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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.

(see Test Methods D2196). The viscosity measured will depend on several parameters related to how the testing is conducted. Important parameters to control include, but are not limited to, the following:

(*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°, 25°, or 37°C, or a combination thereof.

(*2*) *Chitosan Concentration*—The moisture content of the chitosan must be known to prepare correct concentrations of chitosan or chitosan salts.

(*3*) *Ionic Strength*—The viscosity of a chitosan solution is very sensitive to the ionic environment in which the measurement is made. The most important aspect is to keep the ionic content consistent. Typically, viscosity measurements are made either in deionized water or a standardized ionic environment such as isotonic saline.

(*4*) *Molecular Mass*—Viscosity measurements are sensitive to the molecular mass of the chitosan. The following is one suggestion concerning the measurement of chitosan viscosity, but any appropriate method would apply. To measure the apparent viscosity of chitosan or chitosan salts, prepare a solution in deionized water (for chitosan salts) or 1 % acetic acid (for chitosan) with a concentration (w/w, corrected for dry matter content) appropriate for the end use. For example, if the sample has a suspected molecular weight above approximately 50 000 g/mol, prepare a 1 % (w/w) solution; if the suspected molecular weight is less than about 50 000 g/mol, then prepare a 10 % (w/w) solution. 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.

5.2.4.2 *Dry Matter Content*—Various chitosan and chitosan salts are supplied with different moisture contents. The dry matter content determination is based upon the removal of water from the sample. Normally with chitosan, gravimetric techniques are used. They are adapted directly from <731> USP 24/NF19 and use a calibrated drying oven at 105°C.

5.2.4.3 *Ash Content*—The ash content of a sample describes the total amount of inorganic material present. After combustion, the sample contains a mixture of salts. The composition of the ash depends on the temperature used during the combustion of the organic material. For ash content of chitosan, a combustion temperature of 800°C for at least 6 h is recommended. Chitosan or chitosan salts intended to be used in biomedical applications should have a very low ash content.

5.2.4.4 *Insolubles*—The percentage of insolubles describes the total amount of insoluble impurities (insoluble salts, chitosan, or other contaminants) in a chitosan/chitosan salt sample. The determination of insolubles content is based upon dissolving the chitosan in acetic acid, or chitosan salt in water, and filtering the chitosan solution. Then, the insolubles are calculated form the weight of chitosan dissolved and the weight of insoluble particles obtained on a filter. While no specific limits are suggested, chitosan/chitosan salts used in biomedical and tissue-engineered medical products should have as low an insolubles content as possible.

5.3 *Impurities Profile*—The term impurity relates to the presence of extraneous substances and materials in the chitosan powder. Additionally, and dependent upon the end use, a high-molecular-weight chitosan present in a sample of low molecular weight could constitute an impurity. Various processing aids may also be used in the manufacture of chitosan and could constitute an impurity. If there is a concern for the presence of processing aids or other contaminants associated with chitosan, they should be addressed with the supplier. The major impurities of concern include, but are not limited to, the following:

5.3.1 *Endotoxin Content*—Endotoxin contamination is difficult to prevent because it is ubiquitous in nature, stable, and small enough to pass through sterilizing filters. There are several tests to determine the presence of endotoxin in the chitosan salts. These are the gel clot, end point assay, and the kinetic assay. The gel clot test is the simplest and easiest of the Limulus amebocyte lysate (LAL) test methods, although much less sensitive than the kinetic assay. A firm gel that maintains its integrity upon inverting the tube is scored as a positive test. Anything other than a firm gel is scored as a negative test. The end point assay is based on the linear relationship between the endotoxin concentration and the formation of color (chromogenic assay) over a relatively short range of standard dilutions. A standard curve is then constructed by plotting the optical densities of a series of endotoxin standards as a function of the endotoxin concentration. Using linear regression analysis, the standard curve covers an endotoxin range of approximately 1 log (usually 1.0 to 0.1 EU/mL). The most sensitive means of determining the endotoxin content is with a quantitative, kinetic assay. This test uses a LAL and a synthetic color-producing substrate to detect endotoxin chromogenically (such as, but not limited to, BioWhittaker's Kinetic-QCL (Trademarked) methodology, or other equivalent assay). The kinetic assay measures the amount of time required to reach a predetermined optical density (kinetic turbidimetric) or color intensity (kinetic chromogenic), sometimes called the onset optical density or reaction optical density. The Food and Drug Administration (FDA) currently defines linearity as a correlation coefficient of ≥ 0.980. See FDA Guideline DHHS, December 1987. It is important that operators of the LAL method are qualified and that each new lot of reagents is validated. Positive product controls (PPCs) must be added to test inhibition in the sample. Recovery of the known added amount of endotoxin standard must be obtained for a valid assay. It is recommended that endotoxin measurements be performed using an initial 0.1 % concentration of chitosan and 3 dilution ranges (for example, 20, 50, and 100x). The endotoxin level in chitosan will ultimately be critical to its use in biomedical applications where there are regulatory limits to the amount of endotoxin that can be implanted into humans. Relevant FDA guidance for allowable levels and information regarding validation of endotoxin assays should be consulted if human trials are contemplated. See FDA Guideline DHHS, July 15, 1991.

5.3.2 *Protein Content*—Protein content in chitosan or chitosan salts should be assayed using an appropriate method having sufficient sensitivity to detect low levels of contamination. One method, although not the only suitable one, is the Coomassie brilliant blue G dye binding assay as described by Read and Northcote.[13] This method is able to quantitate protein content as low as 3 µg/mL. The protein content should be assayed using a 1 % (w/w) chitosan solution corrected for dry matter content. It is important to confirm that the method chosen is insensitive to materials present in the sample and to validate it against a reference method on a one-time basis. It is the responsibility of the end user to evaluate the chitosan product for the presence of specific proteins that could cause undesirable tissue reactions.

5.3.3 *Heavy Metal Content by the USP Method*—This test is provided to demonstrate that the content of heavy metal impurities does not exceed a limit in the individual product specification in terms of parts per million lead in the test substance. Under the specified test conditions, the limit is determined by a concomitant visual comparison of metals that are colored by sulfide ion with a control prepared from a standard lead solution. Substances that typically respond to this test are lead, mercury, bismuth, arsenic, antimony, tin, cadmium, silver, copper, and molybdenum. This method is based on (231) heavy metals, USP24/NF19. The presence of specific heavy metals may be detected by methods such as atomic absorption spectroscopy using flame or graphite furnace techniques; or by inductively coupled plasma techniques.

5.3.4 *Microbiological Safety*—Bacteria, yeast, and mold are also impurities that can arise in a chitosan sample. The presence of bacteria may also contribute to the presence of endotoxins. The following Microbiological Tests in USP 24 are of particular relevance: Microbial Limit Tests <61>, Sterility Tests <71>, Sterilization and sterility assurance of compendial articles <12211> and the Biological Tests and Assays: Bacterial Endotoxins Tests <85>. The user should also consider other relevant standards, such as, but not limited to, Association for the Advancement of Medical Instrumentation (AAMI) standards and international standards, of which the following are examples: ANSI/AAMI/ISO 11737-1: 1995, ANSI/AAMI/ISO 11737-2: 1998, and ISO 13408-1: 1998. Membrane filtration can be used for the determination of bacteria, yeast and mold in chitosan samples. The chitosan salt is first dissolved in sterile, deionized water, then filtered using sterile techniques through a 0.45-µm membrane filter. The filters are subsequently incubated on tryptic soya agar to determine the presence of bacteria, and on sabouraud dextrose agar to determine the presence of yeast and mold. If chitosan products are intended to serve as a barrier to microorganisms, this function will need to be validated with specific experiments.

6. Product Development Considerations

6.1 *Type of Solvent (that is, acid, medium, or water)*—The conformation of the chitosan molecule will vary with changes in the pH and ionic strength of the solute. Therefore, the apparent viscosity of a chitosan solution may change, depending upon whether the chitosan is dissolved in water, acid, or a salt-containing medium.

6.2 *Stability of Chitosan*—For chitosan, the most relevant stability-indicating parameters are those related to the functionality of the polymer. Dependent upon what function the chitosan will have in the final formulation, parameters such as viscosity (apparent and intrinsic) and molecular weight should be evaluated during a stability study. Storage conditions are of importance, especially for chitosan solutions. The following ICH guidance documents should be consulted for information on stability testing of pharmaceuticals: 62 FR 62922, 61 FR 18199, 62 FR 62472, 59 FR 48746, 61 FR 15360, 61 FR 8153, 63 FR 8983, 60 FR 11278, 62 FR 64259, and Q1A.

6.3 *Methods of Sterilization*—Chitosan powder can be sterilized by gamma irradiation or *E*-beam (with subsequent degradation of the chitosan polymer chain resulting in a reduction in molecular weight) or by ethylene oxide. Solutions of chitosan may be (*1*) filter sterilized if the viscosity of the chitosan solution permits; (*2*) gamma-irradiated with a resulting loss in viscosity (molecular weight); or (*3*) autoclaved (which also reduces the viscosity of the solution). Selection of the method of sterilization will depend upon the viscosity or molecular weight needs of the final application. Use of ethylene oxide will also require testing for residuals. The reader should refer to the relevant standards regarding the sterilization of healthcare products by radiation, steam, and ethylene oxide gas, such as AAMI TIR No. 19—1998, AAMI/ISO 14160—1998, and AAMI ST67/CDV-2: 1999.

7. Safety and Toxicology Aspects of Chitosan

7.1 Chitosan has been included in the Codex Alimentarius Inventory of Processing Aids (ALINORM 91/12, para 104), effective by the 22nd Session of the Codex Committee on Food Additives and Contaminants, Hague, March 20, 1990. This listing, however, does not indicate approval for the use of chitosan in pharmaceutical or biomedical applications, or both.

7.2 The safety of chitosan in biomedical and pharmaceutical applications and in TEMPs should be established according to current guidelines such as ISO 10993 and Practice F748. Suppliers of chitosan or chitosan salts may have such documentation on file. Preclinical safety studies specific to the clinical application under consideration shall also be done in accordance with 21CFR312.

7.2.1 A database generated to support the safety of chitosan-containing pharmaceuticals should reflect consideration of the proposed clinical route of administration and product formulation, although it may be appropriate for certain studies to involve a route of administration or formulation which differs from the clinical situation. Guidance on the need for timing, and conduct of the nonclinical toxicology studies is available in the ICH (International Conference on Harmonization) guidelines on the respective topics. Such studies may include, but are not limited to: acute toxicology testing, repeated dose toxicology testing with a treatment regimen and duration that is relevant to the proposed clinical use (ICH guidance M3), hypersensitivity testing, and genetic toxicology testing (ICH guidances S2A and S2B). Additional studies that

[13] Read and Northcote, *Analytical Biochemistry*, Vol 116, 1981, pp. 53–64.

may be relevant to a proposed pharmaceutical use include reproductive/developmental toxicology testing (ICH guidances S5A and S5B) and carcinogenicity testing (ICH guidances S1A, S1B, S1C, and S1C[R]). Additional testing may be specific to the route of administration, for example, application or injection site irritation, ocular irritation, dermal carcinogenicity testing, or studies of photoirritation and photo co-carcinogenicity potential. Other testing may be appropriate, depending on the results of early studies and the intended clinical use of the product. Specific guidance on the development or marketing of drug products, biologics, or biomedical devices in the United States may be obtained by contacting the Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, or the Center for Devices and Radiological Health, respectively, of the U.S. Food and Drug Administration.

7.3 *Biocompatibility*

7.3.1 Biomaterials are materials of natural or man-made origin that are used to direct, supplement, or replace the functions of living tissues. These materials may be considered biocompatible if the materials perform with an appropriate host response in a specific application.[14]

[14] Williams, D. F., *The Williams Dictionary of Biomaterials* , Liverpool University Press, 1999.

7.3.2 Many materials have been shown to produce a well-characterized level of biological response following long-term clinical use in laboratory animals. When new applications of a material, or modifications to the material or physical forms of the material are being considered, then the recommendations and test methods of the following standards should be considered: Practices F748, F619, F749, F756, F763, F813, F981, F1903, F1904, F1905, and F1906; Guide F1439; Test Method F895; Terminology F1251; and ISO 10993-1, ISO/DIS 10993-9—Part 9, ISO/DIS 10993-17—Part 17, EN 12442-1—Part 1, EN 12442-3—Part 3.

7.4 Chitosan or chitosan salts for use in biomedical and pharmaceutical applications and in TEMPs should ideally be documented in a device or drug master file to which end users may obtain a letter of cross reference from suppliers of chitosan or chitosan salts. Such a master file should be submitted to the U.S. FDA and to other regulatory authorities, both national and international.

8. Keywords

8.1 biomedical; chitosan salts; tissue-engineered medical product applications (TEMPs)

APPENDIXES

(Nonmandatory Information)

X1. RATIONALE

X1.1 The use of naturally occurring biopolymers for biomedical and pharmaceutical applications and in TEMPS is increasing. This guide is designed to give guidance in the characterization and testing parameters for chitosan and chitosan salts used in such applications. Knowledge of the physical and chemical properties of the chitosan, such as degree of deacetylation, molecular weight (or viscosity), counterion, and so forth, will assist end users in choosing the correct chitosan for their particular application. Knowledge of these parameters will also ensure that users can request and obtain similar material from suppliers on reordering. Molecular characterization of chitosan will also assist end users in documentation of their formulation or device. Finally, characterization of the chitosan will allow the functionality of the chitosan to fit the application or end product. Tests outlined in this guide are sufficient for release of chitosan or chitosan salts to the end user. Other validated tests that would accomplish the same purposes as those set forth in this guide may be substituted. The tests may not be suitable for characterization and functionality of the final product.

X2. BACKGROUND

X2.1 Chitosan is a linear polymer that is composed of glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) units linked in a β(1→4) manner. The glucosamine and *N*-acetyl glucosamine are randomly distributed along the polymer chain. Chitosan as such is not soluble in aqueous solution, but can be solubilized using acids such as acetic acid, hydrochloric acid, and solutions of organic acids. In solution, chitosan salts will carry a positive charge through protonization of the free amino group on glucosamine. Reactivity with negatively charged surfaces is a direct function of the positive charge density of chitosan. The cationic nature of chitosan gives this polymer a mucoadhesive property (see Fig. X2.1).

X2.2 *Raw Materials for Chitosan Production*—All current industrial manufacture of chitosan is based on the extraction of the polymer from crustacean shells. Chitosan has also been processed from the pens of squid. Since chitosan is also synthesized as an exocellular material by some mold and yeast, this polymer can be obtained through fermentation.

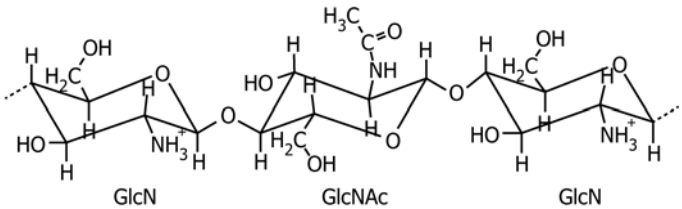

FIG. X2.1 Chitosan

X2.3 *Functional Properties and Applications of Chitosan:*

X2.3.1 The functional properties of chitosan of primary importance for most biomedical applications are the bioadhesive ones. Solubility, swellability, and film-forming properties are other characteristics exploited in biomedical and pharmaceutical applications.

X2.3.2 Gelling properties are a function of the degree of deacetylation.

X2.3.3 Thickening (viscosifying) properties of chitosan are a function of the molecular weight and the conformation of the chitosan molecule in solution. Interactions with other molecules in the solution as well as competition for water at high chitosan concentrations affect the flow properties of chitosan solutions.

X2.3.4 Both gelling and thickening properties of chitosan depend upon the order in which the different materials are added.

X2.3.5 The solubility of chitosan is related to the rate of dissociation of the chitosan molecule.

X2.3.6 Films can be formed from chitosan solutions simply by evaporation of the solvent. The molecular weight of chitosan needs to be above a certain lower limit to achieve film formation and avoid brittleness. Films can be formed easily in situ by spraying a chitosan solution onto a binding surface.