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Wear of implant materials — Polymer and metal wear particles — Isolation and characterization

Usure des matériaux d'implant — Particules d'usure des polymères et des métaux — Isolation et caractérisation

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 17853 was prepared by Technical Committee ISO/TC 150, *Implants for surgery*, Subcommittee SC 4, *Bone and joint replacements*.

This third edition cancels and replaces the second edition (ISO 17853:2010), of which it constitutes a minor revision.

Introduction

The biological responses to wear particles contribute to the failure of joint implants through bone resorption and consequent implant loosening. A standardized method of particle retrieval from the tissues followed by particle characterization is necessary to ensure that the investigations of wear particle effects have a uniform approach. The characterization of the particles generated from implants in joint simulators also provides valuable information on the wear properties and performance of the implant being studied.

In the protocols included in this International Standard, for isolation and characterization of particles from both tissues or test fluids from joint simulators, the particles are isolated and then dispersed using filtration or embedding in resin for scanning electron microscopy (SEM) or transmission electron microscopy (TEM) analysis. An alternative protocol for isolation and characterization of metal particles from implants tested in joint simulators has recently been developed in which the particles are deposited on to wafers for SEM analysis, without filtration or embedding[1]. At the time of publication of this International Standard, this alternative method has not been tested for isolation and characterization of particles from tissues and no direct comparison between the different methods is available. Therefore, the latter method has not been included in detail.

Wear of implant materials — Polymer and metal wear particles — Isolation and characterization

1 Scope

This International Standard specifies methods for sampling wear particles generated by joint replacement implants in humans and in joint simulators. It specifies the apparatus, reagents and test methods to isolate and characterize both polymer and metal wear particles from samples of tissues excised from around the joint replacement implant, obtained at revision surgery or post mortem, and from samples of joint simulator test fluids. Some of these procedures could certainly be adapted for isolation and characterization of particles from human biological fluids (e.g. synovial fluid).

The methods given in this International Standard do not quantify the level of wear the implant produces; neither do they determine the amount of wear from any particular surface. This International Standard does not cover the biological effects of wear particles or provide a method for evaluation of biological safety.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
polymer wear particle
particle generated from the wear of polymeric components of an implant

2.2
metal wear particle
particle and particulate corrosion product generated from the wear of metal components of an implant

2.3
ceramic wear particle
particle generated from the wear of ceramic components of an implant

3 Principle, reagents and apparatus

3.1 Principle

Particles of polymeric and metal wear are isolated from tissue samples and simulator lubricants by digestion. The yield of each particle species is then purified by eliminating any remaining organic debris.

NOTE The methods involved in polymer and metal particle isolation are different and are described in 4.2 and 4.3, respectively.

The particles are collected, and are characterized and counted (where applicable) using scanning electron microscopy (SEM) or transmission electron microscopy (TEM).

3.2 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled water or water of equivalent purity.

All reagent solutions shall be filtered through a filter of 0,2 µm or smaller pore size prior to use, to avoid contamination of the sample by extraneous particles.

3.2.1 Absolute ethanol.

3.2.2 Acetone, 100 % or diluted with distilled water, with a volume fraction of 80 % acetone.

3.2.3 Distilled water.

3.2.4 Fixative, e.g. formalin, diluted with distilled water, with a volume fraction of 10 % formalin.

3.2.5 Hydrochloric acid solution, HCl, $c = 0{,}01$ mol/l.

3.2.6 Isopropanol-water mixtures, $\rho = 0{,}96$ g/cm^3 and $\rho = 0{,}90$ g/cm^3.

3.2.7 Papain solution, at 4,8 U/1,5 ml of 250 mM sodium phosphate buffer containing 25 mM ethylenediaminetetraacetic acid solution (EDTA), pH 7,4.

3.2.8 Sodium phosphate buffer, at 250 mM containing 25 mM of EDTA, pH 7,4.

3.2.9 Proteinase K, 2 g/ml of 50 mM TRIS-hydrochloride (TRIS-HCl), pH 7,6.

NOTE For particles isolated from joint simulator serum lubricant, the quantity should be adjusted depending on the serum percentage of the lubricant and initial serum volume from which particles are isolated. See 5.3.2.

3.2.10 Resin, epoxy, such as EMbed 812.

3.2.11 Sodium dodecyl sulfate (SDS), 2,5 g/100 ml of distilled water or 3 g/100 ml of 80 % acetone.

3.2.12 Sodium hydroxide, NaOH, solutions and pellets, $c = 5$ M.

3.2.13 Sucrose solutions, $\rho = 1{,}35$ g/cm^3, 1,17 g/cm^3, 1,08 g/cm^3, 1,04 g/cm^3 and 1,02 g/cm^3.

3.2.14 TRIS-hydrochloride buffer, TRIS-HCl, at 50 mM, pH 7,6.

3.3 Apparatus

All apparatus shall be cleaned and triple rinsed with distilled water previously filtered through a filter of 0,2 µm pore size (3.3.6) before use to remove any contaminating particles.

3.3.1 Aluminium stub.

3.3.2 Balance, with an accuracy of at least 0,1 mg.

3.3.3 Carbon stickers.

3.3.4 Centrifuge tubes, different sizes.

3.3.5 Centrifuge.

3.3.6 Filters, with a pore size of 0,2 µm for filtering reagents and distilled water.

3.3.7 Filtration unit.

3.3.8 Formvar-coated copper grids, of 200 mesh size for TEM analysis.

3.3.9 Fourier Transform Infrared (FTIR) Spectroscope.

3.3.10 Heating plate.

3.3.11 Lint-free cloth.

3.3.12 Pipettes, micropipettes and tips.

3.3.13 Polarizing light microscope.

3.3.14 Polycarbonate membrane filters, of pore sizes 10 µm, 1 µm, 0,1 µm, 0,05 µm and 0,015 µm, for collecting particles.

3.3.15 Scanning electron microscope, SEM, with an energy dispersive X-ray analysis (EDXA) module.

3.3.16 Sterile Petri dishes, with lids.

3.3.17 Syringe, with wide-bore needle.

3.3.18 Teflon-glass potter tissue grinder.

3.3.19 Transmission electron microscope, TEM, with an energy dispersive X-ray analysis (EDXA) module.

3.3.20 Ultrasonic cell disrupter, equipped with a titanium microprobe.

3.3.21 Ultrasonic bath.

3.3.22 Water bath, agitating temperature controlled.

4 Methods of sampling and analysis of polymer and metal wear particles from tissue samples

4.1 Storage and preparation of samples

Store the tissue at −70 °C (or lower) in a freezer, or at room temperature in a fixative such as formalin (3.2.4), diluted with distilled water (3.2.3), with a volume fraction of 10 % formalin. Thaw the tissue, if applicable, and rinse it thoroughly in distilled water before continuing with the extraction method. Remove excess water from the rinsed tissue by blotting with a lint-free cloth (3.3.11).

Unfixed tissue should be handled under universal conditions.

The nature of surgical instruments used for sample retrieval should be recorded in case of contamination.

NOTE Sampling variability due to specimen origin can occur.

4.2 Procedure for polymer particle isolation

4.2.1 Tissue digestion

There are many published methods for polyethylene particle isolation from periprosthetic tissues. The methods presented here are based on those of Campbell et al.[2], Tipper et al.[3] and Richards et al.[4].

Cut the tissue into smaller pieces using a scalpel and blade before digestion to speed up the digestion times. Extract the lipids from the minced tissue by placing into a 2:1 (volume ratio) chloroform:methanol mixture for 24 h or until the tissue sinks to the bottom of the container. Remove and rinse the tissue with PBS (3.2.8).

Add 5 M NaOH (3.2.12) to the tissue (10 ml of 5 M NaOH to 1 g of tissue) and leave to digest for a minimum of 24 h in an agitating water bath (3.3.22) at 65 °C. Digestion can be judged to be complete when no visible solid pieces of tissue remain in the suspension.

4.2.2 Purification of the polymer particle yield

4.2.2.1 General

The polymer particles can be purified from the digested tissue in a number of ways. Use one of the methods described in 4.2.2.2 or 4.2.2.3.

4.2.2.2 Purification of polymer particles by high-speed centrifugation

This method enables all particle sizes to be collected from the nanometre-size range to several millimetres in length, enabling the total wear volume of particles to be isolated. Cool the digested tissue to 4 °C. Add an equal volume of ice-cold absolute ethanol (3.2.1). At this point, salts might precipitate. If this is the case, add ultrapure water until the salt dissolves. Incubate the solution at 4 °C overnight with stirring. Centrifuge the solution at 20 000 g for 2 h at 4 °C. Decant the supernatant liquid into a clean tube (3.3.4) and dilute with 400 ml of ultrapure water prior to filtration.

4.2.2.3 Purification of polymer particles by ultracentrifugation

Place 2 ml of each sucrose solution (3.2.13) (ρ = 1,35 g/cm^3, 1,17 g/cm^3, 1,08 g/cm^3, 1,04 g/cm^3 and 1,02 g/cm^3) into centrifuge tubes (3.3.4) so that the tubes are roughly three-quarters full, and apply measured aliquots of the digested tissue suspension to the surface of the sucrose solution in each tube. Ultracentrifuge at 100 000 g for 3 h at 5 °C. Carefully collect the top layer into a sterile tube and dilute with distilled water at 65 °C to help dilute the residual sucrose. Ultrasonicate for 10 min to break up the agglomerated particles and then heat for 1 h at 80 °C to dissolve the sucrose.

Apply measured volumes of the suspension to two layers of isopropanol-water mixture (3.2.6) of densities 0,90 g/cm^3 and 0,96 g/cm^3 formed in ultracentrifuge tubes. Ultracentrifuge these at 100 000 g for 1 h at 20 °C. After removing the tubes from the ultracentrifuge rotor, a layer of white particles should be visible at the interface of the two layers. Remove this layer, containing the polyethylene particles, and place into a sterile tube using a fine-tipped glass pipette (3.3.12) inserted through the top isopropanol layer. Ultrasonicate for 10 min to break up any aggregates.

Different ultracentrifugation times and speeds may be used, provided that they have been demonstrated to give the same degree of separation and the results of the verification procedure have been documented.

NOTE 1 The first ultracentrifugation step serves to separate the lighter polyethylene wear particles from the heavier fractions. The second ultracentrifugation step purifies the polyethylene particle yield by putting it through a finer density gradient.

NOTE 2 This method might discriminate against the largest sizes of polyethylene generated, and consequently, the total wear volume might not be isolated.

4.3 Procedure for metal particle isolation

Due to the solubility of metals in strong acids and alkalis, an enzymatic digestion method needs to be used. The method below has been described by Catelas et al.[5] and is similar to the procedure developed earlier by the same authors for particle isolation from joint simulator lubricant[6] (c.f. Clause 5), with only minor differences in the initial steps as well as in the enzyme concentrations to account for the use of tissue instead of serum lubricant.

NOTE 1 Being able to use the same procedure to isolate and characterize particles from tissues and joint simulator lubricant enables direct and accurate comparison of the isolated particles[7], which is important, for example, for joint simulator validation. This is a significant advantage of this procedure.

a) Cut the tissue into small pieces using a scalpel and blade to speed up the digestion time. Resuspend several small pieces (about 2 mm × 2 mm × 2 mm) in 2 ml tubes.

NOTE 2 The tissue weight depends on the overall wear noticed in the patient as well as the piece of tissue used for particle isolation (e.g. granuloma, capsule).

The recommended wet weight is 100 mg to 150 mg, but may be adjusted as necessary.

b) Wash four times for 2 min in sodium phosphate buffer (3.2.8), pH 7,4.

c) Resuspend the tissue pieces in 1 ml of SDS (3.2.11) (2,5 g/100 ml of distilled water) and boil for 10 min. While boiling, homogenize the tissue pieces in solution using a Teflon-glass potter tissue grinder (3.3.18) every 2 min.

d) Cool at room temperature for 10 min.

e) Centrifuge the tubes at 16 000 g for 10 min.

f) Wash once with 1 ml of acetone (3.2.2), diluted with distilled water with a volume fraction of 80 % acetone. Centrifuge at 16 000 g for 10 min.

g) Wash three times with 1 ml of 250 mM sodium phosphate buffer containing 25 mM EDTA, pH 7,4. Centrifuge at 16 000 g for 10 min for each wash.

h) Sonicate in 1 ml of 250 mM sodium phosphate buffer containing 25 mM EDTA, pH 7,4, for 20 s to 25 s, using an ultrasonic cell disrupter equipped with a microprobe, or in a sonicating water bath for 30 min.

Using the ultrasonic cell disrupter is more efficient, but an appropriate apparatus with a clean and undamaged/non-corroded probe tip should be used to avoid potential titanium contamination from the probe tip.

i) Add 0,5 ml of 250 mM sodium phosphate buffer containing 25 mM EDTA, pH 7,4, and papain (3.2.7) (4,8 units per 1,5 ml of sodium phosphate buffer). Incubate in an agitated water bath (3.3.22) for 24 h at 65 °C.

j) Centrifuge the tubes at 16 000 g for 10 min.

k) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

l) Resuspend the pellet in 1 ml of SDS (2,5 g/100 ml of distilled water).

m) Boil for 10 min.

n) Cool at room temperature for 10 min.

o) Centrifuge the tubes at 16 000 g for 10 min.

p) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

q) Wash the pellet with 1 ml of 50 mM TRIS-HCl (3.2.14) (pH 7,6). Centrifuge the tubes at 16 000 g for 10 min. Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

r) Repeat step q) once more.

s) Resuspend the pellet in 1 ml of 50 mM TRIS-HCl and sonicate for up to 30 s using an ultrasonic cell disrupter equipped with a microprobe, or in a sonicating water bath for 30 min.

Once again, using the ultrasonic cell disrupter is more efficient, but an appropriate apparatus with a clean and undamaged/non-corroded probe tip should be used to avoid potential titanium contamination from the probe tip.

t) Add proteinase K (3.2.9) (2 g/ml of TRIS-HCl buffer) and incubate for 24 h at 55 °C in an agitated water bath.

u) Centrifuge the tubes at 16 000 g for 15 min.

v) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

w) Add 1 ml of SDS (2,5 g/100 ml of distilled water).

x) Boil for 10 min.

y) Cool at room temperature for 10 min.

z) Centrifuge the tubes at 16 000 g for 15 min.

aa) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

bb) Wash with 1 ml of 50 mM TRIS-HCl (pH 7,6). Centrifuge at 16 000 g for 15 min and carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

cc) Wash with 0,5 ml of 80 % acetone containing 3 % SDS (3.2.11) (3 g/100 ml solution in 80 % acetone). Centrifuge at 16 000 g for 15 min and carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

dd) Wash with 1 ml of distilled water. Centrifuge at 16 000 g for 15 min and carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

ee) Add absolute ethanol (3.2.1) and store at 4 °C until collecting the particles (see 4.4.2).

NOTE 3 Alternatively, steps g) to i) might be performed using 50 mM TRIS-HCl buffer. The efficacy of the papain enzyme used should then be verified.

4.4 Collection of particles

4.4.1 Polyethylene particles

Collect particles on filters of pore size 0,1 µm followed by a 0,015 µm filter, or similar. For particles isolated by high-speed centrifugation or where the whole wear volume is necessary, filter the entire sample volume obtained in 4.2.2.2 using a vacuum filtration system. For particles isolated by ultracentrifugation (see 4.2.2.3), use between 10 µl and 300 µl aliquots of the particle suspension or larger volumes of diluted suspension.

NOTE The appropriate dilution is achieved when the suspension appears almost clear, usually at dilutions between 1:10 and 1:100. The aim is to produce a concentration of particles on the filter which is not so dense as to make visualization of discrete particles difficult by SEM, while ensuring a reasonable number of particles is available for analysis (at least 100 particles).

If diluting, record the exact dilution for each sample. For filtration, take up a known volume of the particle suspension into a syringe (3.3.17) and attach the end of the syringe to the filtration unit (3.3.7). Apply gentle pressure to the syringe in order to push the water through the filter and out at the other end of the filtration unit at a rate of about one drop (0,045 ml) per second, depositing the particles on the surface of the filter. Change the filter if it becomes blocked. Use the following procedure on each filter. Flush the filter and syringe through with distilled water. Finally, flush the filter with air in the same direction as the filtration before removing the filter from the unit, using tweezers, and leaving it to dry in a sterile, covered Petri dish.

Due to the small size of the particles, filters with a pore size smaller than 0,1 µm should be used, if available. In this case, sequential filtering might be necessary to avoid pore clogging.

4.4.2 Metal particles

Centrifuge particles in ethanol (from the isolation protocol described in 4.3) at 16 000 g for 20 min. Remove supernatant liquid and add 0,5 ml of 100 % acetone:epoxy resin (3.2.10) (1:1). Place the tubes in a tube holder and rotate overnight at room temperature (this step could possibly be shortened for very small pellets). Centrifuge the particles at 16 000 g for 20 min. Carefully remove the liquid using a micropipette and without touching the pellet at the bottom of the tube. Leave the tubes under vacuum for 1 h to remove the remaining acetone. Add 1 ml of pure epoxy resin (3.2.10) and leave the tubes under vacuum for 3 h. Place the tubes at 60 °C for 48 h to allow resin polymerization. Remove plastic tubes to obtain solid resin with the particle pellets at the bottom. This protocol enables resin infiltration of the pellets and particle separation.

Section pellets using a diamond knife and spread the sections (about 100 nm to 120 nm thick) on formvar-coated copper grids of 200 mesh size for TEM analysis[5][6]. Section the pellets such that an even representation of all the particles in the pellets can be ascertained (section the whole pellet if possible).

NOTE Metal particles can also be collected by vacuum filtration on a 0,015 µm filter membrane for analysis using high-resolution SEM[8]. However, when using this method, the user needs to check for potential particle agglomeration and oxidation on the filter and be aware of potential particle loss.

4.5 Particle size and shape characterization

4.5.1 Polyethylene particles

For SEM imaging of particles, attach the filter with particles on it to an SEM mount using a carbon sticker (3.3.3). Coat the filter with gold or other conductive material, e.g. platinum/palladium, to make the particles conductive. The coating thickness shall be 3 nm to 5 nm. Image the polymer particles at an accelerating voltage of not greater than 10 keV.

To characterize particles larger than 0,1 µm, select random, non-overlapping fields on the filter carrying the particles at a magnification of at least ×5 000 until a total of 100 particles has been imaged. For particles larger than 10 µm, use a lower magnification such as ×500.

Characterize the size, shape and area of the particles using a series of predefined descriptions such as length, breadth, equivalent circle diameter (diameter of a circle with the same area as particle), area, perimeter, aspect ratio (length:breadth) and roundness (perimeter $1/4\pi \times$ area), as described in ASTM F 1877-05[9]. The magnification at which size and shape analysis was performed should be stated in the test report.

To characterize particles smaller than 0,1 µm, use high-resolution SEM. Use magnifications up to ×100 000 at an accelerating voltage of 3 keV.

NOTE 1 The differentiation of fibrillar and rounded polymer particles can also be useful.

NOTE 2 Computerized or manual image analysis software can be used to determine particle sizes and shapes.

NOTE 3 Particle analysers might also be used if their limit of resolution is 0,1 µm or less, however, there is a risk of size overestimation due to particle agglomeration.

4.5.2 Metal particles

4.5.2.1 General

As described in 4.4.2, particles are typically analysed by TEM, but they could also be analysed by SEM.

4.5.2.2 TEM analysis

Image metal particles using TEM at an accelerating voltage of around 80 kV and a magnification setting of at least ×21 000 (the magnification should be stated in the report).

Characterize metal particles in terms of size and shape by manual image analysis of representative TEM micrographs (minimum 150 particles, in case of low wear; 300 or more, if permitted). Characterize all particles from each micrograph in order to have a better and unbiased representation of the different particle sizes. Determine the maximum dimension (or length) of each particle image along with the maximum orthogonal dimension (or width). Values of the ratio of length over width, r, give information on particle shape. Particles can be arbitrarily classified as round if $1 \leqslant r < 1{,}5$; oval if $1{,}5 \leqslant r < 2{,}5$; needle-shaped if $r \geqslant 2{,}5$[5][6]. A custom software program can be written for automation of particle data processing.

NOTE TEM accelerating voltage might be adjusted as needed to optimize the particle imaging.

The use of a computerized image analysis software may be considered for particle characterization, but should first be checked for accuracy (e.g. comparing the analysis of at least one sample using both computerized and manual analysis). Manual image analysis remains preferable and is highly recommended.

4.5.2.3 SEM analysis

For SEM imaging of particles, attach the filter with particles on it to an SEM mount using a carbon sticker. Coat the filter with gold or other conductive material, e.g. platinum/palladium, to make the particles conductive. The coating thickness shall be 3 nm to 5 nm. Use high-resolution SEM at magnifications between ×60 000 and ×150 000, and at an accelerating voltage of 3 keV to capture images of particles in a randomly selected area of the filter.

As for TEM analysis, characterize metal particles in terms of size and shape by manual image analysis of representative SEM micrographs (minimum 150 particles). Determine the maximum dimension (or length) of each particle image along with the maximum orthogonal dimension (or width). Values of the ratio of length over width, r, give information on particle shape. Particles can be arbitrarily classified as round if $1 \leqslant r < 1{,}5$; oval if $1{,}5 \leqslant r < 2{,}5$; needle-shaped if $r \geqslant 2{,}5$. Particle area can also be measured. A custom software program can be written for automation of particle data processing.

As for TEM analysis, the use of a computerized image analysis software may be considered for particle characterization, but should first be checked for accuracy (e.g. comparing the analysis of at least one sample using both computerized and manual analysis). Manual image analysis remains preferable and is highly recommended.

4.6 Particle identification

4.6.1 Polyethylene particles

Verify the identity of the retrieved particles as polyethylene using Fourier Transform Infrared (FTIR) Spectroscopy. Particles shall be prepared for FTIR spectroscopy by drying and pressing into potassium bromide (KBr) discs or using a microscope attachment.

Particles shall be considered to be ultra high molecular weight polyethylene (UHMWPE) if the dominant peaks in the FTIR spectra are comparable to those of a reference UHMWPE spectrum, such as the spectrum obtained from medical grade UHMWPE powder.

Particle morphology might be used as an additional basis for identifying UHMWPE particles by reference to published images of UHMWPE particles (see, for example, ASTM F 1877-05[9]).

NOTE Particles from different samples might need to be pooled to provide a sufficient volume of material for FTIR spectroscopic analysis.

4.6.2 Metal particles

The composition of the metal particles shall be determined using energy dispersive X-ray analysis (EDXA). Diffraction patterns can also be used to confirm the identification.

5 Methods of sampling and analysis of polymer and metal particles from joint simulator lubricants

5.1 General

The volume of bovine serum used as test fluids in joint simulators can vary between simulators and between tests. As it is impractical to process the total volume of serum for particle analysis, representative samples of the serum should be taken as aliquots from the well-mixed test fluid and stored frozen until required. Ensure that the sample is representative by scraping deposits from the surfaces and stirring the test fluid before sampling.

5.2 Procedure for polymer materials — For example UHMWPE and polyetheretherketone (PEEK)

5.2.1 General

Due to the absence of tissue, the procedure for particle isolation of simulator lubricant samples differs from the procedure described in 4.2. Two published methods have been established by test laboratories worldwide. The selection of the appropriate method depends on the particle material being isolated.

5.2.2 Serum digestion with hydrochloric acid

The following method has been published by Scott et al.[10] and was originally used for UHMWPE materials only. The isolation of other materials such as PEEK and ceramics has been found to be successful with the acid digestion method, but resistance to hydrochloric acid should be investigated carefully for all materials other than UHMWPE.

a) Add 10 ml of the serum sample to 40 ml of hydrochloric acid (37 % volume fraction).

b) Mix with a stirrer bar for approximately 1 h at 50 °C.

NOTE The fluid turns a slightly purple colour.

c) Add 100 ml of methanol to 0,5 ml of the digestion solution.

5.2.3 Serum digestion with NaOH

The following method is based on those published by Campbell et al.[2] and Richards et al.[4].

a) Lubricant samples should be digested with 5 M NaOH in a 60 °C agitated water bath for a minimum of 24 h, or until fully digested. Cool samples to 4 °C for a minimum of 2 h. Ensure samples are completely cold.

b) Add an equal volume of chloroform:methanol (2:1) to each sample and incubate at room temperature for 24 h.

c) Centrifuge digested samples at 500 g for 10 min at room temperature, and decant the supernatant liquid into a clean tube.

d) Repeat steps b) and c) three to four times until the supernatant liquid is visibly clear.

e) Pool the samples and add an equal volume of absolute ethanol to precipitate proteins.

f) Add ultrapure water until the solution turns transparent and incubate the samples overnight at 4 °C with stirring.

g) Centrifuge the samples at 20 000 g for 2 h at 4 °C.

h) Decant the supernatant liquid into a clean tube and add an equal volume of ultrapure water prior to filtration.

5.2.4 Collection of particles

The particles are collected by filtering through a 0,05 µm polycarbonate filter membrane. Smaller pore sizes such as 0,015 µm might be necessary for materials known to generate nanometre-sized particles. Alternatively, a sequence of progressively smaller filter sizes, e.g. 10 µm, 1 µm and 0,015 µm, should be used for heavily loaded simulator lubricants to enable observation of individual particles.

5.2.5 Particle size and shape characterization

SEM images of the particles should be recorded. The magnification of the images depends on the particle size and typically varies between ×500 and ×150 000. A minimum of 100 particles should be analysed in accordance with the method described in 4.5.1.

5.2.6 Particle identification

The identity of particles shall be determined using Fourier Transform Infrared Spectroscopy, as described in 4.6.1.

5.3 Procedure for metal particles

5.3.1 General

Due to the solubility of metals in strong acids and alkalis, an enzymatic digestion method needs to be used. The method described in 5.3.2 has been published by Catelas et al.[6]. Some minor adjustments were made by the authors compared to the initial procedure they had developed[11] in order to accommodate the use of larger volumes of serum involving more organic contaminants.

The procedure is the same as the one described for isolation and characterization of particles from tissues[5] (c.f. Clause 4), with only minor differences in the initial steps, as well as in the enzyme concentrations to account for the use of serum lubricant instead of tissue.

NOTE 1 Being able to use the same procedure to isolate and characterize particles from tissues and joint simulator lubricant enables direct and accurate comparison of the isolated particles[7], which is important, for example, for joint simulator validation. This is a significant advantage of this procedure.

NOTE 2 The procedure described in 5.3.2 was initially developed for the isolation of particles from 95 % serum lubricant. It can therefore be used for particle isolation from fluids with very high protein content.

NOTE 3 This procedure could certainly be adapted for the isolation and characterization of particles from human biological fluids (e.g. synovial) because of its multiple digestion steps for various types of organic components.

5.3.2 Serum digestion

a) Centrifuge a 15 ml serum sample at 16 000 g for 10 min in a 25 ml glass tube (a glass tube is preferable to avoid particles sticking to the tube walls).

NOTE 1 The volume of serum necessary depends on the total volumetric wear measured from the implant cycled in the joint simulator. A sample of 15 ml of serum from a 100 ml aliquot that contains a total volumetric wear of about 0,4 mm^3 to 0,8 mm^3, provides a pellet of particles. If the volumetric wear is lower, multiple samples of 15 ml can be used and pooled after the digestion step with papain [step o)].

b) Carefully remove the serum leaving about 1,5 ml. Resuspend the pellet in the 1,5 ml of serum and transfer the solution to a 2 ml microcentrifuge tube.

c) Centrifuge at 16 000 g for 10 min. Carefully remove the liquid using a micropipette. Do not touch the pellet at the bottom of the tube.

d) Resuspend the pellet in SDS (2,5 g/100 ml solution in distilled water).

e) Boil the tubes for 10 min.

f) Cool at room temperature for 10 min.

g) Centrifuge the tubes at 16 000 g for 10 min.

h) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

i) Wash the pellet with 1 ml of acetone, diluted with distilled water with a volume fraction of 80 % acetone. Centrifuge the tubes at 16 000 g for 10 min. Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

j) Wash the pellet with 1 ml of 250 mM sodium phosphate buffer containing 25 mM EDTA, pH 7,4. Centrifuge the tubes at 16 000 g for 10 min. Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

k) Repeat step j) twice more.

l) Resuspend the pellet in 1 ml of 250 mM sodium phosphate buffer containing 25 mM EDTA, pH 7,4 and sonicate for 10 s to 15 s using an ultrasonic cell disrupter equipped with a microprobe, or a sonicating water bath for 30 min.

Using the ultrasonic cell disrupter is more efficient, but an appropriate apparatus with a clean and undamaged/non-corroded probe tip shouls be used to avoid potential titanium contamination from the probe tip.

m) Add 0,5 ml of 250 mM sodium phosphate buffer containing 25 mM EDTA, pH 7,4 and papain (4,8 units per 1,5 ml of sodium phosphate). Incubate in an agitated water bath for 24 h at 65 °C.

n) Centrifuge the tubes at 16 000 g for 10 min.

o) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

When multiple serum volumes of 15 ml each are initially used (in case of low wear), the corresponding pellets should be resuspended in distilled water and pooled in one tube in a total volume of 1 ml of distilled water. The resulting tube should then be centrifuged at 16 000 g for 10 min, and the liquid removed using a micropipette without touching the pellet at the bottom of the tube.

p) Resuspend the pellet in 1 ml of SDS (2,5 g/100 ml solution in distilled water).

q) Boil for 10 min.

r) Cool at room temperature for 10 min.

s) Centrifuge the tubes at 16 000 g for 10 min.

t) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

u) Wash the pellet with 1 ml of 50 mM TRIS-HCl, pH 7,6. Centrifuge the tubes at 16 000 g for 10 min. Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

v) Repeat step u).

w) Resuspend the pellet in 1 ml of 50 mM TRIS-HCl and sonicate for up to 5 s using an ultrasonic cell disrupter equipped with a microprobe, or a sonicating water bath for 30 min.

Once again, using the ultrasonic cell disrupter is more efficient, but an appropriate apparatus with a clean and undamaged/non-corroded probe tip should be used to avoid potential titanium contamination from the probe tip.

x) Add proteinase K (0,9 g/ml of TRIS-HCl for an initial serum volume of 15 ml; concentration should be adjusted depending on the initial volume of serum) and incubate for 24 h at 55 °C in an agitated water bath.

y) Centrifuge the tubes at 16 000 g for 15 min.

z) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

aa) Add 1 ml of SDS (2,5 g/100 ml solution in distilled water).

bb) Boil for 10 min.

cc) Cool at room temperature for 10 min.

dd) Centrifuge the tubes at 16 000 g for 15 min.

ee) Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

ff) Wash with 1 ml of 50 mM TRIS-HCl (pH 7,6). Centrifuge the tubes at 16 000 g for 15 min. Carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

gg) Wash with 0,5 ml of 80 % acetone containing 3 % SDS (3 g/100 ml solution in 80 % acetone). Centrifuge at 16 000 g for 15 min and carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

hh) Wash with 1 ml of distilled water. Centrifuge at 16 000 g for 15 min and carefully remove the liquid using a micropipette without touching the pellet at the bottom of the tubes.

ii) Add absolute ethanol and store at 4 °C until collecting the particles (see 5.3.3).

This protocol is efficient for extracting particles from 95 % serum. In case of lower serum percentage, some steps could possibly be removed. However, if comparing particles from joint simulator lubricant with particles from tissues, the same procedure should be used for accurate comparison.

NOTE 2 Alternatively, steps j) to m) might be performed using 50 mM TRIS-HCl buffer, especially when isolating particles from lower serum percentage.

5.3.3 Collection of particles

Centrifuge the particles in ethanol at 16 000 g for 20 min and follow the procedure described in 4.4.2 for particle embedding in epoxy resin and pellet sectioning.

NOTE Alternatively, particles might be collected by filtration, as described in 4.4.2.

5.3.4 Particle size and shape characterization

5.3.4.1 General

As described in 4.4.2, particles are typically analysed by TEM, but they could also be analysed by SEM.

5.3.4.2 TEM analysis

Image metal particles using TEM at an accelerating voltage of around 80 kV and a magnification setting of at least ×21 000 (the magnification should be stated in the report), as described in 4.5.2.2.

Metal particles can be characterized in terms of size and shape by image analysis of representative TEM micrographs (minimum 150 particles, in case of low wear; 300 or more, if permitted), as described in 4.5.2.2.

5.3.4.3 SEM analysis

For SEM imaging of particles, attach the filter with particles on it to an SEM aluminium stub (3.3.1) using a carbon sticker. Coat the filter and observe using high-resolution SEM, as described in 4.5.2.3.

As for TEM analysis, characterize the particles using image analysis, as described in 4.5.2.3.

5.3.5 Particle identification

The composition of the metal particles shall be determined using energy dispersive X-ray analysis (EDXA) as described in 4.6.2. Diffraction patterns may also be used to confirm the identification.

5.4 Procedure for ceramic particles

This International Standard focuses on methods for isolation and characterization of polymer and metal wear particles from both tissues and test fluids from joint simulators. However, some of these methods could also be applied for the isolation and characterization of ceramic particles, taking into consideration the following recommendations.

Due to the density of ceramic materials used in orthopaedics, ceramic wear particles cannot be isolated by the alkali method described above (see 5.2.3) or by the method employing density centrifugation (as described in 4.2.2.3). Therefore, it is recommended that ceramic particles be isolated either by the hydrochloric acid digestion method described above (see 5.2.2), or by using the enzymatic digestion protocol described in 5.3.2. However, if hydrochloric acid digestion is used, resistance to the acid should be investigated carefully.

6 Test report

The test report shall include at least the following information:

a) identity of the test specimens (anonymized patient details in the case of tissue samples, location of tissue samples, identification of post-mortem specimens, test details in the case of joint simulator test fluids);

b) type of implant and details of previous surgery, if available;

c) appearance of the retrieved prosthesis (for information regarding the source of particle generation or implant failure such as impingement of the femoral neck against the socket rim or loosening of the stem), if available;

d) mass of tissue sample or total volume of joint lubricant used;

e) data on particle sizes, shapes and numbers, with distributions;

f) composition of the metal particles;

g) number of particles analysed;

h) magnifications used for SEM or TEM particle counts and particle size and shape analysis;

i) TEM accelerating voltage used to visualize the particles;

j) in the case of tissue samples, the nature of the surgical instruments used for sample retrieval;

k) if contamination is present, a list of possible sources of metal/polymer particles.

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