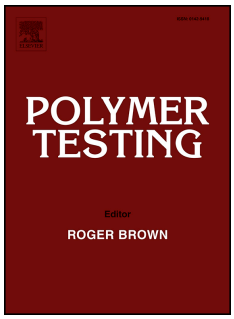

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PII: S0142-9418(19)32011-2

DOI: https://doi.org/10.1016/j.polymertesting.2019.106297

Reference: POTE 106297

To appear in: *Polymer Testing*

Received Date: 29 October 2019

Revised Date: 6 December 2019

Accepted Date: 14 December 2019

Please cite this article as: A.C. Heidenreich, M. Pérez-Recalde, Ana.Gonzá. Wusener, É.Beatriz. Hermida, Collagen and chitosan blends for 3D bioprinting: A rheological and printability approach, *Polymer Testing* (2020), doi: https://doi.org/10.1016/j.polymertesting.2019.106297.

Collagen and Chitosan blends for 3D bioprinting: a rheological and printability approach

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Abstract

Collagen and chitosan are widely employed as biomaterials, including for 3D-bioprinting. However, the use of collagen and chitosan (col:chi) blends as bioinks is still scarce. In this work, the rheology of different hydrogel precursors (0.5-1.50 % w/v chi: 0.18-0.54 % w/v col) was analyzed through frequency and strain sweeps, as well as at different shear rates. Col:chi blends showed a shear-thinning behavior, with viscosity values at low shear rates between 0.35 and 2.80 Pa.s. Considering the strain rate determined by the applied flow in a 3D-bioprinter, precursor viscosities during the extrusion were in the interval 0.5-0.8 Pa.s. Printability (Pr) was measured comparing images of the printed meshes and the corresponding CAD grid design, using photograph analysis. Col:chi 0.36:1.00 was chosen to print mono-layered scaffolds for tissue engineering (TE) because of its suitable viscosity, printability and polymer ratio content. Hydrogels were obtained through $NaHCO_3$ nebulization and 37º incubation, and NHS/EDC were added to obtain scaffolds with improved mechanical behavior. They were stable after 44 h in PBS with collagenase at physiological level and showed no cytotoxic effect in NIH-3T3 fibroblasts.

***Keywords*: 3D bioprinting; hydrogel precursor rheology; collagen; chitosan; bioinks.**

Introduction

Resorbable scaffolds intend to emulate the extracellular matrix (ECM), needed for cell adhesion and proliferation, and thus for tissue regeneration. In fact, they serve as temporary constructs where cells proliferate and produce their own ECM. Methods to obtain scaffolds were profusely inquired in the literature, standing out hydrogel sponges by freeze-dry [1], hydrogels to cell delivery by injection [2] and particles for cell encapsulation [3]. Biodegradability, biocompatibility and a highly interconnected porosity are requirements for a proper performance of these scaffolds [4].

3D bioprinting technology is the newest technique to working with biomaterials and build wet cellular scaffolds [5]. The technique allows materials to be dispensed in a controlled, repeatable and relatively fast manner; in addition, the technique enables to add autologous cells from the patient to be treated [6]. The 3D bioprinting process involves three steps: generation of CAD file to STL of shapes to be printed - which can be set up from medical images, giving personalized scaffolds -, then biomaterials and cell dispensation, and finally printed post-processing. This last step involves crosslinking methods and the assessment of cell viability and functionality [7].

In 3D bioprinters, hydrogels loaded in syringes are the feeding material, accurately connected with the software design. These viscous fluids can be directly printed with cells in suspension - bioinks - or also as cell-free polymers, generating a supporting layer, alternating with cells printed from a second syringe in their culture medium, in a process called indirect bioprinting. Hydrogels have largely proved to be suitable for cell proliferation and represent a high focus research in tissue engineering. Correspondingly, hydrogels are the most used materials for 3D-bioprinting [7]. A limited variety of natural polymers are suitable for bioprinting, standing out collagen, gelatin, alginate, hyaluronic acid, chitosan, dextran and fibrin. As for other hydrogel applications, blends of them have shown improved performances, combining mechanical structure and biocompatibility [8]. Indeed, 3D bioprinting has been used to generate, for example, a heart valve with alginate and gelatin [9], myocardial tissue with alginate and RGD-modified alginates [10], a scaffold for viable hepatocytes with gelatin and chitosan [11] or nervous tissue using soybean, collagen and fibrin [12].

Even this background, as far as we could know there is still a lack of work regarding the use of collagen and chitosan blends as bioinks. Collagen is an animal protein extracted from connective tissues, largely used as biomaterial due to its excellent biocompatibility and availability. Type I collagen is abundant in tendons, skin and ligaments, where its fibrilar organization provides mechanical support to these tissues. Chitosan is an

aminated polysaccharide composed of randomly distributed monomeric units of β-(1-4) D-glucosamine and N-acetyl-D-glucosamine. It is a semicrystalline polymer obtained from the deacetylation of chitin, a polysaccharide mainly extracted from crustacean's exoskeleton.

As mentioned above, two aspects in the 3D ink development must be considered: i) features of the *hydrogel precursor* to achieve proper injectability and shape fidelity to the digital design, and ii) suitable mechanical properties of the *hydrogel* obtained after crosslinking, in order to allow scaffold integrity and cell proliferation. We emphasize, in this contribution, on the rheological properties and shape fidelity - *printability* - of the *hydrogel precursors* containing different proportions of chitosan and collagen.

Both collagen and chitosan were reported as exhibiting pseudo-thinning behavior in diluted solutions [13]. Thus, viscosity in the shear rates at applied stresses during the extrusion becomes relevant since it influences printing accuracy. In these sense, a suitable viscosity range for extruding is between 0.30 and 30 Pa.s, since higher values bring large pressure to hydrogel extrusion out of the nozzle, and the process becomes instable [14].

Printability (Pr), is affected not only by ink features but also by process parameters such as extruder head speed, ejected material volume, extrusion nozzle size and distance between nozzle and substrate. Several studies relate printing parameters to the process results by measurements methods, even if it doesn't exist an only way to determine Pr. One method, for instance, is by printing grids and relating the hole designed area in the grid to the real area obtained by printing; another method is by printing sharp angles and assessing overlap between lines [14]. In other cases, also working with meshes and designed squared holes, Pr can be determined by measuring the circularity of the closed area and the perimeter of the printed square [15].

After printing, the pathway from hydrogel precursor to hydrogel is given by intermolecular forces among the polymer chains. For chitosan, pH neutralization is enough to trigger gelation process; in the case of collagen, pH neutralization and temperature, commonly 37º, is required. In addition, to obtain stronger gels, cross-linking agents such as genipin [16] [17] glutaraldehyde [18] [19], NHS and EDC/EDAC [20] are widely employed, as promoters of covalent links between chains. Finally, the swelling or contraction features in physiological medium are considered so that the deformation of the final construct can be minimized.

Collagen and chitosan blends are very well characterized as biomaterials with excellent features in tissue engineering; studies have strongly focused in dry scaffolds, sponges [1] [19] [20] or microspheres [21] as well as dry scaffolds including hyaluronic acid in triple blends [22][23]. However, the study of these blend properties as viscous fluid, is less explored. A thermal and rheological study for a blend 1:1 ratio col:chi, has been carried out, performing frequency sweeps, and apparent viscosity determinations, in which blends

viscosities values (~ 1-0.1 Pa.s) at different shear rates were between collagen alone (~ 10-0.2 Pa.s) and chitosan alone (~ 0.01-0.006 Pa.s), both three with shear thinning behavior [13]. In another study, col:chi at five different ratios from 1:1 to 50:1 were assessed about injectability as hydrogels carrying endothelial cells, determining the onset of gelation measured as a time-dependent change in viscosity [24]. Reis and coworkers [2] studied col:chi hydrogels with a peptide-modified chitosan, assessing different ratios in final concentrations between 0.25 and 0.50 % w/v, performing rheological assays, cardiomiocytes culture *in vitro* and animal injections with. In another study, col:chi hydrogels with bioactive glass nanoparticles for injectable systems were assessed, using chi 2 % w/v and col 0.20 % w/v in a 70:30 ratio and performing rheological assays to evaluate gel formation at different temperatures [3].

Col:chi blends as inks for 3D bioprinting are still poorly characterized. Indeed, Murphy and coworkers [25] assessed twelve different hydrogels for bioprinting for skin regeneration, one of which was col:chi 0.1% w/v:1.5% w/v. The study comprised cell viability, degradation and gelation; however, rheological studies were not part of this work, neither printability for the chosen col:chi blend. Taking into account the chitosan and collagen features and the wide bibliography about col:chi scaffolds for tissue engineering, the aim of this work is to study rheological features and printability of col:chi blends, seeking for proper inks for extrusion 3D bioprinting.

Materials and Methods

Collagen Extraction

The extraction protocol was made according adaptations of previous works [26][27]. Briefly, fresh tails were placed in 96% ethanol and incubated at -20° for at least 24 hours. The skin was removed, exposing the white tendons, in which their composition is approximately 80-90% collagen fibers. Tendons were detached with a clamp and placed in sterile PBS. Exposed fibers were cut into portions of approximately 1 cm long and were placed in 1:1000 glacial acetic acid, at a volume of 50 ml per tail. They were left under magnetic stirring at 4° during 48 h. A first centrifugation was made at 1000*xg* for 20 minutes at 4° and supernatant was recovered. A second centrifugation was carried out for 15 min at 10000*xg* at 4°, also obtaining the supernatant: a very viscous solution. 2 ml aliquot, by triplicate, was freeze-dried and weighted to know collagen concentration. Fresh collagen was usually used; the rest of solution was freeze-dried and resuspended in 1:1000 acetic acid to obtain desired concentrations. Extraction protocol was repeated three times with similar results. Collagen fibers and fibrils were observed with an Atomic Force Microscope (Supplementary Data Fig. 1).

Collagen:Chitosan (col:chi) Inks

Low molecular weight powder chitosan (Sigma-Aldrich: deacetylation degree 92% and viscosity 46 cps for the 1% solution) was employed to preparing solutions. 0.10 M acetic acid was used as a solvent to achieve a 2 % w/v solution by magnetic stirring at room temperature. Final pH was ~4.50. Col:chi blends were obtained from different volumes of chi 2 % w/v and col 0.72 % w/v stock preparations, to obtain, in % w/v: col:chi 0.36:0.50; col:chi 0.54:0.50; col:chi 0.24:1.0; col:chi 0.36:1.0; col:chi 0.18:1.5 and col:chi 0.45:1.5. Final blends had pH=4.50 and showed excellent miscibility. All of them were used as hydrogel precursors – inks – and stored at 4º.

3D-Bioprinter

A low-cost bioprinter (3-DonorRes, trademark LIFE SI, Argentina) with two syringes, one of them thermostatized, was used for printing. Software parameters allowed to control ejection time, material amount in each dot, and the distance between dots; two first parameters were changed in Printability assays in order to obtain different flows. In all cases, a 25G needle was used and the distance between needle and bed was approximately 1 mm. Temperature during extrusion process was room temperature, both in ejection chamber and in at deposit bed.

Flow estimation

Strain γ imposed by syringe piston during the extrusion process was estimated according to the equation 2, where Δz represents the lengthwise displacement, and R the needle radio (Fig. 1). This simplification is possible because the Reynold's number is under 2000, which is the limit where a laminar flux may be supposed.

$$\gamma = (\Delta z/R) \quad (eq.1)$$

So, strain rate was obtained as the derivative with respect to time, as it is expressed in eq. 3.

$$\dot{\gamma} = 1/R \ \ \Delta z/\Delta t \ \ (eq.2)$$

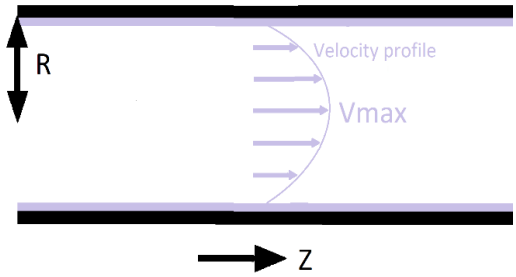


Figure 1: Schematic lumen needle with radio R and distance Z. Laminar flux approach is considered due to Reynold's number under transitional level to turbulence. Adapted from Amer et al [28].

Displaced volume ΔVin the cylindrical geometry of the lumen is related with Δz as indicated with equation 3.

$$\Delta V = \pi R^2 \Delta z \ (eq. 3)$$

By combining equations 3 and 4, shear rate and flow ($\frac{\Delta V}{\Delta t}$) are related according with equation 4.

$$\dot{\gamma} = 1/(\pi R^3)\ \Delta V/\Delta t \ \ (eq. 4)$$

Rheology

A rheometer TA Discovery Hybrid HR-3 was used, with a 40 mm diameter 2-degree cone plate geometry, a truncation gap of 60 um and a solvent trap to prevent drying. Only when total polymer concentration was under 1.00 % w/v concentric cylinders geometry was used. Storage modulus (G′) and loss modulus (G″) were recorded as a function of strain [0.25- 450] % at constant frequency 1 rad/s (oscillatory mode). Frequency sweeps [0.15-10] rad/s at constant strain amplitude 1% (oscillatory mode) was performed to obtain the storage modulus (G′), loss modulus (G″) and complex viscosity (η*). Apparent viscosities were measured as a function of the shear rate $\dot{\gamma}$ [0,015-100]s^{-1} (flow mode). All experiments were made at 25º and at least by duplicate. TRIOS software in the rheometer was used to fit zero rate viscosity with the best model.

pH neutralization and gelation

Immediately after printing, $NaHCO_3$ 0.80 M nebulization was made to neutralize the scaffolds. A San-Up Model 3042/3059 ultrasonic nebulizer was used, which provided drops with diameters between 1.5 and 5.7 μm, at an oscillation frequency of 2.5 MHz and a flow rate of at least 0.5ml / min. Three 5-minutes cycles were performed, controlling the pH increase after each nebulization cycle with pH paper, until pH ~7.50 was

reached; the last control of pH was performed with a pHmeter. Then, nebulized scaffolds were incubated at 37° in a water bath during 30 minutes.

Crosslinkers addition before printing

EDC (1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide) and NHS (N-hydroxysuccinimide), both from Termofisher, were added in powder form into the hydrogel precursor solutions, until 15 mM and 6 mM were achieved, respectively. The crosslinked col:chi mix was vortexed and kept at 0 °C until loaded into the syringe until printing. Subsequently, to achieve the hydrogel the protocol was the same as in the previous section, but with a 10 minutes nebulization cycle and a 5-minutes stabilization.

Mechanical properties

1mm thickness and 12 mm diameter round-trip samples were printed. A dynamic mechanical analyzer (DMA) model Q800 was used (TA Instruments, DE, USA) to determine their mechanical response. Compression tests were performed on the substrates using the 12 mm diameter geometry, in controlled force mode, at 37 º. The preload force was 0,01 N, the force ramp 0,02 N/min and the force limit 1N. The compression modulus (E) was calculated as the slope value in the linear section of the curve "tension vs. deformation" between 5 % to 10 % of deformation (n=3).

Printability

To measure Printability (Pr), a mesh with square holes of 4 mm on each side was chosen (Fig. 2). The strand thickness was 0.3 mm so that the software that commanding the printer, slic3r, generates in the GCODE a single path of the extrusion head per side. The design was made with CAD software. Three different printing conditions - 1, 2, 3 - were assayed taking advantage of the software possibilities, varying speed and material amount, so that flows were 0.19 µL/s, 0.42 µL/s and 0.35 µL/s, respectively.

Pr index by area compares printed area versus those in digital design [6] [7], according to the equation 5, where nearer to 1, better the printing fidelity.

$$Pr = A/A\,theoretical \qquad (eq.5)$$

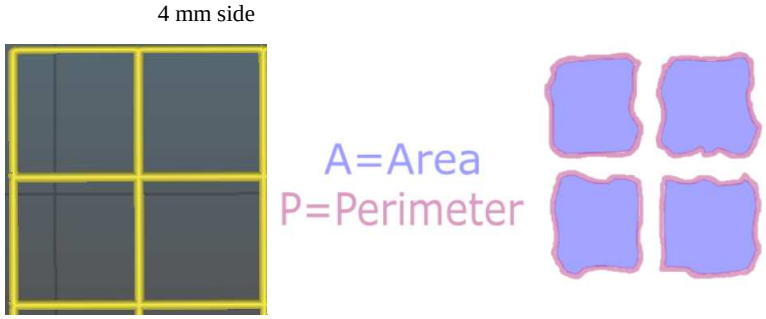


Figure 2: Digital model and parameters measured in each hole from printed grids

A is the printed area, determined by images and the Image-J®, and $A_{theoretical}$ is the grid area according to the design. *P* refers to the printed square perimeter and *A* is the measured area. To construct the meshes, hydrogel precursors were loaded in a 1 ml syringe. A glass slide with a 1-2 mm thickness was used as support for the mesh. Images were acquired immediately after printed. At least 24 squares were measured in order to determine A mean values.

Stability in PBS and in PBS/collagenase.

15mm x 15mm x 1mm height square geometries were printed with approximately 250 µl of ink (n = 5). Samples were weighed to determine their initial weight, W_0, and were immersed in PBS pH=7.4, at 37° during 72 h. Scaffold weight was controlled each day, extracting them from the solution and drying them by draining by gravity, supporting the scaffolds with a piece of paper. Residual mass (M.R.) was calculated as the ratio between the weight of the dry substrate at a time t (W_t) and the mass of the initial test piece (W_0), as $M.R.(\%) = Wt/W0 * 100\%$

In parallel, a solution of PBS pH 7.4 was prepared including 60 µl of 1mg /ml collagenase solution (from *Clostridium histolyticum*, Sigma) each 5 ml of PBS. Samples (n=5) obtained as in the paragraph above, were immersed in 4 ml of collagenase/PBS solution. Two test pieces were used as positive control, printed by the same way but containing only collagen 0.72% w/v. They were incubated at 37° for 48 h, or until their complete breakup in the case of positive controls. Each sample weight was taken at different times after being drained by gravity and by blotting paper. The residual mass percentage was calculated by the M. R. % equation.

Scaffolds Cytotoxicity

Direct Toxicity. NIH/3T3 cells were incubated in direct contact with the col:chi scaffolds. 1.10^5 cells were incubated in a 24-well plate (Corning Costar, MA) at 37° for 24 h in a 5% CO_2 humidified incubator. Samples

and control materials were put in each well, occupying 10 % of the well area. Complete culture medium was used as null control. As a positive control, we used latex rubber. Teflon (DuPont, DE) was used as a negative control, since it has no known *in vitro* cytotoxic effects. Cells were incubated in contact with the samples for 24 h at 37° in a 5% CO_2 humidified incubator. The cytotoxicity was assessed qualitatively. Cells were examined microscopically in a Nikon TE2000-U inverted microscope coupled to an ORCA-ER CCD camera (Hamamatsu). Changes in general morphology, vacuolization, detachment and cell lysis were assessed. All experiments were performed in triplicate.

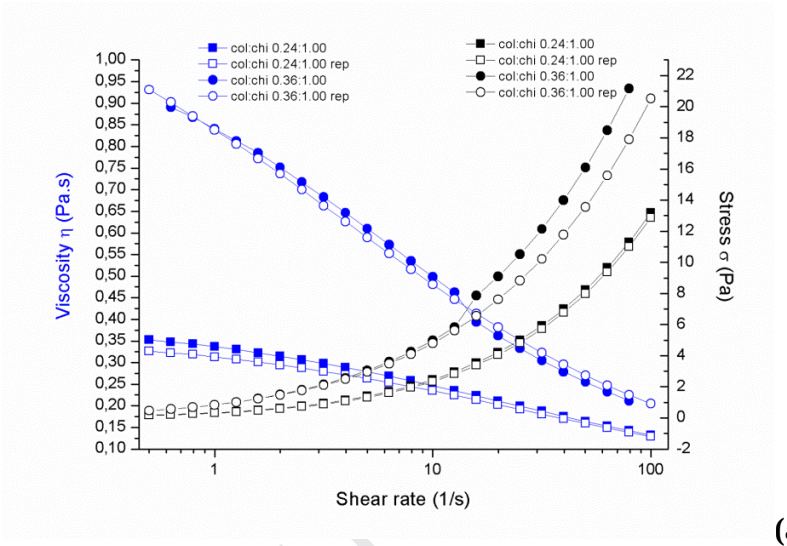
Indirect Citotoxicity. Material extracts were prepared by incubating scaffolds and control samples in complete medium with a material area (cm^2): media (ml) ratio of 6:1, for 72 h at 37° in a humidified atmosphere containing 5% CO_2. Scaffolds extracts were compared with medium control, positive control (latex rubber) extract and positive negative control (Teflon, DuPont, DE). 1.10^5 NIH/3T3 cells were incubated in a 24-well plate (Corning Costar, MA) at 37° in a 5% CO_2 humidified incubator. After 24 hours of incubation, the culture medium was replaced for the pure extract or 1/16 dilution of the extract in complete medium. Cells were incubated with the extracts for 24 h. Cells were examined microscopically in a Nikon TE2000-U inverted microscope coupled to an ORCA-ER CCD camera (Hamamatsu). Changes in general morphology, vacuolization, detachment and cell lysis were assessed. All tests were performed by triplicate.

Results and Discussion

1. Rheology of the inks

Rheological analysis to six col:chi hydrogel precursors were performed, under rotational mode (Fig. 3) and under oscillatory mode (Fig. 4 and 5). Apparent viscosity (η_{app}) and corresponding stress (σ) under different shear rates (0.50 to 100 1/s) are presented in Fig. 3. Shear-thinning behavior was evident for all blends, as viscosities decreased with the shear rate. In Fig. 3.a, two inks with the same chitosan concentration (1.00 % w/v) but differing in collagen content (0.24 and 0.36 % w/v) are presented, with their duplicates. As reported previously in col:chi blends rheological analysis [13], collagen component strongly contributes to the viscosity. Zero rate viscosities were 0.383 ± 0.01 Pa.s (col:chi 0.24:1.00) and 1.16 ± 0.08 Pa.s (col:chi 0.36:1.00) according to our data and to Carreau-Yasuda model. In Figure 3.b, viscosity curves from four blends containing chitosan 0.50 % w/v (col:chi 0.36:0.50 and col:chi 0.54:050) or 1.50 % w/v (col:chi 0.18:1.50 and col:chi

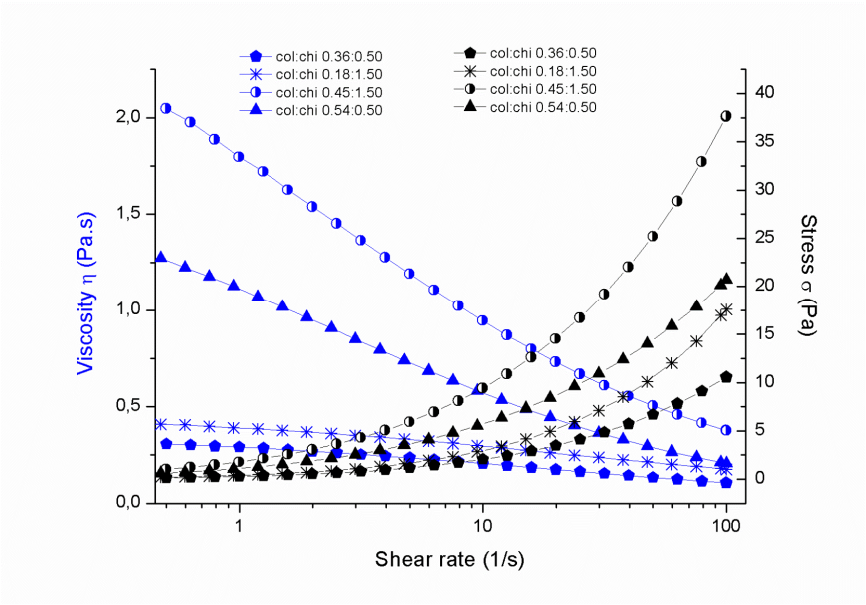
246 0.45:1.50) are presented, in this case one representative sweep of each blend. They exhibited similar behavior
247 than both chitosan 1.00 % w/v inks regarding shear- thinning behavior as well as collagen viscosity influence.
248 Beyond the total polymer concentration, collagen influence may be appreciated, for instance, comparing col:chi
249 0.54:0.50 with higher viscosity curves by comparison with of col:chi 0.18:1.50.



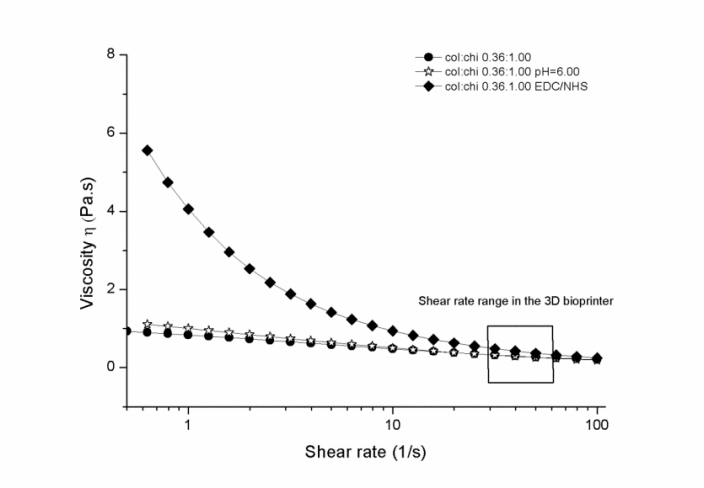
250 **(a)**

251

252



253 **(b)**



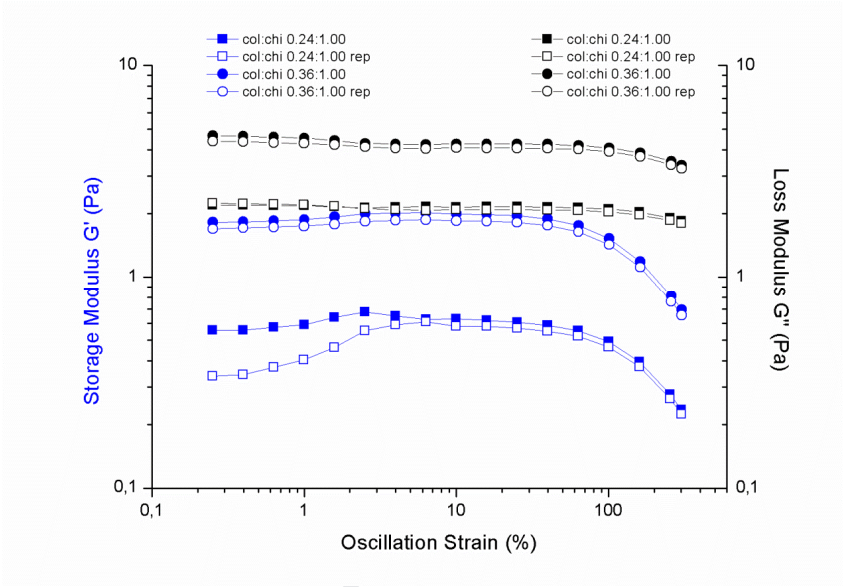
(c)

Figure 3: Apparent Viscosity as a function of shear rate (a) for two pH= 4.50 col:chi blends sharing chitosan composition 1% w/v; (b) for four pH=4.50 col:chi blends, with chitosan 0.50 % w/v or with 1.50 % w/v; for clarity one replicate is shown, and (c) for col:chi 0.36:1.00 in comparison with the same ink after adding in crosslinkers (col:chi 0.36:1.00 EDC/NHS) and after raising its pH to 6.00 (col:chi 0.36:100, pH=6.00).

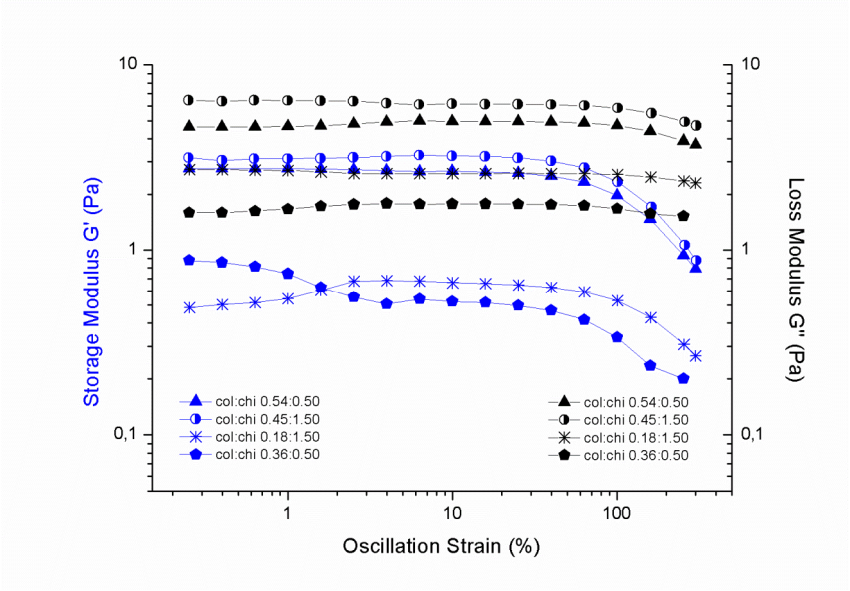
In Fig. 3.c, a comparison between one selected ink (col:chi 0:36:1.00) and its viscosity behavior at pH=6.00 is shown. Considering the possibility of direct bioprinting, hydrogel precursor pH increase becomes necessary: the results indicated that viscosity increased at low shear rates (zero rate viscosity col:chi 0.36:1.00 pH=6.00 2.50 Pa.s) and was almost non variable values at shear rates upper than 40 (1/s). Similar results were obtained with col:chi 0.54:0.50 at pH=6.00 (data not shown). In addition, col:chi 0.36:1.00 including NHS and EDC crosslinking activators is showed in 3.c. In this case, viscosity increase was considerable and the difference with the original ink started decreasing since 20 (1/s). As it was introduced above, viscosity in the shear rates at applied stresses during the extrusion becomes relevant as influences printing accuracy. Fig. 3.c shows the working range shear stress - 30-60 (1/s) - with the 3D bioprinter that will be exposed in Section 2 of Results. Ink treatment with NHS/EDC to improve printed scaffold final mechanical properties will be discussed in Section 3 of Results.

Although low-viscosity precursors are important for cell viability in a direct printing approach, some authors emphasize about the importance of high viscosities to improve printing process [29] suggesting the viscosity

modulation using pre-crosslinking methods; e.g., to partially crosslink increasing viscosity at the hydrogel precursor state. Several works have used this approach tipically with calcium to alginate. In our case, a pre-crosslink by pH increase, was observed in Fig. 3.c. In the same way, a higher effect was observed on the viscosity by adding NHS/EDC crosslinkers into this precursor stage.



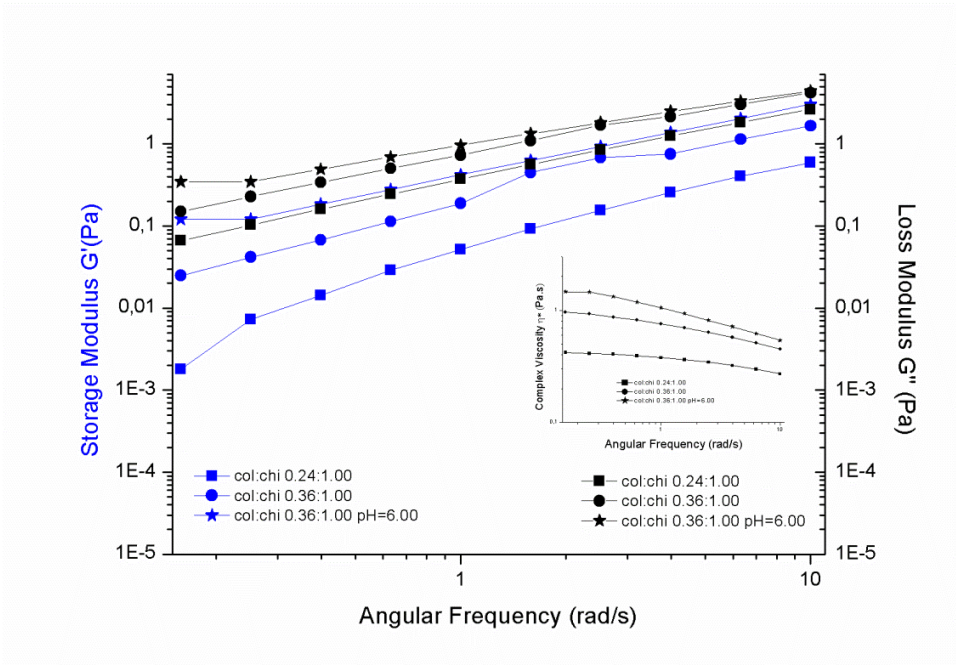
(a)



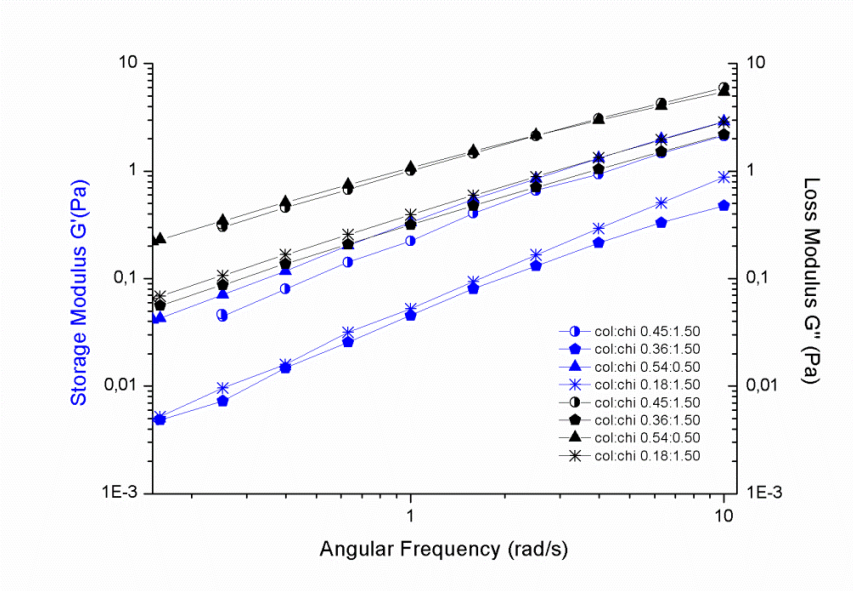
(b)

Figure 4: Storage Modulus (G') in blue and Loss Modulus (G'') in black as a function of the oscillation strain (a) for two pH= 4.50 col:chi blends sharing chitosan composition 1% w/v, by duplicate and (b) for four pH=4.50 col:chi blends, with chitosan 0.50 % w/v or with 1.50 % w/v; for clarity one replicate is shown.

In Fig. 4, G' and G'' from amplitude sweeps of the six col:chi blends are shown. Linear viscoelastic range (LVR) could be observed in G' curve, showing linearity between 5 and 80 % of oscillation strain. Both Fig. 4.a and 4.b graphs exhibit materials with viscous component more important than solid component, according with these low-viscosity hydrogel precursors.



(a)



(b)

Figure 5: (a) Loss modulus (G'') in black , storage modulus (G') in blue and complex viscosity (η*) in the inset as a function of the frequency for two pH= 4.50 col:chi blends sharing chitosan composition 1% w/v and col:chi 0..36:1.00 at pH=6. (b) Loss modulus (G'') in black and storage modulus (G') in blue as a function of the frequency for four pH=4.50 col:chi blends, with chitosan 0.50 % w/v or 1.50 % w/v. For clarity in the graphs only one representative sweep of each ink is showed.

In Fig. 5, frequency sweeps show that in all cases the loss modulus (G´´) was higher than the storage modulus, prevailing the viscous-like behavior in these viscoelastic fluids. pH=6.00 ink in (a) exhibited similar behavior than the original at pH=4.50, but with higher values. Since no crossover between modulus, all of the inks show stability at 25 º under these conditions.

In bioinks research, it is known that shear thinning performance contributes positively to 3D bioprinting, being advantageous for print fidelity and also for cell protection. Shear thinning performance enables a decreasing proportional stress with increasing flow that results in less stress for the cells [29]. Yield stress and recovery time are another interesting aspects to considering when bioinks rheology is deepen [30].

2. Printability

Using eq. 4 from Methods, the shear rate applied to the ink at three different conditions in the 3D bioprinter, has been estimated. Table 1 presents the shear rates values, for a 25 G needle (260 um internal diameter) at the flow imposed in each condition. Even if Printability is usually described as dependent of the hydrogel viscosity [31], it is important to note that for the same ink by different set conditions, e.g. different flow rates, viscosity and printing quality may change. In addition, the high pressure and small nozzle diameters represent possible damages to cells. Material amount and printing speed determine the line width of the construct [31]. Although, low pressures and bigger sized nozzle may be favorable for cell viability after printing, but it can result in a structure with low shape fidelity. So, the advantages and drawback are important to select printing conditions. By last, at one determined condition the needle tip to bed printing distance may influence the final quality; in this work prints were always performed at the constant distance of about 1 mm.

Printing Condition	Flow [ul/s]	Shear rate[1/s]
1	0,19 ± 0,09	30 ± 10
2	0,42 ± 0,04	62 ± 7
3	0,35 ± 0,07	50 ± 10

Table 1. Estimated shear rates for the flows at the working conditions in the 3D-bioprinter, fed with different col:chi inks.

Three inks printability in conditions 1, 2 and 3 were analyzed. Inks were chosen so that they represent the variety regarding polymer contents while those with lowest viscosity were discarded. So, col:chi 0.54:0.50, col:chi 0.36:1.00 and col:chi 0.18:1.5 were selected. In addition, thinking in a future bioink and taking into account the low pH of all these blends, col:chi 0.36:1.00 was used to generate a new hydrogel precursor (col:chi 0.36:1.00 pH ~ 6.00) by carefully adding NaOH 1.00 M drops with fast and enough vortexing. Given that the ratio 1:1 was quite explored in the scaffolds literature, col:chi 0.36:1.00 was selected because its good rheological features (always nearby to col:chi 0.54:0.50, in viscosity above all at the 3D printer shear rates) and because the possibility of more physiologically stable scaffolds – more chitosan content – [1].

From image analysis and according to eq. (6) from Methods, Pr values were obtained (Table 2). Pr as representing a measure about some material tendency to dilatate or to flux, in the case of this design reducing the hole area. In our inks, we could appreciate that the higher the flow - the lowest viscosity during the

extrusion - the worst performance in Pr measurements. These observations were in agreement with the fact that too low viscosities do not allow to maintain the shape in relation to the digital design (see below Fig 6.b).

Ink (col:chi)	Shear rate 30 s^{-1} Flow 0.19 µL/seg **(PC 1)**	Shear rate 62 s^{-1} Flow 0.42 µL/seg **(PC 2)**	Shear rate 50 s^{-1} Flow 0.35 µL/seg **(PC 3)**
	Pr	**Pr**	**Pr**
0.54:0.50 pH=4.50	0.67±0.11	0.68±0.13	0.75±0.12
0.36:1.00 pH=4.50	0.74±0.20	0.49±0.18	0.67±0.13
0.36:1.00 pH=6.00	0.69±0.12	0.72±0.15	0.68±0.19
0.18:1.50 pH=4.50	0.69±0.18	0.56±0.18	0.72±0.15

Table 2. Printability values assessed by square area (Pr) under three different printing conditions (PC) with determined flows and associated shear rates, for four different hydrogel precursors (inks) made with collagen (col) and chitosan (chi).

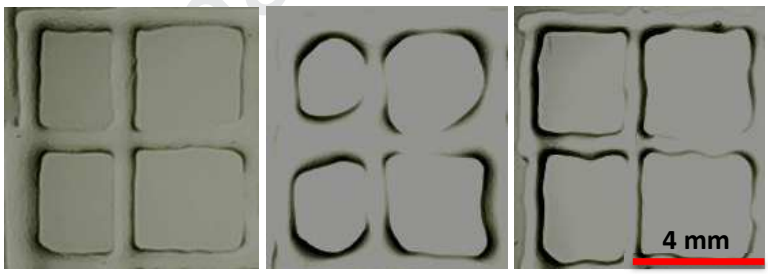


Figure 6. Printed grids with col:chi 0.36:1.00 ink, under conditions 1 (**A**, flow 0.19 ul/s; shear rate 30/s), 2 (**B**, flow 42 ul/s; shear rate 60/s) and 3 (**C**, flow 0.35 ul/s; shear rate 50/s).

Fig. 6 shows printed grids representative pictures under each printing condition, using col:chi 0.36:1.00 ink. Condition 1 (Fig 6.A), is associated with the lowest flow (0.19 µL/s) given that its low ejection speed and a small material amount: this condition seemed to enable the better results, also quantified by Pr index in Table 2. Under condition 2 (Fig 6.B), a filament thickness effect was observed, probably due to a high material amount

needed to achieve the flow 0.42 μL/s, resulting in poor shape fidelity. Finally, in Fig 6.C results of printing condition 3 is observed; in this case, the flow 0.35 μL/s was reached increasing the ejection speed relating to printing conditions 1. A slight line undulation evidenced the higher speed and influenced negatively the printing quality. Depending of the future use of printed forms, these observations could be important if small or linear geometries are required. However, they could be less important in big printed areas (e.g. wound patchs), where probably the insuming time becomes a more critical variable. For the first case, results under the PC 1 and PC 3 seem to be independent from inks viscosities in the range here used (0.50-1.80 Pa.s), showing similar Pr values among inks. Conversely, with the higher flow (PC 2), only inks in the higher range of viscosity values showed acceptable performance, exhibiting their capacity of holding the size without spreading. In this sense, even if the pH increase to the chosen ink col:chi 0.36:1.00 had null effect under PC 1 and PC 3, it had some positive impact according in PC 2, probably because of a viscosity change. According to rheological determinations, for the pH=6.00 ink similar features to the original blend (pH=4.50) were found but greater G' and smaller G''.

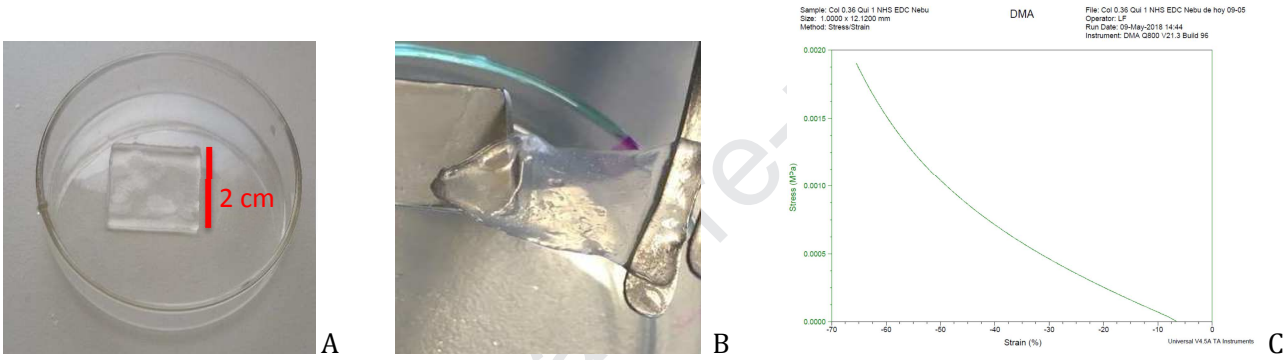
As it was mentioned above, regarding Pr some authors emphasize the pre-crosslinking approach to improve the shape quality, instead of a change in polymer concentration affecting cells. So, to be able to regulate viscosity and shape quality, for example by calcium ions or temperature, in the profusely studied alginate/gelatine ink, for example [15] [29].

3. Substrate for Tissue Engineering

Squares of 1 cm x 1 cm x 1 mm thickness were printed with the selected col:chi 0.36:1.00 ink. Triggering the gelation through $NaHCO_3$ nebulization and 37º incubation, we observed a resulting homogenous hydrogel but fragile and deformable substrates were obtained. This same feature have shown scaffolds obtained from other assayed inks as col:chi 0.54:0.50. Given that, NHS/EDC crosslinkers activators were added to the hydrogel precursors in solid form just before printing. These two agents covalently link carboxyl or phosphate groups to primary amines giving covalent unions amide both between collagen-collagen and between collagen-chitosan. According to the literature, this treatment appears always by immersion in NHS/EDC solutions [20] [32], and as far as we know it is the first report under this approach.

After printing and gelation, obtained substrates had suitable manipulable features (Fig. 7) and their elastic modulus E was estimated as 1.95±0.14.

Even if the printing quality and performance in the syringe were accurate, when we analysed the precursor col:chi 0.36:1.00$_{EDC/NHS}$ by rheological properties, an increase in the ink viscosity (η_{app}) was detected, in comparison with the original ink (see Fig. 3.c). Associated values with shear rates in printing conditions 1, 2 and 3 were 0.52 Pa.s, 0.34 Pa.s and 0.37 Pa.s, respectively, in comparison with 0.35 Pa.s, 0.25 Pa.s, 0.27 Pa.s for the original col:chi 0.36:1.00 Measurements were made immediately after adding the crosslinkers, and during approximately 40 minutes. We noticed a strong tendence to viscosity increase at room temperature, so that keeping on ice was always necessary after the crosslinker addition. Time sweeps at 0º in rheometer have confirmed that at least during 40 minutes gelation does not occur, even if G' approachs to G'' (See Fig.1 Supplementary Data).



A B C

Figure 7. Example of mono-layered substrate from col:chi 0.36:1.00$_{EDC/NHS}$ ink obtained with the 3D-printer (A) Just after printing (B) After gelation by pH (nebulization) and temperature. Representative pictures showing their integrity and manipulability (C) One curve resulted from compression DMA mechanical analysis.

4. Cytotoxicity

Direct and indirect cytotoxicity of the col:chi 0.36:1.00$_{EDC/NHS}$ scaffolds were evaluated according to the international standard ISO10993-5 for biomedical devices. In the direct assay, no alterations in cell morphology were observed, indicating null toxic effect. Monolayers cultivated for 24 hours in direct contact with the substrates are shown in Fig. 8, in comparison with controls. In the indirect cytotoxicity test, the exudate of the construct immersion in culture medium, did not affect the cells, which showed normal morphology, both in pure extracts and 1/16 dilution.

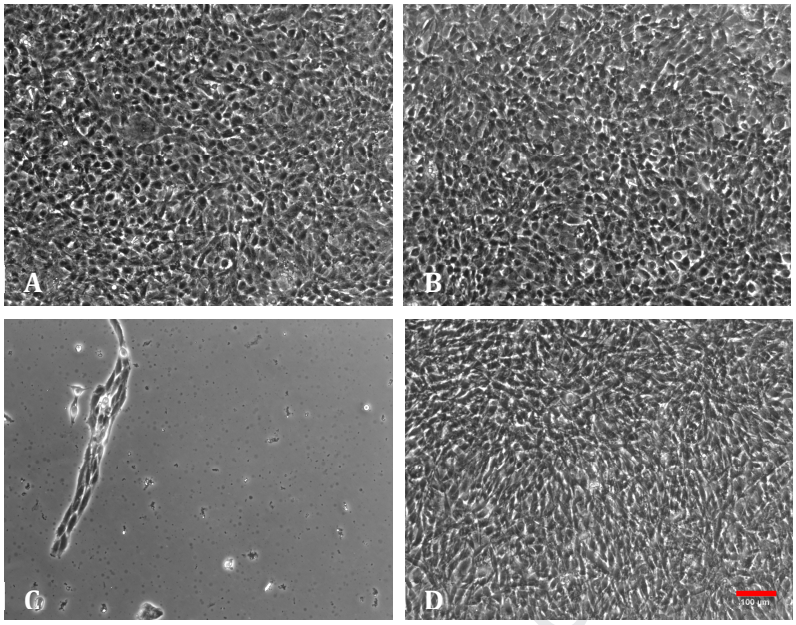


Figure 8. NIH/3T3 fibroblasts monolayers after direct cytotoxicity assay. (A) Null control (culture medium). (B) Negative control (Teflon®). (C) Positive control (Latex®). (D) Hydrogel constructcs made of col:chi 0.36:1 crosslinked with EDC/NHS. Magnification 100x. Scale bar 100 μm.

5. Degradation of scaffolds in PBS and PBS/collagenase

Substrates obtained in 3. from the ink $0.36{:}1.00_{EDC/NHS}$ were subjected to stability tests at 37 ºC in PBS. Fig. 9 shows three curves data: on the first one, % Residual Mass after immersion in PBS at 37 ºC (squares), in the second one, a similar protocol but PBS containing physiological collagenase was used for the incubation (circles). By last, constructs made by the same crosslinking method but with collagen only, were used as comparison and as positive control of enzymatic activity (triangles).

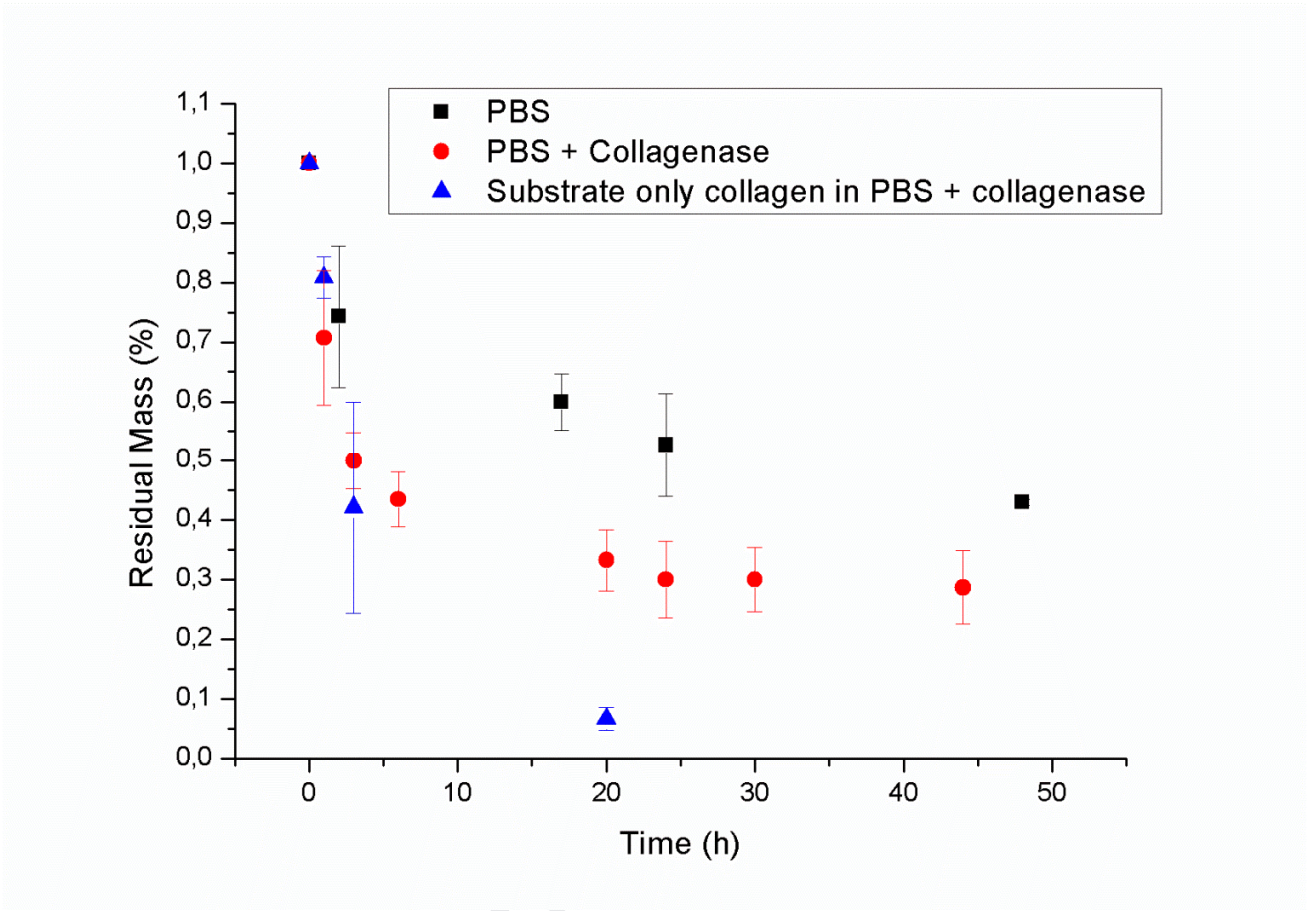


Figure 9. Degradation kinetics (% residual mass, % R. M.) for 5 **col:chi 0.36: 1**$_{EDC/NHS}$ constructs in PBS (black points, squares) and in PBS/collagenase (red points, circles). Collagen substrate, also crosslinked with EDC/NHS, was used as positive control (blue points, triangles). Data represent the average and standard error of 5 determinations.

Mass loss after 48 h in PBS was important, more than 50 %, considering possible uses for tissue engineering. Even with this mass reduction, constructs were perfectly tractable, having kept their integrity. Depending on cell type to be seeded, this feature could be improved by other crosslinking methods or by changing polymer concentrations. We could confirm that most of the loss mass corresponded to water mass, according to a low-viscous hydrogel. By scaffolds freeze-dry before and after incubation determined mass was quite unchanged, being almost all polymer mass, taking into account possible inclusion of PBS salts.

When the PBS curve is compared with PBS+collagenase, the enzyme effect was evident but moderate, considering the % M.R. at 45 h, with also integral and manipulable substrates. In this sense, the collagen construct was fully degraded in 20 h, evidencing the beneficial chitosan content in the selected ratio col:chi 0.36:1.00.

Conclusions

Taking advantage of the chitosan and collagen proven properties as biomaterials, in this work inks for 3D-bioprinting made of both biopolymers were assessed. Hydrogel precursors were evaluated by rheology, exhibiting low viscosities (η_{app}= 0.35-2.80 Pa.s) and shear-thinning behavior.

The extrusion process in the 3D-bioprinter was evaluated both by printability and rheology. For three inks with different polymer ratios (col:chi 0.18:1.50; col:chi 0.36:1.00 and col:chi 0.54:0.50), acceptable Pr values were found under printing flows between 0.19 uL/s and 0.42 uL/s.

Col:chi 0.36:1.00 was selected in this study and evaluated as a biomaterial for 3D constructs for tissue engineering. The possibility of printing with NHS/EDC into the ink was a suitable way of improving the final construct mechanical properties. Other ways should be explored in this sense, taking into account that keeping on ice the mix as a condition to minimize the viscosity increase is also time-dependent.

Regarding the acidic pH, an apparent drawback due to the solubility of both precursors, a final construct at neutral pH by nebulization was achieved, obtaining mono-layered scaffolds suitable for cell seeding. The main goal of this work was to assess Col-Chi formulations seeking proper rheological properties and printability; the best formulation —col:chi 0.36:1.00— was used to print mono-layered scaffolds. Thinking of multi-layered scaffolds, nebulization *in situ* just after printing might be an alternative.

From these results, other blends partially assessed here, such as col:chi 0.45:1.50 or 0.54:0.50, should be considered for further evaluation. In the same way, alternative crosslinking methods for the selected ink col:chi 0.36:1.00 could be assayed in order to obtain different modulus E for applications in tissue engineering. In addition, more stability at physiological conditions and higher Pr values may be inquired.

We consider the results encouraging, taking into account the innovative 3D-bioprinting technique and the extensive knowledge of collagen and chitosan as biomaterials. Since concentrated materials would provide a restrictive environment for cells, these low concentrated inks show a perspective, using pre-crosslinker modulation to achieve higher printability and finally suitable hydrogel scaffolds.

Declarations of interest: none.

Acknowledgements: FAN (Nanotechnology Argentinian Fondation) and Mr Bernando Villares and Ms. Camila Ruiz for the AFM images. LIFE-SI and its developer Adén Díaz Nocera because of his constant support with the 3D-bioprinter.

Funding: This work was supported by the Argentinian Ministry of Education through its Secretary of University Policies (grant *Agregando Valor*, Project: Bioinks). A.C.H. was a Biomedical Engineering student with a fellowship of the National Interuniversity Council. M.P.R was a posdoctoral fellow of CONICET. A.G.W. and E.B.H. are CONICET researchers.

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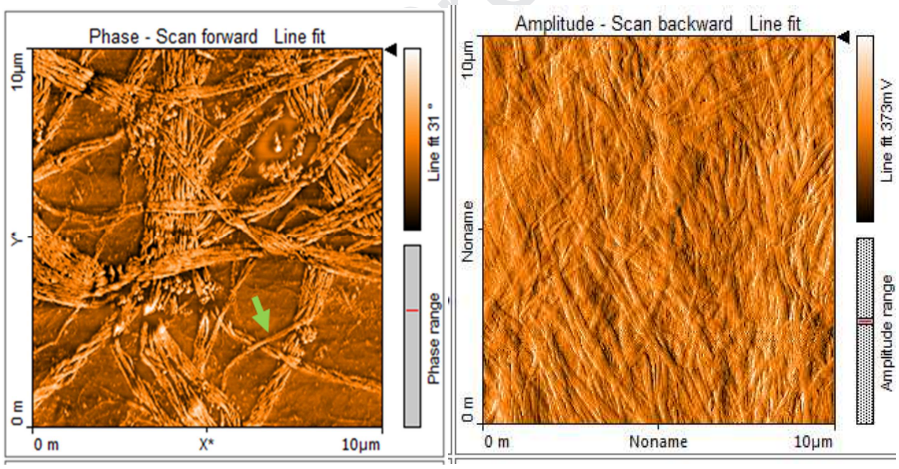
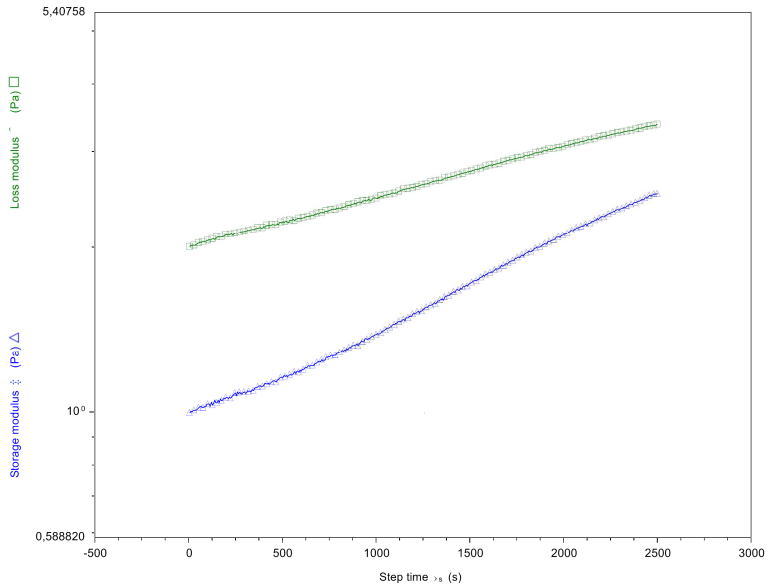
Supplementary Data



Supplementary Data Fig. 1: Collagen fibrils obtained from rat tail by extraction in acetic acid, observed by AFM images. In addition to the morphological observation, diameter sizes were calculated from the image, comparing with those reported for collagen fibrils in literature, 90 - 120 nm. Green arrow shows a representative 100 nm width fibril.



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571 **Supplementary Data Fig. 2**: Time sweep at 0ºC to col:chi 0.36:1.00 $_{NHS/EDC}$. Until 2500 secondes no gelation was
572 observed.

- 3D-bioprinting is a powerful emerging field in which ink composition is a critical issue.
- Collagen and chitosan are very well-known biopolymers.
- Blends of collagen and chitosan composing a bioink are poorly explored.
- Collagen and chitosan blends behavior through a 3D-bioprinter were assessed in this work.
- Printed and crosslinked scaffolds for tissue engineering were obtained from col:chi 0.36:1.00, both % w/v.

Author Statement

Ana Carolina Heidenreich: Conceptualization, Methodology, Investigation, Visualization, Writing – Original Draft.

Mercedes Pérez-Recalde: Conceptualization, Methodology, Writing - Original Draft, Writing - review and Editing, Project Administration.

Ana Gonzalez Wusener: Methodology, Investigation.

Elida Beatriz Hermida: Funding Acquisition, Project Administration, Supervision, Resources.

Buenos Aires, December 6th 2019

Polymer Testing

Associate Editor Dong Qiu

The authors of the current manuscript “Collagen and Chitosan blends for 3D-bioprinting: a rheological and printability approach” declare not to have conflict of interests about this subject. It contains just a scientific work at our National University of San Martin, Argentina.

Yours sincerely,

Mercedes Pérez-Recalde

Corresponding Author