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Influence of loading history on mechanical properties of biomaterials

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Abstract

It is known that the mechanical response to load or displacement of a biological tissue can highly vary from one cycle to the next. Therefore, in order to establish a reference state for repeating data measurements, tissues are subjected to a preliminary step called preconditioning. Previous studies have suggested that the choice of preconditioning may influence the measured properties. To explore this effect, two different preconditioning methods were carried out on polymeric scaffolds and on bovine cartilage. The equilibrium young modulus and the energy dissipation were then measured to examine the change in the mechanical properties. Furthermore, the influence of the recovery time after preconditioning and the order of tests on the measured properties were also investigated. Results showed that the choice of preconditioning had a great impact on the measured properties as differences of more than 36% were observed for the equilibrium young modulus between samples subjected to different preconditioning protocols. The preconditioning method should therefore be informed to allow meaningful comparisons of measured data from different studies. This study also demonstrated the importance of a sufficient recovery period after preconditioning and before mechanical testing as insufficient recovery time led to irreversible damage of the samples.

Contents

1 Introduction

The characterization of the mechanical properties of biological tissues has proven to be a challenging task[1]. Indeed, tissues such as articular cartilage are inhomogeneous and present both a non-linear elastic and time-dependent behavior. In cartilage, the time-dependent behaviour is a result of two distinct mechanisms. First, an intrinsic viscoelasticity is present from the natural viscoleasticity of the collagen and proteoglycans, both components constituting the extra-cellular matrix (ECM) in the cartilage. The second mechanism, referred as the frictional drag force, results from the liquid-solid interaction of the interstitial fluid flowing through the pores of the extra-cellular matrix.
The viscoelastic behaviour of biological tissues leads to unequal measurements of the mechanical properties when subjected to repeated mechanical loading. This stress-softening, also observed in rubbers and called the Mullins effect, is corrected by a sequence of loading and unloading cycles called preconditioning. Preconditioning therefore "provides a known loading history and produces a consistent and reproducible reference state for the period of data recording"[2]. Although the phenomenon is not fully understood, preconditioning is considered to be a necessary step and measured data of specimens which were not preconditioned beforehand are not considered as valid. Despite the importance of preconditioning it has proven to be very difficult to establish a standard protocol as the adaptation of tissues to mechanical loading is not unique. Recent studies have actually demonstrated that the choice of preconditioning load sequence had an influence on the measured properties of spinal cord tissues [3]. Therefore, the first objective of this project was to investigate the influence of the preconditioning method on the measured properties. In this matter, two preconditioning protocols with different loading sequences were tested on bovine cartilage and on polymeric scaffolds whose objective were to mimic the structure of the cartilage.
The mechanical properties chosen to represent the change in the characteristics were the equilibrium young modulus and the energy dissipation. The equilibrium young modulus (E_{eq}) is a static representative of the stiffness of the material whereas the energy dissipation (ED) is an overarching viscoelastic indicator in dynamic loading. Indeed the energy dissipation takes into account all forms of dissipative behaviours related to the solid and liquid phases and is correlated to the microstructure of the polymeric scaffolds and the cartilage[4]. These two mechanical properties are measured by applying a well defined sequence of compressive loading and this may lead to irreversible modifications of the structure and thus influence the following measurements. Therefore, the scope of the second part of this study was to assess if the recovery time as well as the order of tests (i.e measuring E_{eq} before ED and vice versa), or in other words the loading history, have an impact on the measured mechanical properties.

2 State of the art

2.1 Preconditioning

Preconditioning can be considered as the gradual adaptation of the specimen to the mechanical loading. Figure 1 presents a typical preconditioning sequence of load-

ing/unloading cycles of a biological tissue such as cartilage or tendon. During the early cycles the hysteresis curves are very unequal but as the number of cycles increases the tissue observes continuous softening until reaching a saturated state where the hysteresis overlap each-other. Once this saturated state is achieved, the sample is said to be preconditioned.

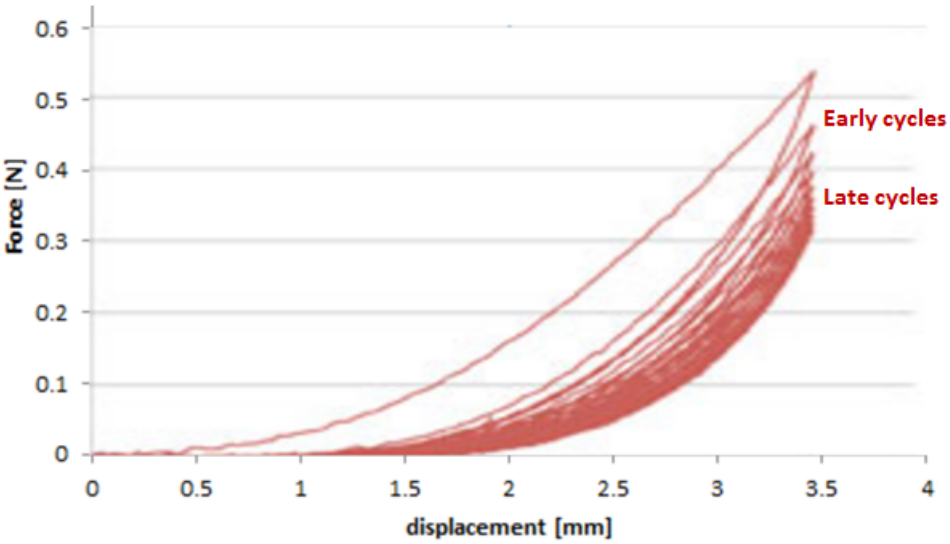


Figure 1: Example of a preconditioning cycle of a biological tissue

The number of preconditioning cycles needed to obtain a repeatable reference state depend on the material being tested and the type of test. For instance, Haut *et al*[5] only required one loading/unloading cycle to achieve the preconditioned state for collagen fibres. For Kwan *et al*, 5 preconditioning cycles were required to precondition the anterior cruciate ligament [6] whereas this number rose up to 30 cycles for the preconditioning of pig aortic valves[7].
The effects of the preconditioning protocols on the mechanical properties have been investigated for number of tissues such as cardiac muscles[8], pig aortic valves[7] and tendons[9]. Cheng *et al*[3] studied the influence of various preconditioning strains on the stress-strain responses of spinal cord tissue. Two groups of samples were preconditioned at 2% and 5% strain and were then both subjected to uniaxial strains of 2%. Results demonstrated significant higher stress-strain responses for samples preconditioned at the lower strain of 2%. It was therefore advised that the preconditioning strain employed should be higher than the strain applied during tests to avoid overestimation of the properties.
The preconditioning effects on the measured mechanical properties were also explored for articular cartilage by Hosseini *et al*[10]. Their study however focused on the interaction between the two time dependent effects present in the cartilage which arise from the intrinsic viscoleasticity of the collagen and the fluid flowing through the porous medium. They discovered that these two time dependent mechanisms competed against each other and may also mask each other's effects. Their relative importance is correlated to the type of loading imposed during the preconditioning step.

2.2 Cartilage and biomaterials

Cartilage is subdivided into three different groups primarily according to the histology :

- **elastic** : most springy and supple type of cartilage. Composed of elastic fibers, it makes up the outside of the ear and the tip of the nose.
- **fibrocartilage** : toughest type of cartilage thanks to a matrix with an enriched collagen fibre content. It is mainly found between the discs and the vertebrae.
- **Hyaline** : Both springy and tough, it is often referred as articular cartilage as it is present in the synovial joints.

Articular cartilage is a highly specialized tissue present in synovial joints with a thickness of approximately 2-4 mm. Its principle function is to provide a smooth and lubricated surface to facilitate both the movement in the articulation and the transmission of loads[11]. The structure of the articular cartilage can be seen as a biphasic medium with a liquid phase and a solid phase. The liquid phase is mainly composed of water which is the most abundant component of articular cartilage contributing to 65-80% of its weight. The solid phase is characterized by a dense and porous extracellular matrix primarily composed of collagen and proteoglycans. Specialized cells called chondrocytes, which are the only resident cells, are dispersed throughout the ECM and are responsible for the development, the maintenance and the repair of the latter.

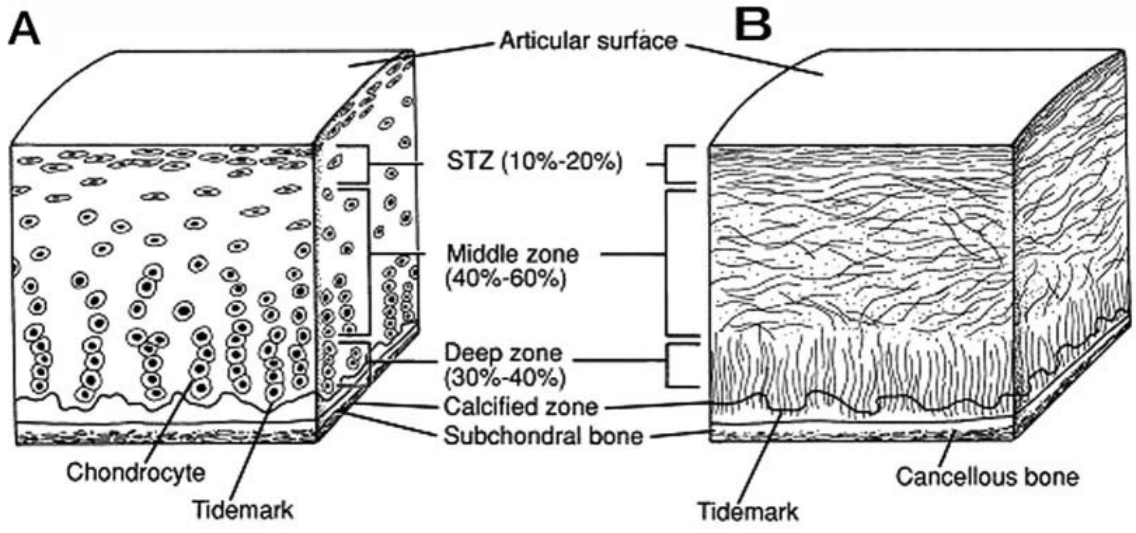


Figure 2: Schematic cross-section of a healthy articular cartilage [11]

The articular cartilage is frequently damaged due to excessive stresses or diseases such as osteoarthritis. However, there are no blood vessels in articular cartilage and thus due to this absence of vascularization it has a very limited self healing capacity. Plus, despite the large number of clinical cases reported and the important on-going research, no therapy capable of restoring the healthy structure of a damaged cartilage

has yet been established. Different tissue-engineering strategies involving biomaterials, combined with cells and growth factors, have therefore been thoroughly investigated to enhance repair in deteriorated damaged cartilage.
In cartilage-tissue engineering, biomaterials act as scaffolds whose primary objective is to replicate the ECM of the cartilage. For a biomaterial to be successful, it should fulfill certain requirements. First, the biomaterial should be biocompatible to avoid inflammatory response from the immune system. Plus, it must provide a favorable environment, such as structural stiffness and bioactivity, for the implantation of chondrocytes cells and have a sufficient permeability to allow the diffusion of nutrients and growth factors. Finally, the biomaterial should present a long term biodegradability for it to be integrated in the tissue remodelling process.
ECM-like biomaterials can be classified as synthetic or natural, in which we distinguish those based on proteins or polysaccharides. Among the synthetic matrices, the commercial Bio-Seed-C (BioTissue Technologies, Freiburg, Germany) mimics the ECM thanks to a porous structure based on polyglycolic acid, polylactic acid and polydioxanone in which chondrocytes are incorporated via a fibrin glue[12]. Natural protein-based biomaterials are generally based on collagen[13] as it is the main component of the ECM. Indeed, type I collagen gel is widely studied and has been successfully used for 3D cell culture and *in vivo* implantation of antologous chondrocytes in case studies [14]. Finally, concerning polysaccharides-based biomaterials, alginate and agarose are extensively studied polysaccharides for cartilage substitute and are extracted from, respectively, brown and Chinese algae. Both have been used *in vitro* for 3D culture of chondrocytes as they maintain the phenotype of the ECM and allow the growth of proteins in the latter[15]. However, when implanted alone in the human body, they both hinder the processus of spontaneous repair[15]. Nevertheless, a hybrid alginate-agarose hydrogel, called Cartipatch (Tissue bank of France, Lyon, France) showed promising results after *in vivo* implantation with antologous chrondrocytes as 8 out of the 13 patients tested presented improvements in the damaged cartilage.
In this study, the synthetic polymer scaffolds aiming to mimic the structure of the cartilage were based on 2-hydroxyethyl methacrylate (HEMA), which was crosslinked with Ethylene glycol dimethacrylate (EGDMA). These polymeric scaffolds have the particularity of being non-degradable; and as the effect of loading history on the mechanical properties was the focus of this project, multiple consecutive mechanical tests could be carried out on these scaffolds without taking into account the degradation factor, observed in other synthetic biomaterials.

2.3 Mullins effect

When a rubber-like material such as soft tissue is subjected to consecutive cycles of uniaxial loading and unloading, the load needed to obtain a given stretch is reduced for the following loading sequence. Such behaviour is sketched in Figure 3. First, the sample is loaded from *a* to *b'* following the *a b b'* path. When unloaded from *b'* to *a*, it follows this time the path *b' B a*. If the sample is reloaded again up to *c'* it follows the *a B c c'* and the subsequent unloading path would be *c' C a*, and so on. This stress softening behaviour is called the Mullins effect.

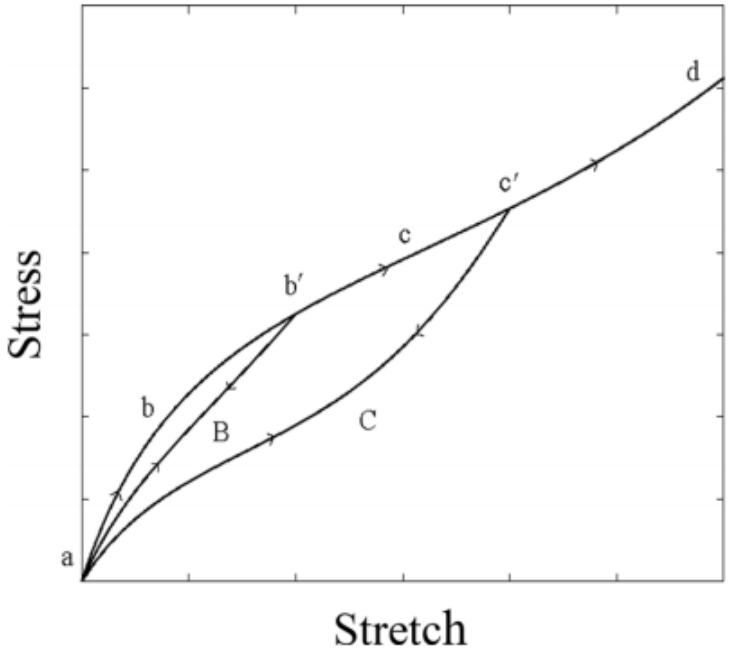


Figure 3: Sketch of a material exhibiting the Mullins effect during uniaxial tensile loading and unloading sequences[16].

After a certain number of loading/unloading cycles at a given strain, the material's responses coincide during the subsequent cycles , i.e the hysterisis overlap each other. Once the material has reached this state, the material is hence said to be preconditioned. The importance of preconditioning is highlighted here since if this step was not carried out, it would lead to an overestimation of the properties during the measurements.
The physical explanations behind the Mullins effect in rubbers is highly reported in the literature[17] and can be divided into 5 physical mechanisms : bond rupture, disentanglement of the polymer chains, molecular slipping, filler rupture and the double layer model. Concerning soft tissues, we can assume that only the first two mechanisms mentioned, bond rupture and disentanglement, are responsible for the Mullins effect.

3 Experimental Protocol

3.1 Materials

- 2-hydroxyethwl methacrylate (HEMA, Sigma-Aldrich)
- Ethylene glycol dimethacrylate (EGDMA, Sigma-Aldrich)
- Sodium Metabisulfite (SM, Sigma-Aldrich)
- Ammonium persulfate (AP, Biorad)
- Sodium Chloride (Fischer)

- Active Aluminum oxide (Merck)
- Bovine cartilage

3.2 Scaffold Preparation

The polymer scaffolds were prepared by the salt leaching method. For this method, sodium chloride was first placed in a teflon mold, sealed at the bottom with a plastic cover. Two types of scaffolds were prepared throughout the project : the *fine* and the *coarse* scaffolds. The first type were obtained by placing salt, with a particle size ranging from 200 to 250 um, in a 4 mm thick mold containing 10 mm diameter holes. Concerning the *coarse* scaffolds, a mixture of salt containing 60% of 355-400 um particles and 40% of 400-500 um particles was poured in a a 6 mm thick mold containing 12 mm diameter holes.
The polymer solutions with different crosslinking densities (4% for the *fine* scaffolds and 8% for the *coarse* ones) were prepared according to the amounts in table 1. First, the EGDMA was added to HEMA and pipet in-out three times for a initial mixing. Next the AP and SM water solutions were added to the solution. Finally, the resulting solution was mixed for 1 minute to obtain a homogenized mixture.

Table 1: Solution preparation for scaffolds with different salt sizes

Scaffold Type	Salt size [um]	pHEMA [um]	EGDMA [um]	AP [um]	SM [um]
fine	200-255	1000	60	41	41
Coarse	355-400 (60%) 400-500 (40%)	1000	125	41	41

The corresponding solutions were then poured on the salts in the teflon molds. These molds were placed in an oven at a temperature of 65^{o}C for 2 hours enhancing the polymerization of the scaffolds. The solidified scaffolds were then placed in deionized water for 4-5 days to remove the salt, which dissolved inside the water. Finally, the scaffolds were punched and cut to obtain a final thickness of approximately 2.8 mm and a diamater of 7.7 mm.

3.3 Cartilage extraction

The mechanical properties of the polymer scaffolds were compared to those of bovine cartilage, obtained from the local butchery in Ecublens (Vaud, Switzerland). Hence, bovine cartilage samples were punched from the patella groove using an 8 mm diameter puncher. Figure 4 gives a quick insight on this method, even though the picture displayed is a femoral head and not a patella groove. Samples were then placed in Phosphate-buffered saline (PBS) to conserve them in humid conditions.

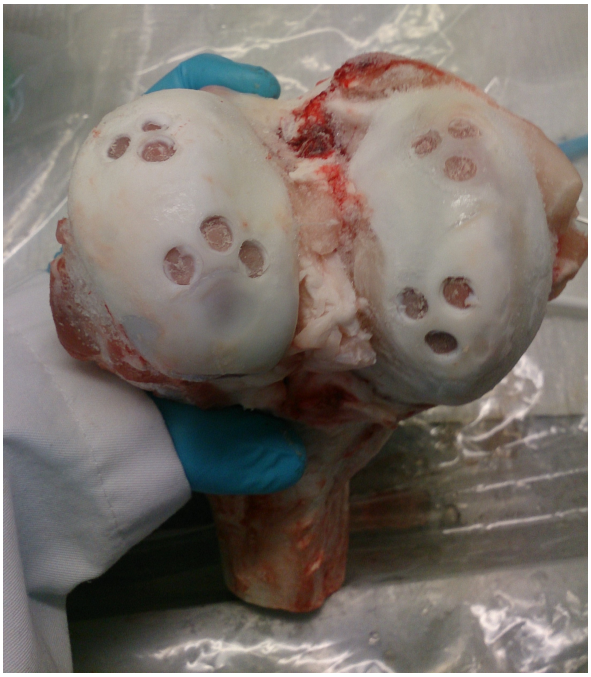


Figure 4: Example of bovine cartilage extraction from the femoral head

3.4 Preconditioning methods

The scaffolds and the cartilage samples were subjected to two different preconditioning protocols, exposed in figure 5, in order to investigate the impact of the preconditioning on the measured mechanical properties. Both preconditioning were made using the Electropuls Dynamic Test System (Instron E3000, Instron, Norwood, Massachusetts, USA).

- Preconditioning 1 : the sample is compressed up to 32% strain at a rate of 0.05 mm/s. The sample is then maintained at 32% strain for 3 s before being fully unloaded at a rate of 10 mm/s. This sequence of steps is repeated five times.

- Preconditioning 2 : The sample is compressed up to 32% strain with a strain rate of 10 μm/s. After a 2 s delay the sample is decompressed up 5% strain and is followed by a cyclic compression of 20 cycles at 1 Hz with a dynamic amplitude of 15%.

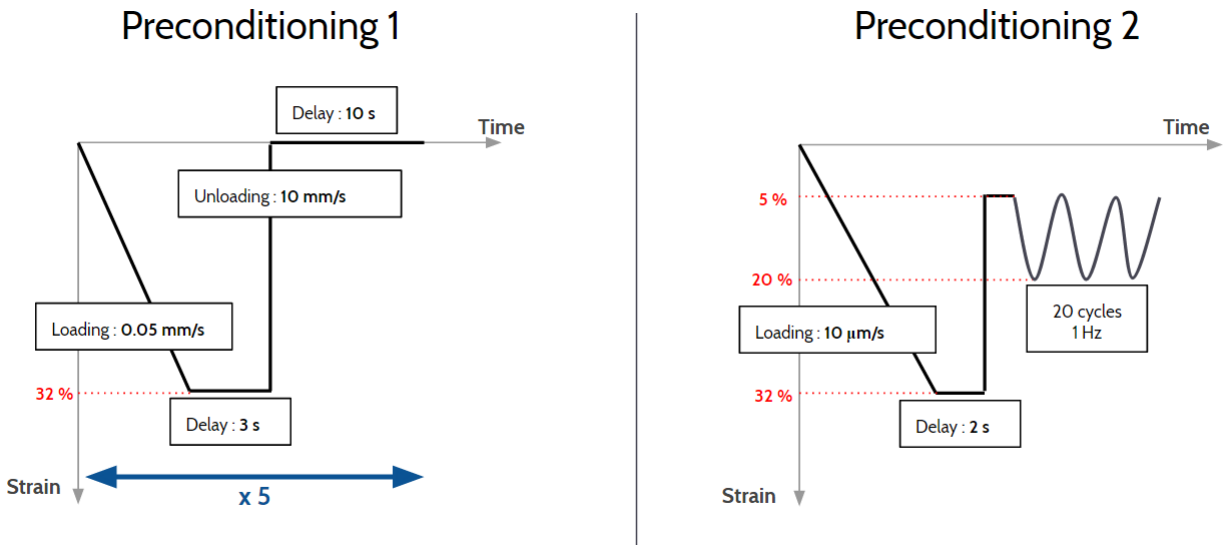


Figure 5: The two different preconditioning protocols

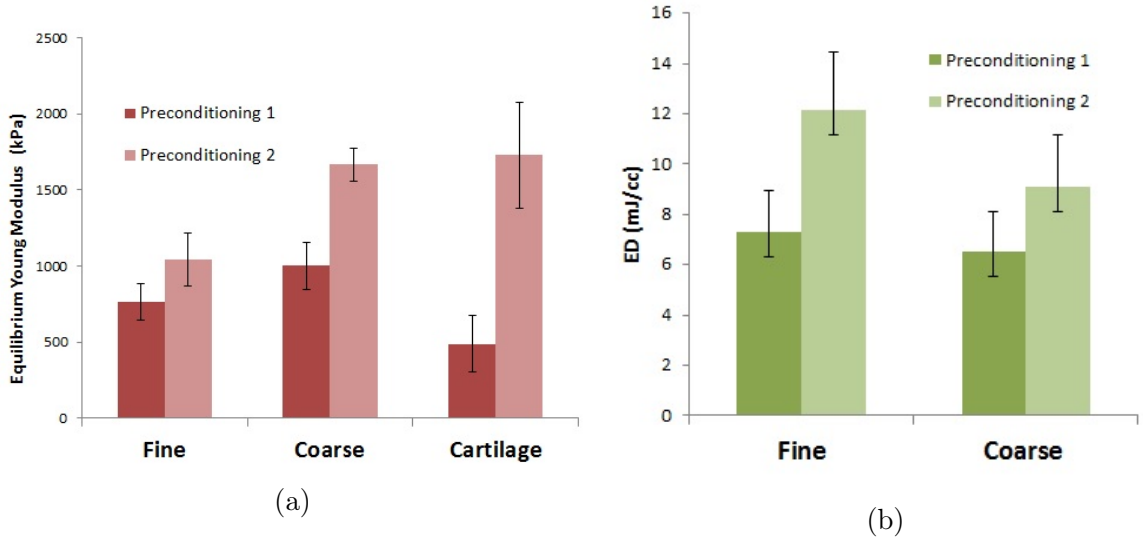
3.5 Mechanical properties

The equilibrium young modulus (E_{eq}) and the Energy dissipation (ED) were both quantified. Unconfined compression tests were carried out using Electropuls Dynamic Test System (Instron E3000, Instron, Norwood, Massachusetts, USA) on the polymer scaffolds and the cartilage samples immersed in deionized water.
For the equilibrium young modulus measurements, samples were first compressed up to 10, 15 and 20% strain and followed relaxation times of respectively 400, 200 and 200 seconds. The stresses obtained at the end of each relaxation periods were extracted and were plotted in a stress-strain graph. The equilibrium young modulus is then obtained by defining the slope of the stress/strain curve.
Concerning the energy dissipation, a pre-strain of 10% was applied and was followed by a cyclic compression of 10% amplitude at a frequency of 1 Hz. The energy dissipation was obtained from the resulting Load-Displacement graphs by measuring the enclosed area inside the hysteresis curve.

4 Results and Discussion

4.1 Influence of preconditioning protocol

In the first part of this study, the objective was to investigate the influence of the preconditioning protocols on the measured mechanical properties. The polymeric scaffolds and the cartilage samples were subjected to either the first or the second preconditioning protocol and were then tested for equilibrium young modulus and energy dissipation after a 24h recovery. A rest period of 24h was chosen as previous studies demonstrated that such rest period was enough for the sample to recover from the viscoelastic effects induced by the prior preconditioning step[18]. The results obtained for E_{eq} and ED are presented below in figure 9.



(a) (b)

Figure 6: the equilibrium young modulus (a) and the energy dissipation normalized by volume (b) after 24h recovery for samples subjected to preconditioning 1 and 2.

One can immediately notice a difference in the measured properties between samples subjected to different preconditioning protocols. Indeed, samples preconditioned with the second method (light red) show an increase in the equilibrium young modulus of 36%, 66% and 255% for, respectively, the *fine* scaffolds, the *coarse* scaffolds and the bovine cartilage in comparison to samples subjected to the first preconditioning protocol (dark red). Furthermore, as expected coarse scaffolds achieved higher E_{eq} than *fine* scaffolds, respectively 1000 kPa vs 765 kPa for precondition 1 samples and 1665 kPa vs 1042 kPa for precondition 2 samples, due to a higher fraction of cross-linker in the polymer solution.
E_{eq} of 490 kPa and 1700 kPa were obtained for bovine cartilage samples preconditioned with, respectively, the first and the second method. Although theses values are similar to those found in the literature[19], the high standard deviations observed can invite one to question the obtained results. These high intrinsic fluctuations between the samples (38% for preconditioning 1 and 20% for preconditioning 2) may be explained by the structural composition of the cartilage's ECM. Indeed, as presented in Figure 7, articular cartilage present 4 different zones : the superficial zone, the middle zone, the deep zone and the calcified zone[11].

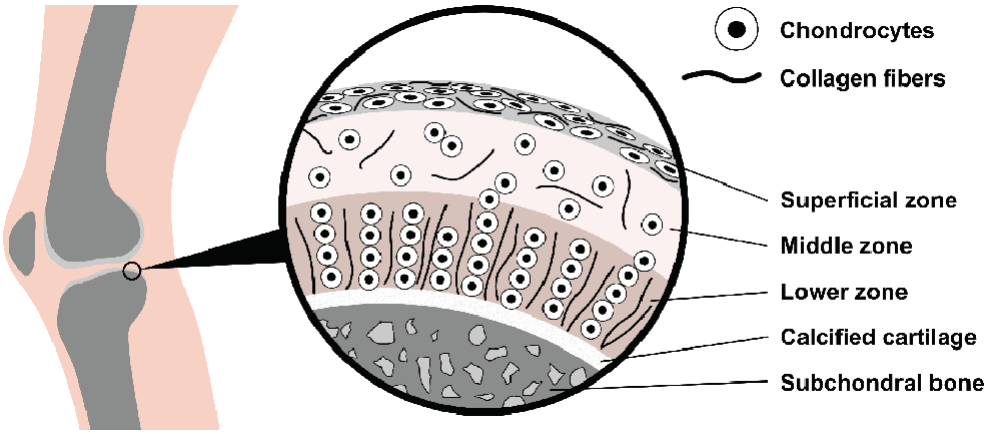


Figure 7: Zone organization of artificial cartilage [11]

In the superficial zones, collagen fibres are parallel to the surface and protect the articular cartilage from the shear stresses. On the other hand, in the deeper zones the collagen fibres are arranged perpendicular to the surface and are thus highly responsible for the compressive resistance of the cartilage. Therefore, the equilibrium young modulus measured may vary depending on which surface of the sample the compressive load is being applied. The importance difference in E_{eq} between the cartilage specimens may also arise from the intrinsic variation of the cartilage itself throughout the patella groove. Indeed, previous studies have demonstrated a distribution of the compressive stiffness of the cartilage across the patella groove[20]. Therefore, a cartilage sample extracted from the medial border will naturally present a higher stiffness than a sample extracted from lateral regions of the patella grove. Unfortunately, both influences of the cartilage's structure and its location in the patella groove were not considered during the tests and should be taken into account in future studies.
Concerning the energy dissipation, *fine* and *coarse* scaffolds both showed a higher energy dissipation when subjected to preconditioning 2. Samples therefore demonstrated both lower equilibrium young moduli and energy dissipation after being preconditioned with the first protocol. This decrease in the mechanical properties was attributed to the "harsher" loading sequence employed in preconditioning 1 method. Indeed, samples underwent 5 loading/unloading cycles up to 32% strain whereas for preconditioning 2, samples were administrated a cyclic compression with a 15% dynamic amplitude around a mean value of 12.5% strain. This harsher method hence leads to greater irreversible modification of the structure of the specimen by inducing cleavage of the crosslinking bonds between the polymer chains. The resulting matrix is thus weakened explaining the lower mechanical properties obtained.
The impact of the preconditioning protocol on the measured mechanical properties was therefore highlighted in this section as a harsher protocol led to lower measured values due to irreversible damage of the sample. It would be interesting in future studies to investigate the microstructure of the samples, for instance with scanning electron microscopy, after preconditioning to highlight the impact of the latter on the morphologies.

4.2 Impact of the recovery time and loading history

In the second stage of this project, the impacts of the the recovery time after preconditioning and the order of tests were more thoroughly explored. 5 groups of *coarse* scaffolds (N=3) were therefore subjected to different sequences of tests with different recovery times, as displayed in figure 8. For instance, samples in *Group 1* were first preconditioned (P2) and then directly, i.e with no recovery time, tested to evaluate the energy dissipation (ED). 24 hours later the energy dissipation was measured once more and finally 24 hours later again (hence 48 hours after preconditioning) the equilibrium young modulus (E_{eq}) was measured. Preconditioning 2 protocol was chosen for the preconditioning step as preconditioning 1 seemed to induce greater damage to the samples, as seen in the previous section.

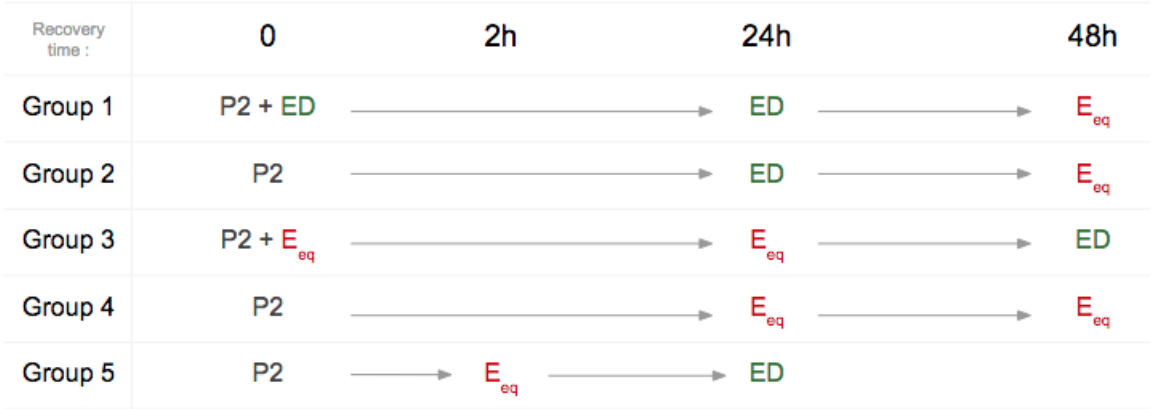
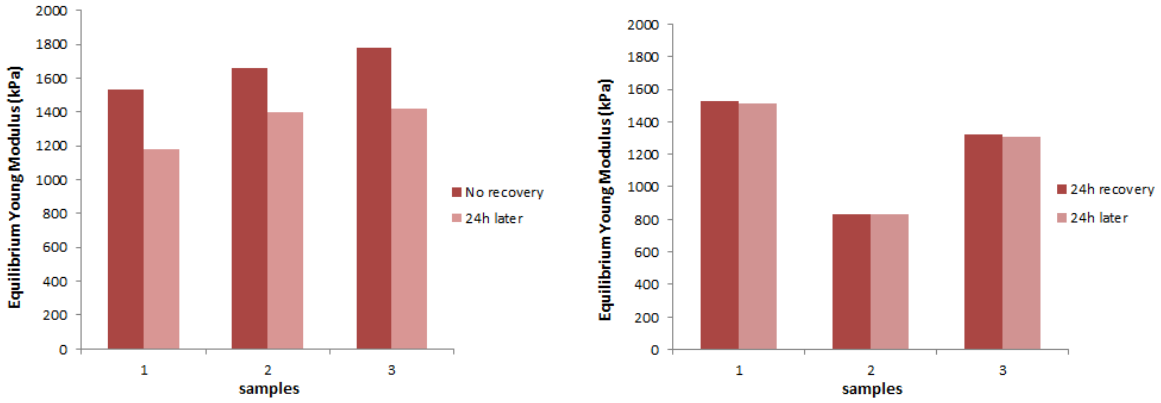


Figure 8: Different sequences of tests with different recovery times applied to *coarse* scaffolds. P2 : preconditioning 2, ED : Energy Dissipation, E_{eq} : Equilibrium young modulus.

Figure 9(a) presents the results obtained for the 3 coarse scaffolds from *Group 3* when the E_{eq} was measured, first, right after the preconditioning step (i.e no recovery) and then 24 hours later. One can notice that all three samples show a decrease in the E_{eq}, respectively 23%, 18% and 20%, when they were measured again 24 hours later. Similar decrease was also noticeable for the energy dissipation of samples from *Group 1* which was also measured instantly after preconditioning, and after a recovery time of 24h. This behavior however was not observed for *Group 4* samples. Indeed, as observed in Figure 9(b), the equilibrium young modulus remains constant when measured first 24 hours after preconditioning and then 24 hours later again. Although the second sample of *Group 4* demonstrates an unexpectedly low E_{eq} (approximatly 820 kPa) and is not considered for the mean value in Figure 11, it is still displayed to highlight the consistency of the modulus.



(a) **Group 3** (b) **Group 4**

Figure 9: The equilibrium young moduli measured for Group 3 (a) and Group 4 (b) samples.

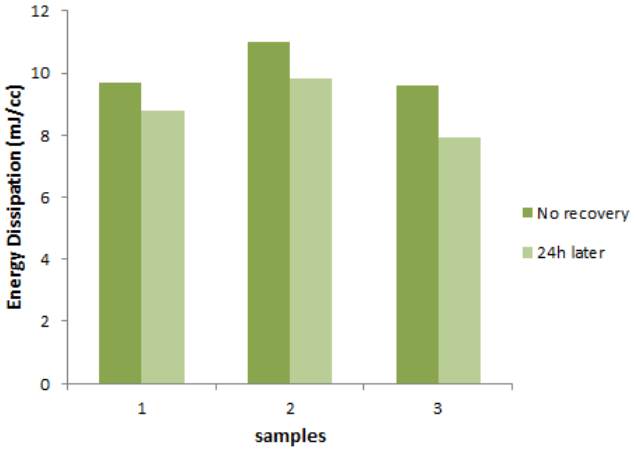


Figure 10: Energy dissipation normalized by the volume for Group 1 samples after 0h and 24h later.

Therefore, as also demonstrated in Conza *et al*'s work[18], a recovery period of 24h after preconditioning is sufficient to remove the viscoelastic effects and to establish a reference state for repeatable measurements. On the other hand, when mechanical testing is performed right after the preconditioning step, subsequent measurements reveal a reduction in the mechanical properties. Hence, immediate testing after preconditioning leads to the irreversible damage of the polymer matrix by cleavage of the cross-linking bonds, as mentioned previously, resulting in a lessened stiffness in subsequent measurements.
In order to investigate in greater depth the influence of the recovery time on the measured properties, samples from *Group 5* observed a intermediate recovery period of 2 hours before being tested for the equilibrium young modulus. Figure 11 displays the mean measured E_{eq} for samples which, respectively, observed recovery times of 0h, 2h and 24h. Lower mean E_{eq} was obtained when samples underwent a recovery period

of 2h instead of the 24h recommended by Conza *et al*'s paper[18], respectively 1150 kPa and 1430 kPa. The reduced stiffness observed for the *2h recovery time* samples was attributed to the partial re-swelling and restructuring of the polymer chains due to an insufficient recovery. The viscolesastic effects from the loading sequences during preconditioning are thus still present after 2 hours and can only be annealed by a longer recovery period, such as 24 hours as demonstrated above.
Nevertheless, one would thus expect to obtain even lower stiffness for samples tested right after preconditioning as the re-swelling is even more incomplete. However, this is not the case as a batch-high mean E_{eq} of 1580 kPa is observed. Although the phenomenon behind this result is not fully understood, since the recovery time is practically null we can assume that the relatively high compressive resistance arises from the polymer chains being still under stress from the preceding preconditioning. The recovery period after preconditioning is therefore an important step as it highly influences the measured mechanical properties. Conza also concluded that "preconditioning without an adequate rest period may increase the complexity of the strain history"[18]and hence the recovery time should be chosen wisely.

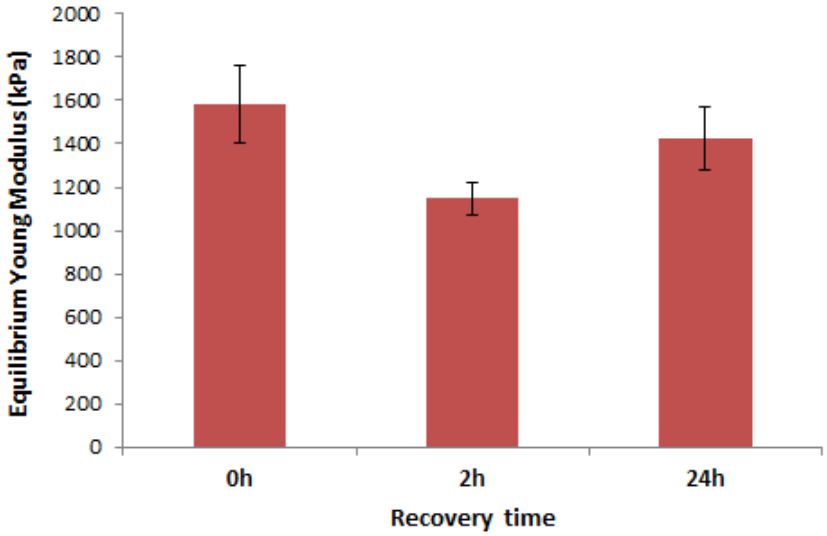


Figure 11: The equilibrium young moduli after a recovery time of 0h, 2h and 24h. (N=3)

5 Acknowledgments

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6 Conclusions

In the first place, the scope of this study was to compare two preconditioning protocols with different loading sequences and their impact on the mechanical properties, measured after a rest period of 24 hours. Results revealed that samples presented much higher mechanical properties when subjected to *preconditioning 2* protocol instead of *preconditioning 1* with differences of equilibrium young moduli (E_{eq}) rising up to 36, 66 and 255% for, respectively, the *fine* scaffolds, the *coarse* scaffolds and the bovine cartilage. These high variations arise from the fact that the first protocol is "harsher" and thus leads to more irreversible damage of the sample by cleavage of the cross-linking bonds. High fluctuations were also observed for the E_{eq} between the cartilage samples. This was attributed to two characteristic of the articular cartilage : (a) the cartilage is composed of 4 zones with different mechanical response and thus the compressive resistance may vary depending on which surface the loading is applied; (b) The mechanical properties of the cartilage depend on its position in the patella groove.
In the second stage of the project, the emphasis was placed on the influence of the recovery time and the loading history on the measured mechanical properties. 5 groups of *coarse* scaffolds were thus subjected to different sequences of tests and recovery periods. The first conclusion drawn was that a recovery time of 24 hours is needed to obtain a reproducible reference state, in accordance with other similar studies [18][10]. Inadequate recovery times led to either irreversible damage of the sample or the incapacity to remove the residual viscoelastic effects. Concerning the order of tests, i.e if the energy dissipation is measured before the young modulus and vice versa, conclusions could not be drawn with the results obtained and requires further investigation. Future studies could be accompanied with a microscopic investigation in order to observe the changes in the microstructure.

7 References

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