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Biomechanical characterization of human soft tissues using indentation and tensile testing

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Corresponding Author:	Michelle Griffin, MBChB MSc MRes MRCS University College London Medical School london, london UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	12michellegriffin@gmail.com
Corresponding Author's Institution:	University College London Medical School
Corresponding Author's Secondary Institution:	
First Author:	Michelle Griffin, MBChB MSc MRes MRCS
First Author Secondary Information:	
Other Authors:	Yaami Premakumar, BSc
	Alexander Seifalian, PhD
	Peter Butler, FRCS MD
	Matthew Szarko, PhD
Order of Authors Secondary Information:	
Abstract:	Regenerative medicine aims to engineer materials to replace or restore damaged or diseased organs. The mechanical properties of such materials should mimic the human tissue it is aiming to replace to be able to sustain the mechanical forces it will experience when implanted at the tissue defect site to provide the required anatomical shape. Although the mechanical properties of tissue-engineered scaffolds are of great importance, many human tissues, which undergo restoration with engineered materials, have not been fully biomechanically characterized. Several compressive and tensile protocols are reported for evaluating materials but with large variability. It is difficult to compare results between studies. Further complicating studies is the often destructive nature of mechanical testing. Whilst an understanding of tissue failure is important, it is also important to have knowledge of the elastic and viscoelastic properties under more physiological loading conditions. This report aims to provide a minimally destructive protocol to evaluate the compressive and tensile properties of human soft tissues. As examples of this technique, tensile testing of skin and compressive testing of cartilages are described. These protocols can also be directly applied to synthetic materials to ensure the mechanical properties are similar to the native tissue. Protocols to assess the mechanical properties of human native tissue, will allow a benchmark by which to create suitable tissue-engineered substitutes.
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TITLE:
Biomechanical characterization of human soft tissues using indentation and tensile testing

AUTHOR:
Griffin, Michelle
Division of Surgery & Interventional Science
University College London (UCL)
London, UK
12michellegriffin@gmail.com

Premakumar, Yaami
Anatomy Department
St Georges University
London UK
yaami.p@gmail.com

Seifalian, Alexander
Division of Surgery & Interventional Science
University College London (UCL)
London, UK
a.seifalian@gmail.com

Butler, Peter Edward
Division of Surgery & Interventional Science
University College London (UCL)
London, UK
And
Plastic & Reconstructive Surgery Department
Royal Free Hospital
London, UK.
pembutler@gmail.com

Szarko, Matthew
Anatomy Department
St Georges University
London UK
mszarko@sgul.ac.uk

CORRESPONDING AUTHOR:
Michelle Griffin
12michellegriffin@gmail.com

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SHORT ABSTRACT:
Tissue biomechanics is important for maintaining cell shape and function and for determining phenotype. This report demonstrates non-destructive mechanical protocols for characterizing elastic and viscoelastic properties of human soft tissues, which can be directly applied to tissue-engineered substrates to allow a close matching of engineered materials to native tissue.

LONG ABSTRACT:
Regenerative medicine aims to engineer materials to replace or restore damaged or diseased organs. The mechanical properties of such materials should mimic the human tissues they are aiming to replace; to provide the required anatomical shape, the materials must be able to sustain the mechanical forces they will experience when implanted at the defect site. Although the mechanical properties of tissue-engineered scaffolds are of great importance, many human tissues that undergo restoration with engineered materials have not been fully biomechanically characterized. Several compressive and tensile protocols are reported for evaluating materials, but with large variability. It is difficult to compare results between studies. Further complicating the studies is the often destructive nature of mechanical testing. Whilst an understanding of tissue failure is important, it is also important to have knowledge of the elastic and viscoelastic properties under more physiological loading conditions.

This report aims to provide a minimally destructive protocol to evaluate the compressive and tensile properties of human soft tissues. As examples of this technique, the tensile testing of skin and the compressive testing of cartilage are described. These protocols can also be directly applied to synthetic materials to ensure that the mechanical properties are similar to the native tissue. Protocols to assess the mechanical properties of human native tissue will allow a benchmark by which to create suitable tissue-engineered substitutes.

INTRODUCTION:
Patients are increasingly waiting for various organ transplantations to treat failing or injured organs. However, with the shortage of suitable donor organs, regenerative medicine is aiming to create alternative solutions for patients with end-stage organ failure. Regenerative medicine aims to meet this clinical need by engineering materials to act as tissue substitutes, including soft tissues, such as cartilage and skin. To create a successful material to restore damaged tissues, the replacement material should mimic the properties of the native tissue it is going to replace[1-2]. Once surgically implanted, the material will need to provide anatomical shape to the tissue defect and thus, the mechanical properties of the material are vital[1]. For example, a material replacing auricular cartilage should have the appropriate mechanical properties to prevent compression by the overlying skin[2]. Similarly, a material to replace nasal cartilage will need to have adequate mechanical properties to prevent collapsing during breathing[3]. However, despite the importance of mechanical properties when manufacturing materials for implantation, little evidence has focused on characterizing the mechanical properties of different human tissues.

Mechanical testing regimes can be used to establish the compressive, tensile, bending, or shear properties of a tissue. Skin is a highly anisotropic, viscoelastic, and nearly incompressible material[4-9]. Commonly-excised skin is tested using uniaxial

tensile methodologies, where a suitably-shaped strip of skin is gripped at both ends and stretched while the load and extension are recorded.[4-9].

Since the major component of all soft tissues is interstitial water, the mechanical response of cartilage is strongly related to the flow of fluid through the tissue[10-11]. Soft tissues such as cartilage have been traditionally tested using compression testing. The methods for testing in compression are quite varied, with confined, unconfined, and indentation being the most prevalent (Figure 1). Within confined compression, a cartilage sample is placed in an impervious, fluid-filled well and loaded through a porous plate. Since the well is non-porous, flow though the cartilage is in the vertical direction[12-13]. In unconfined compression, the cartilage is loaded using a non-porous plate onto a non-porous chamber, forcing the fluid flow to be predominantly radial[12-13]. Indentation is the most frequently-used method for evaluating the biomechanical properties of cartilage[12-13]. It consists of an indenter, smaller than the surface of the specimen being tested, that is brought down onto the specimen. Indentation has many advantages over other methods of compression, including the fact that indentation can be performed *in situ*, enabling the test to be more physiological (Figure 1)[12-13].

To understand the compressive and tensile properties of a tissue, the Young's elastic modulus is typically calculated by analyzing the linear portion of the stress-strain curve, indicating the elastic resistance to compression or tension, irrespective of specimen size[12]. Both tensile and compressive testing regimes can vary according to the load or deformation applied and the rate of both such parameters. At present, there are many different testing protocols to assess tissue mechanics, which makes it extremely difficult to interpret or compare results from different studies[6-13.] Furthermore, many mechanical methods currently focus on characterizing the mechanical properties of the tissue by testing the specimen to destruction. We aim to demonstrate an indentation and tensile protocol that provides direct, non-destructive comparison of human soft tissues and tissue-engineered constructs.

We demonstrate a method that limits the mechanical tests to stress yet still obtains a Young's elastic modulus in compression and tension. The sample is stressed either in tension or compression to a certain value, and once the chosen stress value has been reached, the sample is allowed to relax whilst all the data is recorded. This method captures both the viscoelastic and relaxation properties of the tissue within the same test, which can be applied directly to the synthetic material. We have used the indentation protocol to evaluate human soft tissues, including skin and cartilage[14-16]. Cartilage is assessed using indentation testing and skin is evaluated using tension testing[14-16]. Researchers aiming to engineer materials with similar properties to human soft tissues could consider implementing these protocols.

PROTOCOL:

This protocol follows the ethical guidelines of our institution's human research ethical committee guidelines on the use, storage, and disposal of human tissue. Human tissue samples can be excised from cadaveric bodies that have been consented for research purposes with relevant ethical approvals. Samples can also be discarded tissue from consented patients undergoing surgical procedures, with relevant ethical approval.

1. Preparation of Skin

1.1. Prepare specimens by manually dissecting off the adipose tissue and the thin layer of deep dermis using a scalpel blade and forceps. This step is important to ensure consistency between samples[14].

1.2. Cut the resulting sheet of split-thickness skin into a standardized sample size (*e.g.,* 1 cm × 5 cm samples). Determine the specimen size based on the dimensions of the testing apparatus. If a tissue-engineered construct is also being tested, the specimen size should be appropriate for the material of interest[14]. Dispose of scalpel blades in the appropriate sharps bins.

1.3 To enable completion of the mechanical calculations, measure the thickness of the skin being tested using electronic calipers before and after mechanical testing.

2. Tensile Testing

Note: All materials testing machines should be calibrated according to the manufacturer's guidelines prior to testing.

2.1. Test skin samples in uniaxial tension using a materials testing machine (Figure 2A) at room temperature (22 °C)[14].

2.2. Orientate the skin samples in the same direction for all samples (*e.g.,* perpendicular or in-line with Langer Lines (topological lines drawn on a map of the human body and referring to the natural orientation of collagen fibers in the dermis))[14].

2.3. Immobilize the sample between two clamps (a commercial jig), one affixed to a 98.07 N load cell and the other to an immovable base plate[14]. The resulting area between the clamps tested in uniaxial tension should be 1 cm x 4 cm (Figure 2).

Note: A commercial jig was utilized to avoid non-uniform gripping and damage to the sample before testing. The sample is fixed to a "finger-tight" tightness.

2.4. Cover the sample area (after placement in the apparatus) on both sides with petroleum jelly to prevent specimen desiccation.

2.5. Program the tensile loading and relaxation testing regime into the software as a list of actions, as follows: Zero Load | Zero Position | Find Contact (Tensile loading) | Wait (Relaxation).

2.6. Start the test with the software program. Load the sample under tension to 29.42 N at 1 mm/s. Use a rate and load that does not cause failure of the skin (*e.g.,* 29.42 N at 1 mm/s).

2.7. After the 29.42 N-load is reached, allow the tissue to relax for 1.5 h, a time-point at which there is minimal change in relaxation behavior, controlled by the computer software[14].

Note: The displacement is held constant during the relaxation phase, not the load.

2.8. Calculate elastic and viscoelastic properties as per the analysis section guidelines. The mechanical properties investigated will represent the average properties of the split-thickness skin constituents (epidermis and dermis)[14].

Note: There is no defined tare load, as it is clear from the raw data when deformation is occurring and thus, only these data points are included.

3. Preparation of Cartilage

3.1. Remove the skin and fascia from the cartilage specimen using a scalpel blade and forceps[15,16].

3.2. Divide the cartilage specimens into a standardized sample size (*e.g.,* 1.5-cm blocks) using a scalpel blade and forceps. For all samples, use a semicircular-shaped indenter (Figure 2B) that has a diameter and thickness at least 8 times greater than the size of the cartilage sample. This ratio ensures that the indenter is not affected by any edge effects from specimen preparation[15]. Dispose of scalpel blades in the appropriate sharps bins.

3.3. To enable completion of the mechanical calculations, measure the thickness of the cartilage to be loaded using electronic calipers before and after mechanical testing[15,16].

4. Compressive Indentation Testing

4.1. Compress the cartilage samples using a materials testing machine in a hydrated environment at room temperature. Cover the cartilage sample with phosphate-buffered saline (PBS) prior to and during compression testing to ensure that the sample is hydrated.

Note: PBS does not exactly match the physiological environment, but it allows both the materials and the tissues to be compared equally[15,16].

4.2. Orientate the cartilage sample so the surface is perpendicular to the indenter. This allows the compression to be uniaxial and limits any shear loading[15].

4.3. Program the compressive loading and relaxation testing regime into the software as a list of actions, as follows: Zero Load | Zero Position | Find Contact (Compressive loading) | Wait (Relaxation).

4.4. Start the test using the software program. Load the sample under compression to 2.94 N at 1 mm/s[15,16].

Note: This was determined to be a non-destructive load that is sensitive enough to identify both elastic and viscoelastic properties of cartilage[15].

4.5. After the 2.94-N limit is reached, allow the cartilage to relax for 15 min, a time-point at which there is minimal change in relaxation behavior, using the computer software[15,16].

Note: Figure 2C-D shows a typical set up for the compression and tensile testing of human tissue specimens. The same protocols can then be applied to synthetic biomaterials to match the biomechanical properties to the native tissue being analyzed. For example, Figure 2E-F demonstrates compression and tensile testing of human tissue closely matching a synthetic material's biomechanical properties.

5. Calculation of Young's Elastic Modulus for Indentation and Tensile Testing

5.1. Collect the raw data including time (s), displacement (mm), and load (N) from the materials testing device[14-16].

5.2. Calculate the stress (MPa) and strain (%) using the formulas shown in Figure 3.

Note: If a hemispherical indenter was used during compression testing, dividing the force by the cross-sectional area gives the nominal (average) stress, but not the peak stress.

5.3. Use a linear scatter plot to plot the stress MPa (y-axis) against the strain (x-axis). Determine the linear curve fit. The linear curve fit is equal to $y = mx + b$ with a respective R value.

Note: All data points are included to achieve a minimum R value >0.98. The m value is the slope, which corresponds to the modulus of stress over strain, indicating compressive resistance or resistance to tension in MPa (*i.e.,* Young's Modulus). If the R value is not >0.98, then the assumption of characterizing linear viscoelastic behavior is invalid.

5.4. To identify the viscoelastic properties in which fluid flow from exposure to deformation has reached equilibrium, the ratio of stress over time over the last 200 s of mechanical testing and the final stress level at the end of the experiment are calculated.

Note: With increasing time, the stress level will decrease (relax) as fluid flow reaches equilibrium[17,18]. A fast stress-relaxation response indicates that it is difficult to maintain high stresses within the sample[17,18].

6. Relaxation Properties

6.1. Plot stress in MPa (y-axis) against time in s (x-axis) on a linear scatter plot.

6.2. Determine a linear curve fit to calculate the rate of relaxation. The linear curve fit is equal to $y = mx + b$ with a respective R value of the last 200 s. The m value is the rate of relaxation.

6.3. Include all data points to obtain a minimum R value >0.98. The final stress (MPa) at 1.5 h for skin and 15 min for cartilage is the final absolute relaxation value.

REPRESENTATIVE RESULTS:

Figures 4 and 5 provide examples of data obtained via indentation and tensile testing. Figure 4 demonstrates typical values obtained after human cartilage

indentation testing. Figure 4A is an example of a typical strain-versus-stress plot obtained after indentation testing. To obtain the Young's Modulus, all values are included until the line curve fit has a minimum R value of 0.98 (Figure 4B). The m value is the indicator of Young's Modulus in MPa; for example, in this data, the cartilage has a modulus of 1.76 MPa. Figure 4C shows a typical plot of stress against time to evaluate the relaxation properties of cartilage. The rate of relaxation is calculated from the last 200 s. Similarly, to obtain the rate of relaxation, the m value of a line curve fit in MPa is used. For example, in this data, the cartilage has a rate of relaxation of 8.77×10^{-6} MPa/s (Figure 4D). The absolute final level of relaxation is the final point of stress in MPa. For example, in this data set, the absolute final level of relaxation would be 0.03 MPa (Figure 4D).

Figure 5 shows how to evaluate the viscoelasticity of skin tissue after tensile testing. The analysis is carried out as per compression testing. Figure 5A demonstrates a typical strain-versus-stress plot obtained from the tensile testing protocol. To obtain the Young's Modulus in tension, all values are included until the line curve fit has a minimum R value of 0.98 (Figure 5B). The m value is the indicator of Young's Modulus in MPa; for example, in this data, the skin has a modulus of 0.62 MPa. Figure 5C shows a typical plot of stress against time to evaluate the relaxation properties of skin. The rate of relaxation is calculated from the last 200 s. Similarly, to obtain the rate of relaxation, the m value of a line curve fit in MPa is used. For example, in this data, the skin has a rate of relaxation of 3.1×10^{-5} MPa/s (Figure 5D). The absolute final level of relaxation is the final point of stress in MPa. For example, in this data set, the level would be 0.62 MPa (Figure 5D). The same analysis can then be utilized to analyze biomaterials under compression and tensile testing to match their biomechanical properties to native tissue.

Figure 1: Schematic diagram to illustrate different compression methodologies.
A Indentation Testing. A load is applied to a small area of the cartilage using a non-porous indenter. **B** Confined Compression. The cartilage specimen is placed in an impervious fluid-filled well. The cartilage is then loaded through a porous plate. Since the well is impervious, flow through the cartilage is only in the vertical direction. **C** Unconfined Compression. The cartilage is loaded using a non-porous plate onto a non-porous chamber, forcing fluid flow to be predominantly radial.

Figure 2: Set-up of the mechanical testing machine.
A Illustration of the testing machine. **B** Illustration of the indenter used for the compression testing analysis. **C** Cartilage being analyzed using compression indentation testing. **D** Skin tissue being analyzed under tensile testing. **E** Tensile testing of a synthetic biomaterial. **F** Compression testing of a synthetic biomaterial.

Figure 3: Formulas used to calculate the compressive and tensile mechanical properties of a tissue or tissue-engineered construct.
The formulas used to calculate force (N), stress (MPa), and strain (%).

Figure 4: Example of compression analysis of human cartilage.
A Stress-versus-strain analysis. **B** The m value of the line curve fit equation is the Young's Elastic Modulus in MPa. **C** Stress-versus-time analysis to demonstrate

relaxation properties. **D** The m value of the line curve fit equation indicates the relaxation rate. The final absolute rate is the last point on the graph.

Figure 5: Example of tensile analysis of human skin.
A Stress-versus-strain analysis. **B** The m value of the line curve fit equation is the Young's Elastic Modulus in MPa. **C** Stress-versus-time analysis to demonstrate relaxation properties. **D** The m value of the line curve fit equation equates to the relaxation rate. The final absolute rate is the last point on the graph.

DISCUSSION:
Several tensile and indentation protocols have been published to characterize human soft tissues. We have provided another method, which aims to be more diagnostic and non-destructive. The samples undergoing mechanical testing in this protocol are limited by load rather than by displacement, as transducers are more sensitive to load than to displacement. Therefore, reproductions of the experiment can be more precise across tissues and synthetic materials. Using this technique, we have demonstrated a tensile protocol for evaluating skin tissue and an indentation protocol for analyzing cartilage tissue. Both protocols are easy and simple to implement and could be considered for the characterization of human soft tissues and tissue-engineered constructs.

One of the vital steps of the methodology to obtain a stress-relaxation curve suitable for analysis is to ensure that the sample does not slip during testing. Adequate fixation is required, but this must be balanced against causing any stress on the specimens and ensuring that the indenter is perpendicular to the surface to prevent any shear loading. It is critical that the composition as well as size and shape of the tissue are similar between samples. For cartilage, it is vital to use a repeatable dissection protocol and sample dimensions. For skin samples, it is vital to remove all the subcutaneous tissue in order to obtain a repeatable sample. It is also important to ensure that for all samples, the specimen conditions are identical, including hydration, room temperature, and thawing process, if appropriate.

There are some limitations to the protocols presented. Studies have suggested that deformation characteristics of skin and cartilage are dependent upon specimen orientation[13]. Skin was recognized to be anisotropic as far back as the 19th century, with Langer demonstrating in 1861 that the skin has natural lines of tension, referred to as Langer lines[4]. Thus, when characterizing skin samples, it is important to orientate all samples parallel or perpendicular to the Langer Lines to avoid introducing a methodology bias[4]. Cartilage also shows anisotropic properties and contains Hultkrantz lines, which are equivalent to Langer lines, so the cartilage can deform differently according the direction in which it is loaded[12,19]. Thus, it is important to increase the sample size to allow for the testing of cartilage in different directions. As biomechanical properties of tissue also vary with age and gender, studies should be performed with a representative patient cohort to maintain validity to the clinical setting. Furthermore, some mechanical protocols advocate preconditioning, where the tissue undergoes cyclic loading to ensure that the tissue is in a steady state for subsequent mechanical testing[20]. However, the exact mechanism of preconditioning is unclear and the exact number of cycles needed to produce a consistent and repeatable response varies in different studies[20]. The

researcher should consider whether or not to include preconditioning after evaluating the reason for performing the specific biomechanical test[20].

Skin is a complex, multi-layered material, divided into three main layers: the epidermis, dermis, and hypodermis[4]. The mechanical properties of skin tissue have recently been evaluated using *in vivo* assessments[4]. However, protocols of tensile testing can be utilized to understand the skin biomechanics of excised skin[4]. Such tests can provide information to model stress-strain relationships, since the boundary conditions can be defined[4]. Typically, *in vitro* testing regimes use high strains to characterize the material to failure, whereas *in vivo* systems use low strain ranges[4]. When comparing biomechanical values for excised skin in tension, there is a large variability between different studies, ranging from 2.9-150 MPa[4]. Large differences between subjects are expected due to natural biological variation, but differences in protocol regimes can also compound these natural biological differences. For example, differences in loading rates between protocols will cause variation, as greater loading rates cause less time for the fluid to flow out, resulting in a higher stiffness. The preparation, excision, and handling protocols of the skin tissue will also cause differences in the mechanical properties[4]. This protocol demonstrated for testing skin provides an alternative method for researchers to characterize skin tissue. It provides a few advantages, including the ability to identify the elastic and viscoelastic properties of skin tissue in one mechanical test, allowing for a greater understanding of the skin in a short amount of time. Furthermore, the same test can be applied to tissue-engineered replacements to manufacture constructs with similar biomechanical properties as native skin.

Indentation testing provides an attractive option compared to confined compression testing for understanding the biomechanics of cartilage[21]. Indentation has the ability to preserve the physiological structure of the cartilage and thus provides values that mimic those of a clinical setting. Using indentation, it is also possible to test the cartilage while still attached to the underlying bone. Indentation also allows for physiological testing of cartilage as *in vivo*. When two cartilage surfaces approach each other, the edges surrounding the area of contact “bulge” due to water under the contact area being displaced laterally after compressive deformation occurs[17,21]. Cartilage indentation must be conducted with an indenter with a smaller radius than the cartilage sample to allow for similar bulging. The size of the indenter should also be at least 8 times the sample size to ensure that the cartilage reacts as if it were part of an indefinite sample[22]. Using an indenter much smaller than the radius of the sample diameter eliminates any edge effects present in specimen creation. In addition, indentation avoids possible experimental errors caused by testing cartilage defects damaged by sample extraction. Indentation also does not involve deep sample preparation, such as confined compression, allowing small, thin pieces of cartilage to be tested[17,21]. Furthermore, the non-destructive method of indentation means that it has a potential application in the clinical setting as a diagnostic tool after validation and verification studies have been performed.

There are key assumptions with indentation that the user must ensure for appropriate results. A critical boundary condition in indentation loading requires constant contact between the indenter and the cartilage surface (*i.e.,* that the surface does not deform away from the indenter)[23,24]. Indentation loading also includes the assumed boundary condition that the contact between the cartilage surface and the

indenter is non-destructive (*i.e.,* that the indenter is in contact with the surface but does not go through the surface; the cartilage surface should not fail under the indenter)[25-26]. Studies have shown that this boundary condition can be verified through use of India ink, which will stain damaged areas when applied to the cartilage surface[25,26]. A further boundary condition assumes that the indenter compresses the cartilage perpendicular to the surface of the sample. The perpendicular orientation of the compression is an important boundary condition because compressing at an angle, especially if using cyclic loading, may cause slippage, which may induce shearing components and change the mechanical loading. This condition may be ensured through careful test equipment set up.

After the summarized protocols have been optimized for the soft tissue of interest, it would be useful for researchers to look into dynamic testing of the tissue of interest. Appropriate cyclical loading of specimens should mimic normal physiological limits and behavior, such as mimicking walking or other repetitive movements[27]. In summary, this report demonstrates simple mechanical testing protocols to evaluate human tissues. Implementing these protocols will provide key information on the biomechanical characteristics of tissues, enabling tissue-engineered constructs to better mimic the native tissue.

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The authors have nothing to declare.

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Figure 1

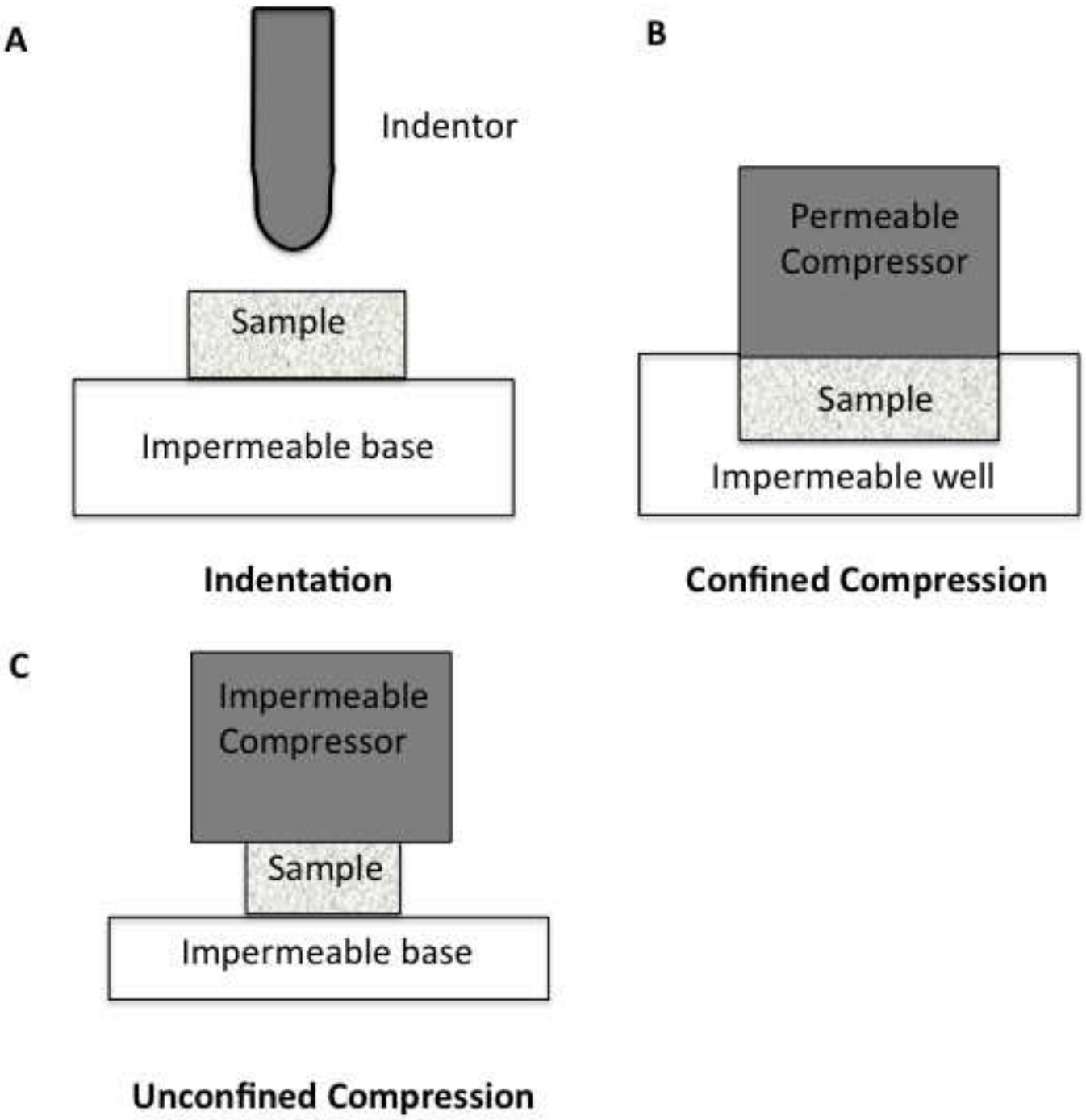

Figure 2

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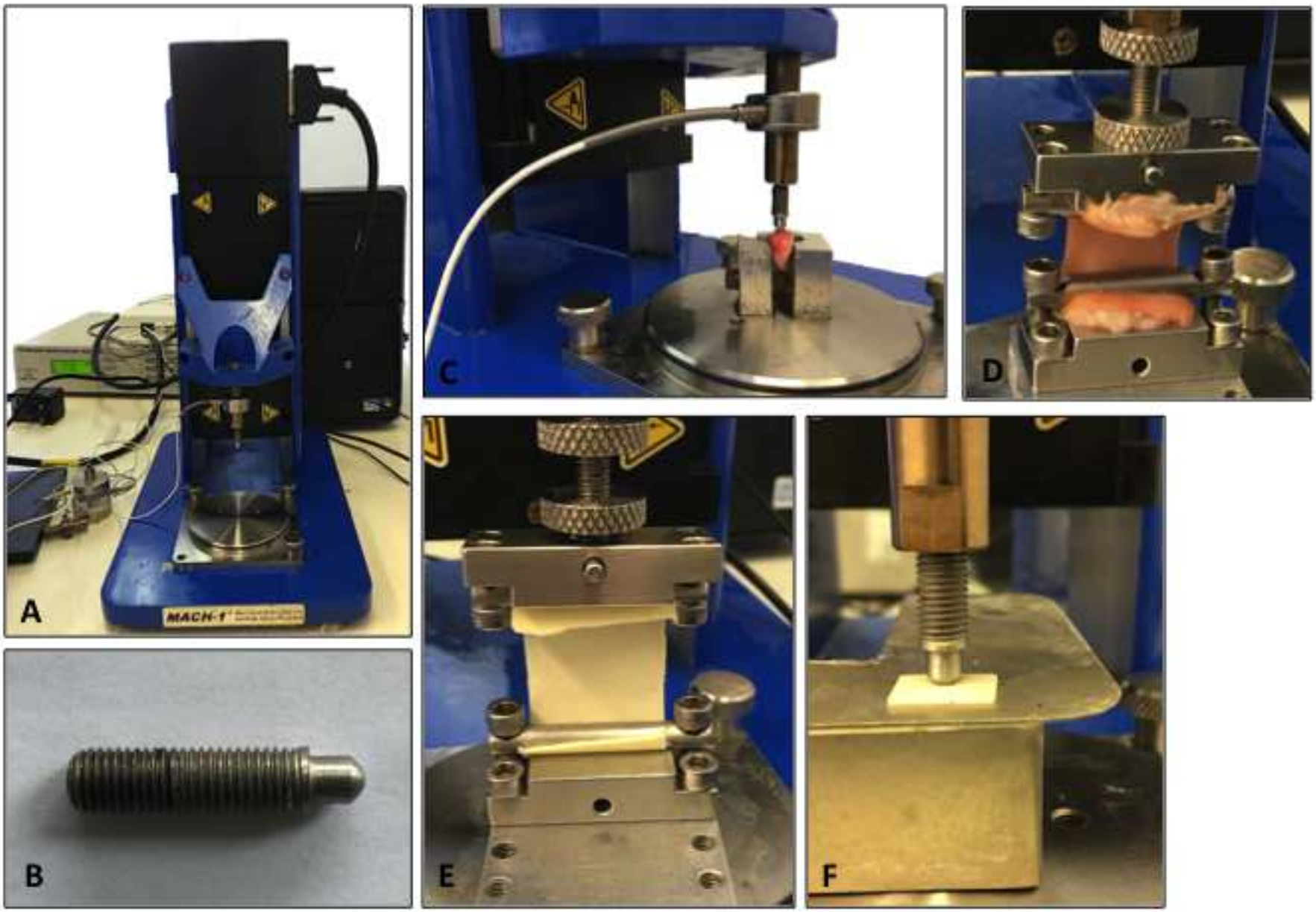

Figure 3

Force (N)

$$= load\ (N) \times acceleration\ \left(\frac{m}{s^2}\right)$$

Stress (MPa)

$$= \frac{force\ (N)}{area\ (mm^2)}$$

$$= \frac{force\ (N)}{cross\text{-}sectional\ area\ of\ indenter\ (mm^2)[compression]}$$

$$= \frac{force\ (N)}{cross\text{-}sectional\ area\ of\ sample\ (mm^2)[tension]}$$

Strain (%)

$$= \frac{length\ (mm)_{New} - length\ (mm)_{Original}}{length(mm)_{Original}}$$

$$= \frac{\Delta L\ (mm)}{L_o(mm)}$$

Figure 4

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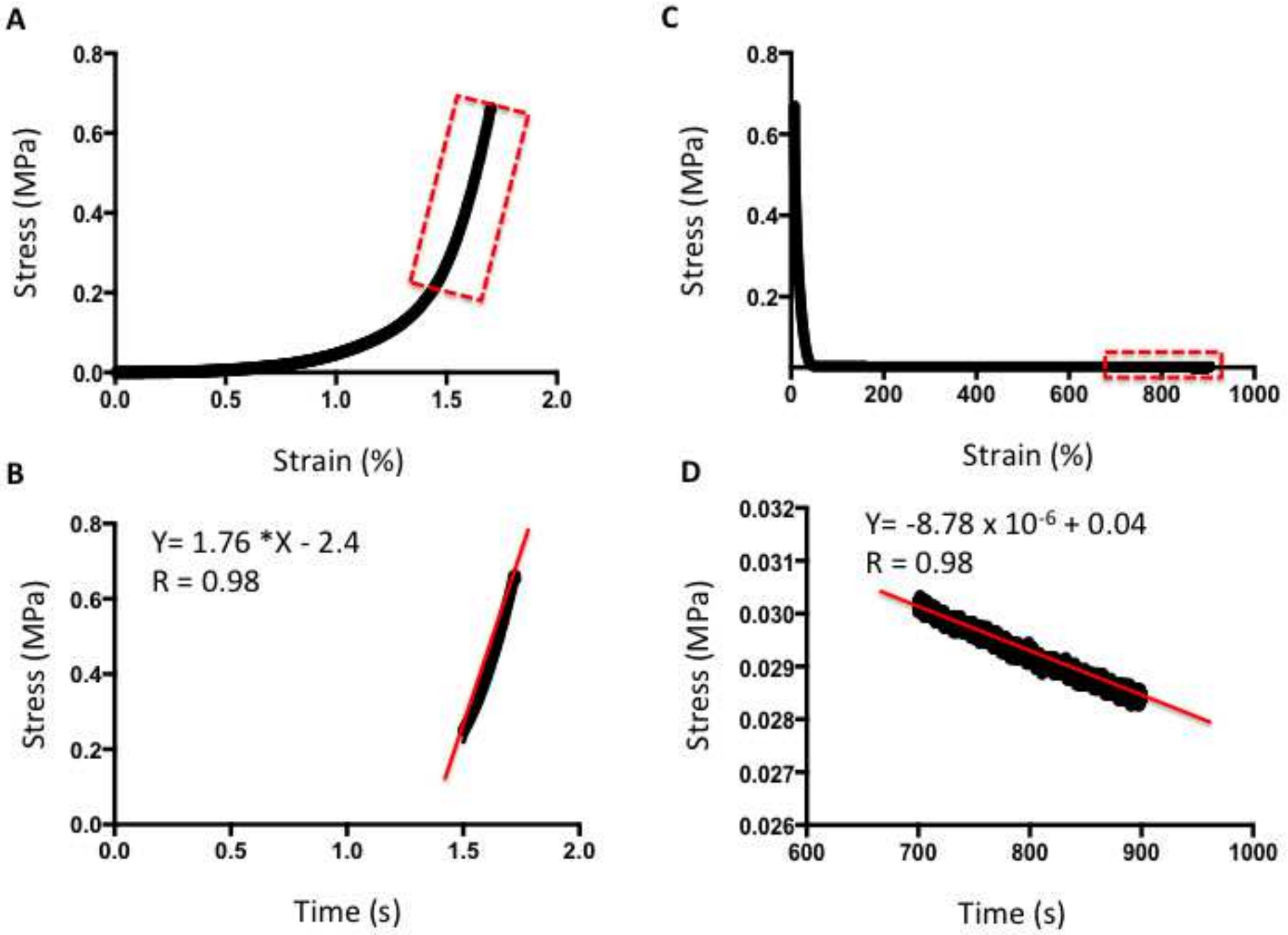

Figure 5

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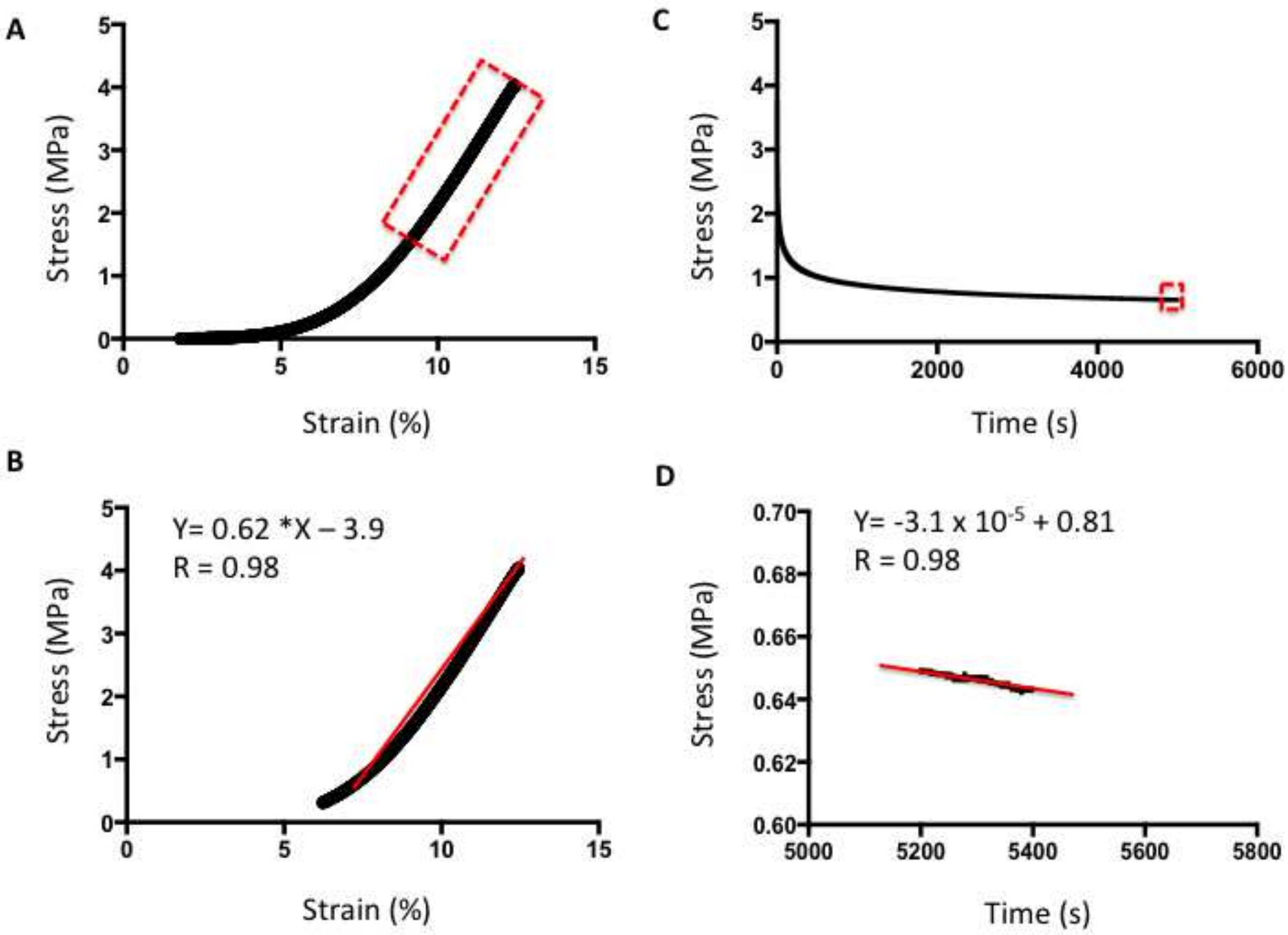

Excel Spreadsheet- Table of Materials/Equipment

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JoVE_Materials-2.xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Digitial Vernier Calipers	Machine Mart	40218046	Digitial vernier caliper is used to measure sample thickness.
Water Bath	Cole Parmer	UY-12504-94	StableTemp Digital Water Bath Flask Holder used to defrost tissues samples if they are frozen.
Mach-1 Material Testing Machine	Biomomentum	V500c	Mechanical Testing Machine used to test the mechancial properties of the tissues.
Scalpel Blade	VWR	233-5335	Scalpel blades using to cut and dissect the tissues.
Forceps	VWR	470007-554	Forceps used to dissect the tissues.
Phosphate Buffered Saline (PBS) pH 7.2	Life Technologies	20012019	PBS is used to hydate the tissue samples

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

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Author(s): Griffin M, Premakumar Y, Seifalian AM, Butler PE, Szarko M.

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CORRESPONDING AUTHOR:

Name:

Department: dr michelle grififfn
UCL Division of surgery and interventional science

Institution: pond street london uk

Article Title: The biomechanical characterisation of hard and soft tissues for the optimisation of generating tissue engineered organ replacements

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University College London.

Centre for Nanotechnology & Regenerative Medicine,

UCL Division of Surgery & Interventional Science,

London,

UK

5.7.2016

Dear Editor,

RE: **The biomechanical characterisation of hard and soft tissues for the optimisation of generating tissue engineered organ replacements**

Thank you for reviewing our paper. We have now formatted the paper according the reviewer comments as below. We have shown our edits in red in the manuscript.

We look forward to hearing from you,

Best wishes

Miss Michelle Griffin

MRC and AMR Plastic Surgery Research Fellow

Centre for Nanotechnology & Regenerative Medicine | Division of Surgery & Interventional Science

University College London | Royal Free Hospital | Pond Street | London | NW3 2QG

Editorial comments:

. 1**. Please be consistent with the usage of indentor vs. indenter.**
Indenter has been used throughout.

2. Formatting: -Please include commas between Last Name, First Name on the title page. -
Commas have now been inserted.

Sections 3 & 4 have the same heading. Please either combine the sections or use different headings. -
Section 3 has been edited to 'preparation of cartilage'.

4.5 – Description of the indentor should appear much earlier in the protocol,
The indentor description has been moved to 3.2.

4.1. -All figure legends should contain a title and a brief description.
The figures legends have been edited.

3. Grammar: -Please copyedit the manuscript for typographical errors.
The paper has now been checked for grammar throughout.

-**Line 200 – Should be "Note:"**
This has now been corrected.

-Line 283 – "Figures 4 and 5 provides" -
This has now been corrected.

Line 293 – "The absolute final level of relaxation is the final point in stress of MPa for example in this data set the level would be 0.03 MPa (Figure 4D)." – Please correct the run on sentence – it should be split into two. -
This has now been corrected.

Please use correct punctuation in the figure legends.
This has now been corrected.

-Line 389 – "still attached the underlying bone"
This has now been corrected to 'still attached to the underlying bone'.

4. Additional detail is required: -From where are specimens obtained? This can be included as a note(s). -2.5,
This has now been added under section 1.1.

4.3 – How is the sample loaded? Is this manual or controlled by software? -3.3 – How was mechanical testing performed?
Explanation that the process is governed by software is provided.

5. Branding (Mach 1) should be removed from the Figure 2 legend.
This has now been removed.

6. Results: Figure 1 legend – Please highlight the panel labels in the figure legend identically. Panel A is missing.
Panel A has now been added.

Reviewer #1:

1. **The abstract and introduction devote too much attention to motivation in the context of tissue engineering, including issues (e.g., stem cell differentiation on substrates of varied stiffness) with no connection to the tissue testing protocol described here. On the other hand, almost entirely missing is a discussion of relevant aspects of tissue mechanical behaviors, which aspects of these behaviors the proposed protocols can and cannot provide insights into, and the relationship between the proposed protocols and common, well established testing methods for tissues of interest. The paper would be stronger if it deleted the extraneous motivation and addressed these issues in detail.**
Thank you for your comment we have taken out non-relevant elements if the introduction and re-written the introduction to include more aspects of mechanical properties of tissue and the nature of different testing protocols.

2. Line 70: any protocol that involves dissection of tissue samples cannot be described as "minimally invasive," which implies in vivo testing with minimal damage. Non- or minimally-destructive may be appropriate.
It has been changed to minimally destructive.

3. Lines 110-112: It is difficult to see how the proposed protocols are relevant to the "clinical setting," as they do not represent physiologic loading. For example, what is the physiological situation simulated by a 1.5 hour stress relaxation test? This does not mean that they are invalid as mechanical test procedures, but the proposed protocols seem to fall under this umbrella characterization.
We have removed this sentence on line 110-112 as we have re-written the introduction. The decision to use 1.5 hour stress relaxation test was due to the time required to show minimal relaxation changes in the skin tissue as indicated later in the manuscript. This terminology and method has been previously peer reviewed and published by the senior author (Wood et al). As our research looks at the long-term subcutaneous implantation of implants long-term stress relaxation of 1.5 hours could be indicative of this. Furthermore, there are many occasions where there is prolonged stress relaxation including standing.

4. Lines 118-122: These descriptions are not completely accurate (the descriptions summarized in the figure captions are better). In confined compression, the walls are rigid and impermeable but either the base or the platen must be porous and present minimal resistance to fluid flow; many authors have described systems with permeable bases and rigid,

impermeable plungers. Unconfined compression is not "identical," as both the top and bottom platens are usually impermeable, forcing flow to be predominantly radial. It isn't clear what "axial deformation is not limited" is intended to convey, but it is not an accurate description of either confined or unconfined compression.
Thank you for our comments, the reviewer is right. We have now re-written the definitions of the three test regimes and adjusted the figures.

5. **Lines 129-30: Three references are provided to support optimization of these protocols; one (#17) appears to be unrelated, as it uses a different protocol for a different tissue/organ. The others are single references (one for tension, one for compression), and do not convincingly establish that these protocols are in any way optimized, superior to the many alternative testing protocols that have been used for similar/identical tissues, or should be viewed as a gold standard (which, while not explicitly stated, appears to be the motivation for this methods manuscript). Again, this does not mean that the proposed methods are not reasonable, but promoting them as a standard procedure requires a higher level of justification and validation.**
We have removed the Boughton et al paper from our group. This was primarily included because it does use the same testing regime and principles of using a stress defined test to obtain a Young's Elastic Modulus. However, we have now removed this to avoid confusion. We have rephrased the introduction and discussion to convey that we do not feel these should be the gold standard, just that these protocols are another technique by which to evaluate soft tissue mechanics that can be easily transferred to tissue-engineered constructs. We have also added another published paper to support our technique, which evaluates the mechanics properties of auricular cartilage.

6. **The inclusion of two very different testing modes for tissues typically viewed as governed by different physical phenomena (elongation of fibrillar networks and intrinsic viscoelasticity for skin in tension; osmotic interactions and flow-dependent dissipation in cartilage) makes this protocol paper somewhat disjointed.**
Cartilage and skin are both soft tissues, so they can be included within the same paper. We have now indicated that the analysis is the same for both the tension and compression testing, to further support the inclusion of both cartilage and skin in the same paper.

Also, the manuscript implies that these procedures can be directly applied to other tissues; this is not the case (as evidenced by the authors' own use of different protocols for other tissues).
The same principles of testing were used in Boughton et al and thus demonstrating the testing could be applied to other tissues. However, we have removed this paper to avoid confusion. We have made it clear that these protocols could be used for characterising human soft tissues throughout the manuscript.

Protocols: 7. 1.2: "use any size of skin": A sample that is too large will exhibit substantial inhomogeneity, and there are likely to be size effects. Some caveats, and discussions of relevant limitations, are called for.
Thank you for the comment. 'Use of any size of skin' has been removed and discussion of relevant limitations has now been inserted.

8. 1.2 and 2.3: Some guidance is required for the method of clamping and the clamping force, as it is possible to damage the sample and alter the observed behaviors. 2.3: It is quite easy to introduce a non-uniform gripping that results in non-uniform loading, with one side of the sample being stretched more than the other. Specific directions for properly mounting the samples are required.
We have provided a note that a commercial jig was used to avoid damaging the sample and unequal gripping in section 2.3 'Note: A commercial jig was utlised to avoid non-uniform gripping and damaging the sample before testing'.

10. 2.5: Here and later, the "load" should be given in units of force (N), not mass (g).
We have changed the load to N throughout the manuscript.

11. 2.5: It is not common to prescribe a load limit to a strain-controlled test. If comparing samples of different material properties, this will result in different peak strains, which may affect both the apparent elastic and viscoelastic behaviors. In this manner, the proposed protocols differ substantially from the vast majority of the tissue testing literature. Other than arguments of convenience (which is essentially the case made later in the manuscript), how is this justifiable?
Yes the reviewer is right, it is not common to load limit and this justifies writing of our protocol. However we have demonstrated that we can use this technique to evaluate the compression and tension of soft tissues with three publications. We have highlighted that the usefulness of our protocol in the introduction on line 129-131 and in the first paragraph in the discussion. Our protocol still provides the researcher with a Youngs elastic modulus as shown in previous publications and in Figure 4 and 5. We have provided data to support that tissue engineered constructs can be tested in the same way in the provided figures. Our protocol is advantageous in that it provides 'direct, non-destructive comparison of human soft tissues and tissue-engineered constructs' and captures both the viscoelastic and relaxation properties of the tissue within the same test.

12. 2.5: Also, it is most common to prescribe a defined tare load and measure deformation/strain relative to that point—the current protocol appears to take the unloaded grip-grip distance as the reference point, which is not as reliable or consistent (due in part to gripping issues noted above).
The reviewer is correct that information will be collected while the indenter is moving onto the object. However, the analysis only includes data when the indenter has made contact with the sample. By applying small strains it is clear when contact has been made with the sample and thus no tare load is

required. This information has been added as a note on line 208. The difference in the loaded specimen size is used for analysis, according to standard literature and we have made this clear on line 165 and line 222 and already in the analysis section.

Finally, many authors employ preconditioning. All of these differences between this protocol (described in a single publication) and standard practices in the literature need to be examined and justified.
Although some mechanical testing may advocate preconditioning, understanding the optimal cycles is still under debate. We have included this as a limitation of this protocol and suggested the researcher should consider including it depending on the reasons for conducting the test on each occasion on line 400-406 with a relevant reference.

13. 2.6: **While 1.5 hours is a substantial relaxation period, the results presented clearly indicate that the tissue is still relaxing and thus not at equilibrium..**
If the reviewer looks at the scale it is a very minimal amount of relaxation. Tissue will always continue to relax. We have rephrased the sentence to 'a time-point at which there is minimal change in relaxation behaviour' to make this clearer.

Also, the manuscript should indicate that the displacement is being held constant, not the load
Thank you for you comment. We have indicated that the displacement is being held constant, not the load as a note on line 201.

14. 3.2: **Here and in some other places, it appears that the protocol describes a specific test that was previously performed, not instructions for future tests. Avoid past tense. 15.**
Past tense has been removed from the protocol section.

3.2: **What is the indenter size and geometry? This should be specified here, along with guidelines in case this size is not available**
The information on the indenter has been moved to section 3.2.

16. 3.2 and later: In addition to being influenced by a cut edge if the sample is not large enough, the indentation stiffness can be affected by the boundary conditions at the base (attached to bone or free) and the sample thickness, as the penetration depth of the deformation field is related to the indenter diameter—if the tissue is not thick enough, tissue thickness will influence the measured response.
Yes the reviewer is right. We have included that the thickness needs to be 8 times greater that the indentor on line 217.

17**. Why do sections 3 and 4 have the same title?**
Section 3 and 4 now have different titles.

18. 4.1: Cartilage samples should be equilibrated in PBS before testing, not just during testing—PBS does not exactly match the physiological

bath, and there will be a transient adaptation that may affect the measured response.
The reviewer is right, it was supposed to read before and during testing the sample is kept in PBS. We have added a note why PBS is justified as PBS allows the comparison of both materials and tissues to be tested.

19. 4.3: Same comment about load limitation on a displacement-controlled test.
This has been changed to N.

20. 4.4 Again, the data indicate that 15 minutes is not adequate to reach equilibrium—change is slow, but ongoing, as could easily be seen by plotting stress vs. log time.
Again if the reviewer looks at the scale it is very minimal amount of relaxation. Tissue will always continue to relax making the time limitations of testing regimes difficult to decide upon. However, we have rephrased ‘a time-point at which there is minimal change in relaxation behaviour’ to make this clearer.’

21. 5.1 (lines 226-229): First, this statement does not make sense: a load cell is completely insensitive to strain because it does not measure strain, it measures load.
This sentence has now been deleted due to making other corrections.

Second, it appears that consistency of the strain history across samples is less valued than avoiding potential damage to an excised sample (which can easily be avoided by setting an appropriate strain limit).
As discussed early we are applying a load limit to be able to perform a biomechanical test, which can be relevant to testing tissue engineered constructs and is the reason for writing this article.

22. 5.2 Clearly indicate how the linear region is defined. Also, there appears to be an error here, as the determination of tensile and compressive properties seems to be mixed and this refers to a section that has not yet been presented (calculation of Young's modulus). In fact, the entire data analysis section seems to address the compressive tests of cartilage.
The data analysis section has now been revised so it is clearer that the Youngs elastic modulus analysis is for indentation compression and tensile testing.

23. Line 247: "Data" is a plural noun ("data are," not "data is"). It is not clear what this sentence means—what happens if all data are included and the r^2 value is less than 0.98? (this also applies to section 7.3)
We have rephrased to include ‘all data points are’. Furthermore, we have stated what would happen if the r value is not less than >0.98. ‘If the R value is not >0.98 then the assumption of characterising linear viscoelastic behavior is invalid’.

24. **Lines 256-264: much of this is unnecessary.**
Most of this note has now been removed.

25. **The approach to determining viscoelastic properties is non-standard, and does not reflect common practices in the literature. Of what physical significance is the rate of relaxation after 1.5 hours of constant tensile strain, and how is this phenomenological measurement related to the viscoelastic material properties (which depend on the selection of a particular material model)**
Yes the reviewer is right, our method is not common and thus the reasons for writing this protocol as explained earlier. The 1.5 hours was chosen as dictated earlier to find a point at which there is a minimal change in relaxation, as tissues will always continue to relax. This time period has also been accepted in a peer reviewed biomechanics journal.

26. 7.3: The stress after 15 minutes of compression is not the equilibrium stress, it is merely the final stress of the test.
As highlighted earlier in the rebuttal letter 'Again if the reviewer looks at the scale it is very minimal amount of relaxation. Tissue will always continue to relax making the time limitations of testing regimes difficult to determine. However, we have rephrased 'a time-point at which there is minimal change in relaxation behaviour' to make this clearer.'

27. 7.4: Outside of the context of a specific experimental design, specification of a particular statistical model is not appropriate.
Thank you for our comment. Section 7.4 has now been removed.

28. **Line 293: the rate should be MPa/s, not MPa.**
Thank you for our comment. This has now been corrected.

29. Figure 2 caption (lines 320-324): This protocol should not be specific to any particular testing system, and there is no need to present/promote the use of the Mach-1 system for materials not even covered directly in this testing protocol.
The references to the Mach-1 system have been removed.

30. **Lines 355-356: what is the relevance of the comment on testing in multiple directions? How is that physiologic, and how would it be implemented In a minimally destructive test?**
It is well recognized that cartilage is anisotropic. Cartilage contains Hultkrantz lines, which are equivalent to Langer Lines. Thus it is important that researchers either have enough samples to account for this (as the reviewer is correct you cannot test the small sample in different directions) or allow for this limitation. We have made this clear on lines 388-397.

31. Lines 380-384: Two points. First, as noted above, the comment about transducers being more sensitive to load than displacement is puzzling. The following statement seems to indicate that the protocols described in this manuscript are designed to work around limitations of the test system—if that is indeed the case, then this is a poor foundation for publishing a protocol (which should be based on best practices, not best ways to work around limitations of a specific system).

As highlighted earlier, having a load limit is not a limitation of the mechanical system, it can be applied to other mechanical material testing machines. We have just described a protocol to obtain the Young's modulus using stress that can be used to test the mechanical properties of tissues and synthetic materials. We have deleted this sentence as we agree with the reviewer that it may confuse the reader.

32. **Little of the discussion addresses tensile testing.**
The discussion includes more about tensile testing on line 408-429.

33. Figure 1: Should indicate whether parts are permeable or not.
Thank you the figure 1 has now been edited to include which is permeable or not.

34. **Figure 3: As noted above, g/kg are not units of force; in most cases, load cells are calibrated to provide output in N. The second line lacks units.**
Load has been changed to N. Strain units have been added to %. Force is now in units.

35. **Figure 3: For a hemispherical indenter, dividing the force by the cross-sectional area gives the nominal (average) stress, but not the peak stress. This should be noted.**
Thank you for this. This has been added under 6.2.

36. **Figures 4-5: Some of the subfigures are very similar to those in prior publications; if they are identical, then they should be regenerated for new data to avoid copyright issues**.
New samples were provided for this manuscript. They may be similar to previously published work as they are similar tissues with the same testing procedures so they would act similar demonstrating the accuracy of the protocol.

Reviewer #2:

Major Concerns: **The manuscript is extremely simplistic and reads more like a standard operating procedure or a user guide for employees rather than a scientific manuscript.**
This is a methods paper and is not supposed to be set out as a scientific manuscript, we feel the reviewer may have not realized it is a method paper. We have made it read like a SOP for all users to be able to understand and repeat easily.

Reviewer #3:

Title: a. The title is not appropriate for this manuscript. I suggest a title with a higher focus on the proposed protocol.
The title has now been revised.

2. Abstract: a. The short abstract is appropriate. However, the long abstract borrows a few sentences from the introduction, which shift the

focus of the manuscript. I suggest editing the long abstract to focus more on the protocol and less on the potential application.
The long abstract has now been revised to concentrate on the testing protocols.

Introduction: a. The first paragraph is awkwardly phrased.
The first paragraph has now been revised.

b. Citation is needed to support claim in lines 86 and 87 c.
Citations for this have now been inserted.

In line 87, it is unclear the presence of "scaffold". A non-expert will be confused and miss the point.
The introduction has now been revised.

The sentence in 89-92 is confusing, and does not really explain the mechanical properties of auricular cartilage.
This sentence has been rephrased 'For example a material replacing auricular cartilage should have the appropriate mechanical properties to prevent being compressed by the overlying skin.'

Citation needed in line 94 f.
This has now been edited.

Awkward phrasing in line 97 g.
The introduction has now been revised.

Substitute "tissues" with "cells" in line 98, as differentiated MSCs are still cells. h.
This has now been deleted as the introduction has been re-written.

The transition between tissue engineering, scaffolds, materials, and the need of appropriate testing protocols is not clear. I suggest to reframe the introduction to focus on the need of better testing setups, and less so about the importance of tissue engineering. 4.
This has now been deleted as the introduction has been re-written due to comments from other reviewers.

Methods: a. Please clarify what is meant with "appropriate testing machine" (Line 151).
This has now been rephrased on line 152.

Maybe providing an example would help in this case. b. Please suggest examples of rates that do not cause failure to skin (Line 167).
The speed has been provided that does not cause failure of the skin on line 194.

c. In the context of researchers that purchases a new testing machine, it would also be appropriate to suggest methods for benchmarking the

equipment to guarantee that any variances in testing are not due to the equipment.
We have provided a note to account for this in 3.1 ‘Note: All materials testing machine should be calibrated according to the manufacture guidelines prior to testing. ‘

a. The word "suggested" is repeated twice (Line 361)
This has now been edited.

. b. **Awkward phrasing in line 379**
This has now been rephrased.

Reviewer #4:

Major Concerns: **There are a large number of prior studies that examine the failure properties and viscoelastic properties of these two tissue types. In addition, there are a number of commercially available, non-invasive mechanical testers for examining skin biomechanics in vivo. It was not clear how the discussed equipment/protocol is an improvement over available technology.**
Yes there are many in vivo skin techniques to analyse skin mechanics. However, the major addition of our techniques is using minimally destructive protocols using small stresses and strains that be correlated to tissue engineered constructs and to gain information on the viscoelasticity and relaxation properties in a single test. This has now been highlighted in the introduction (119-142) and discussion (line 366-375).

Minor Concerns
For example a material replacing auricular cartilage should have adequate mechanical properties to present the deformation of the skin but not be too stiff to cause extrusion through the skin due to the failure to interact with the other connective tissues, causing failure of the implant." Suggest rewording. How it is written is confusing and makes it sound as if the main function of the cartilage biomechanics is to deform but not penetrate the overlying skin.
This sentence has been revised.

"Several cell types have shown to respond to the scaffold's mechanical properties including fibroblasts, muscle cells and neurons by displaying different morphological and adhesion characteristics." Should read "have been shown" "
This sentence has been deleted due to modifying the introduction.

The size of the indentor should be at least 8 times the sample size to ensure the cartilage to react as if it were part of an indefinite sample." Is this supposed to read 1/8th?
This is correct, however the sentence has been checked for grammar.

Reviewer #5:

Major Concerns: **The major concern on this manuscript is that, to this reviewer's knowledge and experience, the range of strain that should be used for calculation of elasticity is the first portion of the stress-strain curve, corresponding to low strain regions where the material exhibits an elastic response. High strains, on the other side, correspond to the viscous properties of the material. The method for analyzing data in this manuscript indicates, however, that the Young's Modulus should be obtained from the data corresponding to higher strain data points. Please correct or explain why high strains are been used to calculate elasticity.**

The reviewer is right you should use the first portion of the stress-stain curve to calculate a Young's Elastic Modulus. As the novelty of the manuscript is to calculate apply small stresses and strains we are calculating the Youngs Elastic modulus a low stain regions as normal literature dictates.

Minor Concerns: **- The title feels too broad and does not indicate clearly what is described in the manuscript.**

The title has been changed to be more specific.

- Please include units for strain (% or mm/mm)? -

Thank you % has been added to the figures and throughout the text to strain.

Please indicate which equations of stress and strain should be used for each testing method.

The Data analysis section has now been revised to make it clear that the analysis is for tension and compression.

Reviewer #6: .

Pages 4-5. A minimum number of specimens to be tested for each tissue and each testing method should be provided.

This is highly dependent on what the researchers is trying to show and examine and thus cannot be given in a protocol. The researchers should be following general guidelines on performing scientific experiments and performing power calculations.

Page 4, Point 3. Should it be Indentation Testing? The indentation speed should also be provided.

It has been changed to compressive indentation testing. The speed has already been provided on line 239.

Page 6. Line 283. "provides" should be "provide".

This has now been changed.

Page 7. Lines 346-347. **Try to revise this sentence as the way to calculating the elastic moduli of synthetic viscoelastic materials is different from the method presented in this paper. It is typically calculated using a different equation from the unloading curve.**

This is not true these protocols could be used for synthetic materials, which is

the essence of the paper and demonstrated in the analysis section. However this sentence has been revised to ‘Both protocols are easy and simple to implement and could be considered for the characterization of human soft tissues and tissue engineered constructs.’