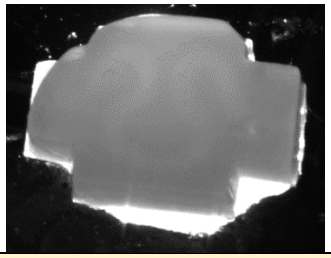
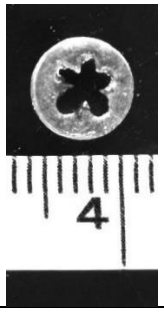
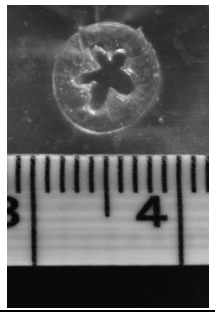
Table XVII.
MAXIMUM FORCE AND MAXIMUM STRAIN FOR EACH BIOINK

Bioink	Max. Force	Max. Strain
GELMA-PEGDA 1:1	0,9 N	22%
GELMA-PEGDA 1:4	1,13 N	25%
PEGDA18%+ALG	1,49 N	27,2%
PEGDA18%+PVA	3,8 N	32,1%
PEGDA18%+PVA+ALG	2,7 N	26,8%
PEGDA10%+ALG	1,11 N	51,5%
PEGDA10%+PVA	2,012 N	56,6%
PEGDA8%+PVA+ALG	0,296 N	47,62%
PEGDA 30% - Homemade	9,05 N	29%

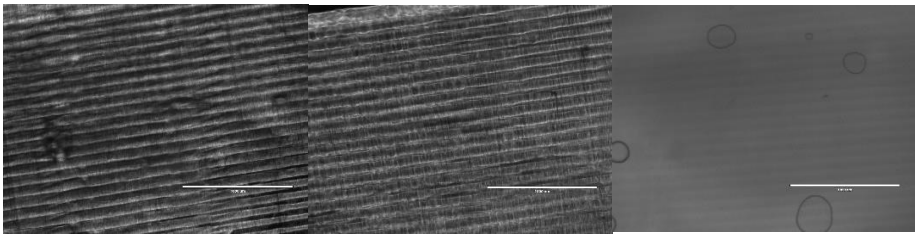
4.6 Printing of more elaborate structures

Prints of more elaborate structures such as those shown in Figure 8 were also made to be able to observe the efficiency of the bioinks when printing more complex models, some of these prints are shown in Table XVIII:

Table XVIII
PRINTING OF MORE COMPLEX DESIGNS WITH DIFFERENT BIOINKS.

Pyramid		
PEGDA 10%- Alginate	PEGDA 8% -PVA-Alginate	PEGDA 10% -PVA
		
Small channels		
PEGDA 10%-Alginate	PEGDA 8%-PVA-Alginate	PEGDA 10%-PVA
		
Cross section of the oviduct		
PEGDA- Homemade	PEGDA-Alginate	PEGDA-PVA-Alginate	PEGDA-PVA
			

Once these impressions were taken, thin slices were made to observe their structure under a microscope using a 10X magnification, and the following could be observed.



A. B. C.

Figure 13. Structure of the impressions under the microscope. A) PEGDA-Homemade. B) PEGDA-Alginate. C) PEGDA-PVA.

5. DISCUSSION

To begin with, in section 4. 1, we started studying the best printing parameters for the PEGDA-Homemade bioink which only contains PEGDA in a 30% concentration, finding that for this bioink the best parameters are found for a power between 65% and 70%, for an exposure time between 12 s and 20 s and a 1st Layer time of 3X, since these parameters allow the result to have a good definition and not to have an over polymerization, that is, that more material is polymerized than desired and the design to be printed loses its shape a little bit. This was visible in table X.

Subsequently, and once the best printing parameters were identified, bioinks with 10%, 15%, 20% and 40% PEGDA concentration were created, and cubes were printed as shown in Table XI. Thanks to these prints, it was possible to notice that the higher the concentration of PEGDA in the bioink, the more rigid it is, the less smooth and the better the definition; whereas the lower the concentration of PEGDA, although the print has a lower resolution and definition, it is smoother and less rigid, which is good to understand for the main objective proposed.

Then, in section 4.2 we show the results when printing with bioinks containing GelMa and PEGDA, being able to observe in table XII the prints achieved. It could be noticed that the GelMa brings enough softness to the printing and decreases the rigidity, to the point of having a great resemblance with the gelatin, but the definition of the printing is quite bad. In addition, when printing the cubes with the printing parameters indicated in Table VI, an over polymerization of material on some sides of the cube could be observed, which also suggests that the parameters used in the printer were not the best, it is possible that to print a more accurate design with these bioinks it is necessary to decrease the value for power, since we worked with 70%, it is possible that this should be decreased to 55% or 60% to achieve better results. It is important to remember that the printers do not always work with the same printing parameters, these depend on the bioinks, and their compounds used.

Following the above, it was thought to reformulate the bioink by adding new components, which in this case were PVA and Alginate, in order to improve the rheological characteristics of the prints, achieving a soft, elastic and well defined print, because until now it was known that when the concentration of PEGDA was reduced, the print was softer, but had less definition, and this last parameter had to be solved by adding other materials to the bioink.

Table XIII shows some observations of the impressions made with the new 3 bioinks containing between 8% and 10% concentration of PEGDA. For this it was found that the new bioinks worked well, there were smooth impressions, with better resolution and apparently more elastic, but during the execution of this step there were two situations that are important to consider. The first is that an attempt was made to work with the Irgacure 2959 photo initiator, but the bioink never polymerized, so no impression was ever obtained, because of which the photo initiator was changed to LAP and work continued with it. However, it would be interesting to understand in the future why the photo initiator did not work, if the error was in the bioink preparation methodology or in the printing parameters.

On the other hand, as it was noticed that the prints were not very transparent, and in the future, it is expected to be able to make certain tests in which it will be necessary to have

good visualization of the inside of the print, we wanted to find a way to reduce the color and opacity of the samples, making them more transparent. For this, it was tested decreasing and removing completely the Orange G which acts as a photoreceptor and it was found that this component for the case of these bioinks is strictly necessary, because when this component is not present, the polymerization during the printing is out of control and the whole bioink is polymerized, instead of only the desired design as shown in Figure 9, while when a minimum of 0.02% concentration of this component is present, the printing is a little clearer and the bioink works perfectly. Therefore, it was understood and visualized that it is a key material for the formulation and efficiency of the proposed bioinks.

Now, having a general idea of the behavior of the proposed bioinks, it was desired to know their resistance to media with different pH values, this test was performed on the bioinks composed of PEGDA, Alginate and PVA. Table XIV shows the results after having the cubic samples immersed in solutions with different pH values for 24 hours. It could be observed that these impressions have an excellent resistance to the different media, since the only pH value that affected them was an alkaline pH of 12, since with this, after 24 hours the impressions had completely crumbled, while, for the other pH values, including acid pH, the impressions did not suffer visible changes.

The above represents a good resistance characteristic for these materials, since, considering the intended application in this study, which is to simulate a female reproductive organ, the fact that these materials can withstand a wide range of pH values is an advantage, since it ensures that the material can last the necessary time to carry out the desired studies.

After analyzing the resistance at different pH values, we proceeded to perform the printing of cylinders, as shown in Table XV, to perform compression tests to the 9 bioinks. As indicated in Table IX, 2 types of compression tests were performed, of which the data obtained from the test where 10 N of force were applied until the impression was brought to its breaking point were finally taken, since when the data were plotted, the Stress vs. Strain graph was much clearer and the behavior of the material was better than with the test where only 10 compression cycles were applied, compressing the sample by 30%.

The values for the modulus of elasticity found in Table XVI show important data such as the following: First, the highest modulus of elasticity corresponds to the PEGDA-Homemade bioink with a value of 4.432 kPa, i.e. this is the one that presents the highest resistance to deformation, it also indicates that it is the one that generates more rigid and less soft prints. Secondly, when components such as GelMa, PVA and Alginate are added to the PEGDA-Homemade bioink, a noticeable change in the stiffness of the impressions can be seen immediately, since the elastic modulus for the other 8 bioinks containing some of the above materials starts to be below 1 kPa, which shows that they are quite soft and not very stiff impressions, which also represents a low level of resistance to deformation. Among these, the bioink that presented the lowest value of modulus of elasticity was the one composed by PEGDA 8%+PVA+Alginate, this makes sense in the moment that it is thought to be the bioink with the lowest concentration of PEGDA, and as it was commented before, the rigidity and softness are highly affected by the concentration of PEGDA in the bioink.

During the development of the study, compression tests were first carried out for the bioinks with a concentration of PEGDA between 8% and 10%, noting that they presented a

very low modulus of elasticity for what was desired, so an attempt was made to integrate more PEGDA to see if this influenced the increase in the modulus of elasticity, testing now with a concentration of 18%. This test showed that the elastic modulus did indeed increase, but not yet sufficiently.

Figures 10, 11 and 12 show the Stress vs. Strain graphs where the bioinks are compared. It is the case of figure 10 where it is possible to notice that GelMa-PEGDA with a 1:4 concentration presents a higher modulus of elasticity, since the force that must be applied is higher than GelMa-PEGDA 1:1 to lead it to rupture, in addition, it has a higher percentage of deformation than GelMa-PEGDA 1:1. Although the modulus of elasticity is higher at a 1:4 concentration, the deformation is also higher, which indicates that in the case of GelMa-PEGDA, the more PEGDA than GelMa, the closer the values of the modulus of elasticity will be to the desired values..

Now, figures 11 and 12 show the behavior of bioinks with PEGDA, PVA and Alginate, the value of the highest modulus of elasticity belongs to the bioink composed of PEGDA 18%+Alginate, with a value of 0.9 kPa, but this bioink reaches the breaking point faster, with a force of only 1.49 N. Table XVII shows the maximum value for the force and percentage of deformation for each bioink, where it can be seen that, although the PEGDA18%+PVA bioink does not have the highest modulus of elasticity, among the new proposed bioinks it is the one that supports more force before reaching the rupture point, needing 3.8 N to reach that point, which indicates that it presents a high rate of deformation and resistance to rupture. At the same time, the PEGDA10% + PVA bioink also has the highest value of maximum force and percentage of deformation among this group of bioinks.

Considering the above and Tables XVI and XVII, the experimental values are compared with the theoretical values corresponding to the modulus of elasticity of the different parts of the female reproductive organ, which are shown in Table I. It is known that the female reproductive organ has great characteristics as mentioned in the introduction, regarding the modulus of elasticity, most of its values are in the range of Mega Pascals, while the values obtained here are in the range of Kilo Pascals. The 3 tissues that present values in kPa are the uterine tissue with 5 kPa, the cervix with 2.17-243 kPa, the cervix with 2.17-243 kPa and the oviduct with 11.5 kPa. Of the values achieved here the one that comes closest is that of the PEGDA-Homemade bio-ink, but it is considered that this in relation to the real tissue is very rigid and not very soft, and it is known that the real tissue is soft and quite elastic, so the Alginate and the PVA have contributed a little to the softness of the impressions.

Knowing the above and seeing the results with each bioink in relation to the behavior according to the different concentrations of materials, it is necessary to continue working in the search for a bioink that allows to achieve the impression of a soft, elastic female reproductive organ with a modulus of elasticity like the real tissue. Here it was possible to give ideas of what happens when high concentrations of PVA, Alginate and PEGDA are added, many of these highly influence the viscosity of the bioink as well, which is an important factor for the printing process. In this case we tested with higher concentrations of PVA and Alginate, but the viscosity of the bioink was very high and the printer did not work well, so when controlling these concentrations and adding new components, this parameter should not be left aside.

Therefore, the task is to continue working on varying the concentrations of the components to achieve a modulus of elasticity closer to the desired ones and to find new components that can contribute to the achievement of the main objective, achieving ideal rheological characteristics and at the same time respecting the viscosity and parameters required by the Lumen X printer.

Finally, prints with greater complexity in the design were made, where it could be seen that the bioinks with only alginate and PEGDA do not allow a good range in the precision and definition of the print in relation to the other bioinks, while when they have PVA the definition improves considerably, although, at the moment of having visibility inside a print, as in the case of the print with internal channels, the bioink without PVA is better, because without it they have greater transparency and allow better visibility. Table XVIII shows impressions of the transversal section of the oviduct, showing however that each material presents in general a good definition. In addition, microscopic images are shown in Figure 13, where the different layers of impression in the transverse section of the oviduct are appreciated, although it is possible to notice that, due to the color of the bioink with PVA, it is more difficult to visualize these layers.

6. RECOMMENDATIONS AND FUTURE WORK

In the present research project, a methodology was proposed and executed to fulfill the proposed objectives. Although the main objective, which was to optimize a bioink for the impression of the female reproductive organ, was not achieved in its entirety, several important advances were made; these were shown in chapter 4, corresponding to the results, and explained in chapter 5, corresponding to the discussion.

In the following sections, ideas and proposals for future work are presented, which, according to the author, can represent a positive impact and relevant advances in the process of reaching the initial objective:

- Search for a suitable methodology to perform tension tests on the bioinks already proposed, since the prints achieved are hydrogels, the grip of the prints at the time of performing this test is difficult, but being able to achieve this characterization is important for the knowledge about the developed bioinks.
- Analysis of the porosity of the impressions. It is known that the characterization of the macro and microporosity of the materials is important information for their characterization and gives an idea of how the material could behave during a cell growth process, so studying this factor could be interesting.
- As indicated in the introduction, one of the printing parameters corresponds to the resolution, which for this case can be 50 µm or 100µm. For all the prints made in this study, a resolution of 100µm was used. But how can we know if the bioink used really allows us to have a print with this resolution? This would be another future study, to be able to determine to what extent the bioink used allows the print to have the desired resolution.
- On the other hand, the photo initiator proposed in the article used as the basis for the proposal of the new bioink was Irgacure 2959 (I-2959), we tried to work with this photo initiator, but we could not achieve the photopolymerization of the bioink in the printer, so it was only possible to work with LAP. The idea of being able to work with a wide range of photo initiators is quite attractive, so finding the right way to be able to use this photo initiator and make it work in the Lumen X printer could be a great idea.
- Since the bioinks proposed here do not yet reach the desired elastic modulus values, it is important to find new components that allow the prints to have the characteristics like the female reproductive organ. The test could be done working with materials such as collagen, fibrin, hyaluronic acid, polypropylene, extracellular matrices, among others [26], as these have been used in other studies as biomaterials for bioprinting in this area.

7. CONCLUSIONS

The printing parameters defined for the Lumen X printer depend on the composition of the bioink to be used. In the case of the bioinks proposed here, the parameters are as follows: 50%-70%, 8s - 20s, 3X. The exact value will depend on the composition of the bioink and each of the parameters are shown throughout this study.

PVA and Alginate are two components that help to improve the rheological properties of the prints made here, improving the softness of the samples, and therefore decreasing the modulus of elasticity. Despite this, it can be concluded that more components still need to be found to maintain the softness but increase the modulus of elasticity to bring it closer to the desired values.

Despite not having the ideal values, the bioinks with PVA showed the best values for the modulus of elasticity, force needed to reach the breaking point and the percentage of deformation of the structure.

Orange G as a photoreceptor is of utmost importance in these bioinks and can be used at a minimum concentration of 0.02%.

The proposed bioinks composed of PEGDA, PVA and Alginate, showed excellent resistance to media with different pH values, alkaline media being the only ones capable of severely affecting the sample after 24 hours of exposure to the medium.

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ANNEXES

For more photos, videos, and Excel data of the compression tests, go to the following link:

- Laura Serrano Shared Files (CTRL + click for following link)