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Standard Guide for Characterization and Testing of Hyaluronan as Starting Materials Intended for Use in Biomedical and Tissue Engineered Medical Product Applications[1]

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INTRODUCTION

Hyaluronan, which in this guide will encompass hyaluronic acid, hyaluronate, and its salt forms, is the simplest of the glycosaminoglycans. Hyaluronan is soluble in water and forms highly viscous solutions. Hyaluronan is found in ubiquitously in the body as part of the extracellular matrix of tissues, with high concentrations in the synovial fluid, vitreous humor, and skin, as well as in cartilage. Hyaluronan has found uses in a variety of products ranging from viscosupplements (treatment of osteoarthritis), adhesion prevention (prevention of post-surgical adhesions), viscoelastics (ocular protection), and dermal implants (lip augmentation and wrinkle removal). New applications, such as scaffolds for tissue engineering, are emerging. The aim of this guide is to identify key parameters relevant to the characterization of hyaluronan for the development of new commercial applications of hyaluronan for the biomedical and pharmaceutical industries.

1. Scope

1.1 This guide covers the evaluation of hyaluronan suitable for use in biomedical or pharmaceutical applications, or both, including, but not limited to, Tissue Engineered Medical Products (TEMPs).

1.2 This guide addresses key parameters relevant to the characterization and purity of hyaluronan.

1.3 As with any material, some characteristics of hyaluronan may be altered by processing techniques, such as cross-linking and sterilization, required for the production of a specific formulation or device. Therefore, properties of fabricated forms of this polymer should be evaluated using test methods that are appropriate to ensure safety and efficacy and are not addressed in this guide.

~~1.4~~

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

1.5 *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 requirements prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D2196	Test Methods for Rheological Properties of Non-Newtonian Materials by Rotational (Brookfield type) Viscometer
F619	Practice for Extraction of Medical Plastics
F748	Practice for Selecting Generic Biological Test Methods for Materials and Devices
F749	Practice for Evaluating Material Extracts by Intracutaneous Injection in the Rabbit
F756	Practice for Assessment of Hemolytic Properties of Materials
F763	Practice for Short-Term Screening of Implant Materials
F813	Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices

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[1] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.42 on Biomaterials and Biomolecules for TEMPs.

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[2] 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.

F895 Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity
F981 Practice for Assessment of Compatibility of Biomaterials for Surgical Implants with Respect to Effect of Materials on Muscle and Bone
F1251 Terminology Relating to Polymeric Biomaterials in Medical and Surgical Devices
F1439 Guide for Performance of Lifetime Bioassay for the Tumorigenic Potential of Implant Materials
F1903 Practice for Testing For Biological Responses to Particles ***In Vitro***
F1904 Practice for Testing the Biological Responses to Particles ***in vivo***
F1905 Practice For Selecting Tests for Determining the Propensity of Materials to Cause Immunotoxicity
F1906 Practice for Evaluation of Immune Responses In Biocompatibility Testing Using ELISA Tests, Lymphocyte Proliferation, and Cell Migration

2.2 *USP Documents:*[3]
USP <61> Microbial Limit Tests
USP <71> Sterility Tests
USP <85> Bacterial Endotoxins Tests
USP <231> Heavy Metals
USP <731> Loss on Drying
USP <1211> Sterilization and Sterility Assurance of Compendial Articles

2.3 *EP Documents:*[4]
EP Monograph 1472 Sodium Hyaluronate
EP 2.6.1 Sterility

2.4 *Other Referenced Documents:*
ISO 10993 Biological Evaluation of Medical Devices[5]
ISO 10993-1 Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing
ISO 10993-7 Biological Evaluation of Medical Devices—Part 7: Ethylene Oxide Sterilization Residuals
ISO 10993-9 Biological Evaluation of Medical Devices—Part 9: Framework for Identification and Quantification of Potential Degradation Products
ISO 10993-17 Biological Evaluation of Medical Devices—Part 17: Establishment of Allowable Limits for Leachable Substances
~~ISO 14160-1998~~ISO 14160: 1998 Sterilization of Single-Use Medical Devices Incorporating Materials of Animal Origin—Validation and Routine Control of Sterilization by Liquid Chemical Sterilants[5]
ISO 11737-1: 1995 Sterilization of Medical Devices—Microbiological Methods—Part 1: Estimation of Population of Microorganisms on Products[5]
ISO 11737-2: 1998 Sterilization of Medical Devices—Microbiological Methods—Part 2: Tests of Sterility Performed in the Validation of a Sterilization Process[5]
ISO 13408-1: 1998 Aseptic Processing of Health Care Products—Part 1: General Requirements[5]
ISO EN 12442-1 Animal Tissues and Their Derivative Utilized in the Manufacture of Medical Devices—Part 1: Analysis and Management of Risk[5]
ISO EN 12442-3 Animal Tissues and Their Derivative Utilized in the Manufacture of Medical Devices—Part 3: Validation of the Elimination and/or inactivation of Virus and Transmissible Agents[5]
~~International Conference on Harmonization (ICH) S2B Genotoxicity~~ICH S2B A Standard Battery for Genotoxicity Testing of Pharmaceuticals (July 1997)[6]
~~International Conference on Harmonization (ICH) Q1A ICH~~ICH Q1A Harmonized Tripartite Guidance for Stability Testing of New Drug Substances and Products (September 2001, Revision 1)[6]
FDA Guideline~~Guideline on~~ on Validation of the Limulus Amebocyte Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Healthcare Products, DHHS, December 1987[7]
~~FDAInterim~~FDA Interim Guidance for Human and Veterinary Drug Products and Biologicals, Kinetic LAL Techniques, DHHS, July 15, 1991[7]
AAMI TIR No. 7: 1999 Chemical Sterilants and High Level Disinfectants: A Guide to Selection and Use[8]
~~AAMI ST67/CDV-2: 1999~~AAMI ST67/CDV–2: 1999 Sterilization of Medical Devices—Requirements for Products Labeled "Sterile"[8]
21 CFR 312 FDA Title 21, Food and Drugs, Investigational New Drug Applications[9]

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[3] Available from U.S. Pharmacopeia (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852.
[4] Available from European Directorate for the Quality of Medicines (EDQM), Council of Europe, BP 907, 67029 Strasbourg, France.
[5] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036.
[6] Available from International Conference on Harmonization (ICH) Secretariat, c/o IFPMA, 30 rue de St-Jean, P.O. Box 758, 1211 Geneva 13, Switzerland.
[7] Available from U.S. Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857-0001.
[8] Available from Association for the Advancement of Medical Instrumentation, 1110 North Glebe Rd., Suite 220, Arlington, VA 22201-4795.
[9] Available from Standardization Documents Order Desk, DODSSP, Bldg. 4, Section D, 700 Robbins Ave., Philadelphia, PA 19111-5098

3. Terminology

3.1 *Definitions:*

~~3.1.1~~

3.1.1 *decomposition*, *n*—structural changes of hyaluronan due to exposure to environmental, chemical, or thermal factors. Decomposition may occur at temperatures as low as 121°C during autoclaving. Decomposition can result in deleterious changes to the hyaluronan.

3.1.2 *degradation*, *n*—change in the chemical structure, physical properties or appearance of a material. Degradation of polysaccharides occurs via cleavage of the glycosidic bonds, usually by acid catalyzed hydrolysis. Degradation can also occur thermally and by alkali. It is important to note that degradation is not synonymous with decomposition. Degradation is often used as a synonym for depolymerization when referring to polymers. Degradation (depolymerization) of hyaluronan may also occur enzymatically by the action of hyaluronidases.

3.1.3 *depolymerization*, *n*—reduction in length of a polymer chain to form shorter polymeric units. Depolymerization may reduce the polymer chain to smaller molecular weight polymers, oligomeric, or monomeric units, or combination thereof. In hyaluronan, acid hydrolysis of the glycosidic bonds is the primary mechanism.

3.1.4 *endotoxin*, *n*—pyrogenic high molar mass lipopolysaccharide (LPS) complex associated with the cell wall of gram-negative bacteria.

3.1.4.1 *Discussion*—Though endotoxins are pyrogens, not all pyrogens are endotoxins. Endotoxins are specifically detected through a Limulus Amebocyte Lysate (LAL) test.

3.1.5 *hyaluronan*, *n*—a polysaccharide with a disaccharide repeating unit composed of D-glucuronic acid and *N*-acetyl-D-glucosamine in β-(1→3) linkage. Each disaccharide unit is attached to the next by β-(1→4) bonds. Hyaluronan is a linear polymer. Other common names are hyaluronic acid and sodium hyaluronate.

~~3.1.2~~

3.1.6 *hydrocolloid*, *n*—a water-soluble polymer of colloidal nature when hydrated.

~~3.1.3~~

3.1.7 *molecular mass average (molecular weight average)*, *n*—the given molecular weight (*Mw*) of hyaluronan will always represent an average of all of the molecules in the population. The most common ways to express the *Mw* are as the number average ($\overline{M}_n$) and the weight average ($\overline{M}_w$). The two averages are defined by the following equations:

$$\overline{M}_n = \frac{\sum_i N_i M_i}{\sum_i N_i} \quad \text{and} \quad \overline{M}_w = \frac{\sum_i w_i M_i}{\sum_i w_i} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i}$$

where:

N_i = number of molecules having a specific molecular weight M_i, and

w_i = weight of molecules having a specific molecular weight M_i.

In a polydisperse molecular population the relation $\overline{M}_w > \overline{M}_n$ is always valid. The coefficient $\overline{M}_w / \overline{M}_n$ is referred to as the polydispersity index, and will typically be in the range 1.2 to 3.0 for commercial hyaluronan.

~~3.1.4*depolymerization*, *n*—reduction in length of a polymer chain to form shorter polymeric units. Depolymerization may reduce the polymer chain to smaller molecular weight polymers, oligomeric, or monomeric units, or combination thereof. In hyaluronan, acid hydrolysis of the glycosidic bonds is the primary mechanism.~~

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~~3.1.6*decomposition*, *n*—structural changes of hyaluronan due to exposure to environmental, chemical, or thermal factors. Decomposition may occur at temperatures as low as 121°C during autoclaving. Decomposition can result in deleterious changes to the hyaluronan.~~

~~3.1.7*pyrogen*, *n*—any substance that produces fever when administered parenterally.~~

3.1.8 ~~*endotoxin*~~non-animal derived, *n*~~—a high molecular weight lipopolysaccharide (LPS) complex associated with the cell wall of gram-negative bacteria that is pyrogenic in humans. Though endotoxins are pyrogens, not all pyrogens are endotoxins.~~ —a term describing the absence of any animal-derived tissue, proteins, or products in the manufacturing process.

3.1.9 ~~*non-animal derived*~~pyrogen, *n*~~—a term describing the absence of any animal-derived tissue, proteins, or products in the manufacturing process.~~ —any substance that produces fever when administered parenterally.

4. Significance and Use

4.1 This guide contains a listing of those characterization parameters that are directly related to the functionality of hyaluronan. This guide can be used as an aid in the selection and characterization of the appropriate hyaluronan for a particular application. This guide is intended to give guidance in the methods and types of testing necessary to properly characterize, assess, and ensure consistency in the performance of a particular hyaluronan. It may have use in the regulation of these devices by appropriate authorities.

4.2 The hyaluronan covered by this guide may be gelled, cross-linked, extruded, or otherwise formulated into biomedical devices for use in tissue engineered medical products or drug delivery devices for implantation as determined to be appropriate, based on supporting biocompatibility and physical test data. Recommendations in this guide should not be interpreted as a guarantee of clinical success in any tissue engineered medical product or drug delivery application.

4.3 To ensure that the material supplied satisfies requirements for use in TEMPs, several general areas of characterization should be considered. These are: identity of hyaluronan, physical and chemical characterization and testing, impurities profile, and performance-related tests.

5. Chemical and Physical Test Methods

5.1 *Identity of Hyaluronan*—The identity of hyaluronan can be established by several methods including, but not limited to the following:

5.1.1 *Sodium Hyaluronate Monograph EP Monograph 1472.*

5.1.2 *Fourier Transform Infrared Spectroscopy (FT-IR)*—Almost all organic chemical compounds absorb infrared radiation at frequencies characteristic for the functional groups in the compound. A FT-IR spectrum will show absorption bands relating to bond stretching and bending and can therefore serve as a unique fingerprint of a specific compound. Direct FT-IR analysis of hyaluronan powder is perhaps the easiest technique to perform. One method utilizes a horizontal attenuated total reflectance (HATR) accessory with a zinc-selenium (ZnSe) crystal (or equivalent) having a sample trough and a pressure plate. Record background and sample spectra between 4000 and 600 cm^{-1} at an appropriate resolution. Label the peaks. Typical frequencies (cm^{-1}) for hyaluronan (sodium salt) are 3275-3390 (b), 1615 (s), 1405 (m), 1377 (m), 1150, 1077, 1045 (s), 946 (m), 893 (w). The peak designators are: sh: sharp; s: strong; m: medium; w: weak; b: broad. A typical FT-IR HATR spectrum is shown in Fig. 1. A reference spectrum can be obtained ~~form~~from the European Pharmacopoeia.[10]

5.2 *Physical and Chemical Characterization of Hyaluronan*:

5.2.1 The composition and sequential structure of hyaluronan can be determined by the following method: High-resolution ^1H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR). Hyaluronan should be dissolved in D_2O. If the resulting solution is viscous, viscosity may be reduced by chemical or enzymatic depolymerization. A typical ^1H-NMR spectrum of hyaluronan is shown below. Hyaluronan is characterized by calculating parameters such as glucuronic acid: N-acetylglucosamine ratio. Some literature references to the determination of composition and structure of hyaluronan are given in the References section **(1-4)**.[11]

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[10] EDQM, European Pharmacopoeia, Council of Europe, B.P. 907, F-67029 Strasbourg France; www.pheur.org

[11] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.42 on Biomaterials and Biomolecules for TEMPs.

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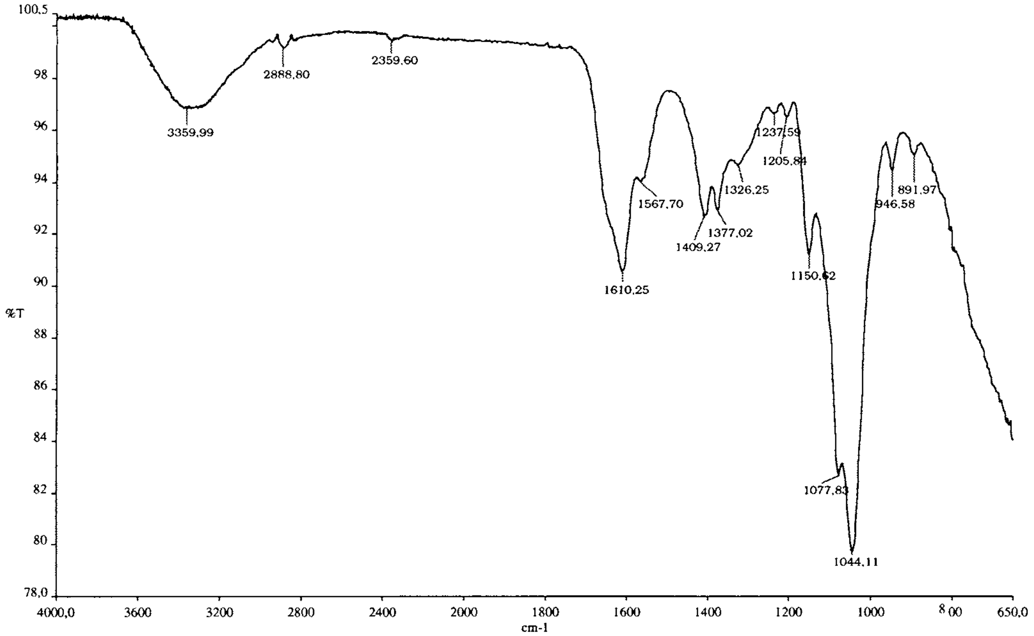

FIG. 1 FT-IR Spectrum of Hyaluronan, Sodium Salt Using Horizontal Attenuated Total Reflectance (HATR)

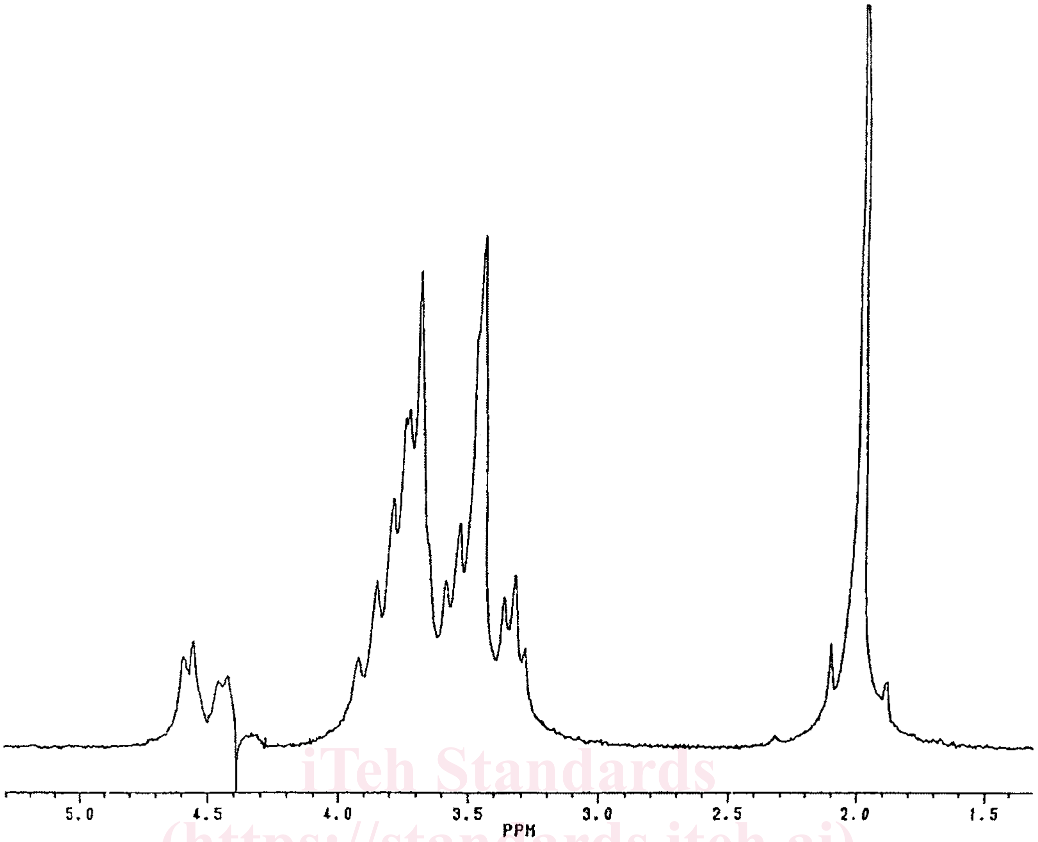

FIG. 2 ^{1}H NMR Spectrum of Hyaluronan from Rooster Comb (Mw ~700 000)

5.2.2 Molecular mass (molecular weight) of hyaluronan will define certain performance characteristics such as viscosity or gel strength, or both. As such and depending on the sensitivity of a particular end use to these variations, determination of molecular mass directly or indirectly may be necessary. Commercial hyaluronan is polydisperse with respect to molecular weight (M_w). M_w may be expressed as the number average (M_N) or the weight average (M_W). Molecular weights may be determined by methods such as, but not limited to the following:

5.2.2.1 *Molecular Weight Determination Based on Intrinsic Viscosity*—The intrinsic viscosity describes a polymer's ability to form viscous solutions in water and is directly proportional to the average molecular weight of the polymer. The intrinsic viscosity is a characteristic of the polymer under specified solvent and temperature conditions; it is independent of concentration. The intrinsic viscosity (η) is directly related to the molecular weight of a polymer through the Mark-Houwink-Sakurada (MHS) equation: $[\eta] = KM^a$. For hyaluronan, K is 0.00057 and the exponent (a) is 0.75 at the following conditions: 0.15 M NaCl in phosphate buffer, pH 7.5, 20°C **(5)**. By measuring the intrinsic viscosity, the viscosity average molecular weight can be determined if K and a are accurately known for the sample: $\log [\eta] = \log K + a (\log M)$, where M is the molecular weight. The intrinsic viscosity is determined by measuring the relative viscosity in an Ubbelohde capillary viscometer. The measurements should be performed in a solvent containing 0.15 M NaCl at a constant temperature of 20°C, and at a sufficiently low hyaluronan concentration. Automatic operation and data acquisition are preferred.

5.2.2.2 *Molecular Weight Determination Based on Differential Pressure*—Alternatively, a Viscotek Relative Viscometer can be used, which is based on Poiseuille's law of capillary flow: the pressure drop of a fluid flowing through a capillary is directly proportional to the viscosity.

$$\Delta P = \eta Q R$$

where:
ΔP = the pressure drop across the capillary measured by the differential pressure transducer (DPT),
η = the viscosity,
Q = the flow rate, and
R = the resistance of the capillary.

Two capillaries are connected in series with the sample injection valve located between capillary one (1) and capillary two (2). The sample is injected in capillary two (2) and the pressure change is detected by the DPT. The relative viscosity is determined by the ratio of the pressures divided by the instrument constant K.

$$\eta_r = P_2/P_1K$$

The instrument constant K is the ratio of the resistances of capillary one (1) and two (2) at the base line where both capillaries contain pure solvent. Specific viscosity, inherent viscosity, reduced viscosity, and intrinsic viscosity values can be calculated from relative viscosity as follows:

$$\eta_{sp} = \eta_r - 1$$

where:
η_{sp} = specific viscosity.

$$\eta_{red} = \eta_{sp}/C$$

where:
η_{red} = reduced viscosity and C is the concentration.

$$\eta_{int} = lim\ (\eta_{sp}/C) \text{ as } C \rightarrow 0$$

where:
η_{int} = the intrinsic viscosity.

From the intrinsic viscosity values, molecular weight can be calculated using the Mark-Houwink-Sakurada equation.

5.2.2.3 *Molecular Weight and Polydispersity Determination by Size Exclusion Chromatography with Multiple Angle Laser Light Scattering Detection (SEC-MALLS)*—The method of choice is to use refractive index coupled to multiple angle laser light scattering detection (MALLS). For separation of the hyaluronan into different molecular weight fractions, a hydrophilic column with the appropriate pore size is required. Such columns include, but are not limited to those mentioned in the techniques below and in Refs **(6, 7)**. The precision of these techniques must be determined as results can vary by 5 to 20 %. Typical methods using these techniques include, but are not limited to:

(1) Using 0.2 *M* NaCl as the mobile phase with separation using TSK 3000 and TSK 6000 columns.

(2) Using 150 *mM* NaCl, 50 *mM* phosphate buffer as the mobile phase with separation using a Biogel column.

5.2.2.4 *Polydispersity*—Depending on the end use and the sensitivity of the application to the molecular mass, the presence of a wide range of hyaluronan fractions may be an issue. In such cases, calculation of the polydispersity will be important. Typically this is between 1.2 and 3.0 for commercial hyaluronan.

5.2.3 Depending on the final use and the required performance control, other characterization assays can include, but are not limited to the following:

5.2.3.1 *Viscosity in Aqueous Solution*—Viscosity is defined as a liquid's resistance to flow. The molecular mass of hyaluronan will determine the extent to which it will thicken an aqueous solution. Therefore, a simple viscosity test may yield information on the relative differences in molecular mass among hyaluronan samples. To allow comparison between laboratories, the viscometer used must be calibrated with traceable standards (see Test Methods D2196). The viscosity measured will depend on several parameters related to how the testing is conducted. Both rotational viscometers and "cone on plate" rheometers may be used. Important parameters to control include, but are not limited to:

(1) Temperature—The temperature at which the measurement is performed is critical. An increase in temperature will, in almost every case, result in a decrease in the viscosity. Consistent and controlled temperature (that is, with a standard temperature bath) is critical to achieving reproducible results. Typically, the temperature used to measure viscosity can be 20°C, 25°C, or 37°C, or combination thereof.

(2) Hyaluronan Concentration—The moisture content of the hyaluronan must be known in order to prepare correct concentrations of hyaluronan (see 5.2.3.2).

(3) Ionic Strength—The viscosity of a hyaluronan solution is sensitive to the ionic environment in which the measurement is made. The most important aspect is to keep the ionic strength consistent. Typically viscosity measurements should be made in a standardized ionic environment of known ionic strength.

(4) Molecular Mass—Viscosity measurements are sensitive to the molecular mass of hyaluronan. The following is one suggestion concerning the measurement of hyaluronan viscosity, but any appropriate method would apply. To measure the apparent viscosity of hyaluronan, prepare a solution in deionized water with a concentration (*w/w*, corrected for dry matter content) appropriate for the end use. The viscosity is measured using a rotational viscometer (for example, Brookfield type) at 20 ± 0.2°C (or other controlled temperature) using the appropriate spindle, spindle rotation speed and a temperature-controlled water bath.

(5) Shear Rate—Hyaluronan is sensitive to shear and the viscosity may vary as a function of the shear rate.

5.2.3.2 *Dry Matter Content*—Hyaluronan from various suppliers may contain different moisture contents. The dry matter content determination is based upon the removal of water and other volatile substances (such as alcohol) from the sample. Normally with hyaluronan, gravimetric techniques are used. They are adapted directly from USP <731> ~~USP 24/NF19, Loss on Drying,~~ and utilize a calibrated drying oven at 105°C, or EP 2.2.32 by drying at 100 to 110°C over diphosphorus pentoxide for 6 h.

NOTE 1—Dried hyaluronan can reabsorb up to 1 % moisture within 5 min.

5.2.3.3 *pH*—Hyaluronan is generally less stable at acidic pH's. The pH of a 0.5 % solution of hyaluronan should be approximately neutral.