Designation: F561 – 13

Standard Practice for Retrieval and Analysis of Medical Devices, and Associated Tissues and Fluids[1]

This standard is issued under the fixed designation F561; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice covers recommendations for the retrieval, handling, and analysis of implanted medical devices and associated specimens that are removed from patients during revision surgery, at postmortem, or as part of animal studies. This practice can also be used for analysis of specimens and lubrication fluids from *in vitro* wear tests and joint simulators. The aim is to provide guidance in preventing damage to the associated specimens which could obscure the investigational results, and in gathering data at the proper time and circumstance to validate the study.

1.2 This practice offers guidelines for the analysis of retrieved implants to limit damage to them, and to allow comparisons between investigational results from different studies. The protocols are divided into three stages, where Stage I is the minimum non-destructive analysis, Stage II is more complete non-destructive analysis, and Stage III is destructive analysis. Standard protocols for the examination and collection of data are provided for specific types of materials in relation to their typical applications. For particular investigational programs, additional, more specific, protocols may be required. If special analytical techniques are employed, the appropriate handling procedures must be specified.

1.3 This practice recommendation should be applied in accordance with national regulations or legal requirements regarding the handling and analysis of retrieved implants and excised tissues, especially with regard to handling devices which may become involved in litigation, as per Practice E860.

1.4 A significant portion of the information associated with a retrieved implant device is often at the device-tissue interface or in the tissues associated with the implant and related organ systems. Attention should be given to the handling of adjacent tissues, so as not to interfere with study of the particles in the adjacent tissue, a chemical analysis for the byproducts of degradation of the implant, or a study of the cellular response to the implant.

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

1.6 *This standard may involve hazardous materials, operations, and equipment. As a precautionary measure, explanted devices should be sterilized or minimally disinfected by an appropriate means that does not adversely affect the implant or the associated tissue that may be subject to subsequent analysis. A detailed discussion of precautions to be used in handling of human tissues can be found in ISO 12891-1. This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

A262 Practices for Detecting Susceptibility to Intergranular Attack in Austenitic Stainless Steels

A751 Test Methods, Practices, and Terminology for Chemical Analysis of Steel Products

C20 Test Methods for Apparent Porosity, Water Absorption, Apparent Specific Gravity, and Bulk Density of Burned Refractory Brick and Shapes by Boiling Water

C158 Test Methods for Strength of Glass by Flexure (Determination of Modulus of Rupture)

C169 Test Methods for Chemical Analysis of Soda-Lime and Borosilicate Glass

C573 Methods for Chemical Analysis of Fireclay and High-Alumina Refractories (Withdrawn 1995)[3]

C623 Test Method for Young's Modulus, Shear Modulus,

[1] This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.15 on Material Test Methods.

Current edition approved Sept. 1, 2013. Published October 2013. Originally approved 1978. Last previous edition approved in 2010 as F561 – 05a (2010). DOI: 10.1520/F0561-13

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

and Poisson's Ratio for Glass and Glass-Ceramics by Resonance
C633 Test Method for Adhesion or Cohesion Strength of Thermal Spray Coatings
C674 Test Methods for Flexural Properties of Ceramic Whiteware Materials
C730 Test Method for Knoop Indentation Hardness of Glass
C1069 Test Method for Specific Surface Area of Alumina or Quartz by Nitrogen Adsorption
C1161 Test Method for Flexural Strength of Advanced Ceramics at Ambient Temperature
C1198 Test Method for Dynamic Young's Modulus, Shear Modulus, and Poisson's Ratio for Advanced Ceramics by Sonic Resonance
C1322 Practice for Fractography and Characterization of Fracture Origins in Advanced Ceramics
C1326 Test Method for Knoop Indentation Hardness of Advanced Ceramics
C1327 Test Method for Vickers Indentation Hardness of Advanced Ceramics
D256 Test Methods for Determining the Izod Pendulum Impact Resistance of Plastics
D412 Test Methods for Vulcanized Rubber and Thermoplastic Elastomers—Tension
D570 Test Method for Water Absorption of Plastics
D621 Test Methods for Deformation of Plastics Under Load (Withdrawn 1994)[3]
D624 Test Method for Tear Strength of Conventional Vulcanized Rubber and Thermoplastic Elastomers
D638 Test Method for Tensile Properties of Plastics
D671 Test Method for Flexural Fatigue of Plastics by Constant-Amplitude-of-Force (Withdrawn 2002)[3]
D695 Test Method for Compressive Properties of Rigid Plastics
D732 Test Method for Shear Strength of Plastics by Punch Tool
D747 Test Method for Apparent Bending Modulus of Plastics by Means of a Cantilever Beam
D785 Test Method for Rockwell Hardness of Plastics and Electrical Insulating Materials
D790 Test Methods for Flexural Properties of Unreinforced and Reinforced Plastics and Electrical Insulating Materials
D792 Test Methods for Density and Specific Gravity (Relative Density) of Plastics by Displacement
D1004 Test Method for Tear Resistance (Graves Tear) of Plastic Film and Sheeting
D1042 Test Method for Linear Dimensional Changes of Plastics Caused by Exposure to Heat and Moisture
D1238 Test Method for Melt Flow Rates of Thermoplastics by Extrusion Plastometer
D1239 Test Method for Resistance of Plastic Films to Extraction by Chemicals
D1242 Test Methods for Resistance of Plastic Materials to Abrasion (Withdrawn 2003)[3]
D1505 Test Method for Density of Plastics by the Density-Gradient Technique
D1621 Test Method for Compressive Properties of Rigid Cellular Plastics
D1622 Test Method for Apparent Density of Rigid Cellular Plastics
D1623 Test Method for Tensile and Tensile Adhesion Properties of Rigid Cellular Plastics
D1708 Test Method for Tensile Properties of Plastics by Use of Microtensile Specimens
D2240 Test Method for Rubber Property—Durometer Hardness
D2842 Test Method for Water Absorption of Rigid Cellular Plastics
D2857 Practice for Dilute Solution Viscosity of Polymers
D2873 Test Method for Interior Porosity of Poly(Vinyl Chloride) (PVC) Resins by Mercury Intrusion Porosimetry (Withdrawn 2003)[3]
D2990 Test Methods for Tensile, Compressive, and Flexural Creep and Creep-Rupture of Plastics
D3016 Practice for Use of Liquid Exclusion Chromatography Terms and Relationships
D3417 Test Method for Enthalpies of Fusion and Crystallization of Polymers by Differential Scanning Calorimetry (DSC) (Withdrawn 2004)[3]
D3418 Test Method for Transition Temperatures and Enthalpies of Fusion and Crystallization of Polymers by Differential Scanning Calorimetry
D3835 Test Method for Determination of Properties of Polymeric Materials by Means of a Capillary Rheometer
D3919 Practice for Measuring Trace Elements in Water by Graphite Furnace Atomic Absorption Spectrophotometry
D4000 Classification System for Specifying Plastic Materials
D4001 Test Method for Determination of Weight-Average Molecular Weight of Polymers By Light Scattering
D4065 Practice for Plastics: Dynamic Mechanical Properties: Determination and Report of Procedures
D4754 Test Method for Two-Sided Liquid Extraction of Plastic Materials Using FDA Migration Cell
D5152 Practice for Water Extraction of Residual Solids from Degraded Plastics for Toxicity Testing (Withdrawn 1998)[3]
D5227 Test Method for Measurement of Hexane Extractable Content of Polyolefins
D5296 Test Method for Molecular Weight Averages and Molecular Weight Distribution of Polystyrene by High Performance Size-Exclusion Chromatography
E3 Guide for Preparation of Metallographic Specimens
E7 Terminology Relating to Metallography
E8 Test Methods for Tension Testing of Metallic Materials
E10 Test Method for Brinell Hardness of Metallic Materials
E18 Test Methods for Rockwell Hardness of Metallic Materials
E45 Test Methods for Determining the Inclusion Content of Steel
E92 Test Method for Vickers Hardness of Metallic Materials (Withdrawn 2010)[3]
E112 Test Methods for Determining Average Grain Size
E120 Test Methods for Chemical Analysis of Titanium and Titanium Alloys (Withdrawn 2003)[3]

E135 Terminology Relating to Analytical Chemistry for Metals, Ores, and Related Materials
E168 Practices for General Techniques of Infrared Quantitative Analysis (Withdrawn 2015)[3]
E204 Practices for Identification of Material by Infrared Absorption Spectroscopy, Using the ASTM Coded Band and Chemical Classification Index (Withdrawn 2014)[3]
E290 Test Methods for Bend Testing of Material for Ductility
E353 Test Methods for Chemical Analysis of Stainless, Heat-Resisting, Maraging, and Other Similar Chromium-Nickel-Iron Alloys
E354 Test Methods for Chemical Analysis of High-Temperature, Electrical, Magnetic, and Other Similar Iron, Nickel, and Cobalt Alloys
E386 Practice for Data Presentation Relating to High-Resolution Nuclear Magnetic Resonance (NMR) Spectroscopy
E407 Practice for Microetching Metals and Alloys
E562 Test Method for Determining Volume Fraction by Systematic Manual Point Count
E663 Practice for Flame Atomic Absorption Analysis (Withdrawn 1997)[3]
E860 Practice for Examining And Preparing Items That Are Or May Become Involved In Criminal or Civil Litigation
E883 Guide for Reflected–Light Photomicrography
E986 Practice for Scanning Electron Microscope Beam Size Characterization
E1188 Practice for Collection and Preservation of Information and Physical Items by a Technical Investigator
E1479 Practice for Describing and Specifying Inductively-Coupled Plasma Atomic Emission Spectrometers
F316 Test Methods for Pore Size Characteristics of Membrane Filters by Bubble Point and Mean Flow Pore Test
F619 Practice for Extraction of Medical Plastics
F981 Practice for Assessment of Compatibility of Biomaterials for Surgical Implants with Respect to Effect of Materials on Muscle and Bone
F1044 Test Method for Shear Testing of Calcium Phosphate Coatings and Metallic Coatings
F1147 Test Method for Tension Testing of Calcium Phosphate and Metallic Coatings
F1854 Test Method for Stereological Evaluation of Porous Coatings on Medical Implants
F1877 Practice for Characterization of Particles
F2102 Guide for Evaluating the Extent of Oxidation in Polyethylene Fabricated Forms Intended for Surgical Implants
F2182 Test Method for Measurement of Radio Frequency Induced Heating On or Near Passive Implants During Magnetic Resonance Imaging
F2214 Test Method for ***In Situ*** Determination of Network Parameters of Crosslinked Ultra High Molecular Weight Polyethylene (UHMWPE)
F2995 Guide for Shipping Possibly Infectious Materials, Tissues, and Fluids

2.2 *Other Document:*[4]
ISO 12891-1, Retrieval and Analysis of Implantable Medical Devices, Part 1: Standard Practice for Retrieval and Handling

3. Terminology

3.1 *Definition of Terms Specific to Issues of Microbial Contamination:*

3.1.1 *antiseptic*—a germicide that is used on skin or living tissue for the purposes of inhibiting or destroying microorganisms.

3.1.2 *decontamination*—a process or treatment that renders a medical device, instrument, or environmental surface safe to handle. Ranges from sterilization to cleaning with soap and water.

3.1.3 *disinfectant*—a germicide that is used solely for destroying microorganisms on inanimate objects.

3.1.4 *disinfection*—generally less lethal than sterilization. It eliminates virtually all recognized pathogenic microorganisms but not necessarily all microbial forms (for example, bacterial endospores) on inanimate objects. It does not ensure overkill.

3.1.5 *sterilization*—use of a physical or chemical procedure to destroy all microbial life; including large numbers of highly resistant bacterial endospores.

4. Summary of Practice

4.1 This practice provides recommendations for collection of clinical data, analysis of adjacent tissues, and the material characterizations to be performed when an implant is retrieved as part of a clinical or an animal study. It also provides for analysis of specimens and lubrication fluids from *in vitro* wear tests.

4.2 The clinical data to be recorded include a case history review, roentgenogram reviews, tissue culture, and observations of the implant site.

4.3 Protocols are provided for the handling of the implant tissue interface, and adjacent tissues and fluids for subsequent analysis. These protocols are intended to facilitate *(a)* histologic and immunohistochemical examination of the tissues, *(b)* chemical analysis of the tissues for identification and quantification of implant corrosion or degradation products, and *(c)* digestion of tissues and fluids for subsequent harvesting and analysis of particulate debris.

4.4 The material characterizations include observation and description of the retrieved device and adjacent tissues, determination of chemical composition, macroscopic and microscopic examinations and mechanical property determinations. The guidelines are separated in three stages. Stage I is considered to comprise an essential minimum analysis for routine examination of all types of materials. Stage II is nondestructive but provides more detail and is intended for special studies of devices with or without impaired function, made of all types of materials. Stage III includes destructive

[4] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

methods for and material-specific protocols for detailed failure, microstructural, and chemical analysis as well as determination of physical and mechanical properties.

5. Significance and Use

5.1 The investigation of retrieved implantable medical devices and adjacent tissues can be of value in the assessment of clinical complications associated with the use of a specific prosthetic device design; can expand the knowledge of clinical implant performance and interactions between implants and the body; provide information on implant performance and safety; and thus further the development of biocompatible implant materials and devices with improved performance. Comparison of wear patterns and wear particle morphology observed with retrievals and those observed with *in vitro* joint simulator tests can provide valuable insight into the validity of the *in vitro* simulation.

5.2 A significant portion of the information associated with a retrieved implant is obtained with detailed studies of the device-tissue interface. Appropriate methods are provided to facilitate a study of the particles in the tissues, and chemical analysis for the byproducts of degradation of the implant, and histologic evaluation of the cellular response to the implant.

5.3 For the analysis to be accurate, it is essential that the device and associated tissues be removed without alteration of their form and structure. It is also essential that the tissues be handled in such a way as to avoid microbial contamination of the work place or the investigator. Standard protocols for the examination and collection of data are provided for retrieval and handling of implantable medical devices, as well as for specific types of materials in relation to their typical applications. For particular investigational programs, additional, more specific, protocols may be required. If special analytical techniques are employed, the appropriate procedures must be specified.

5.4 In order to interpret the analysis of materials and tissues, it is also essential to capture a minimum data set regarding the clinical findings and laboratory studies documenting device performance and reasons for removal.

5.5 Any destructive analysis of implants must be done so as to not destroy any features that may become the subject of litigation, as per Practice E860. This standard recommendation should be applied in accordance with state or national regulations or legal requirements regarding the handling and analysis of retrieved implants and tissues.

6. Interferences

6.1 Some critical features of the retrieved implant, tissue and the interface can only be accurately described by observation at the time of removal, and prior to sterilization or disinfection. Such observation must be made using appropriate aseptic precautions.

6.2 Due to the destructive nature of some of the analysis protocols provided in this practice, their use precludes any other type of analysis. It is therefore essential that handling of the device and tissues be done in concert with the requirements of all of the analyses to be performed, including analyses that may be done in the future. When harvesting tissues for subsequent chemical analysis, it is important to use tools that do not contain the materials or elements of interest in the tissues.

7. Hazards

7.1 The handling of retrieved implants and tissues may involve handling of infectious material.

7.2 It is suggested that individuals handling the devices be vaccinated against Hepatitis B. As a precautionary measure, removed implants should be sterilized by an appropriate means that does not adversely affect the implant.

7.3 There are situations where tissues or implants can not be sterilized or disinfected prior to analysis, for example, requirements of specialized protocols in which sterilization will adversely effect tissue or material properties. In such cases, extreme care should be taken to use aseptic technique and disinfection. Where institutional guidelines for the handling of septic material do not exist, details for handling and sterilizing retrievals, and laboratory practice recommendations can be found in ISO 12891-1.

8. Clinical Information Gathered at the Time of Implant Explantation

8.1 The extent of clinical information to be obtained will depend in part on the type of implant and reasons for removal. Similarly, the amount of information provided about the implant site will depend on the circumstances regarding the removal. A detailed listing and format for documentation of the clinical information associated with removal are provided in Appendix X1. Standard patient evaluation scoring schemes such as those developed by clinical societies may also be utilized.

8.2 As a minimum, the clinical information for device tracking should include the following information:

8.2.1 Date of implantation, and date of explantation.

8.2.2 Identification of hospitals, or physicians' offices, where device implantation and removal was performed.

8.2.3 Confidential, unique, patient ID Code to link to hospitals implantation and removal records.

8.2.4 Device identification (manufacturer's name and device catalogue number).

8.2.5 Device lot and serial number.

8.2.6 Indication for use and reason for explantation (clinical diagnosis).

8.3 For purposes of implant retrieval studies, the following information is considered essential:

8.3.1 Patient or animal age and sex.

8.3.2 A generic statement as to level of patient activity relative to the device.

8.3.3 A statement as to any gross evidence of inflammation, implant site infection, or tissue damage such as osteolysis.

8.3.4 Orientation of the implant relative to the patient. It is suggested that the proximal end of the device be identified with a nondestructive marking scheme.

8.4 More detailed clinical information should be gathered, where feasible, as indicated in Appendix X1. Obtaining an *in situ*, intraoperative photograph of the implant is highly desirable.

8.5 To facilitate subsequent analysis, it is recommended that the device be removed with the tissue interface intact. However, interface preservation should not jeopardize the practice of medicine and patient safety.

8.5.1 In cases of animal studies of tissue responses to implants, the implant should be removed with at least a 4 mm thick layer of adjacent tissue, as per Practice F981.

9. Analysis of the Tissues and the Tissue-Implant Interface

9.1 *Macroscopic Examination of Tissue:*

9.1.1 Record a gross pathologic description of the tissue immediately adjacent to the implant, as to consistency and color, as seen by the naked eye, or with a hand lens or dissecting microscope. Record any differences between the implant-tissue interface and the tissues not in direct contact with the implant. Describe the specimen size either by dimensions or weight.

9.1.2 Since the color of tissue is altered by sterilization and fixation methods, it is recommended that gross observations be made prior to sterilization. Such observations should be made utilizing aseptic techniques.

9.1.3 Where appropriate and feasible, obtain photographic documentation of the explant and adjacent tissue, as well as a photographic record of subsequent dissections.

9.2 *Histopathological Analysis of Tissue:*

9.2.1 Process the excised tissue using standard laboratory procedures for the histological dehydration, embedding and sectioning. These procedures may be for paraffin embedding, methacrylate embedding or other special procedures. Routine staining with hematoxylin and eosin (H & E), or toluidine blue are recommended for light microscopy of soft tissues and bone. Special stains, for example, von Kassa, Masson, Movat pentachrome, may be utilized as indicated and should be fully described.

9.2.2 Provide a detailed histopathologic description of the tissue-implant interface as well as all adjacent tissue specimens, for example, extracellular matrix, necrotic changes, thickness of fibrous capsule, cell types, particulates, hyperplasia, dysplasia, type of inflammatory reaction.

9.2.3 If the implant material is porous, then tissue analysis must include evaluation of the reaction within the pores as well as in the adjacent tissues. This should include the degree and nature of tissue ingrowth, and biological fixation.

9.2.4 For detailed studies of tissue reactions, the use of a quantitative scoring scheme, such as that in Practice F981 is recommended.

9.2.5 Since some polymeric materials, for example, PMMA bone cement, are altered or dissolved by the solutions used for routine histology, special techniques may be indicated, or special note made of voids formerly occupied by the material.

9.3 *Immunohistochemical and Other Special Histopathology Protocols:*

9.3.1 These procedures can be used for identifying specific cell types and extracellular matrix tissue responses to implantable materials and prosthetic devices. This field is constantly changing, and therefore only one such approach is provided as an example.

9.3.1.1 Typical markers chosen are for the presence of immunoglobulins on lymphocytes to indicate B cells or on monocytes/macrophages to indicate activation, the presence of CD2 markers to indicate immature T cells, the presence of CD3 markers to indicate mature T cells, and markers to indicate activated macrophages.

9.3.1.2 The protocols consist of a series of steps or reactions which have been developed to amplify the reactions, and to be cost effective. First, an antibody specific for the CD marker is used (typically mouse anti-human). Then, a biotinilated antibody to the first antibody is applied (typically goat anti-mouse); biotin serves as a marker in this amplification phase of the reactions. Strept-avidine peroxidase is then added to bind to the biotin and immobilize the peroxidase. Finally, a substrate is added which will react with the peroxidase, change color and precipitate. Diaminobenzidine (DAB) is often used, although several substrates are available for different kits or automatic systems. The end result is the peroxidase oxidation of DAB to give a yellow-brown precipitate at the site of the reaction. The sections can be stained with hematoxylin to enhance the visibility of cells.

9.3.1.3 An example of a method to be used is briefly summarized below and is based on standard techniques. Although it was originally described for use on frozen tissues, the use of embedded tissues allows for examination of the same tissue blocks used for routine pathology. This is only one of many approaches.

9.3.2 *Reagents:*

9.3.2.1 The reagents used come from a variety of companies including DAKO, Becton Dickinson, Kirkegaard & Perry, and Oncogene.

9.3.2.2 Antibody for specific markers, for example, CD2, CD3.

9.3.2.3 Biotinilated goat anti-mouse or anti-rabbit IgG.

9.3.2.4 Strept-avidine peroxidase.

9.3.2.5 Diaminobenzidine (DAB), or other suitable substrate.

9.3.3 Sections are deparaffinated in xylene for 5 min twice, and then rehydrated with absolute ethanol for 3 min, 95 % ethanol for 3 min, and then in 70 % ethanol for 3 min.

9.3.4 The sections are then placed in a methanol-hydrogen peroxide solution for 30 min to diminish the background level of peroxidase in the tissue. The sections are rinsed in water, next placed in buffered saline, and then the slide around the section is dried.

9.3.5 The slide is then placed in a humidity chamber, covered with buffer, and the first antibody is added. This will be the antibody specific for the marker (for example, CD2) and will be either of mouse or rabbit origin. This is incubated overnight, then rinsed with buffer, drained, and the slide around the tissue dried.

9.3.6 The second antibody, which is biotinolated, is added. This is usually goat anti-mouse or anti-rabbit IgG. This is

incubated for 30 min, rinsed, the slide dried, and then strept-avidin peroxidase is added.

9.3.7 The strept-avidin peroxidase is incubated for 30 min, rinsed, and then a substrate such as DAB is added. The development of the color is watched under the microscope, the action stopped with water, then the slides are dipped into osmium tetroxide for final fixation. The slides may be counterstained with hematoxylin for visualization of all cells. The slides are processed for mounting with eukitt and can be evaluated for presence of label.

9.3.8 This method can be used to detect the production of cytokines in the cells in the tissues. However, caution should be used in the interpretation of findings, since these are soluble mediators and rapidly leave the site of origin.

9.4 *Chemical Analysis of Tissues By Flame Atomic Absorption Spectroscopy (AAS), Graphite Furnace Atomic Absorption Spectroscopy (GFAAS), by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) or Mass Spectroscopy (ICPMS):*

9.4.1 *Reagents and Materials:*

9.4.1.1 Standard AAS grade solutions (MCB reagents, Fisher, and VWR) are used to make calibration curves. Calibration solutions should be prepared according to Practice D3919, using the same matrix solution as the test specimen. Solutions of low concentration should be made fresh daily. The sensitivity and possible interferences depend on the particular element.

9.4.1.2 Any fixing agents, chemicals and solvents must be of analytic purity. The use of 70 % ethanol is recommended as a transport and storage solution. The use of double distilled, deionized water is necessary.

9.4.1.3 Handling of tissues for subsequent chemical analysis requires special precautions to be taken to insure that the specimens are not contaminated with the elements to be analyzed. Surgical knives or instruments used for tissue excision shall be free of any contamination or loose particulates. The use of ceramic or glass knives is recommended for preparation of specimens associated with metallic implants. Glass knives are not recommended for subsequent silicone analysis.

9.4.1.4 Tissues should be transferred to plastic or glass containers of high quality which have been thoroughly acid cleaned or unused from a lot tested to be free of contamination. Acid cleaning which may etch the glass surfaces is not recommended for subsequent silicone analysis. Tissue transfer should be done in a dust free environment.

9.4.2 Test solutions should be analyzed in triplicate, either as is or after dilution with 1 % nitric acid to a concentration which falls within the standards, and the results averaged. Concentrations are determined in µ/l (ppb), or µ/g of tissue (ppm). Results from solutions of known volumes from *in vitro* studies can be converted to total micrograms in solution.

9.4.2.1 The concentration of metallic species in tissue may vary according to the location of the specimen relative to the implant. It is therefore important to carefully record the location of the specimen.

9.4.3 These methods of analysis require chemical digestion of the tissue samples prior to analysis, and therefore the samples can not be used for any other analysis. The ability to digest tissue is influenced by the method of tissue fixation. It is recommended that tissues be fixed in analytical grade 70 % ethanol in analytical grade water. The methods of digestion depend on the type of tissue to be analyzed.

9.4.3.1 *Blood* samples drawn from patients or animals should be done using polypropylene syringes. The blood can be allowed to clot at room temperature and centrifuged at 1850 g for 30 min to separate serum and clot fractions. Blood may also be drawn in heparinized vacutainer tubes. The blood may be allowed to settle so as to isolate red and white cells, or be centrifuged at 400 g and the plasma supernatant drawn off. Plasma is diluted at least 2× in 1 % nitric acid.

9.4.3.2 *Cells,* either red blood cells or cells from cell culture experiments may receive special treatment, such as separation of cell contents and cell membranes. The cells are washed and centrifuged 3 times with physiologic saline to remove trapped serum or growth media. The cell pellet is then lysed with 1 % Triton X100 to release intracellular contents, centrifuged and the supernatant harvested. This solution is pipetted off, diluted 2× in 1 % nitric acid, and referred to as "cell contents." The pellet of cell membranes is then washed and centrifuged 3 times in saline to remove the Triton and remaining contents. The membranes are then digested in 50 % nitric acid, diluted in 0.5 % nitric acid for analysis as "cell membranes."

9.4.3.3 *Tissue* from implant sites or joint capsules should be weighed and placed in plastic bags.

(1) For the mechanical tissue digestion appropriate bags for the machine should be used. These may then be frozen until use. For preparation, 5 mL of 50 % nitric acid will be added to each bag. The tissue is then homogenized in a mechanical blender.

(2) Alternatively, tissue should be placed in analytical grade 70 % ethanol in analytical grade water in the proper container that will not allow cross-contamination.

(3) The tissue sample may be dried (15 min at 90°C) prior to digestion, to determine the dry weight. Acid digestion of the dry sample can then be accomplished with nitric acid. The dried tissue sample (~100 mg) should be mixed with 5 mL of low trace nitric acid (minimum 70 % HNO_3) and heated for approximately 2 h (or more if needed) at 90°C. Alternatively, the same solution can be placed in a microwave digestion bomb (that is, Parr Microwave Digestion Bombs Model No. 4781 23 mL or 4782 45 mL),[5] which is a chemically inert vessel designed for high heat where venting may be required. These vessels can be placed in a household microwave for more rapid sample dissolution (that is, 2 min at medium power). There are also commercial microwave digestions systems available that operate at higher pressures and temperatures which also have vessels and carrousels as part of the system. (See Note 1.)

(4) The samples are then pipetted into the GFAAS or ICPMS for analysis. Dilution as necessary is done with nitric

[5] The sole source of supply of the apparatus known to the committee at this time is Parr Instrument Co., 211 Fifty Third St., Moline, IL 61265–9984. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

acid. Following the digestion procedure, the solution may need to be diluted to a pre-determined amount (that is, 5:1) using de-ionized water. Dilutions may be necessary if the analytical equipment cannot operate with samples containing high acid concentration.

NOTE 1—Microwave digestion is a good alternative to traditional acid digestion, since microwave dissolution is faster, safer, and simpler, and provides more controlled reproducible conditions than conventional methods. Depending on the power ratings of the microwave, an appropriate level will need to be chosen in conjunction with the adequate time to achieve full digestion of the tissue.

9.4.3.4 *Bone* specimens can be subjected to a two-stage digestion procedure to separate them into two phases, mineralized and demineralized (or organic). The bones are placed in 0.5 N HCl for 48 hrs at 4°C to demineralize them. The rubbery demineralized samples are then placed in 50 % nitric acid to digest the organic portion. Both the HCl and HNO_3 samples are diluted as necessary and analyzed by GFAAS.

9.4.4 Chemical analysis by flame AAS should be done according to manufacturer's instructions, in accordance with E663 and E135.

9.4.5 Analysis with graphite furnace (GFAAS) should be done according to Practice D3919 using the manufacturer's specifications for analyte wavelength and slit width. Calibration standards should be made up in the same matrix as the test specimens.

9.4.5.1 A multi-cycle protocol is recommended to insure complete drying of the specimen prior to charring and atomization; additional steps may be required for post analysis clearing of the tubes.

9.4.5.2 The use of ultra pure argon for clearing is essential.

9.4.5.3 There are two types of graphite tubes used in the furnace: pyrolytic carbon coated graphite tubes, and tubes with L'vov platforms. The analysis for nickel, molybdenum, platinum, titanium, and vanadium are done from the wall of the tubes while cobalt, chromium, and aluminum are done on platforms.

9.4.6 Chemical analysis by ICP should be done in accordance with Practice E1479.

10. Analysis of Tissues and Fluids for Particulate Debris

10.1 Analysis for particulates can be done histopathologically as in 9.2, or by tissue or fluid digestion and particle separation. Tissues subjected to digestion become dedicated to this type of analysis.

10.1.1 Preparation of tissue for drying and digestion will depend on the state of the tissue. If the tissue is embedded in paraffin, the tissue block should be deparaffinized with xylene overnight at room temperature, and then washed with 100 % ethanol. If tissue is fixed in formalin, it should be dehydrated through a series of alcohols using standard histological protocol, and infiltrated with 100 % ethanol. If tissue is fresh, it should be frozen and free of embedding media.

10.1.2 The tissue will be weighed, or the fluid volume determined before digestion. If a wet weight is desired (only applicable for the fresh tissue), 0.3–0.5 g are typically weighed out. For a dry tissue weight, the tissue should be freeze dried, sliced with a ceramic knife, and 0.02–0.03 g weighed out with a microbalance. Weighed tissue should then be placed in acid washed polystyrene vials. Once weighed, the tissue is suitable for digestion.

10.1.3 Several digestion protocols are described in the literature. The choice of protocol depends in part on the type of particle of interest, for example, metal versus polymer, and on the type of tissue. Four methods of digestion are described in this recommended practice.

10.2 *Reagents for Digestion:*

10.2.1 *Ultrapure water*—Distilled H_2O filtered with 0.2 µm filter.

10.2.2 *Phosphate Buffer*—3.55 g Na_2HPO_4, 3.45 g NaH_2PO_4, 0.744 g EDTA, in 100 ml ultrapure water.

10.2.3 *Papain solution*—1 ml phosphate buffer, 100 µL pure papain, 3.26 mg N-acetylcysteine, 9 ml ultrapure water.

10.2.4 *Strong bases*—Tissues have been digested in solutions of sodium hydroxide ranging from 1–10 N NaOH (5–50 ml/g of tissue), or in potassium hydroxide 2 N KOH (10 ml/g of tissue), or 4 M KOH (2 ml/gram of tissue).

10.2.5 *Pronase*—2000 U/ml, 50 mM Tris, 75 mM NaCl.

10.2.5.1 Concentrated nitric acid (HNO_3) has been used when tissues are difficult to digest, especially after fixation and embedding.

10.3 *Procedure for Digestion in Papain:*

10.3.1 Place 1 gram of tissue sample in a clean 50 ml conical tube.

10.3.2 Add 5 ml of papain solution and vortex.

10.3.3 Incubate sample at 65°C for 24 h. If sample does not dissolve add an additional 100 µl papain and vortex.

10.3.4 Centrifuge in an ultracentrifuge for 1 h at 100 000 g. A variable gradient may be used comprising of 2.0 ml each of 5, 10, 20, and 50 % sucrose.

10.3.5 Wash particles in 10 cc of hot filtered water.

10.4 *Procedure for Digestion in Strong Base and Pronase:*

10.4.1 Rinse tissue samples in phosphate buffered saline, and place 1 g in a 15 ml glass tube.

10.4.2 Add 2 ml of 4 M KOH.

10.4.3 Incubate sample at 56°C for 48 h. Mix samples occasionally.

10.4.4 Centrifuge at 1000 g for 1 h. To facilitate sedimentation of debris, add 6 ml of 95 % ethanol to the 2 ml aliquots. Discard clear supernatant, and repeat digestion and centrifugation steps.

10.4.5 Wash debris in distilled water, mix and place in 37°C for 8 h. Mix with excess amounts of ethanol, and centrifuge at 1000 g, 1 h. Repeat washing procedure 3 times.

10.4.6 Digest the organic material with debris in pronase at 37°C for 24 h. During the first 5 min in pronase, ultrasonicate the debris to disaggregate the particles.

10.4.7 Wash the debris 3 times in distilled water.

10.4.8 Resuspend debris (0.5 ml debris/3 ml of ethanol) and add an equal volume of hexane and vortex.

10.4.9 Transfer the cream colored fraction containing the polymeric particles, which stabilized at the hexane-ethanol interface into a clean, sterile tube. Metal debris and bone particles will be in the sediment at the bottom.

10.4.10 Repeatedly add hexane or ethanol to the original tube and vortex. Harvest and pool the polyethylene particles.

10.4.11 To the pooled particles, add 2 ml of ethanol and evaporate any remaining hexane.

10.4.12 Add excess ethanol to the tube and centrifuge at 1000 g for 30 min.

10.4.13 Discard the supernatant and resuspend debris in KOH at 56°C for 8 h.

10.4.14 Add excess ethanol to the tube and centrifuge at 1000 g for 30 min.

10.4.15 Discard the clear supernatant and resuspend particles in 1 ml of distilled water, incubate for 8 h followed by centrifugation in ethanol. Repeat wash procedure three times.

10.4.16 Resuspend approximately 0.5 ml of debris in 2 ml of ethanol. Ultrasonicate for 5 min before transfer to a stub for SEM and particle analysis.

10.5 *Procedure for Digestion in Nitric Acid:*

10.5.1 With this protocol, the digest solution is never filtered, therefore, the submicron particles are not lost and can be evaluated (with SEM and Coulter), without complications that could arise from hemosiderin particles which remain with non-acid digestion protocols.

10.5.2 If paraffin embedded sections are deparaffinated in xylene or toluene, replace the solution with 2 changes of absolute ethanol.

10.5.3 Critical point dry the tissue specimen, slice it and place 0.02 to 0.03 g pieces in plastic tubes.

10.5.4 Add 1.0 ml of concentrated nitric acid.

10.5.5 Digest the tissue for 24 h at room temperature. Shake the tube and sonicate for 2 min. Continue digestion for an additional 24 h.

10.5.6 Centrifuge digestion solution at 9500 g for 5 min. Depending on the amount of debris, there will be a floating band of polymeric debris and a sedimented pellet of metallic debris. These can either be isolated separately, or together.

10.5.7 Separate the fluid from debris by aspirating the clear liquid between the floating band and the pellet.

10.5.8 Add 1 ml of concentrated nitric acid to the debris, sonicate for 2 min and centrifuge at 11 600 rpm for 5 min.

10.5.9 Aspirate the clear liquid and add 1 ml of acetone, sonicate 2 min, and centrifuge at 11 600 rpm for 20 min. Both metallic and polymeric debris will sediment in the acetone.

10.5.10 Aspirate the supernatant and resuspend the debris in 10 µl of dispersant (Coulter I B) and slowly add 1 ml of ultrapure water, with intermittent sonication.

10.6 *Procedure for Tissue or Dried Lubricant or Synovial Fluid Digestion in Sodium Hydroxide:*

10.6.1 For tissue only: mince 2.0–5.0 g of tissue and place in a glass container. To extract lipid, add 2:1 chloroform-:methanol solution and place on an orbital shaker overnight, or until the tissues sink to the bottom of the container. Rinse tissues with 3× filtered (0.2 µm) deionized H_2O.

10.6.2 For extracted tissues and dried (lyophilized) fluid, add 12 ml 5*N* NaOH and incubate at 65°C for 1 to 3 hours on a water bath-shaker.

10.6.3 Allow the digested solution to cool to room temperature, then ultrasonicate for 10 minutes.

10.6.4 Into two clean, particle-free polyallomer centrifuge tubes, place 7 ml each of the digested solution and top off with 5 ml of 5 % sucrose. Ultracentrifuge for 3 hours at 40 000 rpm, 5–15°C.

10.6.5 UHMWPE will rise to the top of each tube to form an opaque layer. Carefully pipette this band into another clean, particle-free vial. Other bands that sometimes appear can be collected separately or pooled with the UHMWPE.

10.6.6 To wash off the sucrose, add 3× filtered deionized H_2O to the collected band until the total volume is 21 ml. Ultrasonicate for 5 min, then heat sample for 1 h at 80°C.

10.6.7 Into three clean, particle-free polyallomer centrifuge tubes, place 7 ml each of the solution and top off with an isopropanol gradient consisting of 3 ml of 0.96 g/cm^3 isopropanol/deionized H_2O and 2 ml of 0.90 g/cm^3 solution. Ultracentrifuge for 1 h at 40 000 rpm, 20–25°C.

10.6.8 Collect the UHMWPE band at the 0.90 and 0.96 g/cm^3 interface into a clean, particle-free vial. Other bands that may appear can be collected separately.

10.7 *Procedure for Sodium Hydroxide Digestion of Protein Solutions from Wear Tests:*

10.7.1 Lubrication fluids harvested during wear or joint simulation tests may be stored frozen at −20°C until digestion.

10.7.2 Digest a minimum volume of 80 ml serum or protein containing solutions by adding KOH pellets to a concentration of 12M. Digest at 60°C for 48 h or until solution clears.

10.7.3 The digested fluids are cooled to 4°C. Lipids and proteins are removed by the addition of an equal volume of chloroform:methanol (2:1). The solutions are then incubated at room temperature for 24 h, and then centrifuged at 2000 g for 10 min at RT. The contaminating lipids and proteins form a layer at the interface of the two solutions. The top layer containing the polyethylene wear particles is then decanted by pipetting into a clean tube and the procedure repeated three times or until the supernatant clears completely, that is, all visible lipids and proteins removed.

10.7.4 Any remaining proteins are removed by precipitation with the addition of an equal volume of ice-cold ethanol. The solutions are incubated at 4°C for 24 h with stirring and then centrifuged at 10 000 g at 4°C for 30 min. The fluid is carefully decanted to a clean vessel to avoid disturbing the protein pellet.

NOTE 2—This protocol will work well for isolation of UHMWPE particles. The enzyme digestion protocol in 10.3 may be found to work better for isolation of metal or ceramic debris in joint simulator fluids.

10.8 *Procedure for Hydrochloric Acid Digestion of Serum Proteins for Harvesting UHMWPE Particles from Wear Test Solutions:*

10.8.1 Lubrication fluids harvested during wear or joint simulation tests may be stored frozen at −20°C until digestion.

10.8.2 Add 4 to 5 parts of 37 % hydrochloric acid to 1 part of the simulator serum solution. (Volumes of 40 to 50 mL of acid to 10 mL of serum are suggested).

10.8.3 Heat the solution to 50 to 60°C for 45 min to 1 h while stirring with a magnetic stir bar at 350 rpm.

10.8.3.1 Digestion of serum protein is generally indicated by fluid becoming clear.

10.8.3.2 The time and temperature for full digestion may depend on the serum type and protein concentration.

10.8.4 Extract 1 mL of solution and add to 100 mL of methanol.

10.8.5 Vacuum filter the solution through a 47 mm diameter polycarbonate filter with a maximum pore size of 0.2 µm.

10.8.6 It has been found that UHMWPE wear particles can be significantly smaller than 0.2 mm in size. Therefore it is recommended that the filter pore size be 0.05 mm or smaller, depending on the application.

11. Analysis of Tissues and Fluids for Particulate Debris using Protocols that Directly Deposit Particles onto the TEM and/or SEM Grid or Wafer

The protocols of this section are adapted from those described in detail and validated in the pair of manuscripts: Billi *et al.* CORR, 2011, Part 1 and Part 2.

11.1 *Procedure for Digestion of Serum Proteins for Harvesting UHMWPE Particles from Wear Test Solutions*

11.1.1 Deionized water is ultrapurified (UP-dH2O) (18.2 MΩ; total organic content, < 5 ppb; pyrogens < 0.001 EU/mL).

11.1.2 All solutions used including UP-dH2O should be filtered through 0.02-µm[6] before use or lyophilization.

Digestion

11.1.3 Lubrication fluids harvested during wear or joint simulation tests shall be stored frozen at –20°C or lower until digestion.

11.1.4 Add 3 mL serum wear lubricant (a volume chosen to provide optimum digestion) to a 50-mL tube containing previously lyophilized 8 mol/L urea, 0.1 mol/L 2-[4-(2-hydroxyethyl)piperazin-1- yl]ethanesulfonic acid (HEPES) buffer (pH 7.5, optimal pH for proteinase activity), and 0.04 % NaN_3 (volume before lyophilization: 6 mL). If the lubricant samples contain ethylenediaminetetraacetic acid (EDTA), add 400 µL 0.5 mol/L $CaCl_2$ to overtitrate the EDTA and improve the activity of proteinase K (pK), which is stabilized in urea in the presence of Ca^{2+} (12–13 mmol/L).

11.1.5 Add 167 µL proteinase K (20 mg/mL) and incubate at 37°C for 18 hours with gyratory mixing (250 rpm).

11.1.6 Remove the sample from the gyrator and sonicate it with a probe four times for 30 seconds, separated by 1 min on ice.

11.1.7 Add 167 µL proteinase K and continue gyratory incubation at 37°C for another 24 hours.

11.1.8 Sonicate the sample again as in 11.1.6, and then add 167 µL proteinase K with gyratory mixing (250 rpm) for another 5 hours.

11.1.9 Repeat the sonication as in 11.1.6, and then add 750 µL 200 mmol/L EDTA (60 mmol/L final concentration) and 850 µL 0.5 mol/L tris(2-carboxyethyl)phosphine (TCEP), 0.1 mol/L HEPES, pH 7.0, 0.04 % NaN3 (final TCEP concentration, 20 mmol/L) to terminate the digestion during 3 additional hours of incubation.

11.1.10 Hold samples at 4°C for 18 hours before purification.

Purification and Display of the Particles

11.1.11 Step 1: Sonicate the sample digest as above and load 7 mL into the bottom of a 14-mL polyallomer centrifuge tube. Overlayer the sample, first with 2 mL buffered 6 mol/L urea and then with 3 mL of a solution containing 20 % sodium lauroyl sarcosine (SLS), 4 mol/L urea, 20 mmol/L EDTA, 50 mmol/L HEPES at pH 7.5, and 0.04 % NaN_3. After centrifugation at 284 000g for 4 hours at 37°C, collect the polyethylene (PE) particles at the liquid-air interface by cutting the tube 1.0 mm below the interface and rinsing the tube/cutter with 1.2 mL SLS/urea solution.

11.1.12 Step 2: Build a continuous isopropyl alcohol (IPA) gradient in a SW40-equivalent tube by sequential layering of 2 mL 20 % IPA, 2 mL 25 % IPA, 1.5 mL 30 % IPA, 1 mL 35 % IPA, and 0.5 mL 100 % IPA and allowing the tube to stand undisturbed for 18 hours at 4°C. Heat the sample from Step 1 at 80°C for 20 minutes and then sonicate without a probe[7] four times for 1 minute with an ice water step of 1 minute between sonications. Next, layer 2 mL 2 % SLS beneath the IPA gradient using a 3-mL syringe attached to a Pasteur pipet, followed by 1.5 mL 20 % SLS in 3 mol/L urea, and finally by 2 mL of the sample from Step 1. Centrifuge the tube at 4446 g for 30 minutes and then at 284 000g for 4 hours, all at 25°C. Collect the PE particles by cutting the tube above and below the isopycnic layer of opaque PE and rinsing the tube/cutter with 1.2 mL 40 % IPA. Dilute the sample with 100 % IPA (2:5) to a volume of 7 mL and hold for 18 hours at 4°C.

11.1.13 Step 3: Sonicate the sample with a probe (any sonicator, cell disruptor, or ultrasonic homogenizer probe should be adequate for this purpose) four times for 30 seconds, separated by 1 minute on ice, and then layer above 50 % (3 mL) and 10 % (2 mL) IPA. Centrifuge the tube at 284 000g for 5 hours at 25°C. Collect the particles at the interface between 50 % and 10 % IPA and store at 4°C for 18 hours.

For morphometric analysis, the particles are deposited onto a 5 × 5 mm silicon wafer that has been coated with a monolayer of marine mussel glue according to the following procedure:

11.1.14 First, clean the wafer by sonication in acetone: IPA (1:1) and then coat it with marine mussel glue.[8]

11.1.15 Add 10 µL marine mussel glue to a microfuge tube, followed by 200 µL 0.2 mol/L HEPES (pH 9.2), 0.15 mol/L NaCl, 0.04 % NaN_3.

11.1.16 Immediately after brief mixing by pipetting, uniformly spread 20 µL of this solution over the silicon wafer and incubate it for 30 minutes at 25°C in a Petri dish to prevent drying.

11.1.17 Remove excess glue by washing with 50 mmol/L HEPES (pH 7.5), 0.15 mol/L NaCl, 0.04 % NaN_3.

11.1.18 Use the wafer either immediately or store it up to 1 hour in 50 mmol/L HEPES (pH 7.5), 0.15 mol/L NaCl, 0.04 % NaN_3.

11.1.19 Sonicate the sample from Step 3 as in Step 3 and mix 125 µL of the sample with 750 µL filtered ultrapurified (UP-dH_2O), and add the mixture to a SW60 (or equivalent) 4.2-mL polyallomer centrifuge tube for flotation of the particles onto an inverted, coated silicon wafer that is positioned at the top of the tube with a custom-made polycarbonate holder (Fig. 1).

[6] Anodisc filters (Whatman International Ltd, Maidstone, UK) has been found satisfactory for this purpose

[7] UIS250v with Vial Tweeter; Hielscher or equivalent.

[8] Cell-Tak BD Biosciences, San Jose, CA have been found satisfactory for this purpose.

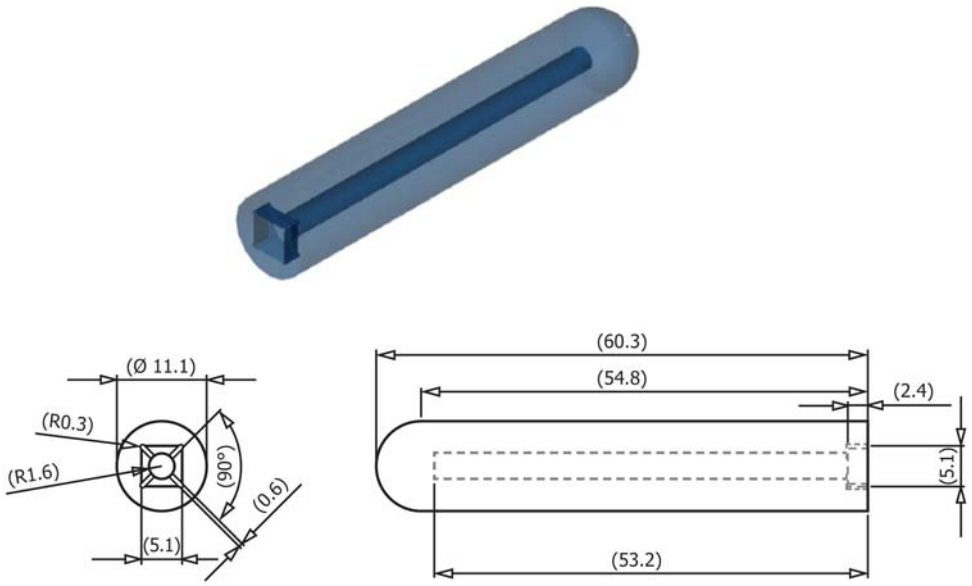

FIG. 1 Polycarbonate Holder for Silicon Wafer (Dimensions in Millimetres)

11.1.20 After centrifugation at 84 000 g for 4 hours, remove the wafer, gently wash it with UP-dH2O water, and dry it in a laminar flow hood to prevent contamination. The volume of the sample might need to be adjusted so that the particles collected on the wafer are well separated, with minimal particle clumping and overlaying.

11.1.21 The particles are verified as PE by the presence on the Fourier transform infrared spectrum of a carbonyl peak located between 1689 and 1756 cm^{-1}.

11.1.22 Given the area analyzed in the images and the cross-sectional area of the centrifuge tube, the approximate total number of particles in the tube can be calculated. Then, taking into account the dilutions, the approximate total number of particles in the original sample can be calculated. Dividing this by the number of cycles experienced by the implant to gives the approximate number of particles generated per cycle.

Rationale

Digestion of lubricant proteins with proteinase K in the presence of urea and calcium leads to more complete proteolytic digestion, and thus, denaturation of proteins due to urea-dependent cleavage of hydrogen bonds without the need for detergents. Inclusion of calcium during digestion partially protects proteinase K from auto-digestion in urea. After digestion, calcium is chelated with excess EDTA to reverse any divalent cation-dependent peptide linkages, and disulfide bonds are broken with TCEP; both steps lead to the smallest possible peptide digestion products. The 37°C temperature assures maximum activity for proteinase K under these conditions.

Purification of the particles is obtained using a three-step ultracentrifugation process. Step 1 utilizes stable reagent layers during centrifugation to minimize handling. Buoyant PE particles move upward in the tube, leaving peptides behind as they enter into the next peptide-free denaturing urea layer. The particles are ultimately deposited in a layer of concentrated detergent (SLS)/urea to solubilize lipids and disperse particles without aggregation. The stable separation of peptide and detergent, and the continuous washing of particles as they pass out of and into the reagent layers, avoids the formation of aggregates of peptides, particles and detergent, which can be difficult to break up.

Step 2 is preceded by heating the particles in detergent/urea to maximize the capacity of SLS to remove any remaining material adsorbed to the particles. In this centrifugation step, particles are floated out of 20 % SLS/urea, through a washing layer of SLS/urea, and through a layer of 2 % SLS. The particles then enter a continuous IPA gradient that serves to strip SLS from the particles and defines the buoyant density of the particles indicated by an opaque band.

Step 3 concentrates particles at the sharp 10 %:50 % IPA interface and further separates the particles from residual detergent. These purified particles are ready for characterization and use in other experimental procedures.

11.2 *Procedure for Digestion of Serum Proteins for Harvesting Ceramic and Metal Particles from Wear Test Solutions*

11.2.1 Deionized water is ultrapurified (UP-dH2O) (18.2 MΩ; total organic content, < 5 ppb; pyrogens < 0.001 EU/mL).

11.2.2 All solutions used including UP-dH2O should be filtered through 0.02-µm[9] before use or lyophilization.

Digestion

11.2.3 Lubrication fluids harvested during wear or joint simulation tests shall be stored frozen at –20°C or lower until digestion.

11.2.4 To concentrate the sample, centrifuge 36 mL (that is, the maximum volume of a SW32 tube) of wear lubricant or particle standard in a siliconized[10] SW32 (or equivalent) polyallomer centrifuge tube for 3 hours at 164 000 g and 25°C.

11.2.5 After centrifugation, remove the supernatant from the centrifuge tube, leaving 4 mL supernatant and the pellet.

[9] Anodisc filters (Whatman International Ltd, Maidstone, UK have been found satisfactory for this purpose.

[10] SurfaSil Siliconizing Fluid; Thermo Fisher Scientific Inc, Waltham, MA have been found satisfactory for this purpose.