# Development of a biocompatible hydrogel based on native temporomandibular joint extracellular matrix powder

Daniela Trindade and Carla Moura*

Centre for Rapid and Sustainable Product Development,
Polytechnic of Leiria,
Leiria, 2030-028, Portugal
Email: daniela.trindade@ipleiria.pt
Email: carla.moura@ipleiria.pt
*Corresponding author

Cecília Calado

ISEL-Instituto Superior de Engenharia de Lisboa,
Instituto Politécnico de Lisboa,
Lisboa, 1959-007, Portugal
and
CIMOSM,
Centro de Investigação em Modelação e
Optimização de Sistemas Multifuncionais,
ISEL, Lisboa, 1959-007, Portugal
Email: cecilia.calado@isel.pt

David Ângelo

Centre for Rapid and Sustainable Product Development,
Polytechnic of Leiria,
Leiria, 2030-028, Portugal
and
Faculdade de Medicina da Universidade de Lisboa,
Lisboa, 1649-028, Portugal
and
Instituto Português da Face,
Lisboa, 1050-227, Portugal
Email: david.angelo@ipface.pt

Nuno Alves

Centre for Rapid and Sustainable Product Development,
Polytechnic of Leiria,
Leiria, 2030-028, Portugal
Email: nuno.alves@ipleiria.pt

**Abstract:** The temporomandibular joint disc (TMJd) is an avascular and fibrocartilaginous tissue with low regeneration capacities. To date there is no permanent solution upon its damage. So, our group hypothesise that the combination of a synthetic material [poly(ethylene glycol) diacrylate, PEGDA] and decellularised matrices may be the answer, since it has the advantage of retaining extracellular matrix (ECM) components. For this purpose, lamb TMJd were decellularised with ethanol (96%)/ acetone (99.5%) followed by an analysis to the ECM obtained. Afterwards, different concentrations of ECM powder were added to the PEGDA to obtain hybrid hydrogels. Their swelling capacities and mechanical behaviour (hydrated or dry) were assessed. Results show a reduction of the proteoglycans in the disc, except in the posterior region. Dried hydrogels with 2% ECM powder are the ones with the closer compressive modulus to the native disc, along with greater viscoelastic capacity, representing a possible approach to a TMJd replacement.

**Keywords:** decellularisation; extracellular matrix powder; poly(ethylene glycol) diacrylate hydrogels; temporomandibular joint disc.

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**Biographical notes:** Daniela Trindade completed her degree in Biomechanics in 2018 at Polytechnic of Leiria and her Master's degree in Biomedical Engineering in 2021 at Instituto Superior de Engenharia de Lisboa. She is a Researcher at the Centre for Rapid and Sustainable Product Development of Polytechnic of Leiria since 2018. Her research activities are focused on the tissue engineering field, through the study of biocompatible materials for temporomandibular joint disc regeneration.

Carla Moura is a researcher at the Centre for Rapid and Sustainable Product Development, since 2009, and an Assistant Professor, since 2017, at the Polytechnic Institute of Leiria. She concluded her PhD of the MIT-Portugal Program in Bioengineering in 2016. She has a Master's degree in Product Development (2011) and a degree in Biomechanics (2008), both from the Polytechnic Institute of Leiria. She works in the fields of medical biotechnology, bioengineering and biomaterials. Her research activity focuses on tissue engineering, especially on the manipulation of materials using mainly additive manufacturing techniques combined with cells, such as mesenchymal stem cells.

Cecília Calado is a Professor at ISEL – Instituto Superior de Engenharia de Lisboa, Instituto Politécnico de Lisboa since September 2014. She is a member of the Pedagogical Council since 2015 and coordinates the BSc and MSc in Biomedical Engineering degrees since 2015 and 2018, respectively, as well as the R&D laboratory in Health & Engineering since 2015. Her academic degrees are PhD in Biotechnology (2003, Instituto Superior Técnico, Technical Lisbon University), MSc in Biochemical Engineering/Biotechnology (1998, Instituto Superior Técnico) and a degree in Biochemistry (five years university degree, 1995, Sciences Faculty, Lisbon University).

David Ângelo is a Clinician at Instituto Português da Face, who presents a great clinical and scientific experience in the area of temporomandibular joint. He is the only Portuguese doctor to join the European Society of Temporomandibular Joint Surgeons, has a Master degree in Medicine from Faculty of Health

Sciences, University of Beira Interior (2004–2010) and a PhD in Medicine, Maxillofacial Surgery from Faculty of Medicine, University of Lisbon (2014–2018). He coordinates and organises the Iberian course on TMJ surgery in Portugal in collaboration with the Faculty of Medicine of the University of Lisbon and has contributed to the development of minimally invasive techniques for the treatment of temporomandibular joint dysfunction.

Nuno Alves is the Director of the Centre for Rapid and Sustainable Product Development, which is dedicated to the transformation of the manufacturing industry through the development and implementation of novel Additive Manufacturing printing technology. He is a Coordinator Professor on Biomechanics at the Polytechnic Institute of Leiria. He received a first degree, an MSc and a PhD degree in Mechanical Engineering, all from Instituto Superior Técnico da Universidade de Lisboa. He was Course Coordinator of the Mechanical Engineering Programme at the School of Technology and Management of the Polytechnic of Leiria and Coordinator of the Socrates/Erasmus Programme for Mechanical Engineering at the same Institute.

## 1 Introduction

In the temporomandibular joint (TMJ), there is a fibrocartilaginous disc (TMJd) fundamental for the normal and correct performance of this joint. It absorbs impact by helping to lessen the incongruity of the involved bone structure. It lubricates the zone and functions as a load-bearing structure. TMJd main biochemical components are collagen type I, elastin and glycosaminoglycans (GAGs) as chondroitin sulphate, dermatan sulphate, hyaluronic acid, keratin sulphate and heparin sulphate (Detamore and Athanasiou, 2003; Shu et al., 2015). The main cells are fibrochondrocytes, where fibroblasts represent 70% of the population, founded on porcine tissue (Acri et al., 2019). The disc is predisposed to damage and degeneration due to its cartilaginous nature, which consequently leads to additional dysfunctions in the remaining bony structures of the joint (Juran et al., 2015). Disc dysfunctions involve its displacement from its normal position, its perforation or thinning, wherein today's advanced cases of disorder, disc removal is the most accepted option (Vapniarsky et al., 2018).

Xenogeneic tissues may be a viable approach to a TMJd construct. They present the necessary ECM components and biochemical signals and adequate three-dimensional architecture important for cell growth. However, for their application in tissue engineering (TE), decellularisation methods are necessary to remove the immunogenic components (Schwarz et al., 2012; Nie and Wang, 2018) and after obtaining the acellular matrix, the tissue can be subjected to a set of methods: frozen, lyophilised and minced to powder. The extracellular matrix (ECM) powder can be solubilised or use as it (Edgar et al., 2018).

Hydrogels are polymeric networks with high water absorption capacity. They allow the transport of water, cellular waste and nutrients. With the use of ultraviolet or visible light, synthetic hydrogels can be chemically crosslinked, termed photopolymerisation, where it is possible to convert a liquid solution into a solid. This procedure also allows encapsulation of cells or biological agents. Poly(ethylene glycol) (PEG) presents great biocompatibility, it is non-immunogenic, promoting the production of ECM by

photoencapsulating chondrocytes in PEG hydrogels (Chung and Burdick, 2008; Zhu, 2010; Cui et al., 2014).

In the TE field of cartilage regeneration, decellularised xenogeneic tissues have been investigated for the production of ECM scaffolds, where proliferation and differentiation of chondrocytes were verified (Yang et al., 2008; Oh et al., 2018). The first attempt to use xenogeneic tissues for TE of the TMJ disc was through the incorporation of porcine urinary bladder powder in sheets of the same material to form a TMJd shape scaffold. Implantation of this acellular scaffold demonstrated tissue formation (Brown et al., 2011, 2012). Recently, different studies have demonstrated the benefit of using the combination of ECM powders with synthetic polymers. For regeneration of the intervertebral disc, small intestinal submucosa powder was solubilised and coated on polylactic-co-glycolic acid, where it was found that pore size and area are fundamental for an appropriate scaffold mechanical performance and ECM synthesis (Kim et al., 2014). For the production of nanofibrous scaffolds for cartilage, solubilised auricular cartilage was mixed with polycaprolactone (PCL), where cartilage regeneration was obtained in vivo (Feng et al., 2020) and nasal cartilage particles were biofunctionalised in Polyhydroxyalkanoate, where collagen formation and chondrogenic markers expression was found (Masaeli et al., 2017).

Poly(ethylene glycol) diacrylate (PEGDA) is a PEG acrylate-derived polymer. In this functionalised form, it can be photopolymerised and in previous investigations of our group, it was shown to present good results when combined with PCL. The produced PEGDA hydrogel presented a compressive modulus within the native disc, and when introduced as a core in the PCL, these properties improved since a better viscoelastic capacity was found (Moura et al., 2020).

To date, a hybrid hydrogel for TMJd combining a synthetic material and decellularised matrices has not yet been developed, demonstrating its potentiality, and, consequently, leading to further work for optimisations. In the present study, different decellularised lamb ECM powders concentrations were combined with PEGDA to obtain a hybrid scaffold. It was hypothesised that the addition of the natural components would mimic the TMJ environment and, consequently, help in its lubrication. Moreover, it will have greater mechanical performance and will support in the synthesis of cartilage ECM. The decellularisation impact on the disc ECM was evaluated, and hydrogels behaviour under compression and their swelling capacity were tested.

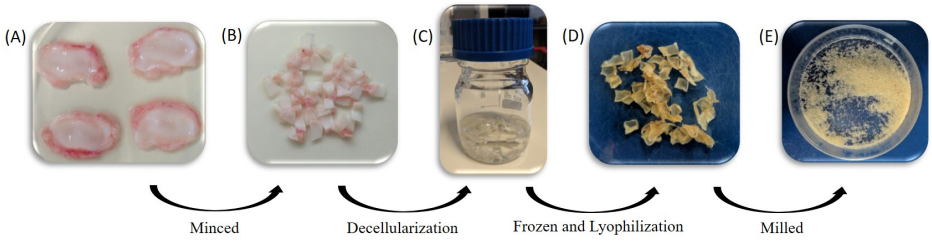
## 2 Materials and methods

### *2.1 Tissue preparation and decellularisation*

TMJ discs were dissected from lamb heads and the retrodiscal tissue and ligaments were carefully removed (N = 15). Before decellularisation, the discs were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich®) and cut into small pieces with a scalpel. Decellularisation was performed with a modification of the protocol by Matuska et al. (2018), where fresh discs were subjected to 1:1 solution of ethanol (96%)/acetone (99.5%) in an orbital shaker at 150 rpm for 24 h, followed by two PBS washes of 45 min to remove the remaining cellular and agent components. Afterwards, samples were frozen at –80°C overnight, lyophilised for 24 h at 0.01 mBar, milled into a powder and filtered

in a 300 µm mesh to uniform the powder (Figure 1). The final powder obtained from the 15 samples weighted 0.6582 g.

**Figure 1** Schematic representation of the different steps performed to obtain the extracellular matrix powder (see online version for colours)




Notes: The native discs (a) were cut into small pieces with a scalpel (B) and subjected to the decellularisation process (C). Afterwards, samples were lyophilised (D), milled into a powder and filtered in a 300 µm mesh (E).

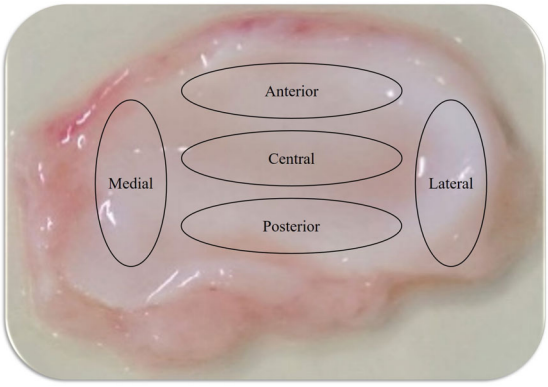
## *2.2 Hydrogel production*

Poly(ethylene glycol) diacrylate (PEGDA) hydrogels were produced according to Moura et al. (2020). Briefly, 20% (v/v) PEGDA hydrogels (MW 575, Sigma-Aldrich®) were dissolved in 0.5 M aqueous solution of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (Sigma-Aldrich®) and 0.1% (w/v) 2, 2-dimethoxy-1, 2-diphenylethanone (DMPA) photoinitiator was added (Sigma-Aldrich®). The mixture was heated at 45°C, at 60 rpm for 5 min and, then, photopolymerised (UV light, $\lambda$ = 365 nm) for 3 min. Concentrations of 1% and 2% (m/v) of decellularised powder were combined with PEGDA hydrogels. The solution was obtained by shaking the components at 100 rpm during photopolymerisation to avoid powder deposition at the bottom of the mould. Each mould had a capacity of 1 ml of solution and a cylindrical shape.

## *2.3 Fourier-transform infrared spectroscopy*

Fourier-transform infrared (FTIR) spectroscopy with an attenuated total reflectance detection mode (Alpha FT-IR, Bruker) was carried out on the native and decellularised discs, with a resolution of 4 $cm^{-1}$ and 64 scans per spectrum. The FTIR spectroscopic analysis was performed in triplicates over five different regions of the disc (N = 3) (Figure 2). Spectra were pre-processed by baseline correction. Quantitative analysis was conducted as Zhou et al. (2018) and Spalazzi et al. (2013), where the collagen and proteoglycans content was determined by integrating the area under the amide I region (1,720 $cm^{-1}$ to 1,590 $cm^{-1}$) and carbohydrate region (1,140 $cm^{-1}$ to 985 $cm^{-1}$) normalised to the amide I peak, respectively. Normalisation was performed to eliminate thickness variations between samples.

**Figure 2** Schematic image of the five regions of the disc evaluated by Fourier-transform infrared spectroscopy (see online version for colours)




## *2.4 Compressive mechanical tests*

PEGDA and hybrid hydrogels were subjected to uniaxial unconfined compression tests using a texture analyser with a 1.2 mm $min^{-1}$ extension rate and a 50 kg load cell (TA.XTplusC, Stable Micro Systems, UK). A set of the produced hydrogels were compressed by submerging them in $dH_2O$ for at least 24h before testing to assure full hydration (N = 4). Another set was tested in the dried form after lyophilisation at 0.1 mBar for 24h (N = 3). Compressive modulus was calculated by the slope of the elastic region of the stress-strain curve.

## *2.5 Swelling ratio assay*

The equilibrium swelling ratio (q) was determined by weighting the hydrogels after photopolymerisation and submerging them for 48 h in $dH_2O$ at 37°C to fully hydrate (N = 4). The weight of the different samples was measured periodically until the reach of the equilibrium state ($W_{swollen}$). Afterwards, the hydrogels were dried by lyophilised for 24h at 0.1 mBar ($W_{dry}$). The q was calculated using equation (1):

$$q = \frac{W_{swollen}}{W_{dry}}. \tag{1}$$

The percentage of water content (WC) of each hydrogel was also calculated using equation (2):

$$WC = \frac{W_{swollen} - W_{dry}}{W_{swollen}} \times 100. \tag{2}$$

## *2.6 Statistical analysis*

Statistical analysis was performed with GraphPad Prism 8 software. Values are presented as mean ± standard deviation. Statistical analysis to the swelling and mechanical tests were assessed with one-way ANOVA with multiple comparisons corrected by the

Dunnett test and FTIR spectra analysis with Mann-Whitney U test. A confidence interval of 95% was used, where significant different values were considered for p-value < 0.05 (*p < 0.05, **p < 0.01, and ***p < 0.001).

## 3 Results and discussion

### *3.1 Characterisation of the decellularised matrices*

Decellularisation is a process that should be optimised for the total removal of cellular components, while the 3D architecture and composition of the matrix are maintained (Lopresti and Brown, 2015). The native TMJd used for the proposed work (N = 15) presented a weighted of 0.343 ± 0.066 g. After the ethanol/acetone treatment, they presented a weight loss of ~16% (0.289 ± 0.064 g).

The type of agents used in decellularisation could have a negative impact on the ECM components, destroying them, which could modify the mechanical properties and necessary biochemical cues (Kim et al., 2019). Few decellularisation agents have been tested for obtaining an acellular TMJd. Matuska et al. (2018) compared 0.1% (m/v) sodium dodecyl sulphate (SDS) and 1:1 solution of acetone/ethanol for the decellularisation of porcine discs and scanning electron microscopy (SEM) and histology staining did not show major differences in the ECM structure when compared to the native disc. Lumpkins et al. (2008) performed SEM analysis on porcine discs after decellularisation and results showed collagen fibres disorganised in decellularisation with 1:3 solution of acetone/ethanol when compared to 1% (m/v) SDS. Despite this, Juran et al. (2015) has observed collagen loss after decellularisation with 1% SDS. Considering these results, the best proposed strategy which did not lead to ECM changes was the 1:1 acetone/ethanol.

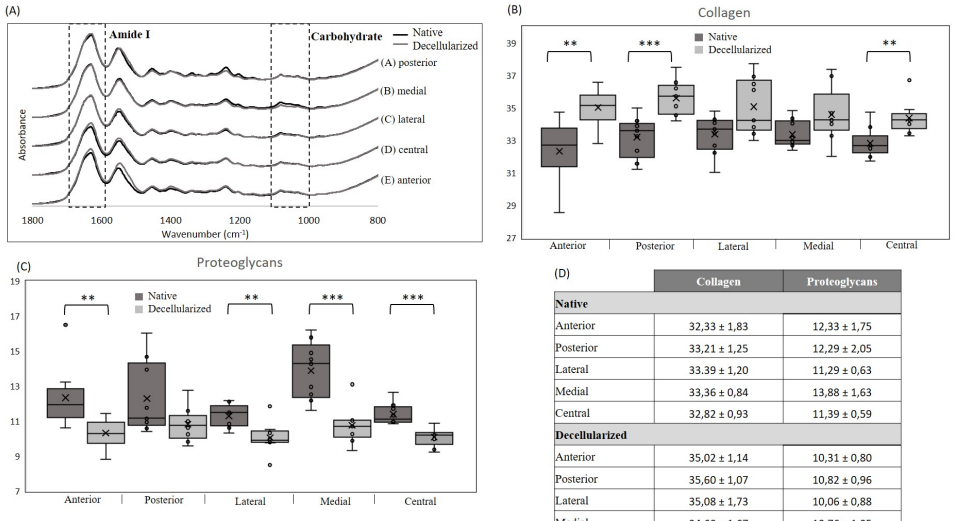
Since the TMJd is composed of 70-80% of collagen type I and 1-10% of GAGs, of the dry weight of the disc (Fazaeli et al., 2016; Acri et al., 2019), the analysis of the impact of ethanol/acetone on the disc ECM components, specifically in the five regions of the disc is important. For this, quantitative analysis by FTIR spectroscopy was performed and the representative spectra of the native and acellular matrices are shown in Figure 3(a). Results show that peaks of the amide I (1,720 $cm^{-1}$ to 1,590 $cm^{-1}$) and carbohydrate (1,140 $cm^{-1}$ to 985 $cm^{-1}$), corresponding to collagen and proteoglycans, respectively, are present in all spectra.

The comparison between the cellular and acellular matrices is shown in Figures 3(b), 3(c) and 3(d). For the collagen content, there were significant differences between the different regions of the disc, where there was an increase in the area of the amide I peak of 8.32% in the anterior region (32.33 ± 1.83 vs. 35.02 ± 1.14, $p < 0.01$), 7.19% in the posterior region (33.21 ± 1.25 vs. 35.60 ± 1.07, $p < 0.001$) and 4.72% the central region (32.82 ± 0.93 vs. 34.38 ± 1.00, $p< 0.01$). For the proteoglycans, also significant differences were found, where there was a decrease of 16.42% in the anterior region (12.33 ± 1.75 vs. 10.31 ± 0.80, $p < 0.01$), 11.47% in the central region (11.39 ± 0.59 vs. 10.08 ± 0.50, $p < 0.001$), 10.95% in the lateral region (11.29 ± 0.63 vs. 10.06 ± 0.88, $p < 0.01$) and 22.49% in the medial region (13.88 ± 1.63 vs. 10.76 ± 1.05, $p < 0.001$).

The increase in the absorbance in the amide I region was not expected, as by Zhou et al. (2018), it was predicted by FTIR spectroscopy a decrease in 19.1% of collagen and

27.6% proteoglycan during decellularisation in rabbit pubic symphysis fibrocartilage. Differences obtained between the present study and the literature cannot be compared as the animal model and cartilage used was different and, also the decellularisation agent was different – 2% SDS. In our case, the increase absorbance of amide I in decellularisation samples could have resulted from denaturation of the collagen fibrils upon contact with decellularisation agents, which consequently leads to an increase in the exposure of the amino acids that absorb the infrared radiation of FTIR spectra. Another reason for the amide I increase with decellularisation, is due to the loss of volume, and water content, resulting in an increase of concentration in the remaining molecules as collagen. If that occurred, the proteoglycan decrease could imply that its decrease was much higher than the gain of its concentration due to water/ volume loss.

**Figure 3** FTIR spectroscopic analysis




(D)

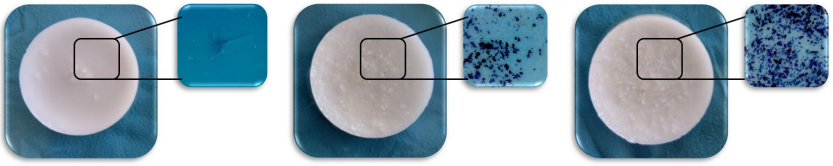
| | Collagen | Proteoglycans |
|---|---|---|
| **Native** | | |
| Anterior | 32,33 ± 1,83 | 12,33 ± 1,75 |
| Posterior | 33,21 ± 1,25 | 12,29 ± 2,05 |
| Lateral | 33.39 ± 1,20 | 11,29 ± 0,63 |
| Medial | 33,36 ± 0,84 | 13,88 ± 1,63 |
| Central | 32,82 ± 0,93 | 11,39 ± 0,59 |
| **Decellularized** | | |
| Anterior | 35,02 ± 1,14 | 10,31 ± 0,80 |
| Posterior | 35,60 ± 1,07 | 10,82 ± 0,96 |
| Lateral | 35,08 ± 1,73 | 10,06 ± 0,88 |
| Medial | 34,60 ± 1,67 | 10,76 ± 1,05 |
| Central | 34,38 ± 1,00 | 10,08 ± 0,50 |

Notes: Representative spectra of the native and decellularised discs across the five regions of the disc (A). Relative collagen (B) and proteoglycan (C) content distributed across the five regions of the disc, determined as the peak areas shown in (A). Mean and standard deviation of peak areas relative to collagen and proteoglycans of the five regions of the disc (D). In (B) and (C), statistical differences are presented by **$p < 0.01$ and ***$p < 0.001$.

## *3.2 Characterisation of the hydrogels*

The hydrogels were successfully produced with the addition of the ECM powders (Figure 4). This powder, being a natural material, will enhance the synthetic polymer due to its bioactive properties and beneficial host tissue responses (Nie and Wang, 2018). The assessment of the swelling and mechanical properties of the hydrogels is important in TE as it can influence the cellular viability and the success of the material implantation, respectively (Bryant and Anseth, 2002).

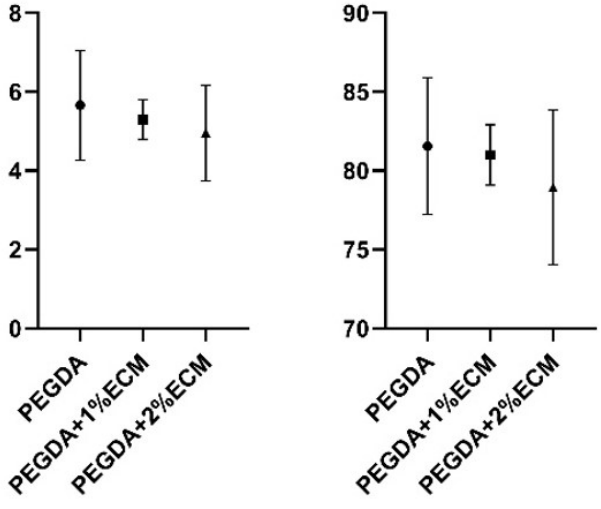
**Figure 4** (a) PEGDA, (b) PEGDA with 1% ECM and (c) PEGDA with 2% ECM, with a closer view stained with methylene blue (see online version for colours)



(a) (b) (c)

Swelling studies were carried out to the PEGDA and hybrid hydrogels to assess whether the addition of ECM powder could enhance or decrease the capacity of the hydrogels to absorb water. PEGDA hydrogels presented an area of 294.09 ± 11.94 $mm^2$ and a thickness of 3.94 ± 0.44 mm, PEGDA hydrogels with 1% ECM presented an area of 287.76 ± 7.99 $mm^2$ and a thickness of 3.52 ± 0.39 and PEGDA hydrogels with 2% ECM presented an area of 291.68 ± 6.72 $mm^2$ and a thickness of 3.87 ± 0.37 mm. For this test, the hydrogels were placed in $dH_2O$ at 37°C for 48 h, where the swelling behaviour was found to be non-statistical different for all specimens (Figure 5). The swelling ratio for the PEGDA hydrogels, PEGDA hydrogels with 1% ECM and PEGDA hydrogels with 2% ECM was 5.66 ± 1.39, 5.30 ± 0.50 and 4.96 ± 1.21, respectively. This means that the hydrogels, without dissolving, can absorb 4.96 to 5.66 grams of water per gram of its weight. Since the disc presents 66% to 80% water content (Detamore and Athanasiou, 2003), the hydrogels must have the same capacity. Therefore, PEGDA hydrogels, PEGDA hydrogels with 1% ECM and PEGDA hydrogels with 2% ECM presented 81.54 ± 4.33%, 80.99 ± 1.92% and 78.95 ± 4.91% of water content, respectively. Although the value for PEGDA hydrogels with 2% ECM is slightly lower, all hydrogels water content is within the values for the native disc.

**Figure 5** (a) Swelling ratio (b) Water content of the PEGDA and hybrid hydrogels (N = 4)



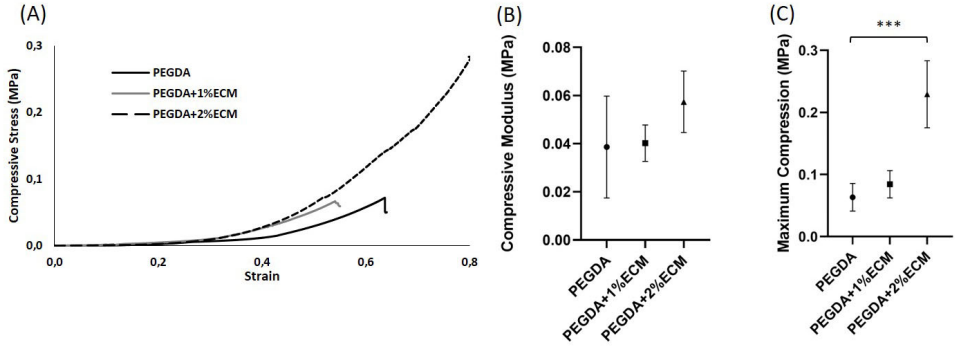

(a) (b)

Notes: No statistical differences were found.

The native TMJd has the ability to restore to its original shape due to the elastic fibres that are parallel oriented to the collagen. GAGs are thought to give the disc the necessary compressive strength. Collagen provides the tensile strength (Detamore and Athanasiou, 2003; Acri et al., 2019), but it has been shown to confer compression properties (Fazaeli et al., 2016). So, the mechanical evaluation of the hydrogels is important to understand their biomechanical behaviour and if the introduction of the powder alter their properties. For this, two scenarios were tested: in the hydrated form where they were placed in $dH_2O$ for 24 h or in the dry form were they were lyophilised.

PEGDA hydrated hydrogels presented an area of 30.85 ± 2.41 $mm^2$ and a thickness of 4.28 ± 0.49 mm, PEGDA hydrated hydrogels with 1% ECM presented an area of 297.89 ± 7.23 $mm^2$ and a thickness of 4.05 ± 0.27 mm and PEGDA hydrated hydrogels with 2% ECM presented an area of 294.75 ± 10.47 $mm^2$ and a thickness of 3.80 ± 0.43 mm. In the hydrated form, all hydrogels presented a compressive modulus lower than the native disc [Figure 6(B)] (compressive modulus of 0.1–10 MPa) (Athanasiou et al., 2009), obtained from de slope of the stress-strain curves [Figure 6(A)]. Moreover, no significant differences were found between the obtained values. PEGDA hydrogels presented a compressive modulus of 0.038 ± 0.021 MPa and PEGDA hydrogel with 1% ECM presented a value of 0.040 ± 0.007. Despite these values, PEGDA hydrogel with 2% ECM presented a slight increase when compared to PEGDA alone (0.057 ± 0.013 MPa). Although these results are not as desired, when the maximum compression at break is analysed, some statistical differences are found [Figure 6(C)]. PEGDA hydrogels and PEGDA hydrogel with 1% ECM presented a maximum compression of 0.063 ± 0.022 MPa and 0.085 ± 0.022 MPa, respectively. When comparing PEGDA hydrogel with 2% ECM (0.230 ± 0.054 MPa) with PEGDA hydrogel, a significant statistical difference is found ($p < 0.001$), where PEGDA hydrogel with 2% ECM has the ability to withstand greater compressive forces over a greater extent.

**Figure 6** PEGDA and hybrid hydrogels behaviour to compression in a hydrated form (N = 4)

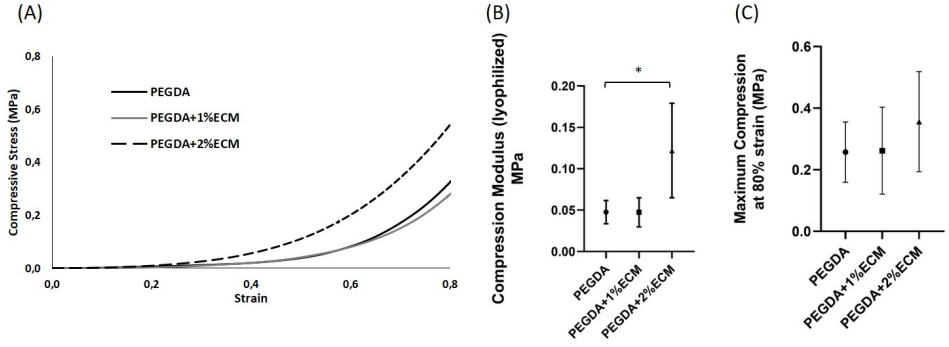



Notes: Stress-strain curve (A) for each hydrogel, and corresponding compressive modulus (B) and maximum compression at break or at 80% (C). Statistical differences were performed with PEGDA hydrogel as control and are presented by ***$p < 0.001$.

Analysing the dried hydrogels, PEGDA hydrogels presented an area of 202.36 ± 6.75 $mm^2$ and a thickness of 3.13 ± 0,39 mm, PEGDA hydrogels with 1% ECM presented an area of 166.29 ± 20.26 $mm^2$ and a thickness of 2.51 ± 0.76 mm and PEGDA hydrated hydrogels with 2% ECM presented an area of 205.49 ± 7.90 $mm^2$ and a

thickness of 3.00 ± 0.10 mm. The maximum compression obtained at 80% strain for all hydrogels presented no statistically significant difference [Figure 7(C)], where the values were 0.257 ± 0.098 MPa, 0.262 ± 0.141 MPa and 0.356 ± 0.162 MPa for PEGDA, PEGDA with 1% ECM and PEGDA with 2% ECM, respectively. Regarding the compression modulus, PEGDA hydrogels and PEGDA hydrogels with 1% ECM presented a compression modulus of 0.048 ± 0.014 MPa and 0.047 ± 0.017 MPa, respectively. Comparing with PEGDA with 2% ECM (0.126 ± 0.051 MPa) statistical differences were found ($p < 0.05$) with PEGDA alone [Figure 7(B)]. In addition to presenting a superior compressive module, is within the values for the native disc.

**Figure 7** PEGDA and hybrid hydrogels behaviour to compression in the dried form (N = 3)




Notes: Stress-strain curve (A) for each hydrogel, and corresponding compressive modulus (B) and maximum compression at 80% strain (C). Statistical differences were performed with PEGDA hydrogel as control and are presented by *$p < 0.05$.

Comparing the results obtained between the hydrated and dried hydrogels is possible to conclude that the hydrated hydrogels have low mechanical capacity and break easily. On the contrary, dry hydrogels have a more sponge-like structure, giving them a more viscoelastic structure with greater mechanical capacity. For hydrogels with 1% ECM, there were no major mechanical improvements over PEGDA, however with a higher percentage, 2%, some improvements were achieved. The compression module was within the native values and with previous investigations (Moura et al., 2020). Moreover, the maximum compression values were also higher compared to the others.

These results may be explained by the fact that the ECM is composed of collagen and GAGs, which, as previously mentioned, are responsible for the compressive properties in the disc. However, as in the decellularisation process there was some loss of these components, the 1% addition in the PEGDA hydrogel was not enough to observe differences in the compressive mechanical properties, unlike the addition of 2%. With this, it is believed that a higher concentration could be a possible strategy to achieve closer mechanical values to the native disc combined with a better protocol for the decellularisation of the TMJd. This correlation between the increase in ECM concentration and the increase in mechanical properties has already been proven for the production of a scaffold from ECM particles (Rowland et al., 2016) or for the production of a hydrogel by solubilisation of ECM particles (Liang et al., 2020).

## 4 Conclusions

Xenogeneic tissues could represent a suitable approach in TE to find viable replacements for the TMJd and cartilage in general. In this study, the ethanol/acetone decellularisation agents were evaluated and a decrease of the proteoglycans was found in the different regions of the disc, except in the posterior one. To prevent unwanted degradation of ECM components, the search for an effective decellularisation process needs to be investigated. Still, the use of 2% powdered decellularised ECM combined with a synthetic polymer, PEGDA, in the dried form, was found to have greater mechanical performance, essential for a proper TMJd implant.

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