Optimization of Nanoindentation Protocols for Hydrogel Mechanical Characterization

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Abstract

Hydrogels offer a range of properties to mimic the native extracellular matrix (ECM), and provide microenvironments to preserve cellular function and encourage tissue formation. ECM mimicking hydrogels may be synthetic or natural in origin and each type offers different advantages and disadvantages when it comes to biocompatibility, mechanical properties and preparation. In this work, we compare a synthetic and a natural hydrogel (poly(ethylene glycol) (PEG) and collagen type I) in terms of mechanical properties as measured by nanoindentation. The nanoindentation technique has been widely used to characterize soft tissues and hydrogels, but the high inter-study variability of hydrogel mechanical testing poses an important problem when characterizing these materials. There is a need for standardized mechanical testing protocols, but more specifically nanoindentation protocols, in order to have confidence on the mechanical characterization of hydrogels. Consequently, this work focuses on pinpointing potential variability sources when performing nanoindentation measurements of such soft materials as well as optimize sample preparation and nanoindentation protocols for production of consistent, bulk measurements. PEG hydrogels showed evidence of poroelastic behaviour but no viscoelasticity, as expected. At small strains, a linear elastic model was considered an acceptable descriptive model for this material. For collagen hydrogels, the time-dependent behaviour is significant and non-negligible. More complex viscoelastic models are needed to characterize this material.

Keywords: Nanoindentation, Hydrogel, Collagen, PEG, Mechanical Characterization, Viscoelasticity, Poroelasticity.

1. Introduction

Interest in hydrogels is growing rapidly due to their potential applications in many active research fields, such as drug-delivery applications [11], wound dressings[5] and tissue engineering [7]. These materials are characterized by the ability to absorb large quantities of water within their polymeric chains without dissolving. Because of their water content, hydrogels are generally very biocompatible when compared to other solid polymer networks [16].

Biological processes in the human body are usually guided by the interaction between cells and their surrounding environment, the ECM. Research has shown that there is a significant difference in cell phenotype when they are cultured in 2D environments with unnatural stiffness, polarity and surface to volume ratio, like petri dishes [17, 23, 1]. As a result, a need has emerged to create 3D microenvironments that mimic *in vivo* conditions, which has led to an increased interest in hydrogels for these specific applications. Additionally, studies have shown that cells are sensitive to the mechanical properties, namely its elastic modulus. The stiffness value of the ECM was shown to greatly influence cell differentiation, migration, proliferation and malignancy [3, 26, 8, 6]. Consequently, determining the mechanical properties of the hydrogels used for ECM-mimicking purposes is of paramount importance.

The focus and application of the hydrogels presented in this work is to serve as multi-dimensional cell culture platforms by mimicking the ECM. The gels described in this study are used as the substrate for *in vitro* vascular invasion assays and 3D Traction Force Microscopy (3D-TFM). In this situation, the mechanical characterization of these hydrogels is of extreme importance: given the mechanical properties of the matrix involving the cells (i.e. the hydrogel), the stress field within the material can be recovered [4].

However, biphasic materials can be particularly difficult to mechanically characterize due to their relatively small elastic modulus, predominant time-

dependent behaviour and the fact that they behave as neither a liquid nor a solid [9, 10]. Due to their nature, hydrogels can be difficult to immobilize for mechanical testing. Also, most equipment is optimized for elastic modulus' a few orders of magnitude greater than that of hydrogels. Finally, its multi-phasic nature makes the mechanical behaviour difficult to test, model and ultimately understand, because they neither behave like liquids or solid.

Nanoindentation has emerged among mechanical testing techniques due to its many advantages in indenting soft materials. Among these advantages are its relatively low sample preparation requirements, ease of use and non-destructive nature. However, there is no standardized protocol for nanoindentation of soft materials and, more specially, hydrogels, which complicates data and result comparison between research groups. This is especially the case for very soft hydrogels (less than 1 kPa) similar to the gels studied in this thesis. Therefore, there is a need for standardized protocols for consistent data recovery. Other techniques to tackle this problem include microindentation, dynamic mechanical analysis, unconfined compression, among others.

The two hydrogels analyzed in this work are poly(ethylene glycol), or PEG, and collagen hydrogels. PEG is a synthetic hydrogel and these types of hydrogels have the advantage of having robust mechanical properties [27]. Additionally, gel formation and degradation can be easily controlled and their mechanical properties are robust and load-bearing. However, these gels are significantly less biocompatible than their natural counterparts and not as good mimics of natural tissue. Moreover, although their covalent bonds may provide several advantages to the material, such as stability and resistance to mechanical loading, they also complicate protein diffusion and cell secretion, which is vital tissue engineering applications [14]. On the other hand, collagen hydrogels are natural hydrogels. These types of gels usually do not exhibit chemical crosslinks but physical entanglements based on weak chemical interactions, which makes these gels more sensitive to mechanical loading. However, their high biocompatibility makes them excellent candidates for ECM mimicking purposes.

In this work, we set out to optimize nanoindentation protocols to obtain robust, bulk measurements for these two different types of hydrogels. Several indentation and sample preparation parameters will be compared and discussed such as indentation velocities, probe tip size and cantilever stiffness, acquisition methods, sample geometry, among others. However, obtaining consistent measurements of mechanical properties of hydrogels has shown to be challenging [21]. With that in mind, this study focuses on decreasing variability by studying its possible sources.

These nanoindentation measurements are to be acquired with the purpose of a later mechanical characterization of these hydrogels. However, even though the mechanical behaviour properties to be measured are discussed and a few simple models are briefly mentioned, the thorough and complete mechanical characterization of these hydrogels is not within the scope of this work. Additionally, the current study reflects on the time-dependent mechanical responses of these materials and suggests appropriate methods of data acquisition to tackle these issues.

2. Materials

2.1. PEG hydrogel synthesis

PEG hydrogels were produced similarly to [22]. This hydrogel formula uses an enzyme mediated crosslinking method by employing coagulation enzyme-activated transglutaminase factor XIIIa (FXIIIa) to crosslink the branches of PEG macromers. The added beads are for later imaging and tracking using the 3D-TFM microscopy technique. Lys-RGD is a bioactive molecule, more specifically a cell adhesive peptide (CAP), that can be added to the synthetic hydrogel's structure to aid in cell adhesion and improve its biocompatibility. EGM (endothelial cell growth medium) is the growth medium later used to facilitate cell seeding and growth in the matrix and S1P, or Sphingosine-1-phosphate, is a blood borne lipid connected with the signaling of angiogenesis. Additionally, the specific PEG used contains the following MMP sensitive peptide sequence: AcFKGG-GPQGIWGQ-ERCG-NH2 (or peptide W) and can be called W-PEG [22]. The addition of this sequence allows for the degradability of the material.

2.2. Collagen hydrogel synthesis

Collagen hydrogels were produced similarly to [15]. Regarding the reagents: BAEM is the cell growth medium used in this solution that will later be used to seed cells; S1P, or Sphingosine-1-phosphate, is a blood borne lipid connected with the signaling of angiogenesis; NaOH is added to adjust the pH of the final solution; bovine skin collagen (collagen G, 4 mg/mL; Matrix Bioscience) and rat tail collagen (collagen R, 2 mg/mL; Matrix Bioscience, Morlenbach, Germany); NaHCO3 (23 mg/mL). The samples were polymerized in an incubator at 37°C, 95% humidity and 5% CO2.

3. Methodology

3.1. Sample preparation

A single droplet of 20 μL for PEG and 60μL for collagen were deposited on a 35 mm petri dish and a

glass 12 mm coverslip was placed on top of the droplet. Before indentation, this coverslip was gently removed from the sample's surface with the help of tweezers. This method allowed for the reproducible characteristics of the samples (thickness and geometry) from trial to trial, reducing variability. Additionally, the coverslip method made the surface of the samples completely flat, reducing the influence of roughness or curvature in the indentation results. All samples were transported in a heated and moisture providing container to avoid dehydration.

3.2. Nanoindentation

A commercial nanoindenter from Optics 11 was used to produce the results in this work (Chiaro model). Each indentation produces a load-indentation curve from where the elastic modulus can be extracted by fitting the curve to a Hertzian contact model. The Poisson's ratio for PEG and collagen was considered to be 0.5. The samples were indented with two different indentation velocities described in Fig. 1. Indentation profile 1 corresponds to an indentation velocity of 5 $\mu m/s$ and indentation profile 2 corresponds to 1.5 $\mu m/s$.

All indentations were performed submerged in PBS to avoid the dehydration of the samples. The probes chosen were:

- **Probe A:** Cantilever stiffness = 0.05 N/m and probe tip radius = 9 μm
- **Probe B:** Cantilever stiffness = 0.45 N/m and probe tip radius = 24.5 μm

Other studies performed to determine the sources of variability of hydrogels' mechanical measurements included an overnight assay (where gels were left in a incubator at 37ºC for 16 - 24 hours), an indentation acquisition method comparison (see Fig. 2) as well as a comparative study of the stiffness value of two concentrations of each gel.

To determine the hydrogel's time-dependent behaviour, both stress and strain relaxation experiments were performed on both gels.

3.3. Statistical Analysis

For the statistical analysis of the data collected in this work, outliers were not considered when calculating the mean values and standard deviation. Statistical significance was determined by the two sample t-test matlab function $ttest2(x, y)$ and the $p - value$ was considered to be 0.05.

4. Results and discussion

It is important to keep in mind that nanoindentation measurements only describe mechanical surface properties of materials. However, good correlations have been established between surface and bulk properties of materials by nanoindentation measurements applying the Hertzian model [20].

4.1. PEG nanoindentation

A typical load-indentation curve for PEG Hydrogel can be seen in Fig.3.

PEG nanoindentation measurements will always present a significant adhesion force. This adhesion is also what makes it possible to indent PEG hydrogels without any lateral movements without any special fixation techniques. Apart from the adhesion force, it is also visible relaxation which leads to the visible hysteresis which is indicative of time-dependet behaviour.

To identify the reaction of the solid component of the load-indentation curve and consequently eliminate the effects of poroelasticity on the results, a series of subsequently lower indentation rates were tested, similarly to [10]. The objective was to minimize the hysteresis seen in 3 until the effect of the fluid flow on the stiffness was negligible. There were several velocities tested, but for the sake of conciseness, only the lowest and highest are compared (n=4).

The indentation velocity of 0.3 $\mu m/s$ (100 seconds of indentation through 15000 nm) was tested and found to minimize hysteresis as much as possible. For confirmation purposes, two types of experiments were performed: one with the relaxation used for the majority of this thesis and consistent with indentation profile 1 (5 seconds), and another with a higher relaxation time of 20 seconds after peak indentation. The resulting indentations can be seen in Fig. 4. These indentations show a striking resemblance to one another and there is no apparent difference in relaxation. However, the loading and unloading curve are not identical and the most likely explanation for this phenomenon is the significant adhesion force present in this material.

On average, indentations with extremely low indentation rates showed to measure an elastic modulus 8.5% lower than the velocity used for the work developed. One question remained as to how high is the intrinsic variability of an indentation. In other words, if we were to indent in the exact same location several times, how variable would the measured elastic modulus be.

To perform this small study, one has to be aware that indentation in the same location is not advised in non-elastic materials because the indentation in itself might change the properties of the material. For n = 9 locations and 4 - 8 indentations per location, the variation coefficient was calculated. Results of this study are described in Table 2.

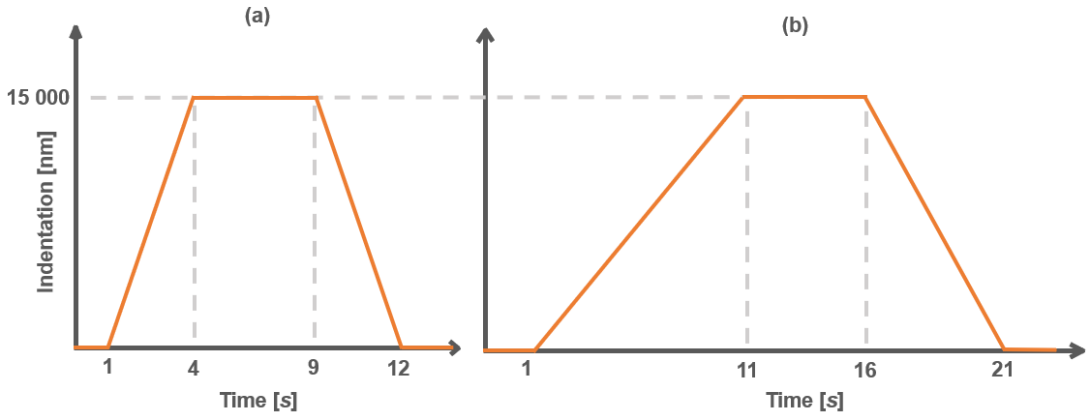

Figure 1: Indentation profiles used in the nanoindentation experiments of PEG and collagen hydrogels. (a) Indentation Profile 1: indentation velocity of 5 $\mu m/s$, relaxation of 5 seconds; (b) Indentation Profile 2: indentation velocity of 1.5$\mu m/s$, relaxation of 5 seconds.

Table 1: Stiffness measurements obtained with indentation rates of 0.3 $\mu m/s$ and 5 $\mu m/s$ of PEG Hydrogels in the same location.

	Stiffness measured with v = 5 $\mu m/s$ (Pa)	**Stiffness measured with v = 0.3 $\mu m/s$ (Pa)**	**Percent difference %**
n = 1	214	196	8.72%
n = 2	130	117	9.95%
n = 3	249	221	11.03%
n = 4	232	222	4.30 %

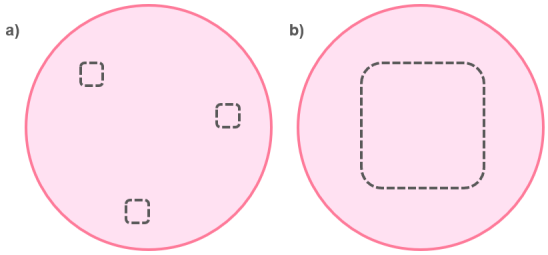

Figure 2: Representation of nanoindentation acquisition methods. a) Method 1: 5x5 points, 50 μm apart b) Method 2: 10x10 points, 300 μm apart.

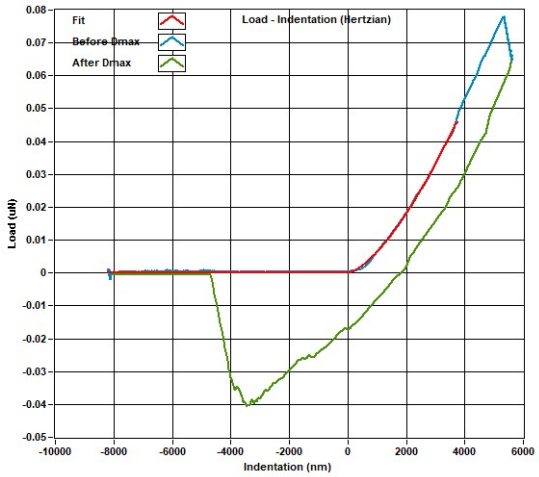

Figure 3: Typical load indentation curve for PEG hydrogel with the indentation rate of 5 $\mu m/s$ and indentation profile 1. The effects of relaxation are visible when maximum indentation is reached. A significant adhesion force when the probe is being retracted is also visible. Indentation performed with Probe A

The variation coefficient was determined according to the following expression:

$$c_v = \frac{\sigma}{\mu} \tag{1}$$

where σ represents the standard deviation and μ represents the mean value.

The average variability coefficient of same location indentations with the same indentation rate was measured as 3.60%. We have used these findings to support the claim that, if indented with a rate of 5 $\mu m/s$, the porous effects of the material on the elastic modulus can be neglected.

Stress and strain relaxation

Stress relaxation assays were performed on n = 3 samples of PEG 1.2% and n = 3 samples of PEG 1.5% with a velocity of 15 $\mu m/s$ and a relaxation time of 100 seconds (see Fig. 5). Additionally, a strain relaxation assay was also performed on PEG 1.5% with a constant stress of 0.05 μN (see Fig.6).

No creep phenomenon was found in these assays and very low stress relaxation on PEG samples was present. These two factors show that there is time-dependency and energy dissipation in this material, but the lack of creep supports the assumption of a very low or nonexistent viscoelastic component. Consequently, we consider modelling PEG hydrogels as a linear elastic material, therefore neglecting the poroelastic and viscoelastic behaviours.

This approximation is particularly justified for the

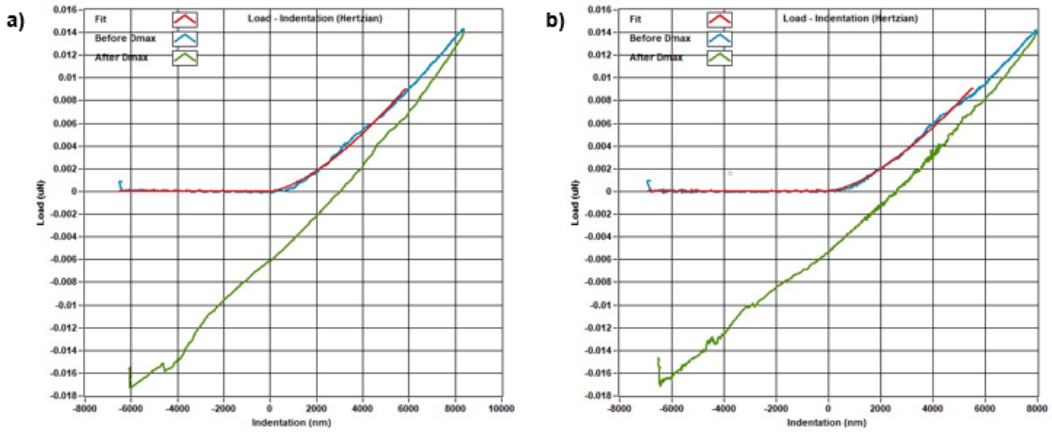

Figure 4: Load indentation curves of PEG Hydrogel samples with an indentation rate of 0.3 $\mu m/s$. a) 5 second relaxation after reaching peak indentation b) 20 second relaxation after reaching peak indentation.

Table 2: Mean stiffness, standard deviation and variation coefficient values for same location indentation of PEG 1.2% hydrogel samples (n=9) with 5 $\mu m/s$ indentation rate.

	Mean stiffness value (Pa)	Standard deviation (Pa)	Variation coefficient %
n = 1	131.71	3.18	2.42%
n = 2	207.59	1.52	0.73%
n = 3	130.41	7.38	5.66%
n = 4	122.70	6.84	5.57%
n = 5	121.20	7.00	5.77%
n = 6	118.91	4.14	3.48%
n = 7	117.28	2.63	2.25%
n = 8	255.81	3.80	1.48%
n = 9	226.57	11.52	5.08%

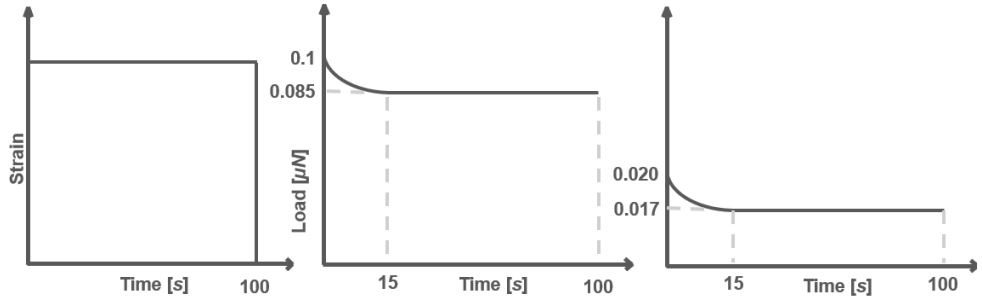

Figure 5: Representative stress relaxation assay results on PEG 1.5% and PEG 1.2%, respectively.

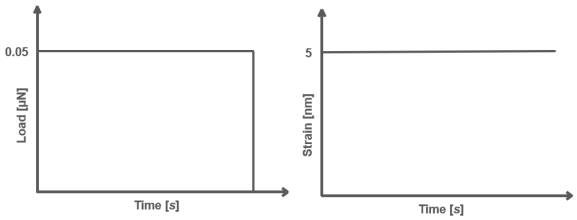

Figure 6: Representative creep assay results on PEG 1.5%.

specific application of this study, 3D-TFM of angiogenic sprouting assays, due to the small rates in question when dealing with tissue engineering and cell migration in angiogenic processes. However, PEG hydrogel is a poroelastic material and at higher strain rates, the linear elastic modulus might not be suitable, especially when considering other applications with higher levels of stress and strain, such as cartilage replacements.

Overnight assay

The swelling of hydrogels over time is a common phenomenon and this swelling can change the stiffness of material. To study the influence of this factor, PEG 1.5% gels were indented right after production and also between 16 - 24 hours later, since the nanoindentation process can take several hours from start to finish (see Fig. 7). The average values of this indentations are described in

The results showed that for day 1, PEG 1.5% average stiffness was of 940 Pa and for day 2 was 986 Pa (standard deviation of 159 and 169 Pa, respectively). This difference is not statistically significant and it is actually opposite to what

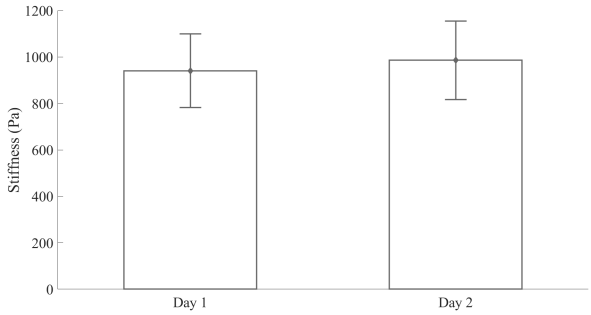

Figure 7: Average stiffness value (Pa) of PEG 1.5% on the day of production (Day 1) and 16 - 24 hours later (Day 2). Indentations performed with Probe A and indentation profile 1.

would be expected for a swelling hydrogel. That being said, even though PEG hydrogels have been shown to swell significantly, this particular formula was chosen in part because it does not. A high swelling coefficient would change the cell's environment from one day to the other, which would not be beneficial for this type of assays.

Indentation method study

Each gel (n=3) was indented with both methods described in 2, and the results of these indentations can be seen in Fig. 8 and Fig. 9.

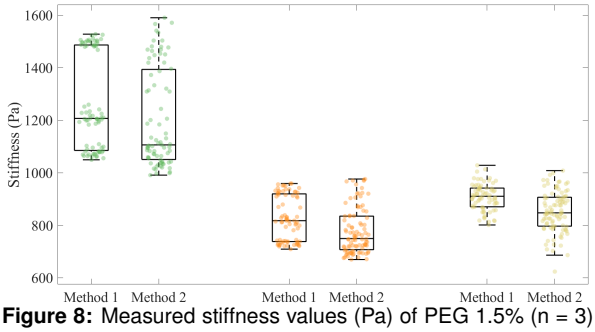

Figure 8: Measured stiffness values (Pa) of PEG 1.5% (n = 3) with indentations acquired via acquisition method 1 and method 2. Indentation rate of 5 $\mu m/s$ (Profile 1) and spherical probe with tip radius 9 μm and cantilever stiffness 0.05 N/m.

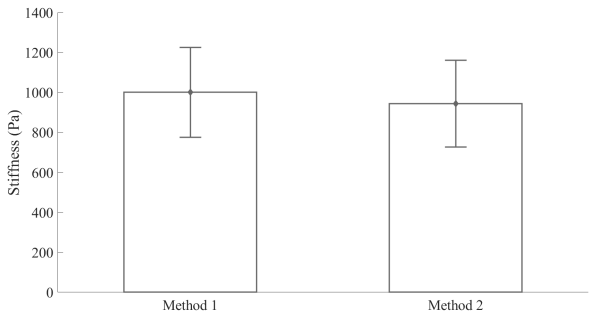

Figure 9: Average stiffness values (Pa) of PEG 1.5% with indentations acquired via Method 1 and Method 2. Indentations performed with Probe A and indentation profile 1.

Overall, the final resulting stiffness is very similar amongst the two methods. However, since this is a variability focused study, the number of indentations performed was extremely high, in some cases even more than 100 indentations per gel. However, other studies using nanoindentation as a mechanical property measurement technique, report as low as 6 indentations per gel [13]. For these types of studies, a method like method 1 would be quite dangerous, because the measured results would be linked to a relatively low variability but they would not be an accurate representation of the stiffness of the whole sample.

Any recommended measurement acquisition method would always require a significant number of indentations (more than 20). For heterogeneous soft tissues like brain, method 1 would be most useful because it can determine the elastic modulus within a certain region with a low variability. When the specimen's structure has different regions and structures (for example, white matter, grey matter, certain specific nuclei, etc.), a grid scan that covers most of the sample's surface would not allow for a specific characterization of each region. On the other hand, for samples where one single stiffness value defines the material, method 2 is the most appropriate. This method can measure the intrinsic variability of the sample as well as provide an appropriate overall stiffness value of the sample. Consequently, this is the method recommended for nanoindentation of hydrogel samples.

Elastic modulus determination

To determine the elastic modulus of this material, each sample was indented 100 times in a 10x10 scan of the sample with each indentation point separated by 300 μm. The indentations from each trial were averaged independently from each other and final results were obtained (see Fig. 10). PEG 1.2% hydrogel measured an average of 213 Pa and PEG 1.5% measured 967 Pa. This stiffness difference was shown to be statistically significant. Taking into consideration the small difference in the formulation of these concentrations of only 0.3%, the resulting stiffness disparity is significant. This difference shows how sensitive this formulation is to PEG polymer quantities and also could be a factor in explaining the variability of the samples from trial to trial.

Collagen nanoindentation

A typical load-indentation curve for collagen hydrogel can be seen in Fig. 11.

Both the energy dissipation mechanisms and the adhesion force are very much present in this

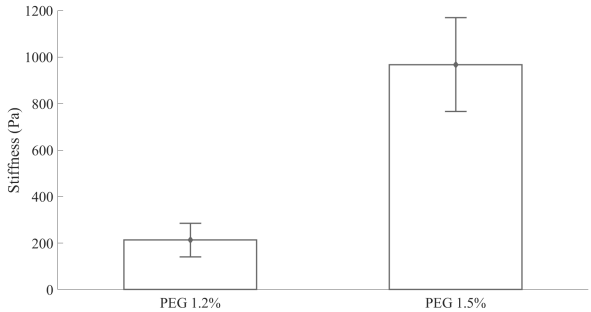

Figure 10: Average stiffness value (Pa) for two PEG 1.2% and PEG 1.5%. Indentation performed with Probe A and indentation profile 1.

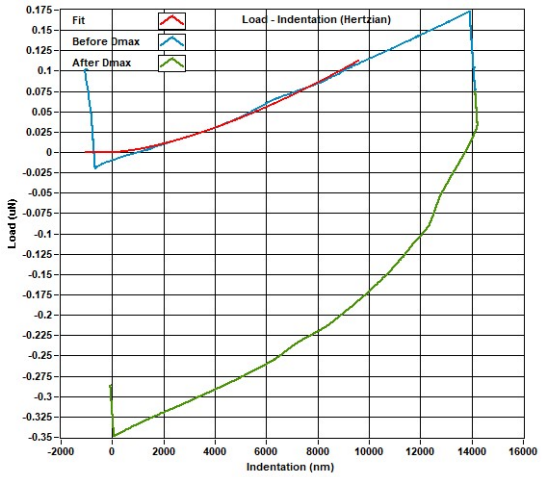

Figure 11: Typical load indentation curve for collagen hydrogel with the indentation rate of 1.5 $\mu m/s$ and indentation profile 2. The effects of relaxation are visible when maximum indentation is reached. A significant adhesion force when the probe is being retracted is also visible. Indentation performed with Probe B.

hydrogel. Being a self-assembled material, collagen hydrogels are expected to show a significant viscoelastic behaviour due to the weakness of their bonds.

Stress and strain relaxation assays

Stress and strain relaxation assays were both performed on 2.4 mg/ml collagen hydrogels (see Fig. 12).

These tests clearly show the time-dependent behaviour of collagen: the strain relaxation test resulted in a distinctive creep behaviour but with asymptotic characteristics, stabilizing at approximately 200 seconds. On the other hand, there is total stress relaxation present in the collagen hydrogel's response, which will certainly be a result of both viscoelastic as well as poroelastic mechanisms.

From these results, a simple viscoelastic model perhaps could be used to describe the material's behaviour. Firstly, the model chosen has to show both stress and strain relaxation. Secondly, the

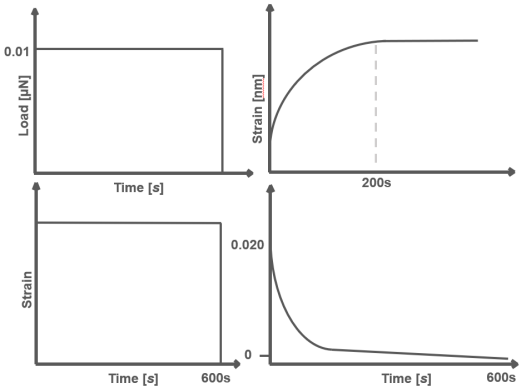

Figure 12: Representative creep and stress relaxation assays of 2.4 mg/ml collagen hydrogel samples.

stress relaxation must be total and the creep is asymptotic and not unbound. A good approach could be a combination of the Standard linear solid (SLS) Model and the Burgers model, since neither of them show the relaxation exhibited from this gel.

However, these models are a very simplistic characterization of this material's behaviour. For one, they do not take into account the poroelastic component of collagen's behaviour and further tests must be conducted to truly separate the poroelastic and viscoelastic contributions [9, 10].

Elastic modulus determination

The method used for collagen hydrogel nanoindentation is a mixture of method 1 and method 2 described in the previous section. Because of the high failure rate of indentations in collagen samples, the indentation specifications have to be altered and optimized several times. Therefore, many smaller matrix scans are recommended. However, the distance between each indentation point is higher (300 μm) to analyze as much of the samples' surface as possible.

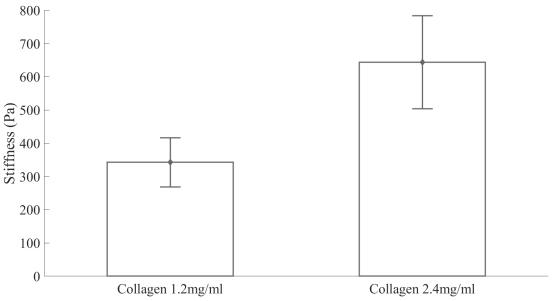

Figure 13: Average stiffness values (Pa) of 2 different concentrations of collagen hydrogels, 1.2 mg/ml and 2.4 mg/ml. Indentations were performed with indentation profile 2 and probe B.

The indentations from each trial were averaged independently from each other and final results were obtained. Collagen 1.2 mg/ml hydrogel measured an average of 343 Pa and collagen 2.4

mg/ml measured 643 Pa, as can be seen in Fig. 13.

5. Conclusions

Assumptions made throughout this study such as the isotropic behaviour of hydrogels or the 0.5 value for the poisson's ratio are potential sources of inter-study variability that can affect the results greatly. These characteristics were not tested in this study and are not tested in many studies [18, 25, 2, 24, 13, 19, 12] and the logical next step would be to acquire reliable measurements and test for these characteristics.

It was shown that the indentation acquisition methodology can greatly impact the results obtained. To minimize these effects, a high number of indentations is recommended. The reason for this recommendation is explained by the intrinsic hydrogel variability shown in this work. Depending on the location chosen, mean stiffness values can vary significantly and that is why, for soft materials like hydrogels that are supposed to have a somewhat homogeneous structure, we recommend an acquisition method that covers most of the sample's surface to obtain a more accurate measurement.

In terms of the potential of nanoindentation for hydrogel mechanical characterization, it is important to have in mind that this depends on the intended application of the material. In this case, all decisions are made under the assumption of small deformation because it fits the intended application of this work. Cells in vascular invasion assays will move at slow rates, cause small deformations and exert small loads on the hydrogel environment.

In the future, perhaps nanoindentation simultaneously coupled with a high resolution imaging technique could explain the reasons behind the high indentation failure rate found when indenting collagen gels as well as shed some light on the behaviour of the polymeric structure when subjected to loading. With this improvement, forces could be recovered by using the specific mechanical characteristics of the hydrogel sample used in that particular assay.

It is our conclusion that the nanoindentation technique is able to provide enough consistent data for a reasonable and reliable mechanical characterization of PEG. On the other hand, collagen mechanical characterization is much more complex and nanoindentation alone cannot fully characterize the viscoelastic mechanisms underlying the behaviour of this material. However, nanoindentation can be a complementary tool for collagen hydrogel mechanical characterization when coupled with other techniques, like shear rheology, dynamic mechanical analysis or uniaxial tension tests.

It is important to note that a full mechanical characterization was not performed because that is beyond the scope of this work.

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