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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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ε[1] NOTE—Mercury warning was editorially added in April 2008.

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 **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.

1.4 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,*

[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.

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

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

[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.

[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.

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 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

[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.

[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.

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*. 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:

[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.

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.

5.17 DNA sequence data on recombinant or transgenic source cells: Verify sequence data for expression gene, that is, COL1A1 or COL1A2.

5.18 The collagen material shall have specifications for an extensive set of chemical and physical properties such as, but not limited to, those listed in Table 1. The table represents methods which may or may not be appropriate for characterizing a particular collagen sample. Not all the methods listed may be required to characterize the sample, and the specificity and sensitivity vary among the methods listed. The user should be familiar with the limitations of the appropriate test methods.

5.19 *Analysis for Type II, IV, and Other Collagens*—Tissues commonly used to isolate Type I collagen typically contain some Type III collagen which coexists in many tissues. Type II collagen is found primarily in cartilage, while Type IV collagen is found in basement membranes and has been associated with Goodpasture's Syndrome (Wieslander, J., et al, (**18**)). The purity of collagen is important in determining the potential for safety problems and providing criteria for the consistency of the manufacturing process. For example, skin collagen is composed of approximately 90 % type I, 8–10 % type III, and the reminder is made up of trace amounts of the less abundant collagens, primarily type IV, V, and VI (Holbrook and Smith, (**19**)). As all collagens contain triple helical domains, the properties of different collagen types can be very similar. Detection by Western Blot analysis, therefore, requires the use of antibodies that recognize epitopes in the more diverse

TABLE 1 Characterization Methods for Type I Collagen

Characterization Method	Applicable to
Chemical	
Appearance	Soluble or Insoluble
Concentration	Soluble or Insoluble
Purity	Soluble or Insoluble
Amino acid analysis	Soluble or Insoluble
Peptide mapping	Soluble or Insoluble
Impurities profile, includes Heavy Metal Analysis	Soluble or Insoluble
Carbohydrate analysis	Soluble or Insoluble
Trypsin resistance	Soluble or Insoluble, Mainly Insoluble
Collagenase resistance	Soluble or Insoluble, Mainly Insoluble
pH of implantable	Soluble or Insoluble
Additives (cross-linkers, lubricants, drugs, sterilents)	Soluble or Insoluble
Physical	
Shrink Temperature (DSC)	Insoluble
Viscosity	Mainly soluble
TEM	Insoluble
SDS-PAGE	Soluble or Insoluble
Moisture Content (5 to 20 %), dependent on storage environment	Insoluble
Electron Micrograph (native banded 640 Å structure for fibrils)	Insoluble
Biochemical	
Endotoxin level	Soluble or Insoluble
Bioburden	Soluble or Insoluble
% Type I collagen/Total Protein	Soluble or Insoluble
% Other Types Collagen and List of which Types present	Soluble or Insoluble
Total DNA (ppm or %)	Soluble or Insoluble
Total Lipid	Soluble or Insoluble
% native collagen (by trypsin resistance, circular Dichroism)	Soluble or Insoluble

Abbreviation in Table:
DSC = Differential Scanning Calorimetry
TEM = Transmission Electron Microscopy
SDS-PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

non-helical regions. Antibodies are available for Types I–VI collagens, and possibly others, for use in Western Blot analyses following SDS gel electrophoresis. The extent of analysis required will depend upon the risk of other collagen types being present as impurities in a particular collagen product.

6. Product Development Considerations

6.1 *Storage Conditions/Shelf Life Stability of Collagen*—For collagen, the most relevant stability-indicating parameters are those related to the functionality of the polymer. Dependent upon what function the collagen will have in the final formulation, parameters such as viscosity (apparent and intrinsic) and biological activity, along with other parameters deemed relevant, may also be considered. Storage conditions are of importance, especially for collagen solutions. International Conference on Harmonization (ICH) guidance documents should be consulted for information on stability testing of pharmaceuticals (that is, ICH Q1A ICH).

6.2 *Sterilization Method, if Applicable, and Effects of Sterilization on Product*—The user should verify that the sterilization method does not adversely effect the collagen end product. Collagen can be sterilized by gamma irradiation, electron-beam, or by ethylene oxide, or prepared using aseptic processing steps. Potential degradation of the collagen or sterilization residuals should be evaluated to determine the impact on the product. Solutions of collagen may be *(1)* filter sterilized if the viscosity of the collagen solution permits; or *(2)* gamma-irradiated. Any changes in viscosity may reflect an alteration of the molecular mass and should be evaluated. The method of sterilization is primarily dictated by the effect on the product effectiveness. The method chosen must be validated to determine the effectiveness of sterilization. The reader should refer to the most current version of 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; 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).

6.3 *Sourcing*—The criteria to consider for safe sourcing include appropriate human or animal donor selection and the tissue collection procedures to assure that the source material is unlikely to contain TSE infectivity. Additional information can be obtained from the following documents: EN 12442–1, EN 12442–2, EN 12442–3; 21 CFR Parts 207, 807, and 1271, 21 CFR Part 820, and 21 CFR Part 1271, Part C; Federal Register Vol. 43; Federal Register Vol. 62; Federal Register Vol. 66, No. 5, January 8, 2001, pp. 1552–1559; Federal Register, Vol. 66, No. 13, January 19, 2001, p. 5447; ISO 13408–1. Council Directive 93/42/EEC with respect to medical products using tissues of animal origin; Commission Directive 2003/32/EC with respect to medical products manufactured using tissues of animal origin; 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. Additional documents may be available. The user should verify the most current version of the document.

6.3.1 For further information, the user is referred to Appendix X2, Sourcing Issues: X2.1 Tissue for collagen or collagen-containing devices; X2.2 Requirements for a closed herd; X2.3 Documentation for the tissue.

6.3.2 The collagen can be isolated from tissues or cell cultures by any method, including, but not limited to extraction by dilute acids or dilute salt solutions or by enzymatic digestion of the tissue **(20-23)**. The user should be aware that even though Type III collagen is less abundant, it is often associated with Type I, except in bones and tendons. Type V collagen is also associated with Type I.

6.4 *Viral and Transmissible Spongiform Encephalopathy (TSE) Agent Inactivation*—Viruses and TSE agents can be introduced into a product as a result of raw materials sourcing or through adventitious means. Appropriate measures should be taken so that the resultant product is free from viruses and TSE agents. For further guidance on viral or TSE clearance, or both, the user is directed to the references throughout this guide as well as USP 30/NF 19 <1050>, and other pertinent references, as appropriate. Additional information may be found in the following FDA Guidance Document, FDA points to consider and International Conference on Harmonization (ICH)

documents: Guidance for Industry and for FDA Reviewers: Medical Devices Containing Materials Derived from Animal Sources (Except for *In Vitro* Diagnostic Devices); 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; 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. The European Pharmacopoeia 5.0 has a monograph describing methods to minimize TSE risks (5.2.8 Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Human and Veterinary Medicinal Products).

6.4.1 *Viral Clearance*—The sources of raw materials from humans or animals should be screened for known viral pathogens to reduce or eliminate the potential infectivity. Viral clearance methods can include, but not be limited to methods such as detergent treatment, high or low pH, urea treatment, other chemical treatments, and filtration or other purification methods. However, even these harsh treatments may not ensure complete viral inactivation. Viral clearance should be demonstrated by an appropriately validated viral clearance study protocol. The user should verify that the viral clearance procedure is compatible with the starting material or the configured end product. For human tissue sources for manufacturing into collagen, the observance of Good Tissue Practices should be considered.

6.4.2 *TSE Clearance*—Due diligence should be made to the sourcing of raw materials, process design to remove potential TSE agents and treatments to inactivate TSE agents for those products which can withstand the harsh treatments required to inactivate TSE agents. The user is referred to the "Meeting Report, International Workshop on Clearance of TSE Agents from Blood Products and Implanted Tissues," **(24)** and the FDA Guidance Document "Guidance for Industry and for FDA Reviewers: Medical Devices Containing Materials Derived from Animal Sources (Except for *In Vitro* Diagnostic Devices)" for additional guidance on recommended practices for sourcing and for TSE clearance. Technology is under development for quantitation of TSE agents in biological materials. The following references are cited as examples of two of the many methods for detecting TSE agents **(25, 26)**. The user should be aware that although detection of the protease resistant form of the ubiquitous prion protein in a tissue generally indicates that it contains the transmissible agent and is not suitable for preparing collagen for human or animal implantation, the converse is not necessarily true. Therefore, a negative test for the protease resistant prion alone may not be sufficient to assure that the source material is safe for producing collagen.

6.4.3 *Source Documentation*—Guidance for viral inactivation validation and CBER guidances. See also the following Reference Sections for additional information: Section 2.1 (Guide E 1298); Section 2.3 (EN 12442–3); Section 2.4 (USP 30/NF 19); Section 2.6 (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); and Refs **(27, 28)**. Additional resources may be available, as this list is not comprehensive. The user should use the current version of all documents.

7. Safety and Toxicology Aspects of Collagen

7.1 The safety of collagen in biomedical and pharmaceutical applications and in TEMPs should be established according to current guidelines such as ISO 10993 and Practice F 748. Suppliers of collagen may have such documentation on file. Preclinical safety studies specific to the clinical application under consideration must be done in accordance with 21 CFR 312.

7.1.1 A database generated to support the safety of collagen-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 differ 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 M3), hypersensitivity testing, and genetic toxicology testing (ICH S2A and ICH S2B). Additional studies that may be relevant to a proposed pharmaceutical use include reproductive/developmental toxicology testing (ICH S5A and ICH S5B) and carcinogenicity testing (ICH S1A, ICH S1B, ICH S1C, and ICH 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. For instance, the user may consider the following, among other documents: Practice F 2148, or Draft Guidance for Preclinical and Clinical Investigations of Urethral Bulking Agents Used in the Treatment of Urinary Incontinence. Specific guidance on the development or marketing of drug products, biologics, or medical 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.

7.2 *Biocompatibility*:

7.2.1 Many materials have been shown to produce a well-characterized level of biological response following long term clinical use in laboratory animals. There are few interspecies

differences in the structure of Type I collagen. The extensive similarity in primary and higher-order structure of Type I collagen may explain why collagen obtained from animal species is acceptable as a material for human implantation (**8**). 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 F 619, F 748, F 749, F 756, F 763, F 813, F 981, F 1439, F 1903, F 1904, F 1905 and F 1906, as well as Test Method F 895, Terminology F 1251, and ISO 10993–1, ISO 10993–9, Part 9, ISO 10993–17, EN 12442–1, EN 12442–3.

7.3 *Immunogenicity*—The immunogenicity of the collagen may vary depending on the source material (that is, extracted versus recombinant collagen), though, for reasons noted in the preceding paragraph, immune reactions due to species differences alone are uncommon. Products from different manufacturers may vary in properties such as, but not limited to, fiber quality and percentage hydroxyproline. The user should be aware that differences in collagen structure and chemistry may result in potential variability to the immunological responses. The manufacturer should ascertain to what degree modifications of the structure of native collagen (for example, by chemical crosslinking or methods not limited to biomimetics) may modify (enhance or reduce) immunogenicity when implanted *in vivo*. When new applications of a material, or modifications to the material or physical forms of the material are being considered, then the immunogenicity testing should include the following standards for testing potential skin sensitizers: Practice F 720 and ISO 10993–10.

7.4 Collagen for use in biomedical and pharmaceutical applications and in Tissue Engineered Medical Products (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 collagen. Such a Master File should be submitted to the US FDA and to other regulatory authorities, both national and international. ISO 14971 should also be referenced when appropriate.

8. Keywords

8.1 biomaterials; characterization; collagen; natural materials; TEMPs

APPENDIXES

(Nonmandatory Information)

X1. BACKGROUND

X1.1 Background

X1.1.1 This short review of the collagen family of proteins is largely based on two review papers (**1**, **2**). Please refer to these articles and their listed references for further details.

X1.2 Overview/Introduction

X1.2.1 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. This classification will be detailed in a later section of this appendix. Collagen types are indicated by Roman numerals (for example, collagen Type I), the associated polypeptide chains (or subunits) as alpha chains with arabic numbers (for example, alpha2(I)) and the genes by COL[arabic numeral]A[arabic numeral] (for example, COL1A2).

X1.2.2 Several inherited and acquired diseases have been linked to these proteins and emphasize the importance of collagen molecules (**3**). Among those diseases are osteogenesis imperfecta, some forms of Ehlers-Danlos syndrome, epidermolysis bullosa, Goodpasture syndrome, Alport syndrome and relapsing polychondritis.

X1.2.3 The basic structural entity contains three polypeptide chains of left-handed helices that form a right-handed triple helix. In order to form such a triple helix, the basic building block of the polypeptide chain is an amino acid triplet of the general sequence Gly-X-Y with prolines often occupying the X position, and hydroxyproline in the Y position. The high amount of proline and hydroxyproline allows for the formation of the left-handed helix (polyproline helix II). In order to form a triple helix, every third position has to be taken by glycine, since the size of any side chain other than a hydrogen atom would interfere sterically with the close proximity of the three chains. Hydroxyproline, forming intramolecular hydrogen bonds, is important for the stability of the triple helix. Underhydroxylation of prolines results in lowering the temperature at which the collagen triple helix destabilizes or "melts."

X1.2.4 It is important to clarify that other proteins contain collagenous domains, but are not classified as collagen family members. Among these proteins are the complement subunit C1q, acetylcholine esterase, Type I macrophage scavenger receptor and the mannose-binding protein.

X1.2.5 The biosynthesis of collagen molecules has been studied in most detail in fiber forming collagens (Type I, II, III, V, and XI). Collagen polypeptide chains are synthesized in the rough ER; hydroxylation of proline and lysine side chains occurs concomitantly with chain elongation and continues until the triple helix forms. This formation in the ER requires cis-trans isomerization of the hydroxyproline residues and is catalyzed by an enzyme. Non-collagenous domains are found at the N- and C-termini of the formed triple helices. Collagen molecules are glycosylated and exported to the extracellular space. Further processing, consisting of lysyl oxidation and proteolytic cleavage of the non-collagenous N- and C-termini, is catalyzed by specific extracellular enzymes and results in the

formation and stabilization of collagen fibers. Crosslinks form non-enzymatically between lysine and oxidized lysine residues and connect alpha chains of different triple helices. See (1) for further details. Not all of these processing steps occur for all collagen types.

X1.3 Collagen Classification

X1.3.1 As indicated above, the members of the collagen family are grouped according to protein domain structures, macromolecular assemblies and exon structures of the corresponding genes. Six groups are currently identified (2):

X1.3.2 *Fibrillar Collagens*—This group contains collagens Type I, II, III, V, and XI, important for the mechanical support of multicellular organisms. These collagens (formed by either homotrimers [Types II and III] or alpha chain heterotrimes) participate in the formation of staggered fibrils of varying diameter. The central triple helical domain (around 100kDa size) usually contains 300 repeats of the Gly-X-Y triplet, forming long, rigid structures. After extracellular processing, the single collagen molecules aggregate to fibrils. Extent of processing may be important in determining the diameter of the fibril.

X1.3.3 *FACIT Collagens*—The fibril-associated collagens with interrupted triple helices (FACIT) collagen group is comprised of collagen Types IX, XII, XIV, XVI, XIX, and XX. Collagen IX has been shown to be associated with Collagen II and XI fibrils. Collagen Types XII and XIV have been associated with Collagen I fibrils. FACIT collagens contain large non-collagenous domains connecting relatively short triple-helical domains. Their associations with fibrils are thought to be important in regulation of fibril diameter and connection of fibrils with other extracellular molecules.

X1.3.4 *Short Chain Collagens*—Collagen Types VIII and X contain short triple helical domains of 50 to 60kDa flanked by globular domains. Collagen Type VIII forms the backbone of the hexagonal network in Descemet's membrane, Type X is found in hypertrophic cartilage matrix. Both are thought to provide open structures resisting compressive forces.

X1.3.5 *Basement Membrane Collagens*—Collagen Type IV forms the network structures found in basement membranes. Triple helical domains are frequently interrupted by non-collagenous domains, allowing for flexibility within the rod-like molecules. The triple helices interact at the N- and C-termini forming a three-dimensional network; collagen Type IV also directly binds to other basement membrane molecules like laminin and BM-40. Six different alpha chains have been identified for Collagen Type IV. The most prevalent collagen Type IV is formed by a heterotrimer of two alpha1 and one alpha2 chain. Specific basement membranes (kidney glomerular basement membrane, neuromuscular junction, and so forth) contain heterotrimers containing the alpha3-6 chains. The exact composition of these heterotrimers is not clear. Goodpasture and Alport syndromes have been associated with Collagen IV (4, 5). Collagen Type VII (see below), although not classified as a basement membrane collagen, forms tight associations (anchoring fibrils) with basement membranes in skin, the oral mucosa and the cervix.

X1.3.6 *Multiplexins*—Collagen Types XV and XVIII have been grouped as Multiplexins (collagens containing multiple-triple-helix domains with interruptions). Both collagens are expressed widely. A fragment of Collagen XVIII, called endostatin, has been shown to inhibit angiogenesis.

X1.3.7 *MACITs*—Two members form the group of membrane-associated collagens with interrupted triple helices. Types XIII and XVII both contain triple helices in the extracellular domain and globular domains with transmembrane domains, attaching the molecules to the cell surface. Type XIII is widespread, and Type XVII is found in hemidesmosomes. It also presents as an autoantigen in the blistering disease bullous pemphigoid.

X1.3.8 *Other Collagens*—Collagen Types VI and VII do not belong to the other classifications and have been grouped separately. Collagen Type VI is the major component of beaded microfibrils. The heterotrimeric molecule contains a central triple helix flanked by globular domains. Dimers and tetramers are formed by disulfide exchange. Collagen Type VII is the major component of anchoring fibrils connecting the basement membrane in stratified squamous epithelia with adhesion plaques in the papillary dermis. The acquired and dystrophic forms of epidermolysis bullosa are associated with collagen VII (15).

X1.4 Occurrences

X1.4.1 Table X1.1 summarizes the location of major collagen types (1). Collagen Types XIII–XX are less well characterized and are excluded.

X1.5 Sources

X1.5.1 Major sources of collagens are skin, tendon, cartilage and placenta, both from animals (bovine and porcine for larger quantities) and human tissues. More recently, human cell cultures and recombinant collagen from yeast and insect cell cultures have become potential additional raw material sources.

TABLE X1.1 Occurrences of Types I to XII Collagen

Collagen Type	Alpha Chains	Distribution
I	a1-2(I)	widespread; skin, bone, tendon, cornea, etc.
II	a1(II)	cartilage, vitreous body of the eye
III	a1(III)	skin, tendon, aorta, cornea
IV	a1-6(IV)	all basement membranes
V	a1-3(V)	widespread; skin, bone, tendon, ligament, etc.
VI	a1-3(VI)	widespread; skin, bone, cornea, etc.
VII	a1(VII)	skin, oral mucosa, cervix
VIII	a1-2(VIII)	Descemet's membrane
IX	a1-3(IX)	cartilage, vitreous body
X	a1(X)	hypertrophic and mineralizing cartilage
XI	a1-3(XI)	cartilage
XII	a1(XII)	all collagen type I containing tissues

X2. SOURCING ISSUES

X2.1 Tissue for Collagen or Collagen-Containing Medical Devices

X2.1.1 Tissues which have been obtained to produce collagen or collagen-containing medical products must be carefully selected, tested, and controlled. The age of the tissue may affect the degree of crosslinking of the collagen as well as the quantity of collagen. Using tissues from the same species and age will provide better process controls for collagen production. The safety of the animal tissues is also of utmost concern. Reference to 9 CFR 113 (FDA) for animal sourced material should be considered, as well as reference to ISO 12442 (current version). The United States Food & Drug Administration implemented new guidelines in 2007 to minimize the danger of contamination of transmissible spongioform encephalopathies (TSE's), Proposed Rule: Use of Materials Derived from Cattle in Medical Products Intended for Use in Humans and Drugs Intended for Use in Ruminants (Federal Register, Vol. 72, Number 8, Jan 12, 2007, pp. 1581-1619). These include:

X2.1.1.1 Elimination of risk materials including skull, brain, trigeminal ganglia, eyes, vertebral column, spinal cord, dorsal root ganglia of animals over 30 months of age and the small intestine and tonsils of cattle of all ages.

X2.1.1.2 Any material from "downer" cattle—those that cannot walk.

X2.1.1.3 Use of advanced meat recovery methods to prevent spinal cord contamination.

X2.1.1.4 Prevention of air-injection stunning.

X2.1.2 Animals must be subjected to ante and postmortem inspection and be fit for human consumption.

X2.2 Requirements for a Closed-Herd

X2.2.1 Tissues obtained from animals should be obtained from well documented herds. It is highly desirable to use closed cattle herds to maximize biosecurity. A closed cattle herd preferably includes the following:

X2.2.1.1 Enclosed property which is chosen to minimize exposure to environmental hazards around the land including the soil and water supply.

X2.2.1.2 Animals born, raised and live their entire lives in the closed herd in isolation from other cattle, sheep, pigs, and deer and elk.

X2.2.1.3 The source and lineage of each animal is documented.

X2.2.1.4 The female parent of each new animal is a member of the herd.

X2.2.1.5 Artificial insemination from registered stock is the primary breeding method.

X2.2.1.6 Animals forage on closed herd pastures. Only purchased grain or hay from selected sources may be used to supplement closed herd pasture forage.

X2.2.1.7 The animals have never been fed ruminant (animal-derived) protein and therefore have not been exposed to the primary suspected source of BSE infection.

X2.2.2 Animal parts are harvested and controlled under documented procedures to minimize contact with brain and spinal cord tissues.

X2.3 Documentation

X2.3.1 Animal tissues serving as the raw material for downstream processing or fabrication into medical products should be well documented. A description of the animal species, the specific tissue used, and the geographical history of the animal need to be fully disclosed. Maintenance of the herd is important to the consistency and quality of the raw material. As such, information on the long term health of the herd, frequency and type of veterinarian inspections, breeding history, animal traceability, animal feed history records, absence of TSE disease, and standard vaccinations such as live modified viruses which could co-purify in the desired tissue should be documented. Animal feed has the potential for introducing adventitious agents and the animal feed composition, diet, and labeling of feed composition at distribution locations should all be documented.

X2.3.2 When the animal is sacrificed the age of the animal should be documented, as well as the USDA status of the slaughter house, measures taken to reduce the risk of contaminating non-TSE tissues with material from tissues that could contain TSE, and the results of the pre and/or post mortem inspection. The test used to release the tissue for further processing or incorporation into other tissues or medical products should be disclosed as well as the Certificate of Analysis. Records of the test results for each lot of material should be maintained at the manufacturing facility and submitted in regulatory documents when appropriate. Methods for maintaining records of the source material and testing should be disclosed in regulatory submissions.

X3. RELATED MATERIAL

X3.1 Kielty, C. M., Hopkinson, I., and Grant, M. E., "Collagen: The Collagen Family: Structure, Assembly, and Organization in the Extracellular Matrix, in Connective Tissue and Its Heritable Disorders: Molecular, Genetic and Medical Aspects," P. M. Royce and B. Steinmann, Editors, 1993, Wiley-Liss, Inc., New York, pp. 103–149.

X3.2 Olsen, B. R., and Ninomiya, Y., "Collagens, in Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins," T. Kreis and R. Vale, Editors, 1999, Sambrook &

Tooze Publication at Oxford University Press: Oxford, pp. 380–408.

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