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

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INTRODUCTION

Biopolymers from marine sources have been studied and used in commercial applications and product development for a number of years. Chitosan, a linear polysaccharide consisting of glucosamine and *N*-acetyl glucosamine derived mainly from crustacean shells, has been used in many technical applications such as water purification (as a flocculant), in cosmetics, and recently as a proposed fat-binding weight control product. In solution, the cationic nature of chitosan gives this polymer a mucoadhesive property. Chitosan salts can be used as a matrix or scaffold material as well as in non-parenteral delivery systems for challenging drugs. Chitosan salts have been shown to increase the transport of polar drugs across the nasal epithelial surface. The purpose of this guide is to identify key parameters relevant for the functionality and characterization of chitosan salts for the development of new commercial applications of chitosan salts for the biomedical and pharmaceutical industries.

1. Scope

1.1 This guide covers the evaluation of chitosan salts 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 for the functionality, characterization, and purity of chitosan salts.

1.3 As with any material, some characteristics of chitosan may be altered by processing techniques (such as molding, extrusion, machining, assembly, sterilization, and so forth) required for the production of a specific part or device. Therefore, properties of fabricated forms of this polymer should be evaluated using test methods that are appropriate to ensure safety and efficacy.

1.4 **Warning—**Mercury has been designated by EPA and many state agencies as a hazardous material that can cause central nervous system, kidney, and liver damage. Mercury, or its vapor, may be hazardous to health and corrosive to materials. Caution should be taken when handling mercury and mercury-containing products. See the applicable product Material Safety Data Sheet (MSDS) for details and EPA's website (http://www.epa.gov/mercury/faq.htm) for additional information. Users should be aware that selling mercury or mercury-containing products, or both, in your state may be prohibited by state law.

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

1.6 *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:*[2]

D2196 Test Methods for Rheological Properties of Non-Newtonian Materials by Rotational (Brookfield type) Viscometer

F619 Practice for Extraction of Medical Plastics

[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.

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
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 (Withdrawn 2012)[3]
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 (Withdrawn 2011)[3]
F1906 Practice for Evaluation of Immune Responses In Biocompatibility Testing Using ELISA Tests, Lymphocyte Proliferation, and Cell Migration (Withdrawn 2011)[3]

2.2 *Ph. Eur. Document:*
Ph. Eur. Monograph Chitosan Chloride, Nov. 2000[4]

2.3 *ISO Documents:*
ISO 10993 Biological Evaluation of Medical Devices[5]
ISO 10993-1 Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing[5]
ISO 10993-3—Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity[5]
ISO 10993-9—Part 9: Framework for Identification and Quantification of Potential Degradation Products[5]
ISO 10993-17—Part 17: Methods for Establishment of Allowable Limits for Leachable Substances Using Health-Based Risk Assessment[5]
ISO 13408-1: 1998: Aseptic Processing of Health Care Products—Part 1: General Requirements[5]

2.4 *ICH Documents:*
International Conference on Harmonization (1997) Guidance for Industry M3 Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals 62 FR 62922[6]
International Conference on Harmonization (1996) Guideline for Industry S2A Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals 61 FR 18199[6]
International Conference on Harmonization (1997) Guidance for Industry S2B Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals 62 FR 62472[6]
International Conference on Harmonization (1994) Guideline for Industry S5A Detection of Toxicity to Reproduction for Medicinal Products 59 FR 48746[6]
International Conference on Harmonization (1996) Guidance for Industry S5B Detection of Toxicity to Reproduction for Medicinal Products: Addendum on Toxicity to Male Fertility 61 FR 15360[6]
International Conference on Harmonization (1996) Guideline for Industry S1A The Need for Long-term Rodent Carcinogenicity Studies of Pharmaceuticals 61 FR 8153[6]
International Conference on Harmonization (1998) Guidance for Industry S1B Testing for Carcinogenicity of Pharmaceuticals 63 FR 8983[6]
International Conference on Harmonization (1995) Guideline for Industry S1C Dose Selection for Carcinogenicity Studies of Pharmaceuticals 60 FR 11278[6]
International Conference on Harmonization (1997) S1C[R] Guidance for Industry Addendum to Dose Selection for Carcinogenicity Studies of Pharmaceuticals: Addition of a Limit Dose and Related Notes 62 FR 64259[6]
International Conference on Harmonization (ICH) Q1A ICH Harmonized Tripartite Guidance for Stability Testing of New Drug Substances and Products (September 23, 1994)[6]

2.5 *FDA Documents:*
FDA Guideline 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]
FDA Interim Guidance for Human and Veterinary Drug Products and Biologicals. Kinetic LAL Techniques DHHS, July 15, 1991[7]

2.6 *ANSI Documents:*
ANSI/AAMI/ISO 11737-1: 1995 Sterilization of Medical Devices—Microbiological Methods—Part 1: Estimation of Bioburden on Product[5]
ANSI/AAMI/ISO 11737-2: 1998 Sterilization of Medical Devices—Microbiological Methods—Part 2: Tests of Sterility Performed in the Validation of a Sterilization Process[5]

2.7 *AAMI Documents:*
AAMI TIR No. 19—1998: Guidance for ANSI/AAMI/ISO 10993–7: 1995, Biological Evaluation of Medical Devices—Part 7: Ethylene Oxide Sterilization Residuals[8]
AAMI/ISO 14160—1998: Sterilization of Single-Use Medical Devices Incorporating Materials of Animal Origin—

[3] The last approved version of this historical standard is referenced on www.astm.org.
[4] Available from EDQM, Publications and Services European Pharmacopoeia, BP 907 226, avenue de Colmar, F-67029 Strasbourg Cedex 1, France.
[5] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.
[6] Available from ICH Secretariat, c/o IFPMA, 30 rue de St-Jean, PO Box 758, 1211 Geneva 13, Switzerland.
[7] Available from Food and Drug Administration (FDA), 5600 Fishers Ln., Rockville, MD 20857, http://www.fda.gov.
[8] Association for the Advancement of Medical Instrumentation, 111 N. Glebe Rd., Suite 220, Arlington, VA 22201–4795.

Validation and Routine Control of Sterilization by Liquid Chemical Sterilants[8]

AAMI ST67/CDV-2: 1999: Sterilization of Medical Devices—Requirements for Products Labeled "Sterile"[8]

2.8 *EN Documents:*

EN 12442-1 Animal Tissues and Their Derivative Utilized in the Manufacture of Medical Devices—Part 1: Analysis and Management of Risk[9]

EN 12442-Part 3: Validation of the Elimination and/or Inactivation of Virus and Transmissible Agents[9]

3. Terminology

3.1 *Definitions:*

3.1.1 *chitosan, n*—a linear polysaccharide consisting of β(1→4) linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcN).

3.1.1.1 *Discussion*—Chitosan is a polysaccharide derived by *N*-deacetylation of chitin.

3.1.2 *decomposition, n*—structural changes of chitosans as a result of exposure to environmental, chemical, or thermal factors, such as temperatures greater than 200°C.

3.1.2.1 *Discussion*—Decomposition can result in deleterious changes to the chitosan.

3.1.3 *degradation, n*—change in the chemical structure, physical properties, or appearance of a material.

3.1.3.1 *Discussion*—Degradation of polysaccharides occurs by means of cleavage of the glycosidic bonds, usually by acid—catalyzed hydrolysis. Degradation can also occur thermally. Note that degradation is not synonymous with decomposition. Degradation is often used as a synonym for depolymerization when referring to polymers.

3.1.4 *degree of deacetylation, n*—the fraction or percentage of glucosamine units (deacetylated monomers) in a chitosan polymer molecule.

3.1.5 *depolymerization, n*—reduction in length of a polymer chain to form shorter polymeric units.

3.1.5.1 *Discussion*—Depolymerization may reduce the polymer chain to oligomeric or monomeric units, or both. In chitosan, hydrolysis of the glycosidic bonds is the primary mechanism.

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

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

3.1.7 *molecular mass average (molecular weight average), n*—the given molecular weight (Mw) of a chitosan 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 ($\bar{M}_n$) and the weight average ($\bar{M}_w$). The two averages are defined by the following equations:

$$\bar{M}_n = \frac{\sum_i N_i M_i}{\sum_i N_i}$$

and

$$\bar{M}_w = \frac{\sum_i W_i M_i}{\sum_i W_i} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i}$$

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 $\bar{M}_w > \bar{M}_n$ is always valid. The coefficient $\bar{M}_w / \bar{M}_n$ is referred to as the polydispersity index, and will typically be in the range 1.5 to 3.0 for commercial chitosans.

3.1.8 *pyrogen, n*—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 chitosan. This guide can be used as an aid in the selection and characterization of the appropriate chitosan or chitosan salt for a particular application. This standard 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 chitosan. It may have use in the regulation of devices containing chitosan by appropriate authorities.

4.2 The chitosan salts covered by this guide may be gelled, extruded, or otherwise formulated into biomedical devices for use as 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 include identity of chitosan, physical and chemical characterization and testing, impurities profile, and performance-related tests.

5. Chemical and Physical Test Methods

5.1 *Identity of Chitosan*—The identity of chitosan and chitosan salts can be established by several methods including, but not limited to the following:

5.1.1 Chitosan chloride monograph Ph. Eur.

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. Cast a chitosan film from a 0.25 % (w/v) solution of chitosan (in 1 % acetic

[9] Available from European Committee for Standardization, CEN Management Centre, 36 rue de Stassart, B-1050 Brussels, Belgium.

acid) or chitosan salt (dissolved in water) by drying approximately 500 µL of the sample onto a disposable IR card[10] for 3 to 4 h at 60°C. Record a background spectrum between 4000 and 400 cm-1 using 128 scans at a resolution of 4 cm^{-1}. Record the IR spectrum of a dried blank IR card, then record the IR spectrum of the sample using 128 scans at a resolution of 4 cm^{-1}, percent transmission mode. Label the peaks. Typical frequencies (cm^{-1}) for chitosan are as follows:

Chitosan Base (as Acetate)	Chitosan Chloride	Chitosan Glutamate
3362b	3344b	1555b
1556	1605	1396
1406	1513	1154
1153	1379	1085s
1083s	1154	
	1086s	

The peak designators are: sh: sharp; s: strong; m: medium; w: weak; and b: broad.

5.2 *Physical and Chemical Characterization of Chitosan:*

5.2.1 The composition and sequential structure of chitosan can be a key functional attribute of any chitosan or chitosan salt. Variations in the composition or the sequential structure, or both, may, but not necessarily will, cause differences in performance of a chitosan in a particular end use. This information may be determined by the following method: High-resolution ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR).

5.2.2 The degree of deacetylation of chitosan can be established using a number of techniques including, but not limited to, the following:

5.2.2.1 *High-resolution ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectroscopy (NMR)*—Chitosan salts should be dissolved in D_2O and partially degraded to a degree of depolymerization of 20 to 30 using sodium nitrite before recording proton or carbon NMR spectra.[11]

5.2.2.2 *Determination of the Degree of Deacetylation by UV Spectroscopy*—This method is based upon that reported by Muzzarelli et al.[12] The method is actually a quantitative measure of the number of amine functional groups in the polymer. The method uses a standard curve produced from varying concentrations of *N*-acetyl glucosamine. The degree of deacetylation is calculated from recordings of the first derivative of the UV spectra of *N*-acetyl glucosamine and of chitosan samples at 202 nm.

5.2.3 Molecular mass (molecular weight) of a chitosan will define certain performance characteristics such as viscosity. 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 chitosans are polydisperse with respect to molecular weight (M_W). Molecular weight 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:

5.2.3.1 *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:

$$[\eta] = KM^{a}$$

where:

K = a constant,
M = viscosity derived average molecular weight, and
a = an empirical constant describing the conformation of the polymer.

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 a Ubbelohde capillary viscometer. The measurements should be performed in a solvent containing 0.1*M* NaCl (a non-gelling, monovalent salt) at a constant temperature of 20°C, and at a sufficiently low chitosan concentration. Automatic operation and data acquisition are preferred.

5.2.3.2 *Molecular Weight and Polydispersity Determination by Size Exclusion Chromatography with Multiple Angle Laser Light Scattering Detection (SEC-MALLS)*—As there are no chitosan standards currently available, refractive index detectors cannot be adequately calibrated. It is not sufficient to only use pullulan standards as a calibration material. Therefore, the method of choice is to use refractive index coupled to MALLS. For separation of the chitosan 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 following techniques. The precision of these techniques must be determined as results can vary by 10 to 20 %. Typical methods using these techniques include, but are not limited to: using 0.01*M* sodium acetate/acetic acid buffer, pH 5.5 as the mobile phase with separation using TSK 3000, TSK 4000, and TSK 5000 columns.

5.2.3.3 *Polydispersity*—Depending on the end use and the sensitivity of the application to the molecular mass, the presence of a wide range of chitosan fractions may be an issue. In such cases, calculation of the polydispersity will be important. Typically, this is between 1.5 and 3.0 for commercial chitosans.

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

5.2.4.1 *Viscosity in Aqueous Solution*—Viscosity is a liquid's resistance to flow. The molecular mass of a chitosan 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 chitosan samples. To allow comparison between laboratories, the viscometer used must be calibrated with traceable standards

[10] No suitable commercially available IR cards are available for the IR analysis of chitosan glutamate salt. Alternative methods are under investigation.

[11] Vårum, K. M., Anthonsen, M. W., Grasdalen, H., and Smidsrod, O., *Carbohydrate Research*, Vol 211, 1991, pp. 17–23.

[12] Muzzarelli, R. A. A., Rochetti, R., Stanic, V., and Weckx, M., *Chitin Handbook*, R. A. A. Muzzarelli and M. T. Peters, Ed., Atec Grottammare, 1997.