Journal of Visualized Experiments

The Quantification of Injectability by Mechanical Testing

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61417R1
Full Title:	The Quantification of Injectability by Mechanical Testing
Section/Category:	JoVE Engineering
Keywords:	Injectability; Testing Method; Biomaterial; Extrusion; Mechanical Testing; Force; Hydrogel; Cement
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Additional Information:	
Question	**Response**
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TITLE:

The Quantification of Injectability by Mechanical Testing

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KEYWORDS:

injectability, testing method, biomaterial, extrusion, mechanical testing, force, hydrogel, cement

SUMMARY:

Presented here is a protocol for quantitatively evaluating the injectability of a material through a syringe-needle system using a standard mechanical testing rig.

ABSTRACT:

Injectable biomaterials are becoming increasingly popular for the minimally invasive delivery of drugs and cells. These materials are typically more viscous than traditional aqueous injections and maybe semi-solid, therefore, their injectability cannot be assumed. This protocol describes a method to objectively assess the injectability of these materials using a standard mechanical tester. The syringe plunger is compressed by the crosshead at a set rate, and the force is measured. The maximum or plateau force value can then be used for comparison between samples, or to an absolute force limit. This protocol can be used with any material, and any syringe and needle size or geometry. The results obtained may be used to make decisions about formulations, syringe and needle sizes early in the translational process. Further, the effects of altering formulations on injectability may be quantified, and the optimum time to inject temporally changing materials determined. This method is also suitable as a reproducible way to examine the effects of injection on material, to study phenomena such as self-healing and filter pressing or study the effects of injection on cells. This protocol is faster and more directly applicable to injectability than rotational rheology, and requires minimal post processing to obtain key values for direct comparisons.

INTRODUCTION:

Biomaterials are often studied and used as scaffolds for cell-based tissue regeneration and depots for targeted, sustained delivery of therapeutics[1]. Within this field, injectable biomaterials

are growing in popularity as they are minimally invasive, which reduces the risk of infection, pain and scarring associated with implantation[2]. Further, because they are usually applied as fluids, they conform perfectly to tissue defects, and drugs and cells may be mixed into them immediately prior to the application[3–5]. As such, while injectable biomaterials may be manufactured as pre-loaded syringes, they are often prepared by clinicians directly prior to application. For example, cements begin to set once the powder and liquid phases are mixed, and so cannot be stored for long periods before use[6]. The characterization of these materials is thus time dependent and inextricably linked to their preparation.

Common injectable biomaterials include calcium cements, polymethyl methacrylate, bioglasses, and various polymeric hydrogels[3,7]. Unlike traditional injections of drugs, which have the same rheological properties as water, these injectable biomaterials are typically more viscous, non-Newtonian, may have some elastic character, and may also change over time. Therefore, the injectability of these materials cannot be assumed but must be assessed experimentally. By quantifying the force required for injection and correlating it to the ease of injection, early decisions about which biomaterial formulations, syringe, and needle sizes to take forward may be made early in the developmental process[8]. Such experiments may also quantify the effects of changing formulations on injectability[9].

There are several methods to assess the properties of injectable materials. Rotational rheology is often utilized to assess viscosity, non-Newtonian behavior, post-shear recovery, setting time, and other properties of these materials[10–12]. Whilst this type of test is useful to establish fundamental properties of the materials, these properties do not correlate directly to injectability. For a Newtonian fluid and cylindrical syringe and needle, the injection force can be estimated from a form of the Hagen–Poiseuille equation[13]:

$$F = \frac{8{R_s}^2 LQ\eta}{{R_n}^4} + F_f$$

Where F is the force required for injection (N), R_s is the internal syringe radius (m), R_n is the internal needle radius (m), L is the needle length (m), Q is fluid flow rate ($m^3\ s^{-1}$), η is the dynamic viscosity (Pa.s) and F_f is the friction force between the plunger and barrel wall (N). Thus, if the viscosity is measured via rotational rheology, the dimensions of the syringe and needle are known and the flow rate estimated, the injection force can be estimated. However, this equation does not account for the conical end of the syringe or any other geometries, such as off-center outlets, and F_f must be estimated or found experimentally by mechanical testing. Further, biomaterials are typically not Newtonian, but exhibit complex rheological properties. For a simple shear thinning fluid, the equation becomes[14]:

$$F = 2^{n+2}\pi^{1-n}\, L\, {R_s}^2\, K Q^n R_n^{-(3n+1)} \left(\frac{3n+1}{2n+1}\right)^{n-1} + F_f$$

Where n is the power index (-) and K is the consistency index ($Pa.s^n$) from the Ostwald de Waele expression: $\eta = K\dot{\gamma}^{n-1}$, where $\dot{\gamma}$ is the shear rate (s^{-1}). The complexity vastly increases for materials whose rheological properties cannot be characterized by two values, and particularly

for time-dependent materials such as setting cements. Additionally, if the material properties are shear dependent, then the material must be tested at the shear rate expected in the needle, which may far exceed the range of a rotational rheometer[15].

Another quantitative method for measuring injectability involves attaching pressure and displacement sensors to a syringe while performing an injection, either by hand or using a syringe pump. This equipment is relatively inexpensive, however, requires users to generate scripts and calibration curves to convert into force data[16]. Further, a syringe pump may not possess sufficient torque to compress the plunger at a precise rate if high forces are required to extrude viscous or semi-solid materials. Alternatively, utilizing these sensors when injecting by hand may be useful as they can be used in a real clinical scenario, during clinical procedures[17]. However, this will take much longer and may introduce user bias, and will, therefore, need larger numbers of repetitions with different users to obtain reliable results. This may, thus, be more appropriate for materials that are further down the translational pipeline, or products already in clinical use.

In this protocol, a mechanical tester is used to compress the plunger at a set rate, and measure the force required to do so. This type of mechanical tester is common in materials laboratories and has been used to quantify injectability for various biomaterials[18–24]. This test can be used with any size and geometry of syringe and needle, containing any material. Further, in the case of biomaterials that are made immediately prior to the use, the exact formulation procedure that would be used in the clinic or surgery can be followed prior to testing. A further advantage of this procedure is that it is relatively fast; once the mechanical tester is set up, tens of samples can be studied in an hour, depending on extrusion speed and syringe volume. This is in contrast to rotational rheology, which typically takes at least 5 – 10 minutes per test, plus loading, equilibration and cleaning time. Using a mechanical tester produces a reliable extrusion rate equally over the plunger, which is particularly advantageous for viscous formulations or those with time dependent properties. Following testing, minimal post-processing of data is required to pull out important values for objective comparisons.

PROTOCOL:

1. Sample Preparation

1.1. Prepare the sample and load it into the syringe.

1.1.1. To simulate a pre-loaded syringe, prepare the sample in advance, load it into the syringe, and attach the needle. Store as required, until testing. This may be suitable for hydrogels and materials that do not change with time.

NOTE: For example, to prepare 2% alginate solutions, dissolve 2 g of alginic acid sodium salt in 100 mL of deionized water, by stirring at room temperature. Aspirate the solution into 5 mL syringes, and store for 24 h at room temperature.

1.1.2. Alternatively, to simulate an injection formulated directly prior to the application, prepare the sample in the same way it would be made in the clinic, allowing for any setting times. Load

into the syringe and attach the needle. This may be suitable for cements, and materials whose properties change with time.

NOTE: For example, to prepare calcium sulfate cement, manually mix 4 g of calcium sulfate hemihydrate into 5 mL of deionized water with a spatula for 1 min. Remove the plunger from the syringe and load the cement into the syringe barrel with the spatula. Begin the mechanical testing after 4 min.

CAUTION: Needles pose a safety risk, use blunt needles if possible. If the material contains cells or other biological materials, extra care should be taken to prevent sharps injuries.

2. Set up the mechanical tester

2.1. Attach flat platens (for compression testing) to the mechanical tester.

2.2. Manually equip the mechanical tester with a load cell with a maximum load of 200 N.

NOTE: A larger load cell may be used, provided it has sufficient precision at the 1 – 200 N range. Samples that are more viscous and not intended to be injected by hand may require a larger load cell.

2.3. Separate the plates, using the manual control buttons, to allow for sufficient space for the needle, syringe and plunger (around 30 cm will be sufficient).

2.4. Create a testing protocol.

2.4.1. Open the test wizard and set the test type to uniaxial compression.

2.4.2. Set the pre-load. This is the measured force value at which testing will begin. 0.5 N is sufficient.

2.4.3. Set the **speed to pre-load** to 5 mm/min. This is the speed the crosshead will move down until it encounters the pre-load.

2.4.4. Set the **loading to displacement control** and select an appropriate test speed. 1 mm/s is an appropriate speed for a standard 5 mL syringe.

2.4.5. Set an **upper force limit** at which to stop the test, e.g., 200 N. This is primarily for safety reasons. The test may also be stopped automatically at a given displacement, e.g., the length of the syringe.

3. Set up the clamping system

3.1. Attach two sets of clamps to two stands, with grips large enough to securely ensconce the

chosen syringe.

3.2. Place the grips between the crosshead and baseplate, with enough space below the grips for the syringe and needle.

3.3. Line up the centers of the two grips, and line these up with the center of the crosshead.

NOTE: Alignment of the clamp grips with each other and the center of the crosshead may take some time and iteration to achieve, but is important to acquire high quality data.

3.4. Ensure the clamps are secured firmly so that there is no movement in the clamps when a downward force is applied.

3.5. Place a dish onto the bottom plate to collect the extruded material.

4. Run the injectability protocol

4.1. Insert the syringe into the clamp grips and close them. The grips should hold the syringe in place, but allow it to move up and down without resistance.

4.2. Ensure the syringe and plunger are perpendicular to the crosshead. This ensures that only uniaxial compression of the material will be measured.

NOTE: An empty syringe should be used to ensure checkpoints 4.4 and 4.5.

4.3. Lower the top plate to a position just above the plunger, using the manual movement buttons.

NOTE: It may be possible to select a '**Start position**' in the mechanical tester protocol, such that the original position above the plunger is reached automatically and is consistent throughout testing.

4.4. Zero the measured force by clicking '**Zero Force**'.

4.5. Run the testing protocol by pressing '**Run**'.

CAUTION: The experimenter should always be present to observe each trial, and ready to activate the emergency stop in case of a mishap.

4.6. Raise the plates to a sufficient height, using the manual movement buttons, such that the syringe can be removed.

4.7. Repeat step 4 for each sample.

NOTE: At this point, the syringe and extruded sample can be discarded if no further analysis is required, but maybe kept in order, to examine filter pressing, self-healing, the effects on cells, etc.

5. Data collection

5.1. Save the data from each trial in a format from which a table of force and displacement values can be generated (.txt, .xls, .xlsx).

5.2. Plot the results from each trial, with displacement on the x-axis and force on the y-axis.

5.3. Read the maximum force (if it exists) and plateau force from the graphs.

REPRESENTATIVE RESULTS:

The set-up of the mechanical tester and clamping system is shown in **Figure 1A**. This protocol generates a table and graph of force versus displacement for each tested sample. A typical force displacement curve consists of three sections (**Figure 1B**): an initial gradient, as the plunger overcomes friction from the barrel and the material is accelerated, a force maximum, and a plateau, as the material is extruded at a steady state.

However, a distinct maximum only exists where the plateau force is lower than the force required to accelerate the plunger. As such, peaks are only seen for inviscid samples passing through wide needles. For viscous samples passing through a more narrow orifice, the force needed to inject the sample at constant speed is greater than the force required to overcome friction in the barrel and accelerate the material, and no distinct peak is seen (**Figure 1C**). For highly viscous samples or very narrow needles, the force required to extrude the material may be so great that the syringe buckles and fails, often with very little extrusion of the material (**Figure 1D**). If the material being injected contains particles or is undergoing setting, such as cement, filter pressing (preferential expulsion of the liquid phase) or bulk setting may occur, leading to incomplete injection (**Figure 1E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sample curves generated by this protocol. (**A**) Set up of the mechanical tester for this protocol. (**B**) Typical force-extrusion curve. (**C**) Force-extrusion curve with no distinct maximum peak. (**D**) Force-extrusion curve for syringe failure. (**E**) Force-extrusion curve for a setting cement. This figure is adapted from Robinson et al.[8].

DISCUSSION:

Mechanical testing is perhaps the simplest and most reliable way to quantify injectability. A key advantage of this protocol is that no special equipment is required, other than the mechanical tester, which is common in materials laboratories. This protocol is highly versatile; any material, needle gauge and syringe size can be used, provided the syringe can be accommodated by the clamps. This has been verified in this protocol for syringes up to 10 mL. Further, the material can be prepared exactly as it would for the real-world application[25]. Finally, this procedure is very

fast, taking only up to a few minutes per sample, allowing tens of samples to be processed per hour.

For samples that give typical curves, two values can be extracted the maximum force and the plateau force curves. The maximum force is arguably more objective and can be extracted computationally from the data table for each sample. Conversely, the plateau force may be more representative, as this will be the force experienced for the greatest amount of time and, as an average, is less affected by curves with large fluctuations. These fluctuations may be caused by air bubbles or particles in the material causing intermittent changes as they are extruded, or by low instrument precision for small force measurements. However, it is notable that, for many samples, there is no maximum force peak, and so the maximum and plateau value are the same. Objective comparisons between injection forces can be made so long as a consistent value is used.

The data obtained can be used in several ways. The injectability force values may be compared to ease of injection, to establish which formulations, syringe and needle sizes are viable for translation[8]. Alternatively, comparing between samples allows for the quantification of changes to formulations on injectability. For example, in cements, changing the viscosity of the liquid phase, the particle size distribution, and adding additives such as citrate to alter the colloidal properties, can have large changes in injectability[9]. These tests may also inform formulation protocol for cements, for example mixing time, time to loading and time to application, for optimum injection and post-injection performance. In addition, this method may be used to test the initial feasibility of novel bioinks for 3D printing.

This protocol can be modified in several ways. The clamp system may be replaced with a bespoke 3D printed construct to hold the syringe, which may make it easier to ensure the syringe and plunger are perpendicular to the crosshead, and the syringe held securely. The needle can be replaced with a cannula or any device that extrudes material by compression of a plunger and can be of any size and geometry. In order to increase the fidelity of the results, the tip of the needle can be placed into a tissue or hydrogel, in order to more accurately simulate clinical injection. However, this adds further complexities to the protocol, as tissue/gel composition and needle depth must be kept constant. Further, this protocol utilizes displacement-controlled extrusion, to measure the force required to inject at the specified speed. Alternatively, the injection force can be specified, and the amount of extrusion can be measured against time. This may be useful for materials with time dependent properties, such as cements. For example, by using a correlation between injection force and ease of injectability to select a force[8], this protocol may be used to establish whether the entire volume of cement can be injected with this speed prior to setting. Finally, this protocol can easily be combined with other experiments, in order to test the effect of injection on the material properties and examine phenomena such as filter pressing and self-healing, or the effect of injection on cells.

The main limitation of this protocol is that a universal mechanical tester is required. While these are common in materials testing labs, they are expensive to purchase if the user cannot access one. Further, the mechanical tester provides uniaxial compression at either a set force or rate of

displacement, whereas the applied force and injection speed may vary over the course of injection by hand. This protocol is also unsuitable for replicating some real world injections, such as injections into complex tissues in theatre, or injecting at different angles. To quantify the force of injection in the clinic, force and displacement transducers may be a better method.

ACKNOWLEDGMENTS:

This work was funded by the EPSRC CDT for Formulation Engineering in the School of Chemical Engineering at the University of Birmingham, UK, Grant reference EP/L015153/1, and the Royal Centre for Defence Medicine.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

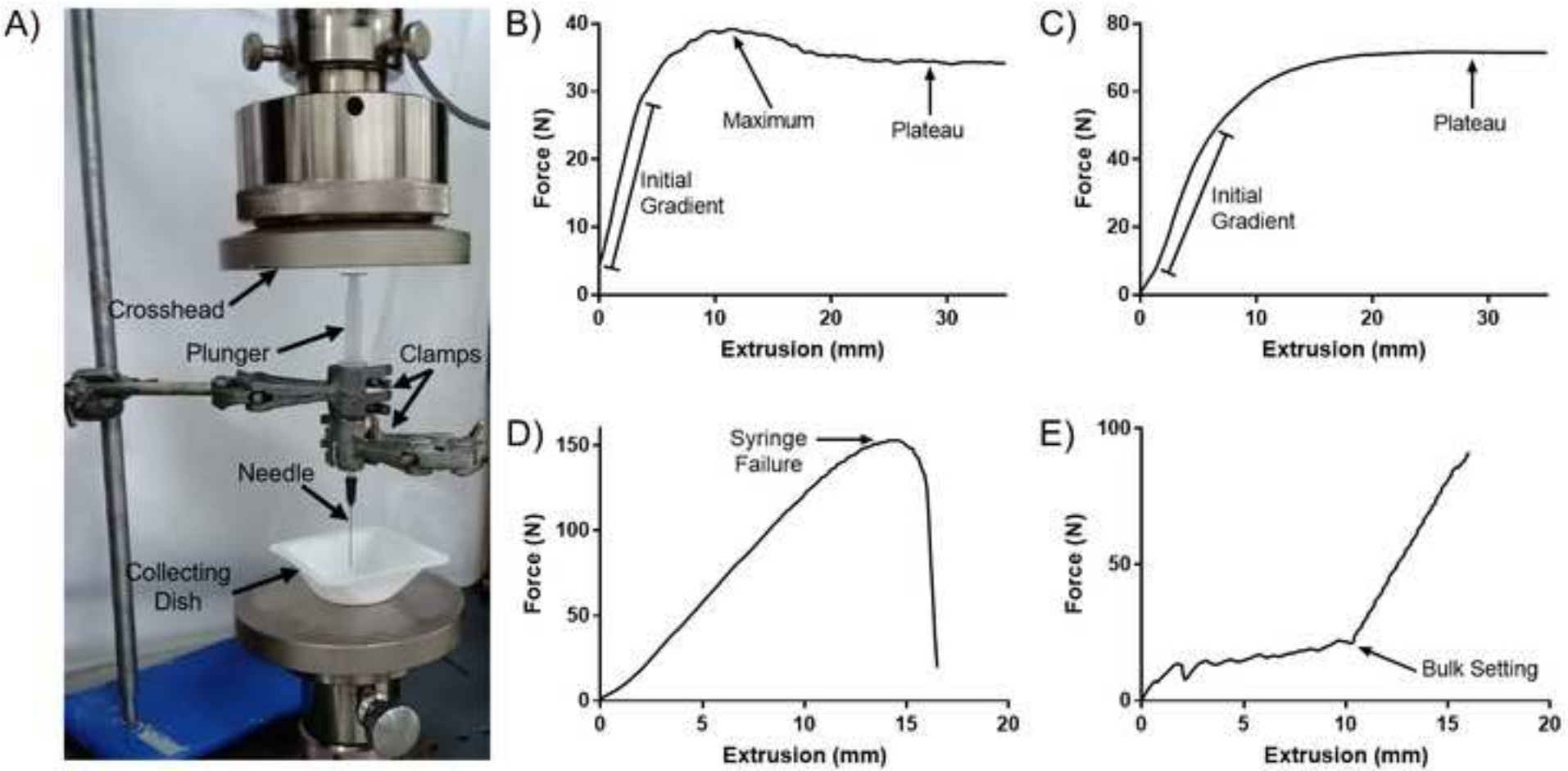

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alginic Acid Sodium Salt	Sigma	A2033-100G	
Blunt Needles	Needlez	NB19G1.5	Any size may be used, depending on application
Calcium Sulphate Hemihydrate	Acros Organics	22441.296	
Clamp stand	Eisco	MTST5	Two required
Clamps	R&L Enterprises	41	Two required, should have flat tops
Syringes	BD	307731	Any size can be used, depending on application
Universal Mechanical Tester	Zwick Roell	Z030	

7th April 2020

School of Chemical Engineering,
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Dear Dr Bajaj,

Thank you for your email, we are delighted that our manuscript *"The Quantification of Injectability by Mechanical Testing"* has been recommended for publication in JoVE following revisions. We have carefully reviewed and fully addressed the reviewer's comments, and altered the manuscript accordingly, using tracked changes to show these. Point by point responses are given below.

We hope that this revised version is now suitable for publication, and we look forward to hearing from you.

Yours sincerely,

Mr Thomas E Robinson and Dr Sophie C Cox

7th April 2020

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please use American English throughout.

We have proofread the manuscript.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have formatted the manuscript into the JoVE template style.

3. Please check with your funding source regarding PMC deposition. We do not deposit articles into PubMed Central on behalf of the authors. However, authors can self-deposit into PMC if required by their funding source.

We have noted this.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

We have rephrased the summary to fit this description:
'Presented here is a protocol for quantitatively evaluating the injectability of a material through a syringe-needle system using a standard mechanical testing rig.'

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

All text is in the imperative tense and in complete sentences.

6. The Protocol should contain only action items that direct the reader to do something.

All actions contain precise instructions.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

Steps are discrete and there are no large paragraphs.

8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
Each step contains a maximum of 3 actions

9. Please ensure you answer the "how" question, i.e., how is the step performed?

Added phrases to instructions 2.3, 2.4.1, 4.6, 4.7, and 4.8 to make these steps more clear

10. 1.1. To make this a stand-alone protocol, please include how samples are prepared? Please include what kind of sample, concentration, and other relevant details.

We have added examples of how to prepare a formulation for a pre-loaded syringe:
'1.1.2 For example, to prepare 2% alginate solutions, dissolve 2 g of alginic acid sodium salt in 100 mL of deionized water, by stirring at room temperature. Aspirate the solution into 5 mL syringes, and store for 24 hours at room temperature before testing.'

As well as a formulation to be prepared directly before application:
'1.1.4 For example, to prepare calcium sulphate cement, manually mix 4 g of calcium sulphate hemihydrate into 5 mL of deionized water with a spatula for 1 minute. Remove the plunger from the syringe, and load the cement into the syringe barrel with the spatula. Begin the mechanical testing after 4 minutes.'

11. 2.2, 2.2: Are these done manually?

Yes, this is physical set up of the machine, we have added 'Manually' to instruction 2.2 to make this more clear.

12. 2.4: Please include how each step is performed. Please include button clicks in the software, knob turns etc.

We have included an 'open the test wizard' step into 2.4.1., the remaining steps in this section are selections and numbers to be typed into this window in the software

13. 4: Where is the injection being performed? Please include all details associated with your experiment.

The injection is performed by the mechanical tester; the clamps are set up between the crosshead and the base plate, as per Figure 1A. We have added an extra line in the protocol (3.5) to make this clearer.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The highlighted section is less than 2.75 pages

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16. Please write the result section in a paragraph style only.

Changed the numbered section to a paragraph style

17. How do you generate the sample curves? Please include the details in the protocol

Curves are generated by plotting the results with displacement on the x-axis and force on the y-axis (Step 5.5)

18. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion contains all of these elements.

19. Please do not abbreviate the journal titles in the references section.

We have changed the citation style for full journal names

20. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.

We have updated the table with all necessary information

7th April 2020

Reviewer #1:

Manuscript Summary:

This paper reports on a protocol for evaluating the injectability of injectable biomaterials using a standard mechanical tester.

We thank reviewer 1 for their time and effort in reviewing our manuscript.

Major Concerns:

The methods for testing injectability of injectable biomaterials have been extensively reported (Ref. 18-24 in the paper). The protocol provided in this paper does not show any advancement, compared with that reported by other researchers. The protocol does not address any weakness of testing methods for injectability either.

We do not claim to report a new protocol in this manuscript. To be clear, the focus of this article is to explain this widely used methodology in an unprecedented level of detail, something not found in biomaterials literature. Further novelty is also achieved through discussing the advantages of this methodology compared to other characterisation methods, such as rotational rheology and pressure transducers. Finally, it is notable that we have suggest various experimental modifications and make recommendation on what conclusions may be drawn from the data generated. We therefore believe that this manuscript will be extremely useful to the biomaterial's community, especially to those new to injectability from non-engineering backgrounds.

We agree with the reviewer that we have not sufficiently highlighted the limitations of this protocol, and to address this have added the following paragraph to the end of the discussion:
'The main limitation of this protocol is that a universal mechanical tester is required. While these are common in materials testing labs, they are expensive to purchase if the user cannot access one. Further, the mechanical tester provides uniaxial compression at either a set force or rate of displacement, whereas the applied force and injection speed may vary over the course of injection by hand. This protocol is also unsuitable for replicating some real world injections, such as injections into complex tissues in theatre, or injecting at different angles. To quantify the force of injection in the clinic, force and displacement transducers may be a better method.'

Minor Concerns:

The clamps are inconvenient to operate and not easy to ensure precise fixation of syringe.

We agree that the setting up and alignment of the clamps is perhaps the most challenging part of this protocol, and requires some iteration. We have therefore added a note into the protocol:
'NOTE: Alignment of the clamp grips with each other and the center of the crosshead may take some time and iteration to achieve, but is important to acquire high quality data.'
However, once aligned, syringes can be placed in and removed from the clamps without upsetting their aligned position. We have found little difficulty using clamps for this protocol, and have described a protocol using clamps because they are ubiquitous in many laboratories. However, one could conceive of a bespoke 3D printed construct to hold the syringe, which would remove the iterative steps to set up the clamps and immediately achieve a right angle. We have added a sentence to this effect in line 281 of the discussion:
'The clamp system may be replaced with a bespoke 3D printed construct to hold the syringe, which may make it easier to ensure the syringe and plunger are perpendicular to the crosshead, and the syringe held securely.'

Reviewer #2:

Manuscript Summary:
Robinson et al. describe how to utilise a mechanical tester to measure force required to compress a syringe plunger at a set rate for the assessment of injectability of biomaterials for clinical applications. The manuscript is well written and easy to follow.

We thank reviewer 2 for their time and effort in reviewing this manuscript, and for their kind commendations.

Major Concerns:
1. "Using a mechanical tester produces a reliable extrusion rate equally over the plunger, which may be difficult to achieve with a syringe pump, especially for viscous formulations or those with time dependent properties." This is emphasized throughout the paper but it is unclear why this is, especially for a reader with a limited engineering background. What is the difference in the mechanics of a mechanical testing rig versus a modern syringe pump, given that a syringe pump also places a specific force on the plunger to achieve a certain flow rate, and are accurate enough to use for drug dispensing applications?

We agree with the reviewer that we have stated this without sufficient explanation. The concern we are trying to convey is that syringe pumps are designed for delivery of aqueous drug solutions, which have the same material properties as water, and therefore may not be designed with sufficient torque to extrude viscous or semi-solid materials at a reliable rate, especially if the material is hardening as in a setting cement. However, we agree that this statement as written is too vague, and somewhat based on our experience – some syringe pumps may indeed possess sufficient mechanics for this purpose. We have therefore changed line 90 to:
'Further, a syringe pump may not possess sufficient torque to compress the plunger at a precise rate if high forces are required to extrude viscous or semi-solid materials.'
And line 109 to:
'Using a mechanical tester produces a reliable extrusion rate equally over the plunger, which is particularly advantageous for viscous formulations or those with time dependent properties.'

2. "any syringe size, needle gauge and material can be used" is a very broad claim. It's not mentioned if this protocol has been carried out for a range of syringe sizes and needle gauges - if so what is the specific range that this protocol has been verified for?

Our group has used this protocol mainly for syringes between 1 and 5 ml, and 19 – 30G needles, and other studies (citations 18 – 24) have used similar procedures for up to 10 ml syringes and 10G needles, though the 1 – 5 ml range is most common. However, there is no reason that a larger syringe could not be used, the clamps would simply have to be widened to accommodate it. Only in the case of uncommonly large syringes would this be an issue, if the clamps could not be widened further. We have therefore added a caveat to this statement:
'This protocol is highly versatile; any material, needle gauge and syringe size can be used, provided the syringe can be accommodated by the clamps. This has been verified in this protocol for syringes up to 10 ml.'
We are certainly not suggesting that any combination of material, syringe and needle will be injectable, but believe that they could be tested in this protocol.

3. "requires minimal post processing to obtain key values" - The graphs in Fig 1 are very useful for the reader to understand what results will look like, but no calculations, equations..etc. There is no information on post-processing required to achieve a quantification of injectability

as the title states, i.e. values. This is needed to understand how this protocol is any better than all the other methods detailed in the introduction, especially that all these methods give a quantification of material properties that contribute to injectability. Discussion states that the forces can then give an 'indication' of ease of injectability - this is different to what the title of the manuscript states, and clarification is needed as to why is this any better than other methods.

The only post processing required is to read the max or plateau force from the graph, we have mentioned this in the discussion, but have added it as a step in the protocol for added clarity:
'5.3 Read the maximum force (if it exists) and plateau force from the graphs.'
This gives a number for injectability, and is therefore quantitative, rather than qualitative. If the user wishes to know whether their formulation is injectable by hand, they are directed to reference 8, where a correlation between the force values generated by this protocol and ease of injectability, i.e. easy, difficult but possible, uninjectable, exists. However, we consider this step to be outside the scope of this protocol, because there are several other ways of interpreting the force data, as outlined in paragraph 3 of the discussion. It is not the fact that this protocol generates a number that is uniquely advantageous; it is that this number is directly related to injectability, whereas rheological and material properties are not.

Minor Concerns:
Add a caution about sharps injuries if used with materials mixed with cells.

We thank the reviewer for highlighting this important safety concern, we have added a caution: 'If the material contains cells or other biological materials, extra care should be taken to prevent sharps injuries.' to line 138 in the protocol.

7th April 2020

Reviewer #3:

Manuscript Summary:

The article deals with injectable biocements, which are important for the minimally invasive delivery to bone defects. However, it is currently difficult to assume the injectability of such cement pastes since no objective test methods are existing. The manuscript describes an experimental approach to test cement viscosity by a standardized test protocol. The method is in my view very well structured and will definitely result in a testing regime, which can be reproduced by other researches working with the injectable materials.

We thank reviewer 3 for their time and effort in reviewing this manuscript, and for their positive comment on our work.

Major Concerns

none

Minor Concerns:

I would only recommend adding some comments on the type of syringe used for the experiment, e.g. syringes may have either central outlets or outlets located more on the edge. This may have an effect on injectability (especially for cement pastes) and should be mentioned and considered for the experiment.

We agree with the reviewer, and indeed syringes with non-central outlets require larger forces for extrusion, a factor not captured by rotational rheology. We have therefore added a specific comment to this effect in line 75 of the introduction:

'However, this equation does not account for the conical end of the syringe or any other geometries, such as off-centre outlets'

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Figure 1 is adapted from Robinson *et al* (2020): 'Filling the Gap: A Correlation between Objective and Subjective Measures of Injectability'
https://onlinelibrary.wiley.com/doi/full/10.1002/adhm.201901521

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