FINAL
DRAFT

TECHNICAL SPECIFICATION

ISO/DTS 7833

ISO/TC **229**

Secretariat: **BSI**

Voting begins on:
2023-11-07

Voting terminates on:
2024-01-02

Nanotechnologies — Extraction method of nanomaterials from lung tissue by proteinase K digestion

Nanotechnologies — Méthode d'extraction de nanomatériaux d'organes par digestion par protéinase K

Reference number
ISO/DTS 7833:2023(E)

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Published in Switzerland

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Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

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Introduction

Quantification of nanomaterials deposited in organs is important to evaluate the lung burden in an inhalation toxicity study and organ distribution in toxicokinetics studies[3][4][5]. Owing to the long retention period of nanomaterials deposited in the alveoli in an inhalation setting, the OECD revised the subacute and subchronic inhalation test guidelines, TG 412 and TG 413 respectively, to include lung burden analysis when testing poorly soluble nanomaterials[3][4]. However, the lung burden analysis method varies depending on the nanomaterials, thus the development of standard methods is highly needed. In addition, a new or revised OECD toxicokinetics test guideline (i.e. TG417) is needed to accommodate nanomaterials[6][7]. Furthermore, OECD launched a new project (1.10) for developing a guidance document on the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies[8]. ISO/TR 22019 addresses considerations for performing toxicokinetic studies with nanomaterials. However, standard methods to measure concentrations of nanomaterials deposited in organs are needed to complement TG 412, TG 413 and ISO/TR 22019.

Quantification of nanomaterials in organs can be divided into two steps:

a) collection of nanomaterials from organs;

b) quantification of nanomaterials using instrumental analysis.

To collect nanomaterials deposited in organs, chemical or enzymatic digestion methods can be used. The ultimate goal of step a) is to collect the particle in particle-form (i.e. the same material that animals were exposed to) rather than the ionic counterparts. However, many of the chemicals used for digestion such as hydrogen chloride, nitric acid, and hydrofluoric acid can ionize some nanomaterials or damage their structure[9][10]. Among nanomaterials, metals or metal oxides can be dissolved by chemicals for digesting organs. Thus, the measured amount of metal ions in organs treated with these digestion chemicals would not be the amount of nanomaterials inhaled. It could be the ionic counterparts of these nanomaterials as well as the same metal present as endogenous ions in the organ[11][12]. Although carbon-based nanomaterials such as carbon nanotubes (CNTs), graphene, and nanodiamonds are not dissolved by chemical digestion, the structure of the carbon-based nanomaterials can undergo alterations including defects and oxidation[9]. The second step is the quantification of nanomaterials by instrumental analysis including methods such as inductively coupled plasma mass spectrometry (ICP-MS), fluorometry, and optical absorbance spectrometry. Because the instrumental analysis is diverse and needs to correspond to the physicochemical properties of the nanomaterial analysed, this document focuses on the method of extracting nanomaterials from organs.

In contrast, the enzymatic digestion of the mixture of powderised lung tissue and nanomaterials in vitro, and lung tissue instilled nanomaterials in vivo using proteinase K (PK) can successfully dissolve tissues with less alterations of the structure of carbon-based nanomaterials and many metal oxides compared to the chemical digestion method[13]. This method allows to collect nanomaterial particles separately from their ionic counterparts dissolved in supernatants. In a previous study, the PK digestion successfully digested lung tissues, and it was possible to separately collect carbon-based nanomaterials including carbon black, carbon nanotube, carbon nanofibre, graphene, and nanodiamond[13]. Other studies have also demonstrated the use of this method to successfully collect and quantify single-walled carbon nanotubes (SWCNTs) instilled into mouse lung, CNTs spiked into rat lung tissue, and microplastics in marine invertebrates species[9][14][15]. However, misleading or inaccurate results may occur if nanomaterials are dissociated or dissolved during the process of enzymatic digestion. The examples of nanomaterials for which the PK digestion method is applicable or not applicable are listed in Annex A. Although this document focuses on the lung tissue digestion, it can be further applicable to other tissues. However, organs besides the lung should be tested for their validity based on this document because the efficacy of PK for tissue digestion varies by the organ-specific nature. Therefore, an optimized procedure to extract nanomaterials from lung tissue is highly needed as a part of recommendations and guidelines on how to conduct lung burden analysis or toxicokinetic studies.

Nanotechnologies — Extraction method of nanomaterials from lung tissue by proteinase K digestion

1 Scope

This document provides an extraction method using the proteinase K (PK) for nanomaterials deposited in the lung. This document specifies the advantages of the PK digestion method and examples of nanomaterials to which it can be applied. This document focuses on extracting nanomaterials from lung tissue and separating nanoparticles from their ionic counterparts. This method is potentially (or theoretically) applicable to any particles that are insoluble during the PK digestion process.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in and ISO 80004-1 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
nanoparticle
nano-object with all external dimensions in the nanoscale

Note 1 to entry: If the dimensions differ significantly (typically by more than three times), terms such as nanofiber or nanoplate are preferred to the term nanoparticle.

[SOURCE: ISO 80004-1:2023, 3.3.4]

3.2
nanotube
hollow nanofibre

[SOURCE: ISO 80004-1:2023, 3.3.8]

4 Symbols and abbreviated terms

PK	proteinase K
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectroscopy
sp-ICP-MS	single particle inductively coupled plasma mass spectrometry
TEM	transmission electron microscopy
EDS	energy-dispersive X-ray spectroscopy
DMSO	dimethyl sulfoxide
CNT	carbon nanotube
SWCNT	single-walled carbon nanotube
MWCNT	multi-walled carbon nanotube
CB	carbon black
ND	nanodiamond
rGO	reduced graphene oxide
UV-Vis	Ultraviolet–visible
PBS	phosphate-buffered saline
ALF	artificial lysosomal fluid
SDS	sodium dodecyl sulfate

5 Materials - PK digestion buffer and optimal concentration for lung tissue digestion

Because PK requires activators such as Ca^{2+}, the addition of $CaCl_2$ in the digestion buffer act as an activator of PK[16]. The protein denaturing agents such as SDS and urea can stimulate the activity of PK[16]. To select an optimal recipe for PK digestion buffer, four recipes were tested by incubating at 56 °C for 24 h of homogenised lung tissue with PK at 10 µg (equivalent to 0,2 U to 0,3 U) per milligram dry mass of lung tissue homogenates (see Annex B). Then, the absorbance of digested samples was tested at 750 nm wavelengths. From this experiment, an optimal buffer recipe was selected as 30 mM Tris-HCl, 10 mM EDTA, 1 % SDS, 5 mM $CaCl_2$, and pH 8,0 (see Annex B). Then, with the selected PK digestion buffer, the optimal concentration of PK for lung tissue digestion was selected by incubating various concentrations of PK with the 20 mg dry mass of lung tissue homogenates (see Annex C). The result showed that the optimal concentration was 10 µg, which is equivalent to about 0,2 U to 0,3 U. One unit of enzyme liberates Folin-positive amino acids and peptides, corresponding to 1 µmol in 1 min at 37 °C using denatured hemoglobin as substrate[17].

6 Technical equipment

6.1 Vessels

A sterile microcentrifuge tube (1,5 ml or 2 ml) can be used. All tubes and glassware are required to be resistant to protein adsorption. Based on the amount of tissue, 15 ml or 50 ml tube can be used. A Petri dish (90 mm × 15 mm) can be used for drying tissue slices.

6.2 Heat block or water bath

Heat block or water bath with controlled temperature (56 °C ± 1 °C) can be used.

6.3 Drying oven

Any drying oven that controls 60 °C can be used. In addition, a lyophilizer that can dry sliced tissue is optional technical equipment.

6.4 Micro ball mill

The micro ball mill can be used to homogenise (or powderise) tissues.

6.5 Microcentrifuge or ultracentrifuge

Microcentrifuge which can centrifuge at 21 000 g or higher is recommended. Because the speed and duration of centrifugation are related to physicochemical characteristics of the nanomaterials such as size and density, an optimal speed and duration should be determined based on whether it can completely spin down nanomaterials. For nanomaterials that cannot be pelleted by conventional microcentrifuge, a higher speed centrifuge such as ultracentrifuge can be used.

6.6 Bath sonicator

The bath sonicator can be used. The power of the bath sonicator can vary but normally, 400 W and 40 kHz are acceptable.

6.7 Pipettes

The single-channel pipette can be used.

7 Procedures

7.1 Preparation of lung tissue sample for digestion

7.1.1 Sampling and drying for lung tissue samples

The collected lung tissue should be weighed before and after drying to report the concentration of nanomaterials per wet or dry mass of lung tissue. Lung tissue should be sliced into pieces with a diameter of about 2 mm. The size of tissue slices can vary. The sliced tissues places in a Petri dish and dried at 60 °C for 2 d in a drying oven. The other drying options such as lyophilization can also be considered. The drying time can be shortened if the lung tissue are dried earlier than the expected time. The drying and powderisation process is highly recommended in this protocol because the lung remnant after 2 d of incubation with PK was minimal in the dried and powdered sample (see Annex D).

7.1.2 Homogenisation of dried tissue slices

The dried tissue slices are weighed and homogenised using a micro ball mill at 1 300 r/min for 40 s. The speed and duration of homogenisation can vary by the types of tissues. The vessel for a micro ball mill

should be a microcentrifuge tube and 0,08 g/tube is general acceptance. The homogenisation process using micro ball mill can be omitted, but this process can reduce the period and efficacy of tissue digestion by PK (see Annex D).

7.2 Tissue digestion by PK

The homogenised tissues should be mixed with PK solution, which is obtained by dissolving PK into a buffer solution, as specified in Annex B. The amount of PK should be 10 µg which is equivalent to 0,2 U to 0,3 U per 1 mg dry mass of tissue homogenates and incubated at 56 °C for 24 h using a heat block or water bath. After 24 h incubation, the solution should be centrifuged at a certain speed, which should be enough for pelleting nanomaterials. After centrifugation, fresh PK solution at the same concentration should be added and should be dispersed nanomaterials using a bath sonicator. The condition of sonication such as powder and duration should be adjusted for optimal dispersion of nanomaterials. The re-dispersed suspensions should be incubated at 56 °C for 24 h using a heat block or water bath.

7.3 Collection of nanomaterials and preparation for instrumental analysis

The suspension of nanomaterials after the second incubation with PK should be centrifuged at a certain speed, which should be enough for pelleting nanomaterials. After centrifugation, the dispersion medium for instrumental analysis should be added to the collected nanomaterials and further treatment such as sonication is needed. If the ICP analysis is applied, the nanomaterials should be dissolved by incubating with acids. Otherwise, the sp-ICP-MS, UV-Vis spectrophotometer, fluorimeter, or relevant instruments can be applied for the quantification of particle-form of nanomaterials.

8 Methodological considerations for the digestion by PK

8.1 Separative collection of nanomaterials from their ionic counterparts

The separation of nanomaterials from their ionic counterpart is possible by collecting the supernatants of the two centrifugation steps. An instrumental analysis such as ICP-MS can be applied to the supernatant to measure the concentration. If the nanomaterial is dissolving in organs but the nanomaterial-composing elements are absent in organs, the concentration of supernatant must be the concentration of the dissolved ions from nanomaterials. If the nanomaterial-composing elements are detected in organs, the subtraction from the concentration in organs of vehicle control can provide the dissolved ions from nanomaterials.

8.2 Types of nanomaterials applicable to this method

This method aims to collect nanomaterials in their particle form, in the lung tissue, and is applicable to nanomaterials that are least affected by the neutral environment during the PK digestion. For example, this method is not applicable to zinc oxide and copper oxide as they dissolve during the PK digestion process (see Table A.1). However, this method can be used for PEGylated AgNPs (AgNP-PEGs) but not pristine AgNPs, as AgNP-PEGs are stable during the PK tissue digestion process. Therefore, the applicability of this method is not directly related to the nanomaterial solubility potential in the biological media. In this regard, the changes in morphology and chemical composition of nanomaterials during the PK digestion process of nanomaterials-spiked lung tissue should be evaluated using TEM (as shown in A.2) and EDS analysis. In addition, this method is also applicable to evaluate the biotransformed nanomaterials such as Ag to Ag_2S[18][19] and “sea urchin” like rare earth oxide nanomaterials[20], if these particles are not dissolving in the PK digestion process. A list of nanomaterials that can be used with this method is described in Annex A.

8.3 The impact of blood in organs on this method

The perfusion process is not required in this method but is recommended if the investigator's objective is to determine the quantity present in the organ tissue itself and not to include the contribution of