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Standard Guide for Characterization of Type I Collagen as Starting Material for Surgical Implants and Substrates for Tissue Engineered Medical Products (TEMPs)[1]

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INTRODUCTION

Collagen-based medical products are becoming more prevalent, especially in the area of soft tissue augmentation. The use of collagen in surgery dates back to the late 1800s, with the use of catgut sutures, human cadaveric skin, and fascia. More recently, collagen has been used in hemostatic sponges, dermal equivalents, injectables for soft tissue augmentation, as a matrix for cell-based products and as a vehicle for drug delivery. It is because of the versatility of collagen in medical applications that specific characterizations should be performed as a way to compare materials.

1. Scope

1.1 This guide for characterizing collagen-containing biomaterials is intended to provide characteristics, properties, and test methods for use by producers, manufacturers, and researchers to more clearly identify the specific collagen materials used. With greater than 20 types of collagen and the different properties of each, a single document would be cumbersome. This guide will focus on the characterization of Type I collagen, which is the most abundant collagen in mammals, especially in skin and bone. Collagen isolated from these sources may contain other types of collagen, for example, Type III and Type V. This guide does not provide specific parameters for any collagen product or mix of products or the acceptability of those products for the intended use. The collagen may be from any source, including, but not limited to animal or cadaveric sources, human cell culture, or recombinant sources. The biological, immunological, or toxicological properties of the collagen may vary depending on the source material. The properties of the collagen prepared from each of the above sources must be thoroughly investigated, as the changes in the collagen properties as a function of source materials is not thoroughly understood. This guide is intended to focus on purified Type I collagen as a starting material for surgical implants and substrates for tissue engineered medical products (TEMPs); some methods may not be applicable for gelatin nor for tissue implants. This guide may serve as a template for characterization of other types of collagen.

1.2 The biological response to collagen in soft tissue has been well documented by a history of clinical use (**1, 2**)[2] and laboratory studies (**3, 4, 5, 6**). Biocompatibility and appropriateness of use for a specific application(s) is the responsibility of the product manufacturer.

1.3 The following precautionary caveat pertains only to the test method portion, Section 5, of this guide. *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:* [3]

E 1298 Guide for Determination of Purity, Impurities, and Contaminants in Biological Drug Products
F 619 Practice for Extraction of Medical Plastics
F 720 Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test
F 748 Practice for Selecting Generic Biological Test Methods for Materials and Devices
F 749 Practice for Evaluating Material Extracts by Intracutaneous Injection in the Rabbit
F 756 Practice for Assessment of Hemolytic Properties of Materials

[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] The boldface numbers in parentheses refer to the 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.

F 763 Practice for Short-Term Screening of Implant Materials
F 813 Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices
F 895 Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity
F 981 Practice for Assessment of Compatibility of Biomaterials for Surgical Implants with Respect to Effect of Materials on Muscle and Bone
F 1251 Terminology Relating to Polymeric Biomaterials in Medical and Surgical Devices
F 1439 Guide for Performance of Lifetime Bioassay for the Tumorigenic Potential of Implant Materials
F 1903 Practice for Testing For Biological Responses to Particles ***in vitro***
F 1904 Practice for Testing the Biological Responses to Particles ***in vivo***
F 1905 Practice For Selecting Tests for Determining the Propensity of Materials to Cause Immunotoxicity
F 1906 Practice for Evaluation of Immune Responses In Biocompatibility Testing Using ELISA Tests, Lymphocyte Proliferation, and Cell Migration
F 1983 Practice for Assessment of Compatibility of Absorbable/Resorbable Biomaterials for Implant Applications
F 2148 Practice for Evaluation of Delayed Contact Hypersensitivity Using the Murine Local Lymph Node Assay (LLNA)

2.2 *ISO Standards:*[4]
ISO 10993–1 Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing
ISO 10993–3 Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity
ISO 10993–9 Framework for Identification and Quantification of Potential Degradation Products
ISO 10993–10 Biological Evaluation of Medical Devices—Part 10: Tests for Irritation and Delayed-Type Hypersensitivity
ISO 10993–17 Methods for Establishment of Allowable Limits for Leachable Substances Using Health-Based Risk Assessment
ISO 13408–1 Aseptic Processing of Health Care Products—Part 1: General Requirements
ISO 14971 Medical Devices—Application of Risk Management to Medical Devices

2.3 *EN (European Norm) Documents:*[5]
EN 12442–1 Animal Tissues and their Derivatives Utilized in the Manufacture of Medical Devices—Part 1: Analysis and Management of Risk
EN 12442–2 Controls on Sourcing, Collection and Handling
EN 12442–3 Validation of the Elimination and/or Inactivation of Virus and Transmissible Agents

2.4 *U. S. and European Pharmacopeia Documents:*[6]
United States Pharmacopeia (USP), Edition XXX (30)
USP 30/NF 19 Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin
European Pharmacopeia 5.0

2.5 *Code of Federal Regulations:*[7]
21 CFR 312 Investigational New Drug Application
21 CFR Part 820 Quality System Regulation
Federal Register Vol. 43, No. 141, Friday, July 21, 1978
21 CFR Parts 207, 807, and 1271 Human Cells, Tissues and Cellular and Tissue-Based Products, Establishment Registration and Listing
Federal Register, Vol. 66, No. 13, Jan 19, 2001/Rules and Regulations, p. 5447
Federal Register, Vol. 72, No. 8, Jan. 12, 2007, pp. 1581–1619, Proposed Rule: Use of Materials Derived from Cattle in Medical Products Intended for Use in Humans and Drugs Intended for Use in Ruminants
21 CFR Part 1271, Part C Suitability Determination for Donors of Human Cell and Tissue-based Products, Proposed Rule
Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products, Inspection and Enforcement. Proposed Rule. Federal Register/Vol. 66, No. 5/January 8, 2001/Proposed Rules, pp. 1552-1559
Guidance for Screening and Testing of Donors of Human Tissue Intended for Transplantation, Availability. Federal Register/Vol. 62, No. 145/July 29, 1997/NoticesDraft Guidance for Preclinical and Clinical Investigations of Urethral Bulking Agents used in the Treatment of Urinary Incontinence. November 29, 1995. (ODE/DRARD/ULDB), Document No. 850
Guidance for Industry and for FDA Reviewers, Medical Devices Containing Materials Derived from Animal Sources (Except for *In Vitro* Diagnostic Devices), November 6, 1998, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Devices and Radiological Health
CFR 610.13(b) Rabbit Pyrogen Assay

2.6 *ICH Documents:*[8]
ICH M3 Guidance for Industry M3 Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals 62 FR 62922 (1997)
ICH S2A Guideline for Industry S2A Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. 61 FR 18199 (1996)
ICH S2B Guidance for Industry S2B Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals

[4] Available from International Organization for Standardization (ISO), 1 rue de Varembé, Case postale 56, CH-1211, Geneva 20, Switzerland, http://www.iso.ch.

[5] Available from European Committee for Standardization (CEN), 36 rue de Stassart, B-1050, Brussels, Belgium, http://www.cenorm.be.

[6] Available from U.S. Pharmacopeia (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

[7] 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.

[8] Available from International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), ICH Secretariat, c/o IFPMA, 15 ch. Louis-Dunant, P.O. Box 195, 1211 Geneva 20, Switzerland, http://www.ich.org.

62 FR 62472 (1997)

ICH S5A Guideline for Industry S5A Detection of Toxicity to Reproduction for Medicinal Products. 59 FR 48746 (1994)

ICH S5B Guidance for Industry S5B Detection of Toxicity to Reproduction for Medicinal Products: Addendum on Toxicity to Male Fertility. 61 FR 15360 (1996)

ICH S1A Guideline for Industry S1A The Need for Long-term Rodent Carcinogenicity Studies of Pharmaceuticals. 61 FR 8153 (1996)

ICH S1B Guidance for Industry S1B Testing for Carcinogenicity of Pharmaceuticals. 63 FR 8983 (1998)

ICH S1C Guideline for Industry S1C Dose Selection for Carcinogenicity Studies of Pharmaceuticals. 60 FR 11278 (1995)

ICH 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 (1997)

ICH Q1A ICH Harmonized Tripartite Guidance for Stability Testing of New Drug Substances and Products (September 23, 1994)

U.S. Food and Drug Administration (FDA and Committee for Proprietary Medicinal Products (CPMP), 1998 International Conference on Harmonization (ICH), Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin, Consensus Guideline ICH Viral Safety Document: Step 5

2.7 *FDA Documents:*[9]

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

U.S. Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER), 1993 Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals

U.S. Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER), 1997 Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use, 94D-0259

FDA Interim Guidance for Human and Veterinary Drug Products and Biologicals, Kinetic LAL techniques, DHHS, July 15, 1991

2.8 *AAMI Documents:*[10]

ANSI/AAMI/ISO 11737-1: 2006 Sterilization of Medical Devices—Microbiological Methods—Part 1: Estimation of Bioburden on Product

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

AAMI TIR No. 19-1998 Guidance for ANSI/AAMI/ISO 10993-7: 1995, Biological Evaluation of Medical Devices—Part 7: Ethylene Oxide Sterilization Residuals

AAMI/ISO 14160-1998 Sterilization of Single-Use Medical Devices Incorporating Materials of Animal Origin—Validation and Routine Control of Sterilization by Liquid Chemical Sterilants

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

2.9 *Other References:*

Draft Guidance for Preclinical and Clinical Investigations of Urethral Bulking Agents Used in the Treatment of Urinary Incontinence, November 29, 1995. (ODE/DRARD/ULDB), Document No. 850[11]

Council Directive 93/42/EEC, with Respect to Medical Devices Using Tissues of Animal Origin[12]

Commission Directive 2003/32/EC, with Respect to Medical Devices Manufactured Using Tissues of Animal Origin[12]

EMEA/410/01-rev.2, Committee for Proprietary Medical Products, Note for Guidance on Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medical Products[13]

The European Agency for the Evaluation of Medicinal Products, (EMEA), Committee for Proprietary Medicinal Products (CPMP) Guidance Document for Decision Trees for the Selection of Sterilisation Methods (CPMP/QWP/054/98 corr 2000) and Annex to Note for Guidance on Development Pharmaceutics (CPMP/QWP/155/96)[14]

3. Terminology

3.1 *Definitions:*

3.1.1 *adventitious agents*, *n*—an unintentionally introduced microbiological or other infectious contaminant. In the production of TEMPs, these agents may be unintentionally introduced into the process stream or the final product, or both.

3.1.2 *biocompatibility*, *n*—a material may be considered biocompatible if the material performs with an appropriate host response in a specific application (**7**).

3.1.3 *collagen*, *n*—Collagens form a family of secreted proteins with predominantly structural function. At least twenty genetically different family members have been identified so far. Several groups of collagen molecules have been classified based upon protein domain structures, macromolecular assemblies, and exon structures of the corresponding genes. All collagens have a unique triple helical structure configuration of three polypeptide units known as alpha-chains. Proper alignment of the alpha chains of the collagen molecule requires a highly complex enzymatic and chemical interaction *in vivo*.

[9] Available from Food and Drug Administration (FDA), 5600 Fishers Ln., Rockville, MD 20857, http://www.fda.gov.

[10] Association for the Advancement of Medical Instrumentation, 1110 N. Glebe Rd., Suite 220, Arlington, VA 22201–4795.

[11] Available from the FDA, 5600 Fishers Ln., Rockville, MD 20857. http://www.fda.gov/cdrh/ode/oderp850.html.

[12] Available from Office for Official Publications of the European Communities—European Law, 2, rue Mercier, L-2985, Luxembourg, http://eur-lex.europa.eu/en/'index.htm.

[13] Available from European Medicines Agency (EMEA), 7 Westferry Circus, Canary Wharf, London E14 4HB, U.K., http://www.eudora.org/emea.html, and http://www.emea.europa.eu/pdfs/human/bwp/TSE%20NFG%20410-rev2.pdf.

[14] Available from European Medicines Agency (EMEA), 7 Westferry Circus, Canary Wharf, London E14 4HB, U.K., http://www.eudora.org/emea.html, and http://www.emea.europa.eu/pdfs/human/qwp/005498en.pdf.

As such, preparation of the collagen by alternate methods may result in improperly aligned alpha chains and, putatively, increase the immunogenicity of the collagen. Collagen is high in glycine, L-alanine, L-proline, and 4-hydroxyproline, low in sulfur, and contains no L-tryptophan. When heated (for example, above 60°C), the helical structure of collagen is denatured irreversibly to single α chains with some β and γ bands (gelatin). At each end of the chains are short non-helical domains called telopeptides, which are removed in some collagen preparations. Through non-covalent interactions with sites on adjacent helixes, fibrillogenesis is achieved. Subsequently, non-reducible cross-links are formed. This guide will focus on the characterization of Type I collagen, which is the most abundant collagen in mammals. Type I collagen is part of the fibrillar group of collagens. It derives from the COL1A1 and COL1A2 genes, which express the alpha chains of the collagen. Type I collagen can be associated with Type III and Type V collagen and also with the other non-collagenous proteins like elastin and other structural molecules like glycosaminoglycans and complex lipoproteins and glycoproteins.

3.1.4 *degradation*, *n*—change in chemical, physical, or molecular structure or appearance (that is, gross morphology) of material.

3.1.5 *endotoxin*, *n*—a high molar mass 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.

3.1.6 *medical product*, *n*—any diagnostic or therapeutic treatment that may be regulated as a device, biologic, drug or combination product.

3.1.7 *microorganism*, *n*—bacteria, fungi, yeast, mold, viruses, and other infectious agents. However, it should be noted that not all microorganisms are infectious or pathogenic.

3.1.8 *solubility*, *n*—a measure of the extent to which the material can be dissolved. Any colloidal system without obvious phase separation can be considered soluble. In the context of collagen, refers to the dissociation of the fibrillar aggregates of collagen molecules into a solution. Native Type I collagen, which is soluble in dilute acids, but not soluble at physiological conditions, is termed "insoluble" or "acid soluble," while simple aggregates of non-fibrillar collagen soluble in neutral salt solutions are termed "neutral salt soluble." Post translational surface charge modifications may alter the solubility of collagen in neutral pH condition.

3.1.9 *sterilization*, *n*—the destruction or removal of all microorganisms in or about an object, as by chemical agents, electron beam, gamma irradiation, or filtration. If the medical product collagen permits, terminal sterilization is preferential to aseptic processing.

3.1.10 *suspension*, *n*—the dispersion of a solid through a liquid with a particle size large enough to be detected by purely optical means.

4. Significance and Use

4.1 The objective of this guide is to provide guidance in the characterization of Type I collagen as a starting material for surgical implants and substrates for tissue engineered medical products (TEMPs). This guide contains a listing of physical and chemical parameters that are directly related to the function of collagen. This guide can be used as an aid in the selection and characterization of the appropriate collagen starting material for the specific use. Not all tests or parameters are applicable to all uses of collagen.

4.2 The collagen covered by this guide may be used in a broad range of applications, forms, or medical products, for example (but not limited to) medical devices, tissue engineered medical products (TEMPs) or cell, drug, or DNA delivery devices for implantation. The use of collagen in a practical application should be based, among other factors, on 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 The following general areas should be considered when determining if the collagen supplied satisfies requirements for use in TEMPs. These are source of collagen, chemical and physical characterization and testing, and impurities profile.

4.4 The following documents relating to the production, regulation and regulatory approval of TEMPs products should be considered when determining if the collagen supplied satisfies requirements for use in TEMPs:

FDA CFR:
21 CFR 3: Product Jurisdiction:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=3
21 CFR 58: Good Laboratory Practice for Nonclinical Laboratory Studies:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=58

FDA/CDRH CFR and Guidances:
21 CFR Part 803: Medical Device Reporting:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=803
21 CFR 812: Investigational Device Exemptions:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=812
21 CFR 814: Premarket Approval of Medical Devices :
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=814
21 CFR 820: Quality System Regulation:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=820
Design Control Guidance for Medical Device Manufacturers:
http://www.fda.gov/cdrh/comp/designgd.pdf
Preproduction Quality Assurance Planning Recommendations for Medical Device Manufacturers (FDA 90-4236):
http://www.fda.gov/cdrh/manual/appende.html
The Review and Inspection of Premarket Approval Applications under the Bioresearch Monitoring Program—Draft Guidance for Industry and FDA Staff:
http://www.fda.gov/cdrh/comp/guidance/1602.pdf

FDA/CDRH Search Engines:
CDRH Guidance Search Engine:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfggp/search.cfm
CDRH Premarket Approval (PMA) Search Engine:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMA/pma.cfm
CDRH 510(k) Search Engine:
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm
CDRH Recognized STANDARDS Search Engine :
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfStandards/search.cfm

FDA/CBER CFR and Guidances:
21 CFR 312: Investigational New Drug Application : http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312
21 CFR 314: Applications for FDA Approval to Market a New Drug: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=31
21 CFR 610: General Biological Products Standards: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=610
21 CFR 1271: Human Cells, Tissues and Cellular and Tissue-Based Products: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=1271
Cellular & Gene Therapy Guidances and Other Publications: http://www.fda.gov/cber/genetherapy/gtpubs.htm
Human Tissue Guidances and Other Publications: http://www.fda.gov/cber/tissue/docs.htm
CBER Product Approval Information: http://www.fda.gov/cber/efoi/approve.htm
21 CFR 600, 601 BLA Regulations: http://www.access.gpo.gov/nara/cfr/waisidx_07/21cfrv7_07.html
21 CFR 210, 211 GMP Regulations: http://www.access.gpo.gov/nara/cfr/waisidx_07/21cfr210_07.html

5. Chemical and Physical Characterizations

5.1 These methods are suggested assays; however, other validated assay methods may be used. Selection of assay systems will vary depending on the configuration of the collagen (that is, soluble or insoluble). The user should ensure that the method selected is reliable and commonly accepted in protein chemistry. A review of collagen materials may be found in Li, 2000 (**8**), while a review of the collagen family of proteins may be found in Refs (**9-14**). When selecting an appropriate test method, the user should note that impurities in highly purified collagen are low or lower than 1 to 2 %, so sensitive test methods need to be utilized. For soluble collagen, the following represents a non-inclusive list of assay systems available: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE); peptide mapping; and physicochemical analysis. A similar list for insoluble collagen may include, but not be limited to, assay methods for hexosamine (that is, detection of glycoproteins), lipid, total sugar, desmosine (that is, elastin), and amino acid composition (that is, collagen composition profile; non-collagenous amino acids). Additionally, methods such as transmission electron microscopy may be helpful in characterizing the collagen fibers or collagen superstructure.

5.2 The concentration of collagen should be expressed in mass/volume or mass/mass. Colorometric assays or amino acid analysis for hydroxyproline are commonly used methods to measure collagen content.

5.3 Amino acid analysis will provide information on the composition of the amino acids of collagen (that is, the amino acids must be within the range of published data for highly purified collagen preparations, generally in the acid soluble form). Amino acid analysis is routinely performed on hydrolyzed collagens by reverse phase High Performance Liquid Chromatography (HPLC). This method can be used to quantify hydroxyproline, tyrosine, tryptophan, and cysteine. There are other methods available for amino acid analysis.

5.4 Purity of soluble collagen can be analyzed by SDS-PAGE, either on the collagen directly or after digestion of the collagen with purified bacterial collagenase to detect any remaining proteins.

5.5 *Elastin Assay*—Elastin can be a component of the impurities in an insoluble collagen preparation. One method to assay for elastin, although other methods are available, involves the detection of desmosine (**15**). These impurities can be detected by Western blots, ELISAs, and other types of assays.

5.6 Peptide mapping is one possible method to identify Type I collagen. The most commonly used peptide mapping method utilizes Cyanogen Bromide (CNBr) digestion. The digest can be analyzed by SDS-PAGE or HPLC.

5.7 *Impurities Profile*—The term impurity relates to the presence of extraneous substances and materials in the collagen. These impurities can be detected by Western blots, ELISAs, GC-MS, and other types of assays. The user is also directed to Guide E 1298 for additional information. If there is a concern for the presence of processing aids or other impurities associated with the collagen, they should be addressed with the supplier. The major impurities of concern include, but are not limited to the following: endotoxins, glycosaminoglycans, elastin, lipids, improperly aligned collagen molecules, host cell contaminants, cell culture contaminants, heavy metals, bioburden, viruses, transmissible spongiform encephalopathy (TSE) agents, cross-linking and enzymatic agents, and components used in extraction or solubilization (for example, acids, surfactants, solvents, and so forth). Type III collagen may also be associated with Type I collagen. While its presence may have no adverse effect on product quality, levels should be evaluated and controlled for lot-to-lot consistency. At minimum, any protein impurity of greater than 1 % in the final collagen preparation should be identified and quantified.

5.8 *Crosslinking Reactions with Collagen*—Collagen is a very stable protein due to its triple-helical structure, imparting resistance to most proteolytic enzymes. It is still sensitive to collagenase, however. The stability can be enhanced by crosslinking the molecule by physical or chemical means. Both inter- and intrachain crosslinking can occur due to propensity of collagen fibers to naturally crosslink. Crosslinking agents and methods include aldehydes, carbodiimides, epoxides, diisocyanates, non-enzymatic glycosylation, dehydrothermal treatment (DHT), radiation (for example, gamma, electron beam) and ultraviolet light. For chemical crosslinking, excess crosslinker should be removed and quantitated before or at the final product stage. A crosslinker may be cytotoxic and any component in the final product needs to be quantitated. There are several methods available, including, mass spec (MS), GC/MS, or other assays. A cytotoxicity assessment will also provide a measure of acceptable crosslinker levels. Physical crosslinking may result in unwanted changes to the structure of the collagen molecule and should be assessed with qualification assays appropriate to the clinical indication under consideration. Direct measurement of collagen crosslinking can be performed looking at the altered amino acid composition and using methods appropriate for the crosslinker. One method, for example, (other methods exist) to measure degree of crosslinking when lysine residues are involved include detecting free lysines and hydroxylysines by labeling the ϵ-amino acid groups with 2.4.6 trinitrobenzenesulfonic acid (TNBS), where the TNBS-labeled amino acids absorb at 345 nm with a molar absorptivity of 1.46×10^4 L/mole × cm. Amino acid composition can also be examined by analysis of sodium borohydride-treated collagen. The thermal denaturation characteristics can also be measured by Differential Scanning Calorimetry (DSC) (**16**). The thermal denaturation characteristics can sometimes be correlated with the crosslink density. The % water uptake (% swell), using the equation $(W_W–W_D)/W_W$, where W_D = dry weight and W_W = wet weight, is also an indirect measure of collagen crosslinking. The tensile strength can be altered by crosslinking. Measurements using an Instron or a rheometer will note a change in properties after crosslinking. Collagen crosslinking imparts a resistance to the proteolytic enzyme collagenase. Collagenase is the one enzyme that will digest triple-stranded collagen. When collagen is crosslinked, it is more resistant to breakdown and extensive crosslinking will afford the greatest resistance to collagenase.

5.9 *Endotoxin Content*—Endotoxin contamination is difficult to prevent because it is ubiquitous in nature, stable and small enough to pass through sterilizing filters (0.22 µm). Endotoxin tests for collagen include the gel clot, endpoint 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. The quantitative kinetic assay, which measures the amount of time required to reach a predetermined optical density, is the most sensitive (Food and Drug Administration, 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). Each new lot of reagents should meet acceptance criteria established by appropriate qualification or validation studies (for investigational or licensed/cleared products, respectively). The endotoxin level in collagen 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 (Interim Guidance for Human and Veterinary Drug Products and Biologicals). The user is also directed to CFR 610.13(b) for information pertaining to the rabbit pyrogen assay.

5.10 *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. This method is based on <231> Heavy Metals, 1st and 6th Supplement USP-NF. Substances that typically respond to this test are lead, mercury, bismuth, arsenic, antimony, tin, cadmium, silver, copper, and molybdenum. 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. Additional heavy metal contaminants may be present due to processing. If necessary, the user may detect these contaminants by various methods that may include, but are not limited to, spectrographic, chromatographic, and flame atomic absorption techniques.

5.11 *Microbiological Safety*—Bacteria, viruses, and fungi are also contaminants that can arise in an a biological sample. User will validate sterilization and characterize its effect on the product. The presence of bacteria may also contribute to the presence of endotoxins. The following Microbiological Tests in USP 30 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: 2006; ANSI/AAMI/ISO 11737-2: 1998; and ISO 13408–1. The collagen is first dissolved in a sterile, aqueous solution, then filtered using sterile techniques through a 0.45 µm membrane filter. The filters are subsequently incubated on Tryptic Soy Agar to determine the presence of bacteria, and on Sabouraud Dextrose Agar to determine the presence of yeast and mold. If collagen products are intended to serve as a barrier to microorganisms, this function will need to be validated with specific experiments.

5.12 Carbohydrate analysis of collagens can be carried out by classical gas-liquid chromatographic methods or spectrophotometric methods. If using a novel source, then glycosylation of proteins may need to be considered.

5.13 Trypsin Susceptibility will detect that portion of collagen that has been denatured during purification steps such as acid and base treatment, solvent treatment, and so forth. Trypsin will digest that portion of the collagen and can be measured by assaying the hydroxyproline content of the supernatant. Triple helical collagen is resistant to digestion by most proteases. Susceptibility to trypsin or other appropriate proteases is determined by exposing the collagen to the enzyme and assaying the digest for degradation. There are several methods for this test.

5.14 Differential Scanning Calorimetry (DSC) determines dissociation temperature of collagens in fibrils, as well as detecting microfibrils and denatured collagen at lower melting temperatures. (See also 5.8, crosslinking reactions with collagen).

5.15 Viscosity is more applicable to gels or suspensions but may be useful with collagen configured in forms such as, but not limited to, pastes or films (**17**). Viscosity of collagen-based materials depends on a number of factors which may include, but are not limited to, the following: solution or dispersion/suspension, concentration, temperature, operating condition, and so forth. It is not feasible to determine the viscosity of films. This is a routine test performed with a viscometer (not a u-tube). The user must clearly state the conditions of the test.

5.16 Transmission electron microscopy may be used to show the quality of collagen fibers. Unraveling or changes in banding will be obvious.