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Nanotechnologies — Assessment of nanomaterial toxicity using dechorionated zebrafish embryo

Nanotechnologies — Évaluation de la toxicité des nanomatériaux au moyen d'embryons déchorionés de poisson zèbre

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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The committee responsible for this document is ISO/TC 229, *Nanotechnologies*.

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Introduction

Fish assays are important and widely used tools for evaluating the toxicity of chemicals in the aquatic environment. However, there are animal welfare concerns regarding the use of vertebrate animals for chemical testing, including fish. The use of early life stage embryos, instead of adult or juvenile fish, is considered an alternative assay because there are animal welfare benefits to testing fish embryos as an alternative to the clear distress caused by testing more developed juvenile fish, e.g. by using OECD TG 203.

Nanotechnology is positively affecting many commercial sectors, but there remain concerns regarding the potential adverse environmental effects from nano-enabled products. The OECD test guideline using fish embryos to evaluate acute toxicity (see OECD TG 236) states that some substances having a molecular weight ≥ 3 kDa, a very bulky molecular structure, and substances causing delayed hatching might be inappropriate for testing using that method. The presence of the chorion could also confound assessment of the nanomaterials biological activity. The chorion is the outmost acellular envelope of a fish embryo and it can serve as an exposure barrier for some chemicals or nanomaterials. It is currently not possible to predict which nanomaterials might be blocked by the chorion. Using dechorionated embryos for toxicity assessments may not provide direct ecotoxicological information, but may help to better identify potentially hazardous nanomaterials. Accordingly, many researchers around the world have developed a number of methods for removing a chorion from early life stage zebrafish embryos[1][2]. There are two ways to remove chorion from embryos: by enzymatic or mechanical method. The enzymatic dechorionation method has some advantages over the mechanical dechorionation method (see Annex A), including time and labour efficiency by easy preparation for dechorionation, no mechanical embryonic damage, and the ability to simultaneously prepare a large number of dechorionated embryos for a high throughput-based approaches. On the other hand, there is a disadvantage of variability in pronase activity that could influence the success rate of chorion removal. Numerous groups have used dechorionated embryos for the assessment of chemical and nanomaterial toxicity[3][4][5][6]. However, these methods have not yet been fully standardized[7][8][9][10].

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Dechorionated zebrafish embryos toxicity assay can serve as a surrogate system to detect potentially hazardous nanomaterials for other vertebrate systems. As the use of higher organism animal models for toxicity testing is being refined, there is an increasing need for alternative test methods. Early life stage zebrafish (up to independent feeding, e.g. 120 HPF) could be an excellent alternative model of in vivo toxicity[22][23][24][25][26][27]. Compared with other animal models, zebrafish have a number of advantages for assessing toxicity, including the relative ease of rearing and breeding, high fecundity (external fertilization, 200 embryos to 300 embryos from a female), short generation time (approximately 3 months to adulthood), availability of genomic resources (complete zebrafish genome sequence), and genetic similarity to humans. About 70 % of human diseases have at least one zebrafish orthologue and 84 % of the human genes associated with disease have orthologues in zebrafish[11]. Therefore, the use of zebrafish to assess chemical toxicity is increasing.

This document provides an optimized procedure to remove chorions along with recommendations on how to conduct toxicity assays using dechorionated zebrafish embryos. It also discusses the advantages of the fish toxicity assay using dechorionated embryos.

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Nanotechnologies — Assessment of nanomaterial toxicity using dechorionated zebrafish embryo

1 Scope

This document specifies a method for rapidly assessing nanomaterial toxicity (fish early life stage, 0 HPF to 120 HPF). It includes information on the importance of acellular chorion removal, detailed chorion removal procedures, and a complete protocol for the toxicity assessment of nanomaterials using dechorionated zebrafish embryos. The focus of this document is on testing nanomaterial toxicity.

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/TS 12805, *Nanotechnologies — Materials specifications — Guidance on specifying nano-objects*

ISO/TR 13014, *Nanotechnologies — Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment*

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ISO/TS 17200, *Nanotechnology — Nanoparticles in powder form — Characteristics and measurements*

ISO/TR 18196, *Nanotechnologies — Measurement technique matrix for the characterization of nano-objects*

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ISO 22412, *Particle size analysis — Dynamic light scattering (DLS)*

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

OECD. *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*. OECD Guidelines for the Testing of Chemicals. Section 2. OECD Publishing, Paris, 2013

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 12805, ISO/TR 13014, ISO/TS 17200, ISO/TS 80004-1, ISO/TS 80004-2, OECD TG 236 and the following apply.

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

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

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

3.1
solubilizing agent
solvent or dispersant that can disperse and stabilize nanomaterials in solution

3.2
spawning
releasing the eggs into the water for fertilization

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3.3
positive control
any well-characterized material or substance that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately positive or reactive response in the test system

[SOURCE: ISO 10993-10:2010, 3.14]

3.4
range-finding test
abbreviated acute test that exposes test organisms to a broad range of nanomaterial testing solutions to establish the range of concentrations to be used in the definitive test

Note 1 to entry: The test includes at least five concentrations of the nanomaterial and untreated control.

[SOURCE: OECD TG 236:2013, modified]

3.5
LC_{50}
concentration of a toxic substance that is lethal to half of a group of test organisms (50 %)

Note 1 to entry: Usually, the exposure to the substance is continuous and the LC_{50} is defined by reference to a specified exposure period.

[SOURCE: ISO 6107-3:1993, 39, modified]

4 Abbreviated terms

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DPF day post fertilization

EM embryo media

HPF hour post fertilization

NOAEL no observed adverse effect level

DO dissolved oxygen

5 Materials

5.1 Organism (zebrafish, *Danio rerio*).

Zebrafish (*Danio rerio*, see Figure 1) is a tropical freshwater teleost belonging to the Cyprinidae, naturally distributed throughout India, Pakistan, Bangladesh, Nepal and Burma. The average size of an adult is 2 cm to 3 cm and has blue stripes, similar to a zebra on the side of the body. The adult male zebrafish has a thinner body, with blue and gold hue stripes. The female has a whitish belly, larger than male and silver stripes instead of gold. This species can be kept and bred in aquaria. The lifespan of zebrafish is approximately 2 to 3 years. Organogenesis of zebrafish is completed within 5 DPF and it is an adult within approximately 3 months.

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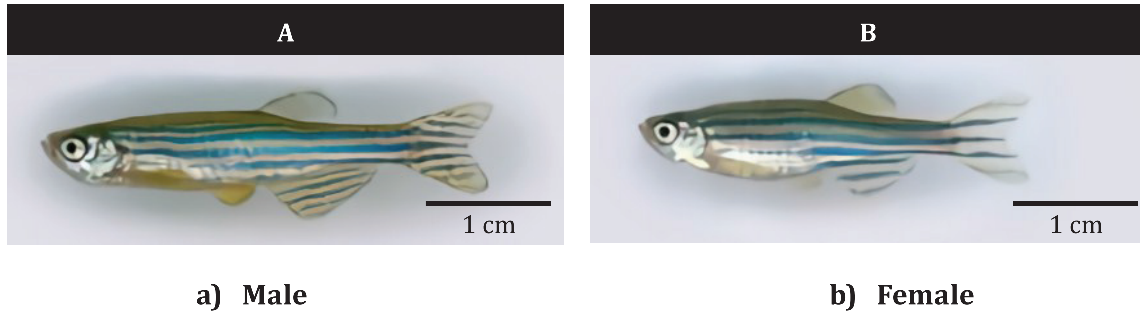

a) Male

b) Female

Figure 1 — Zebrafish

5.2 Stock solution, freshly prepared with purified water.

5.3 Positive control, 3,4-dichloroaniline (3,4-DCA) (CAS# 95-76-1).

6 Apparatus

6.1 Technical equipment

6.1.1 Vessels.

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Multi-well plates (6 wells or 12 wells) can be used. To prevent evaporation during exposure, vessels should be sealed with a biocompatible substrate (i.e. parafilm or transparent self-adhesive foil).

6.1.2 Incubator, with controlled temperature at (27 ± 1) °C and photoperiod (10:14, light:dark).

6.1.3 Microscope, stereo- or inverted with a capacity of at least 80-fold magnification.

6.1.4 Spawning tanks, of any dimensions and design, typically consisting of a mating cage and a slightly larger container.

Mating cages with mesh bottom of grid size (2 ± 0,5) mm allow freshly released eggs to fall down to avoid being eaten by the parent fish.

6.1.5 Pipettes.

A single channel pipette can be used.

6.2 Analytical instruments

Use the analytical instruments given in ISO/TR 18196 and the following.

6.2.1 Scanning electron microscopy (SEM).

Scanning electron microscope that produces magnified images of the nanomaterials by scanning the surface with an electron beam.

6.2.2 Transmission electron microscopy (TEM).

Transmission electron microscope that produces magnified images of the nanomaterials by an electron beam, which passes through the sample and interacts with it.

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6.2.3 Dynamic light scattering (DLS).

DLS instrument to determine the size distribution profile of nanomaterials in liquids.

6.2.4 Static multiple light scattering (SMLS).

SMLS instrument to analyse particle size variation in concentrated dispersions.

6.2.5 Inductively coupled plasma – mass spectrometry (ICP–MS).

ICP–MS instrument for elemental analysis, used for the detection and quantification of metal ions at concentrations as low as one part in 10^{15}.

7 Procedures

7.1 Culture

7.1.1 Zebrafish strain

The strain of zebrafish shall be reported. Adults should be free of externally visible disease and infection, and not treated with any pharmaceutical treatment for 6 months. Adults should be kept under optimal conditions and density to maintain health.

7.1.2 Feeding regime

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Adult zebrafish are fed twice daily with live brine shrimp nauplii (*Artemia* sp.) or commercially available dry diets. Overfeeding should be avoided. Any remaining food should be removed daily for optimal water quality. Larval and juvenile fish are fed thrice a day with dry diets. The diets used shall be reported.

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7.1.3 Photoperiod

A lighting regime of 10 h of light and 14 h of dark is recommended. Deviations from this photoperiod shall be reported.

7.1.4 Temperature and pH

A water temperature of 26 °C to 28 °C is suggested for the maintenance of zebrafish and held near a neutral pH. The actual water temperature and pH shall be reported.

7.1.5 Tank material

Tanks used to hold zebrafish should be made of high-quality glass, polycarbonate or acrylic.

7.2 Spawning stimulation

Embryos are obtained by a spawning group aged between 3 and 12 months. Individual pair or group spawning of adult zebrafish can be used to generate embryos. Several commercially available apparatus or custom-made units are available. Adult fish are placed into the mating cage at a sex ratio of 2:1 or 1:1 (female:male). The full spectrum white light is turned on to stimulate spawning behaviour. Embryos are collected within 1 h and rinsed several times with EM. Unfertilized embryos (opaque and/or ruptured cells inside the chorion) are visually inspected and removed (see Annex B).

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7.3 Dechorionation of embryos

7.3.1 Methods

There are two types of dechorionating methods commonly used: mechanical and enzymatic dechorionation. Embryos can be mechanically dechorionated using two forceps under a stereomicroscope. In general, mechanical dechorionation requires only short preparation time and it does not require the use of enzymes. However, it is both time and labour intensive, which substantially limits the number of embryos that can be dechorionated at one time. It is particularly challenging to remove chorion of early life stage embryos (0 HPF to 10 HPF) as manual manipulations can cause unforeseen damage leading to defects later in life. Enzymatic dechorionation generally uses a protease extracted from bacteria species and allows for simultaneous dechorionating large numbers of embryos.

7.3.2 Enzymatic dechorionation method

Preparation of chemicals, supplies and equipment.

— For 1 l of EM, dissolve 5,0 mM NaCl (0,292 g), 0,17 mM KCl (0,013 g), 0,33 mM CaCl (0,044 g), 0,33 mM MgSO4 (0,081 g) in distilled water and adjust pH to ~7,4 using 0,01 N NaOH or 0,01 N HCl.

— Collect fertilized embryos and separate as many as needed (include 20 % more than needed) all at the same 4 HPF developmental stage.

— Prepare the enzyme [Pronase (EC 3.4.24.4; CAS# 9036-06-0), which is a mixture enzyme of several non-specific endo proteases and exoproteases that digest proteins down to single amino acids, from *Streptomyces griseus*] stock solution on ice using distilled water and into single-use microtubes in small volumes (~100 µl) and store in −80 °C until ready for use.

— Treat chorionated embryos (~1 000) with the enzyme at 19,1 U into 25 ml (0,764 U/ml) of EM in a 90 mm glass petri dish.

— Gently agitate for 5 min or 6 min followed by 6 min rinse with at least a total of 500 ml of EM, then a final mix (without pronase) for 3 min.

— After the rinse, allow the embryos to rest for 20 min to 30 min at (27 ± 1) °C. Following the rest, the weakened chorions left in a petri dish are removed by another rinse cycle (for 6 min) with EM.

— Do not let the embryo contact air or plastic during the process of dechorionation. The use of glass wide bore polish pipettes is recommended.

The method is shown in Figure 2.

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