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Standard Guide for Characterization of Hydrogels used in Regenerative Medicine[1]

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1. Scope

1.1 Hydrogels are water-swollen polymeric networks that retain water within the spaces between the macromolecules; and maintain the structural integrity of a solid due to the presence of cross-links (**1-3**).[2] They are mainly used in regenerative medicine as matrix substitutes, delivery vehicles for drugs and/or biologics, and environments for cell culture. In these applications, hydrogel efficacy may depend on the ability to: support the permeation of dissolved gases, nutrients and bioactive materials; sustain cell growth and migration; degrade; release drugs and/or biologics at an appropriate rate; and maintain their shape.

1.2 Hydrogels used in regenerative medicine can be composed of naturally derived polymers (for example, alginate, chitosan, collagen (**4, 5**)), synthetically derived polymers (for example, polyethylene glycol (PEG), polyvinyl alcohol (PVA) (**4, 5**)) or a combination of both (for example, PVA with chitosan or gelatin (**6**)). In clinical use, they can be injected or implanted into the body with or without the addition of drugs and/or biologics (**7**).

1.3 This guide provides an overview of test methods suitable for characterizing hydrogels used in regenerative medicine. Specifically, this guide describes methods to assess hydrogel biological properties, kinetics of formation, degradation and agent release, physical and chemical stability and mass transport capabilities are discussed.

1.4 The test methods described use hydrated samples with one exception: determining the water content of hydrogels requires samples to be dried. It is generally recommended that hydrogels that have been dried for this purpose are not rehydrated for further testing. This recommendation is due to the high probability that, for most hydrogel systems, the drying-rehydration process can be detrimental with possible alterations in structure.

1.5 This guide does not consider evaluation of the microstructure of hydrogels (for example, matrix morphology, macromolecule network structure and chain conformation).

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D4516 Practice for Standardizing Reverse Osmosis Performance Data

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F895 Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity

F2027 Guide for Characterization and Testing of Raw or Starting Biomaterials for Tissue-Engineered Medical Products

F2064 Guide for Characterization and Testing of Alginates as Starting Materials Intended for Use in Biomedical and Tissue Engineered Medical Product Applications

F2103 Guide for Characterization and Testing of Chitosan Salts as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Product Applications

F2150 Guide for Characterization and Testing of Biomaterial Scaffolds Used in Tissue-Engineered Medical Products

F2214 Test Method for*In Situ* Determination of Network Parameters of Crosslinked Ultra High Molecular Weight Polyethylene (UHMWPE)

F2315 Guide for Immobilization or Encapsulation of Living Cells or Tissue in Alginate Gels

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[2] The boldface numbers in parentheses refer to a list of references at the end of this standard.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

F2347 Guide for Characterization and Testing of Hyaluronan as Starting Materials Intended for Use in Biomedical and Tissue Engineered Medical Product Applications
F2383 Guide for Assessment of Adventitious Agents in Tissue Engineered Medical Products (TEMPs)
F2450 Guide for Assessing Microstructure of Polymeric Scaffolds for Use in Tissue-Engineered Medical Products
F2739 Guide for Quantitating Cell Viability Within Biomaterial Scaffolds

2.2 *ISO Standards:*[4]
ISO 10993 Biological Evaluation of Medical Devices
ISO 22442 Medical Devices Utilizing Animal Tissues and Their Derivatives

2.3 *ANSI/AAMI Standards:*[4]
STBK9–1 Sterilization—Part 1: Sterilization in Health Care Facilities
STBK9–2 Sterilization—Part 2: Sterilization Equipment
STBK9–3 Sterilization—Part 3: Industrial Process Control
ST72 Bacterial Endotoxin—Test Methodologies, Routine Monitoring and Alternatives to Batch Testing

2.4 *Federal Regulations:*[5]
21 CFR 210 Current Good Manufacturing Practice in Manufacturing, Processing, Packaging or Holdings of Drugs, General
21 CFR 221 Current Good Manufacturing Practice for Finished Pharmaceuticals
21 CFR 610 General Biological Products Standards
21 CFR 820 Quality System Regulation

3. Terminology

3.1 *Definitions:*

3.1.1 *adventitious agents, n*—unintentionally introduced microbiological or other infectious contaminant. In the production of tissue engineered medical products (TEMPs), these agents may be unintentionally introduced during the manufacturing process or into the final product or both.

3.1.2 *biocompatibility, n*—the ability of a foreign material to fulfill its intended function with an appropriate host organism response.

3.1.3 *conductivity, n*—property of a substance's (in this case, water and dissolved ions) ability to transmit electricity.

3.1.3.1 *Discussion*—Conductivity is the inverse of resistivity.

3.1.3.2 *Discussion*—Conductivity is measured by a conductivity meter.

3.1.3.3 *Discussion*—The units of conductivity are Siemens per metre (Sm^{-1}).

3.1.4 *hydrogel, n*—a three-dimensional network of polymer chains that retains water within the spaces between the macromolecules.

3.1.5 *loss (viscous) modulus, n*—quantitative measure of energy dissipation, defined as the ratio of stress 90° out of phase with oscillating strains to the magnitude of strain.

3.1.6 *mechanical properties, n*—those properties of a material that are associated with elastic and inelastic reaction when forces are applied and released. These properties are often described in terms of constitutive relationship between stresses, strains, and strain rates.

3.1.7 *permittivity, complex, n*—a material property deduced from the ratio of the admittance, *Yp*, of a given electrode configuration separated by that material, to the admittance of the identical electrode configuration separated by a vacuum or air for most practical purposes, *Yv*.

3.1.8 *regenerative medicine, n*—a branch of medical science that applies the principles of regenerative biology to restore or recreate the structure and function of human cells, tissues, and organs that do not regenerate adequately.

3.1.9 *relaxation modulus, n*—the modulus of a material determined using a strain-controlled (relaxation) experiment at temperature *T* and time *t*, which may also be expressed using reduced time as $E(T_{ref}, \xi)$.

3.1.10 *storage (elastic) modulus, n*—quantitative measure of elastic properties defined as the ratio of the stress, in-phase with strain, to the magnitude of the strain.

3.1.11 *tan delta, n*—ratio of the viscous (loss) modulus to the elastic (storage) modulus in a sinusoidal deformation; mathematically, the tangent of the loss angle, δ.

3.1.12 *tomography, n*—any radiologic technique that provides an image of a selected plane in an object to the relative exclusion of structures that lie outside the plane of interest.

4. Significance and Use

4.1 This guide describes methods for determining the key attributes of hydrogels used in regenerative medicine (that is, their biological properties, kinetics of formation, degradation and agent release, physical and chemical stability and mass transport capabilities). See Table 1.

5. Key Factors for Hydrogel Characterization

5.1 In regenerative medicine, hydrogels can be used with the addition of drugs or biologics, or both (for example, as drug delivery devices or for cell encapsulation (**4**)) or without (for example, as tissue scaffolds or barriers (**4**)). Although characterization of hydrogels requires consideration of the individual

[4] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[5] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

TABLE 1 Key Factors and Attributes to be Considered in Hydrogel Characterization

NOTE 1—Example standards with relevant content are also indicated.

	Key Factors for Hydrogel Characterization			
	Biological Properties (Sections 5 and 6)	Kinetics (Section 7)	Physical and Chemical Stability (Section 8)	Mass Transport (Section 9)
Attribute	Biocompatibility (ISO 10993, F895)	Gelling time (F2315)	Environmental stability (D4516)	Cell migration (F2315)
	Adventitious agents (F2383, ST72, ISO 22442, 21 CFR 210, 21 CFR 221, 21 CFR 610, 21 CFR 820)	Swelling rate (ISO 10993, F2214)	Mechanical properties (F2150)	Transport of nutrients and waste (F2450)
	. . .	Matrix degradation (F2150)	Cell encapsulation (F2315)	Release rate of bioactive agents (F2450)

composition and application the hydrogel will be used for, there are common generic requirements that can be summarised as follows:

They need to be biocompatible **(8)**.

Their mechanical properties should enable them to be compatible with their intended clinical use.

They should be capable of swelling and potentially degrading at a rate that meets the needs of the intended clinical use **(9, 10)**.

They should be sufficiently permeable to promote and maintain cell viability, nutrient and waste product transport, or release therapeutic agents, or combination thereof.

Hydrogels, when combined with drugs or biologics, including any seeded or encapsulated cells, should not negatively alter the functional characteristics of the biologic or the drug through physical or biological interactions arising from the presence of hydrogels.

Ease of handling and delivery must also be considered since it is of importance for clinical use **(4)**.

Variability in the composition of the starting polymer material used in hydrogels impacts the hydrogel final properties. It is therefore necessary to characterize the starting material, particularly for polymers derived from natural sources due to the inherent variability of their composition. Guidance on characterization of starting biomaterials for TEMPs can be found in Guides F2027, F2064, F2103, and F2347. Further, when hydrogels used in regenerative medicine are prepared under broad manufacturing conditions, the effect of variable co-agent concentrations and the effect of variable manufacturing conditions (for example, pH, temperature, ionic strength) on the final hydrogel properties should be considered and measured as appropriate.

5.2 The degree of hydrogel crosslinking is an important parameter affecting hydrogel physical properties and performance. Correlation between the stoichiometric and effective degree of crosslinking in the final hydrogel is a direct indication of the extent of reaction. In some cases determination of crosslinking is not trivial and it is therefore recommended that an estimation of percent post-gelation extractables should be performed. This will serve as a direct indicator for the crosslinking efficiency and will provide information on potentially harmful leachables.

5.3 Sterilization processes can affect the properties of the hydrogel or any active or inactive added components, or both (for example, drug, excipient, and so forth), all of which need to be taken into account when considering characterization data. In order to appropriately assess hydrogel characterization data, it is recommended that a sterilization summary is provided along with the sample. Further guidance on sterilization strategies can be found in Guide F2150, STBK9–1, STBK9–2, and STBK9–3. It is noted, however, that a particular sterilization method may not be applicable to a certain type of hydrogel. In this case, provision of non-sterile hydrogels in a medium that contains antimicrobial agents or producing hydrogels under aseptic conditions may need to be considered with additional controls such as bioburden reduction and sterility testing.

5.4 It may be possible to assess the hydrogel response to *in vivo* conditions through the use of suitable *ex vivo* models. Factors that such models should take into consideration include: tissue-specific mechanical loading; tissue-specific metabolic activity; tissue-specific pH; relevant chemistry to the site/condition of implantation; temperature; oxygen content; and tissue-specific cell types present. For example, the testing of ionically crosslinked hydrogels which are susceptible to ion exchange during implantation (for example, Ca^{2+} crosslinked alginates) should be done in media resembling the physiological environment at least in terms of electrolytic content and osmolarity and the relevant chemistry such as divalent cation delivery. For temperature-sensitive hydrogels, the hydrogel gelling temperature is key to assess *in vivo* performance, and tests should be carried out at 37°C. For gels implanted in interstitial fluid, fat tissues, and so forth, in addition to maintaining a physiologically relevant electrolytic balance, testing may need to be done in serum, or at least in the presence of proteins and lipids, and so forth.

5.5 Consideration should be given to hydrogel stability during storage and transportation. It may be necessary to maintain hydrogels in a controlled environment with factors such as temperature and pH regulated.

5.6 To adequately assess the suitability of a candidate hydrogel for use in regenerative medicine it is necessary to consider the key factors identified in Table 1. Each factor can be assessed through measurement of different hydrogel properties that will be discussed below. In all cases measured quantities should be reported in the relevant SI units.

6. Biological Properties

6.1 *Biocompatibility*—The biocompatibility of the hydrogel product shall be established. Currently there are no standards

that describe protocols specifically for hydrogels; however, guidance on test methods can be found in Practice F748 or in ISO 10993.

6.2 *Adventitious Agents*—Hydrogels containing polymers derived from natural sources and those that contain biologics need to be assessed for safety associated with adventitious agents and their by-products. Guidelines for microbiological testing of aerobic and anaerobic bacteria, fungi, mycoplasma, endotoxins and viruses are given in 21 CFR 610. Test methods for determining the presence of bacterial endotoxins are also given in ST72. The ISO 22442 series is also useful as it provides guidance on risk management related to hazards such as contamination by bacteria and viruses as well as materials responsible for undesired pyrogenic, immunological, or toxicological reactions. Many potential compromises to product safety can be minimized through the adherence to current Good Manufacturing Practice (for example, 21 CFR 210, 21 CFR 221, 21 CFR 820).

7. Kinetics

7.1 Hydrogel kinetics covers the process of hydrogel formation, delivery of the hydrogel to the site of use, hydrogel degradation and release of active agents. Currently there are no guidelines on how to assess the kinetics of hydrogels; however, measurement of hydrogel gelling time, swelling rate and degradation rate, and the release rate of bioactive agents can be useful.

7.2 *Gelling Time:*

7.2.1 Hydrogel gelling time is of practical importance particularly for systems designed to gel in situ. Although there are no protocols for determining gelling time for hydrogels used in therapeutic applications, there are techniques used in the food and polymer industry, which may have applicability (**11-13**). Both the Bloom and Sag tests, which are used to assess the quality of gelatin and pectin gels respectively, could be adapted to investigate hydrogel gelling time (**12, 13**). Gelling time could also be investigated using simple tests such as the tube tilt test and the falling ball test (**14, 15**). In the tube tilt test, a tube containing the hydrogel is inverted periodically and the gelling time determined as the time at which inversion of the tube does not result in observable movement of the hydrogel. The falling ball test is based on determining the time taken for a ball to sink to the bottom of a container filled with hydrogel. Both the tube tilt test and the falling ball test do not require specialist equipment. Care should be taken in selection of tube geometry for both tests as small bore tubes could give different results than large bore tubes.

7.2.2 Alternative methods that have fast sampling rates need to be used for hydrogels that gel rapidly. Monitoring changes in optical turbidity as well as dynamic and static light scattering can be used to determine the gel points as for many hydrogels both turbidity and scattering increase significantly when it is reached (**16-18**). It is noted, however, that optical techniques can become problematic for systems that scatter light strongly prior to reaching the gel point due to lack of a detectable signal.

7.2.3 An alternative approach to determine the gelling time of a hydrogel is to monitor time dependent changes in mechanical properties (**19**). In practice, oscillatory rheometry is often used to determine the mechanical properties (**19-21**). The gelling time can be assessed in a number of ways, for example, by the point at which the storage modulus becomes greater than the loss modulus. The frequency independence of the viscoelastic loss factor, tan delta, can be used to indicate network formation. Additionally, gelling time can be derived from assessment of the power-law dependence of the relaxation modulus. When performing rheological studies on hydrogels care must be taken in both the loading of the sample, choice of the sample volume, and selection of sample test geometries (for example, parallel plate, cone and plate). It is also important to ensure that the sample does not dehydrate during the test. Care should also be taken to limit slippage between the plates and the sample. Various approaches have been followed including using roughened plates as well as loading the sample when it is in a liquid state and leaving it to gel in the rheometer. Regardless of the protocol used to load the sample, a record of the sample measurement geometry used, oscillation frequency, temperature and pre-load conditions should be made as these will impact on the results obtained.

7.2.4 For some hydrogels rheological determination of the gelling time is complicated by their mechanical weakness, strain sensitivity and non-equilibrium behavior. In these instances ultrasonic methods have applicability as they can be used to generate small strain mechanical waves in a sample of interest (**22-24**). Specifically, the propagation of ultrasonic waves is affected by the material mechanical properties. In practice, the ultrasonic speed of propagation and attenuation are measured, from which the complex elastic modulus can be determined (**22**). Dielectric spectroscopy can reveal information about molecular mobility and, as such, has application in determining the hydrogel gelling time. Dielectric spectroscopy typically involves measurement of the frequency dependent complex permittivity and conductivity (**25, 26**). Both ultrasonic and dielectric methods, however, require a skilled operator in order to obtain reliable results.

7.3 *Swelling Rate:*

7.3.1 The swelling behavior of hydrogels depends on their external environment. Abrupt changes in the swelling behavior can be observed in response to changes in pH, temperature and electromagnetic radiation (for example, light, gamma radiation, and so forth) (**27-30**). For these reasons, assessment of hydrogel swelling rate, that is the swelling ratio as a function of time, is important. This is particularly so when considering the range of physiological environments that exist within the body and how they change with time, disease state, age, and between individuals. In this context swelling rate takes into consideration the swelling that occurs following cross-linking. Established methods for assessing swelling rates are referred to in ISO 10993 Part 19. The primary method for assessment of hydrogel swelling rate is determining the equivalent water content as a function of time (**31**). In practice, this involves placing the hydrogel in a solution for a known period of time, after which it is removed and weighed. This can be repeated over a predetermined time period (**31**). The swelling rate is then determined from the rate of mass uptake of the hydrogel. In addition to the rate of swelling the equilibrium degree of

swelling can also be determined. In this case, the degree of swelling is typically expressed either as the equilibrium volume swelling ratio or the equilibrium weight-swelling ratio. These parameters are determined from the ratio of the fully swollen to the dry hydrogel volume or mass respectively. Care must be taken here to ensure the hydrogel is completely dry and that the drying process does not degrade the hydrogel.

7.3.2 Alternative approaches, which do not require removal of the hydrogel from solution, are those that involve tracking changes in hydrogel shape over time. A number of methodologies could be used to assess these changes, including mechanical probes and video microscopy coupled with image analysis and proximity sensors (see Test Method F2214). Tomography techniques, including optical, ultrasound and X-ray, may be needed if the surface of the hydrogel is not readily accessible (for example, when the hydrogel is used to fill a porous material). Regardless of the method chosen to monitor hydrogel shape, the hydrogel should be inspected periodically for the presence of any cracks that may have formed during the swelling process, as this will impact on the validity of data obtained. Conductivity measurements can also be used to study swelling in charged hydrogels through determination of the liquid content dependent electrical conductivity **(32)**. Selection of techniques to measure the hydrogel-swelling rate should be based on both the robustness of the hydrogel to handling and the required measurement accuracy.

7.4 *Matrix Degradation:*

7.4.1 Degradation behavior is integral to the function of many hydrogel products. For some applications, it is desirable for the hydrogel to degrade at a rate matched with new tissue formation or at a controlled rate to regulate drug delivery. For others where the hydrogel is designed to act as a permanent barrier or support, degradation is not desirable. Degradation studies should be carried out in conditions that mimic, or reflect, the *in vivo* parameters that have a direct impact on hydrogel degradation behavior. Consideration should also be given to assessment of degradation in relevant pre-clinical models. Further, in selection of suitable test methods, consideration should be given to the underlying mechanism of degradation, which includes, hydrolysis, enzymatic digestion, oxidative degradation; bulk degradation and surface degradation, as this will affect both the required measurement rate and sensitivity.

7.4.2 On a bulk scale techniques to monitor hydrogel swelling rate also have applicability for investigating matrix degradation. For example, periodic assessment of hydrogel mass loss can be used in addition to volume changes. Here it is important to consider whether the measurement methodology enables the mass of the matrix to be resolved from the mass of the water. Alternatively, the hydrogel can be placed in a solution for a known period of time and degradation inferred from changes in the solution pH or the presence of degradation products.

7.4.3 Matrix degradation products can also be assessed through the use of chromatography including gel permeation chromatography (GPC) **(33)**, high-performance liquid chromatography (HPLC) **(34)** and affinity monolith chromatography (AMC) **(35)**. In these techniques, molecules with different masses can be separated with a high degree of sensitivity.

7.4.4 Changes in chemical structure can also be indicative of degradation which can be probed using infra-red spectroscopy **(36, 37)**, Raman spectroscopy **(38, 39)** and nuclear magnetic resonance (NMR) spectroscopy **(31, 39)**.

8. Physical and Chemical Stability

8.1 The stability of hydrogel shape and structure are important for the correct function of many products. Assessment of hydrogel physical and chemical stability can be made through measurement of hydrogel properties (for example, swelling, mechanical properties and ability to encapsulate cells) in response to environmental changes (including temperature, pH, and osmotic conditions), mechanical properties and the ability to support cells (for example, cell seeding, cell migration, cell adhesion). Again it is recommended that measurement protocols include specification of suitable biologically relevant measurement environments.

8.2 *Environmental Stability*—Hydrogel stability can be affected by the biochemistry and thermodynamics of the local environment in which it is placed, and this needs to be characterized.

8.2.1 For some hydrogel products, lack of osmotic stability has deleterious consequences to their function. For such systems, it is imperative that they be tested in osmotic environments that match those of the site of implantation, otherwise there is a risk that liquid will either be driven out of or into the hydrogel. There are also hydrogels that are specifically designed to undergo osmotic swelling such as space filling osmotic expanders. In either case, it is important that the osmotic swelling of these hydrogels is characterized. Further, time course studies should be performed to assess any changes in either the local environment or the matrix itself that may compromise osmotic stability. Techniques described to study hydrogel swelling also have applicability to the study of osmotic stability (that is, the absence of additional swelling provides evidence of stability).

8.2.2 Some hydrogels are designed specifically to undergo changes in state when certain stimuli (for example, changes in pH, ionic strength, temperature, mechanical loading) are applied **(27, 29, 36)**. For these systems, it is important to assess their stability in the environment of intended use and the reproducibility of any changes in state affecting their function. For example, a primary function of some injectable scaffolds that are initially in a liquid state is to gel once in the body. Techniques described to study the gelling time of hydrogels will have applicability in assessing their stability in their intended environment and changes in hydrogel phase in response to applied stimuli.

8.3 *Mechanical Properties:*

8.3.1 Mechanical properties are particularly important in the study of hydrogels designed to function as physical barriers or support structures, or both. Due to the inherent weakness of many hydrogels, characterization of their mechanical properties can be problematic.

8.3.2 One relatively simple approach to the investigation of hydrogel mechanical properties is indentation testing. Indentation tests can be used to study the force required to move a probe a defined distance into a sample constrained in an open ended container. For some hydrogels, these tests can be carried out using commercially available texture analyzers (which are widely used in the food industry) (**11**). It is noted, however, that texture analyzers will not be able to resolve small probe forces in the case of very weak hydrogels (that is, samples are easily fractured and are unable to support their own weight). Further, care must be taken in the selection of the probe geometry as the contact area has a major impact on results.

8.3.3 Time dependent indentation studies can also be useful in the characterization of mechanical properties. The scale of investigation can be chosen through appropriate selection of the indentation probe. Nano-indentation and micro-indentation studies can be carried out using commercially available equipment which involves the application of a spherical indenter tip to the sample (**40**). A ramp-hold displacement-time profile in which the applied load is ramped up for a given amount of time and then held at a constant value for a defined period of time can be used. The displacement of the sample with time is recorded. In these experiments, the results are sensitive to the thickness of the hydrogel with the reliability of results going down with decreasing hydrogel thickness. An alternative approach is to perform unconfined compression tests in which a hydrogel sample is compressed between two plates and a known load applied. Often an oscillatory load is used. In this approach, it is recommended that a static preload be applied to the sample to ensure that the sample is held in compression throughout the test (**40**). Regardless of the measurement strategy used, it is important that the correct physical model is used to interpret the experimental data when extracting mechanical properties, for example, viscoelastic and poroelastic analysis (**40**).

8.3.4 For sufficiently robust hydrogels, mechanical testing can be carried out using tensile or compression testing. These studies can be performed under either static or dynamic conditions as well as in either constrained or unconstrained geometries. Care must be taken, however, in the mounting of samples. In the case of tensile testing, clamping of the sample can be difficult (for example, it may be necessary to use pressure clamps). It is also suggested that hydrogels be formed in a dumbbell shape rather than uniform strips to reduce the risk of sample breakage at the specimen-clamp junction.

8.3.5 Rheometry can also be applied to the characterization of hydrogel mechanical properties, as detailed in 7.2.3. In some instances, hydrogels will be too weak to undergo rheological or mechanical testing. In these instances, ultrasonic methods, such as sonoelastography, have applicability as they can be designed to determine the hydrogel mechanical properties through the application of small strains. Sonoelastography is an ultrasound imaging technique that applies strain to a sample using a low-amplitude, low-frequency shear waves (less than 0.1 mm displacement and less than 1 kHz frequency) (**41**). The resulting strain in the sample can be determined in real time using Doppler ultrasound to image the vibration pattern. Alternatively, mechanical properties can be assessed through measurement of ultrasonic speed of sound propagation through a sample of known thickness and density as discussed in 7.2.4.

8.3.6 The development of protocols for determining hydrogel mechanical properties must take into consideration the effects of sample loading, measurement geometry, and possible sample dehydration on results.

8.4 *Cell Encapsulation:*

8.4.1 Encapsulation of cells in a hydrogel is important in the development of tissue substitutes and environments for three-dimensional (3D) cell culture. Details of strategies for cell encapsulation in hydrogels are given in Guide F2315. One approach to assess cell encapsulation is to apply optical imaging to study the spatial location of cells in the matrix over time. Selection of a suitable imaging approach will be governed primarily by the opacity of the hydrogel. It may be possible in some instances to fluorescently label cells prior to incorporation into the hydrogel and subsequently form images based on cell fluorescence. This could be realized through the use of confocal microscopy or optical coherence tomography. The depth of investigation will, however, be dependent on the optical properties of the hydrogel. Alternatively, the ability of cells to migrate through the matrix could be determined by placing the hydrogel between the two chambers of a Boyden cell, where one chamber contains a chemical agent, which will promote cell migration, and the other contains cells (**42**). The concentration of cells in each chamber can subsequently be monitored to determine the cell population within the hydrogel and the population that has passed through the hydrogel.

9. Mass Transport

9.1 Here mass transport relates to the ability of cells, nutrients and waste, and bioactive agents to move within and through a hydrogel. In tissue engineering applications, maintaining cell viability (Guide F2739) and functionality is of paramount importance. It depends greatly on the ability of the hydrogel to transport a sufficient supply of oxygen, essential nutrients, and active biomolecules, as well as allow adequate removal of waste. Further, it may be desirable for the hydrogel to promote cell migration into the network. There is also a need to characterize the mass transport in hydrogels used for controlled drug release. In this instance, the movement of drugs and biologics out of the hydrogel should be characterized. For all applications, hydrogel mass transport should be assessed over a range of sizes (for example, small molecules, proteins, and cells). It is recommended that both active (for example, due to fluid flow) and passive (for example, diffusion) transport behaviors of hydrogels be considered. Further, samples should be tested under conditions representative of those the final product will be exposed to (for example, sample geometry, time period of study, biological components).

9.2 *Cell Migration:*

9.2.1 Cell migration relates to the ability of cells to actively migrate into and within hydrogels. A number of contributing mechanisms need to be considered, including cell-matrix interactions, matrix structure and chemical gradients. Other factors, especially those relating to encapsulation of cells in hydrogels, are discussed in Guide F2315. In practice, cell

migration studies can be carried out through modification of a Boyden cell, as described in 8.4.

9.2.2 Hydrogels could also be placed in a cell suspension and a measurement of cell penetration into the hydrogel matrix made over time using a confocal microscope. Alternatively, the hydrogel could be fixed and sliced using microtoming to enable offline image analysis.

9.3 *Transport of Nutrients and Waste:*

9.3.1 Diffusion is a primary mechanism for the passive transport of small solutes such as nutrients and waste in hydrogels. In some cases, this movement is enhanced by flow, as can occur if the material is subject to periodic stress, for example, that resulting from movement of the body. Measurements of nutrient and waste transport can be made using methods that detect either passage through or movement within the hydrogel. Simple twin chambered devices (diffusion chambers) are widely used in the study of nutrient and waste transport in hydrogels. These measurements can either be carried out under static conditions (passive diffusion) or with a positive fluid flow (active transport) through the hydrogel.

9.3.2 A useful method to study the diffusion of ions through a hydrogel is to measure the electrical conductivity of the hydrogel (**43**, **44**). Other studies have monitored nutrient movement through measurement of changes in hydrogel optical density over time or ultraviolet (UV) and visible absorbance spectroscopy. In these studies, key nutrients can be bound to optical dyes. Additional advanced techniques are also available including Fluorescence Recovery After Photobleaching (FRAP) which, involves measurement of the recovery of fluorescence following photo-bleaching (**45**), and NMR techniques using a pulsed field gradient stimulated echo pulse sequence to measure the translational diffusion of water in the hydrogel (**46**). It is considered that for the majority of users a simple diffusion chamber, optical absorbance or electrical conductivity measures will provide sufficient information to study nutrient and waste transport.

9.4 *Release Rate of Bioactive Agents:*

9.4.1 In applications where hydrogels are used as delivery vehicles for bioactive agents, the release rate of these agents must be studied. In practice, the release profile of agents can be controlled by the degree of swelling, crosslinking density, agent binding affinity to the matrix and degradation rate (**4**). In the case of naturally occurring hydrogels delivery is often mediated by degradative action, which can be difficult to control (**4**). Hydrogels made of synthetic polymers offer greater chemistry control and flexibility. Thus, the chemistry can be tailored to produce systems that provide initial release followed by sustained delivery (**47**, **48**).

9.4.2 Measurement protocols should take into consideration the rate at which agents are released and the duration of release. Further, it is recommended that studies be carried out in conditions that mimic the *in vivo* environment as well as in the conditions of any relevant pre-clinical models. In practice, test methods could involve placing hydrogels in a biochemically relevant solution whose composition is analyzed over time. This could be performed either by periodically assaying aliquots of the bulk solution or by performing non-invasive measurements of the bulk solution (for example, fluorescence measurements). In the case of hydrogels, which provide a burst release, periodic measurements of the bulk solution composition should be made more frequently than those that provide a sustained release. Further, it may be necessary to perform bioactivity assays to evaluate if any adverse effects on encapsulated bioactive agents have occurred due to the burst release.

10. Summary

10.1 Table 2 summarizes the test methods discussed in this guide. For each hydrogel attribute, there are a number of test methods that may be applied to characterize hydrogels used in regenerative medicine. Care must be taken in correct technique selection and usage to ensure the reliability and repeatability of data. In particular, selection of suitable test methods can be aided by consideration of whether: quantitative data can be obtained; the measurement method is non-invasive; the technique is suitable to study weak hydrogels and how sensitive the resulting data is to operator variability, equipment variability and sample geometry. Information to aid this decision process is also given in Table 2.

11. Keywords

11.1 gel; hydrogel; regenerative medicine; tissue engineered medical products (TEMPs)

TABLE 2 Candidate Test Methods and Suitability for Hydrogel Characterization.

Attribute	Aspect	Example Test Methods		Quantitative Results	Non-invasive Method	Suitable for Weak Hydrogels	High Sensitivity to Operator Variability	High Sensitivity to Equipment Variability	High Sensitivity to Geometry
Biological Properties	Adventitious Agents	PCR assays, sterility tests		Y	N	Y	Y	N	N
		Bacterial endotoxin test		Y	N	Y	Y	N	N
Kinetics	Gelling Time	Tube tilt test, falling ball test		Y	Y	Y	N	Y	Y
		Optical Turbidity, Scattering		Y	Y	Y	N	N	N
		Rheology		Y	N	N	Y	Y	Y
		Ultrasonic methods and Dielectric Spectroscopy		Y	Y	Y	Y	Y	N
	Swelling Rate	Equivalent solvent content study		Y	N	Y	Y	N	Y
		Video microscopy		Y	Y	Y	Y	N	N
		Conductivity measurement		Y	Y	Y	Y	N	N
	Matrix Degradation	Mass loss measurement		Y	N	Y	Y	N	N
		Mechanical Testing		Y	N	N	Y	Y	Y
		Optical and NMR Spectroscopy		Y	Y	Y	N	N	N
	Release Rate of Bioactive	Optical Spectroscopy		Y	Y	Y	N	N	N
		Biochemical analysis of aliquots		Y	N	Y	Y	N	N
	Rate of Change of Material Phase	Tube tilt test, falling ball test		Y	Y	Y	N	Y	Y
		Optical Turbidity, Scattering		Y	Y	Y	N	N	N
		Rheology		Y	N	N	Y	Y	Y
		Ultrasonic methods and Dielectric Spectroscopy		Y	Y	Y	Y	Y	N
Physical and Chemical Stability	Osmotic Stability	Equivalent solvent content study		Y	N	Y	Y	N	N
		Video microscopy		Y	Y	Y	Y	N	N
		Conductivity measurement		Y	Y	Y	Y	N	N
	Mechanical Properties	Indentation test		Y	N	N	Y	Y	Y
		Tensile/Compressive Testing		Y	N	N	Y	Y	Y
		Rheology		Y	N	N	Y	Y	Y
		Sonoelastography		Y	Y	Y	Y	N	N
	Cell Encapsulation	Optical microscopy		N	Y	Y	Y	Y	N
		Modified Boyden cell		Y	N	N	Y	Y	Y
Mass Transport	Cell Migration	Optical microscopy		N	Y	Y	Y	Y	N
		Modified Boyden cell		Y	N	N	Y	Y	Y
	Transport of Nutrients and Waste	Diffusion chamber		Y	N	N	Y	Y	Y
		Optical density studies		Y	Y	Y	N	N	N
		Chromatography		Y	N	Y	Y	N	Y
		NMR Techniques		Y	Y	Y	N	N	N
	Release of Bioactive	Diffusion chamber		Y	N	N	Y	Y	Y
		Biochemical analysis of aliquots		Y	N	Y	Y	N	N

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