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Nanotechnologies - Use and application of acellular in vitro tests and methodologies to assess nanomaterial biodurability

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Nanotechnologies — Use and application of acellular in vitro tests and methodologies to assess nanomaterial biodurability

Nanotechnologies — Utilisation et application des tests in vitro sur cellules et méthodes pour évaluer la biodurabilité des nanomatériaux

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Foreword

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Nanotechnologies — Use and application of acellular in vitro tests and methodologies to assess nanomaterial biodurability

1 Scope

This document reviews the use and application of acellular *in vitro* tests and methodologies implemented in the assessment of the biodurability of nanomaterials and their ligands in simulated biological and environmental media.

This document is intended to focus more on acellular *in vitro* methodologies implemented to assess biodurability and, therefore, excludes the general review of relevant literature on *in vitro* cellular or animal biodurability tests.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions 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 http://www.iso.org/obp

3.1
bioaccumulation
process of accumulation of a substance in organisms or parts

[SOURCE: ISO/TR 13329:2012, 3.3]

3.2
biodegradation
degradation due to the biological environment

Note 1 to entry: Biodegradation might be modelled by *in vitro* tests.

[SOURCE: ISO/TR 13329:2012, 3.4]

3.3
biodurability
ability of a material to resist *dissolution* (3.6) and mechanical disintegration from chemical and physical clearance mechanisms

[SOURCE: ISO/TR 13329:2012, 3.5, modified]

3.4
biopersistence
ability of a material to persist in a tissue in spite of the tissue's physiological clearance mechanisms and environmental conditions

[SOURCE: EN 18748:1999]

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3.5
dispersion
microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state

[SOURCE: ISO 26824:2013, 16.5]

3.6
dissolution
process of obtaining a solution containing the analyte of interest

Note 1 to entry: Dissolution is the act of dissolving and the resulting species may be molecular or ionic.S

[SOURCE: ISO 17733:2015, 3.4.10, modified]

3.7
ligands
atoms or groups joined to the central atom

[SOURCE: IUPAC Recommendations 1994]

3.8
nanomaterial
material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material.

Note 2 to entry: See also engineered nanomaterial, manufactured nanomaterial and incidental nanomaterial.

[SOURCE: ISO/TS 80004-1:2015, 2.4]

3.9
nanoparticle
nano-object with all three external dimensions in the nanoscale

Note 1 to entry: If the lengths of the longest to the shortest axes of the nano-object differ significantly (typically by more than three times), the terms nanorod or nanoplate are intended to be used instead of the term nanoparticle.

[SOURCE: ISO/TS 27687:2008, 4.1]

3.10
specific surface area for powders
absolute surface area of the sample divided by sample mass and is therefore expressed in units of m^2/g or kg

[SOURCE: ISO 9277:2010, 3.11, modified]

3.11
suspension
heterogeneous mixture of materials comprising a liquid and a finely dispersed solid material

[SOURCE: ISO/TS 80004-6:2013, 2.13]

4 Symbols and abbreviated terms

AAS	Atomic absorption spectroscopy
AF4	Asymmetrical flow field flow fractionation
AgNPs	Silver nanoparticles

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ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
AuNPs	Gold nanoparticles
$CdSe_{core}/ZnS_{shell}$	Cadmium-selenium (core) and zinc sulfide (shell)
CE	Capillary electrophoresis
CFS	Continuous flow system
CNTs	Carbon nanotubes
CPE	Cloud-point extraction
CuONPs	Copper oxide nanoparticles
Da	Dalton
DMEM	Dulbecco's Modified Eagle's Medium
DOM	Dissolved organic matter
EPA	US. Environmental Protection Agency
FFFF	Flow field flow fractionation
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography–mass spectrometry
GPC	Gel permeation chromatography
HPCIC	High-performance chelation ion chromatography
HRP	Horseradish peroxidase
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectrometry
SNMS/TOF-SIMS	Secondary neutral mass spectrometry/time-of-flight secondary ion mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectroscopy-mass spectroscopy
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
m/z	Mass-to-charge ratios
MWCNTs	Multi-wall carbon nanotubes
NaCl	Sodium chloride
NP	Nanoparticle
NIOSH	U. S. National Institute for Occupational Safety and Health
NSL	Nanosphere lithography
PAP	Pulmonary alveolar proteinosis

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PBPK	Physiologically-based pharmacokinetic
PSF	Phagolysosomal simulant fluid
QDs	Quantum dots
QQQ	Triple quadrupole
QTOF	Quadrupole time-of-flight
REACH	Registration, evaluation, authorisation and restriction of chemicals
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SiO_2	Silicon dioxide
SLF	Simulated lung fluid
spICP-MS	Single particle inductively coupled plasma-mass spectrometry
SPE	Solid-phase extraction
SPF	Simulated physiological fluids
SPR	Surface Plasmon Resonance
SS	Simulated saliva
SSW	Simulated sweat
SUF	Simulant ultrafiltrate fluid
SWCNTs	Single-wall carbon nanotubes
TBOs	Tungsten blue oxides
TiO_2NPs	Titanium dioxide nanoparticles
UV-Vis spectroscopy	Ultraviolet-visible spectroscopy
WO_3	Tungsten trioxide
ZnONPs	Zinc oxide nanoparticles

5 Background including need for assessing the biodurability of particles

The tendency of a given inhaled particle or fibre to cause chronic disease is strongly related to the duration of residence time in the pulmonary environment. Biopersistent particles and fibres are defined as materials that resist clearance from the body through physical and chemical means. Resistance to physical clearance may result through their resistance to phagocytosis by alveolar macrophages. Resistance to clearance by chemical and physical means, also known as biodurability, may result through their resistance to chemical dissolution. Particles and fibres that are amenable to dissolution may release components which may contribute to their adverse health effects. Similar resistance to chemical dissolution and/or biodegradation of the attached ligands in environmental media may also produce biodurable nanomaterials.

Historical experiences with micrometre-scale mineral particles and fibres provide a useful model for understanding the relationship between exposure, dose, and effect of nanomaterials in the human lung. It has been shown that the residence time of mineral particles and fibres in the lung depends on their

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mechanical clearance and the dissolution rates. The dissolution rate is governed mainly by particle and fibre chemistry and the properties of the biological fluid of the tissue/cell environment in which they are found.

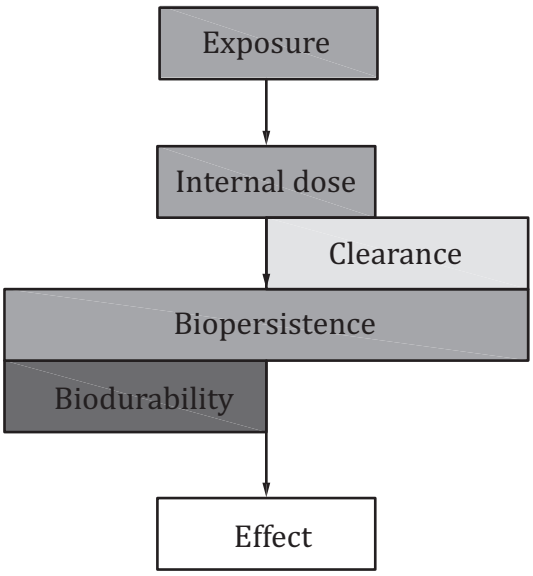

Figure 1 — Relationship between exposure, dose, and effect of nanomaterials[1]

Figure 1 presents a general framework proposed by Oberdörster et al.[1] to evaluate potential adverse effects from exposure to particles and fibres. According to this framework, biopersistence of the particles and fibres is considered to be central to the production of health effects.

The respiratory tract may be divided into different zones, including the ciliated nasal and tracheobronchial regions and the non-ciliated alveolar regions. Particles that deposit in each zone interact with different cell populations with substantially different retention times and/or different clearance pathways[2]. The behaviour of inhaled particles in the respiratory tract and their alternative fates of either deposition or exhalation in these various functional zones may depend upon the chemical composition and the physical behaviour of the aerosol particles.

The importance of airway ciliated cells and alveolar macrophages in the clearance of micrometer-sized particles from the lung surface has long been known. Due to the limited capability of macrophages to recognize nanomaterials, inadequacy of this key clearance mechanism in peripheral lungs was demonstrated[3][4]. Concurrently, due to endocytotic processes and trans-cellular transport mechanisms by other cells, translocation of nanomaterials to extra pulmonary organs has become prominent. Biopersistence of nanomaterials through their resistance to clearance by alveolar macrophages and also to their biodurability through resistance to breakage or dissolution and leaching may therefore lead to their bioaccumulation. With their ability to translocate nanomaterials may accumulate and be retained in critical target organs with the subsequent production of adverse health effects. Translocation (disposition), accumulation and retention are, therefore, important aspects that need to be considered when investigating the long-term toxicity of nanomaterials.

It is now well accepted that the toxicity of nanomaterials may very much be related to their physicochemical properties including size, surface area, and surface characteristics including surface chemistry. Once inside the cell, these and other physicochemical characteristics (surface composition, surface activity) will determine their interaction with biological surroundings. The latter, in turn, will determine the stability and biodurability of the surface and core of nanomaterials. Given the strong sensitivity of many nanomaterial properties to their local environment, it should be noted that biologically relevant changes in the physicochemical properties of a nanomaterial between administration and deposition may have a significant impact on observed responses. As a result,

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biodurability of nanomaterials in their local biological environments may impact on their long-term toxicity.

Although the biodurability of certain larger particles can also be confirmed in different environmental media, the importance of such biodurability in their long-term effects might not be of relevance in aquatic organisms. This is shown to be the case with crystalline silica (quartz and cristobalite) for the fact that they do not bioaccumulate in these biorganisms due to their very limited potential for uptake through the gill or gut of fish[5][6]. This might not be true for nanomaterials as the bioaccumulation of multiple-wall carbon nanotubes (MWCNTs) has been shown in *Daphnia magna*, an aquatic invertebrate[7], of gold nanoparticles (AuNPs) in clams marine bivalve *Scrobicularia plana*[8](Pan *et al.*, 2012) and zinc oxide nanoparticles (ZnONPs) in *S. plana* and *Nereis diversicolor*,[9] copper oxide nanoparticles (CuONPs) in freshwater snail[10][11], titanium dioxide nanoparticles (TiO_2NPs) in *Daphnia magna*[12], and silver nanoparticles (AgNPs) in deposit feeder such as the annelid *Platynereis dumerilii*[13] have been shown.

6 Aims and objectives

Different nanomaterials are reported to be biopersistent as they resist breakage or dissolution and clearance which in turn can lead to their bioaccumulation and subsequently their translocation and distribution. It is, therefore, of great relevance to assess this property of nanomaterials and determine their degradation half-lives in addition to their other properties. It is also of great relevance to study the impact of surface coating and functional groups on biodurability in biological and environmental surroundings.

The aim and objectives are

— the identification of tests performed and the methodologies implemented in the literature, and

— the description of the identified methodologies in the assessment of the biodurability of different nanomaterials in different biological and environmental media.

The ultimate goal is that, by applying the identified methodologies, it is possible to assess the biodurability of nanomaterials and their surface ligands. This is relevant in the assessment of their long-term effects.

7 Approaches for assessment of micrometre mineral particle and fibre biodurability

7.1 General

The toxicity of inhaled fibres is believed to be related to dose, biodurability and to their dimensions, with long, thin fibres being potentially more carcinogenic than short, thick ones and to their biopersistence, as fibres which dissolve rapidly in the lung are unlikely to induce long-term pathological changes[14]. The concept of biopersistence – resistance to clearance and to chemical dissolution - has therefore been proposed as a key concept in the toxicity of mineral or synthetic fibres.

The critical role of dissolution on the potential health effects of inhaled fibres is well established[15]. Over the last several decades, there have been numerous publications on the relation between various physicochemical characteristics, including chemical composition and diameter, of a synthetic vitreous fibre and its dissolution rate in physiological saline solution[16][17][18].

Numerous animal *in vivo* experiments are described in the literature to assess clearance and chemical instability, through their solubility, of different mineral and man-made synthetic fibres. For example, particle clearance has been studied as a measure of physical persistence - the actual amount of fibre remaining in the tissue and their solubility has been assessed through the release of metal constituents in experimental animals[19][20][21][22][23].

Also, *in vitro* cellular systems have been described to study the chemical stability of particles using macrophages, epithelial, and mesothelial cells where differences could be observed in the efficiency

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of these cell types to change the chemical structure of the engulfed fibres emphasizing the differences between phagosomal conditions between these cells[24][25][26][27].

In vitro acellular systems mimicking physiological conditions have been developed to study the release of chemical constituents from fibres. Using different biological media simulants, cell-free chemical dissolution assays were thought to provide valuable information on the behaviour of respired dusts in biological surroundings.

A combination of the *in vitro* dissolution tests, cell-based assays (mono- and co-culture systems), *in silico* methods, and information from existing *in vivo* studies, can provide a measure of biopersistence of mineral particles and fibres. This document is limited to *in vitro* acellular systems and their applications to nanomaterials.

7.2 Dissolution of nanomaterials versus their dispersion and biodegradation

The terms dissolution and dispersion have been defined with a distinction made between these two terms[28]. In this document, dissolution "denotes to the process of obtaining a solution containing the analyte of interest. Dissolution is the act of dissolving and the resulting species may be molecular or ionic" and dispersion "refers to the microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state". As per this definition, dissolution of nanomaterials will entail release of ions to the surrounding solvent where the rate of dissolution will be dependent on size, chemistry, solvent composition, and surface coating or functionalization of nanomaterials.

Dissolution of nanomaterials is an important property and is often a critical step in determining their safety[29]. Dissolution of metal- and metal oxide-based nanomaterials follows thermodynamic and kinetic rules where size, shape, surface coating, aggregation state, and solution chemistry such as pH, ionic components, and dissolved organic matter (DOM) affect the rate of their dissolution. As the toxicity of the dissolved form can have different toxicological effects than the particulate, monitoring dissolution kinetics is important[30]. Subsequently, the determination of dissolution and dissolution kinetics are recommended as part of the minimum requirements for nanomaterials to be characterized.

Biodegradation of organic and carbon-based nanomaterials on the other hand, is achieved through enzymatic catalysis with subsequent complete breakdown and loss of nanomaterials characteristics. The amenability to biodegradation seemed to be dependent on the type of surface functional groups that they may carry[31].

8 Need for the assessment of nanomaterial biodurability

Biopersistence is one of the characteristics which are seen to determine the toxicity/pathogenicity/carcinogenicity of ultrafine particles and also of nanomaterials[32]. These characteristics are also seen to alter their fate and biological distribution. Due to their small size, nanomaterials are likely to translocate beyond the epithelial barrier into the interstitium where they accumulate for a long time due to their resistance to phagocytic uptake[33][34][35]. Those which are biodurable may cause pulmonary inflammation, fibrosis, and cancer. Some of the interstitialized nanomaterials could be translocated into the systemic circulation and some may induce impairment in extrapulmonary organs. Once translocated, evidence seems to suggest that nanomaterials preferentially deposit in liver and spleen[36] resulting in prolonged retention and in some instances producing significant hepatotoxicity.

Biodurable nanomaterials maintain their particulate state which might increase the potential for their bioaccumulation[37]. Release of ions from nanomaterials that are soluble have also been shown to be strongly associated with their toxicity while acute toxicological responses arise from nanomaterials with high dissolution through released ions[38], low dissolution and non biodegradable nanomaterials might provoke a range of long-term effects including carcinogenicity[39]. When the sparingly soluble nanomaterials are made soluble through surface functionalization or surface coating, when in contact with body fluids, they might also disintegrate/dissolve to eventually exposing the biodurable core.

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9 Influence of different types of ligands and coatings on nanomaterial biodurability

The importance of surface properties of micrometre mineral particles and fibres was recently emphasized as an indirect but critical factor in the manifestation of pathogenic activity as well as in their biodurability under conditions of *in vitro* dissolution in biological fluids. Subsequently, in a publication by the US. National Institute of Occupational Safety and Health (NIOSH), surfaces of mineral particles and fibres were thought to be a controlling factor in their biopersistence, which is seen to be a critical aspect in the mechanisms of continuing irritation or inflammatory response in causing fibrosis or neoplastic transformation[40]. For example, it could be shown that surface composition and surface-associated activities of asbestos fibres were the major contributors to their potential to induce disease. Similar observations have been made for crystalline silica in which surface modification by chemical means has also been shown to alter their cytotoxicity[41][42][43].

Manipulation of surface properties of nanomaterials have also been investigated through surface functionalization with a range of molecular groups for a number of reasons including an increase in water dispersability to an otherwise non-water dispersible nanomaterials and for the increase of their intracellular uptake with their subsequent accumulation in cellular lysosomes. As noted previously, surface chemistry is an important nanomaterial property that will influence interactions with biological fluids. The surface chemistry of nanomaterials can be changed drastically upon immersion in a biological fluid[44]. It is now well recognized that modification of nanomaterial surface chemistry can yield novel properties and behaviours. Such modifications can also be used to promote biodurability and minimize health risks. For example, accumulation in cellular lysosomes could be shown for dextran-coated magnetic iron oxide nanoparticles as well as for single-wall carbon nanotubes (SWCNTs) where they could be enzymatically degraded by lysosomal R-glucosidase, horseradish peroxidase, myeloperoxidase, and heme oxygenase-1. This type of enzymatic degradation, however, might not be possible for inert nanoparticles such as gold and therefore once inside the cell, they can deposit for longer periods[45]. Similar studies with carbon nanotubes (CNTs) have indicated that carboxylated CNTs are more susceptible to biodegradation compared to other CNTs[46].

Loss of ligands from the surface of semiconductor nanocrystals in gastrointestinal fluids at low pH was also reported with the subsequent increase in their toxicity[47]. Moreover, the effect of this biological fluid on the PEGylated quantum dots (QDs) was also investigated and showed that the fate of PEGylated Cadmium-selenium (core) and zinc sulfide (shell) ($CdSe_{core}/ZnS_{shell}$) QDs depended on pH, ligand chain length and presence of proteins and subsequently affecting their toxicity[48][49].

10 Review of methodologies to assess micrometre mineral particle and fibre biodurability

10.1 General

Adaptation of traditional dissolution rate studies to mimic the physical and chemical conditions encountered in biological tissues and organs allows investigators to estimate biodurability through calculation of residence times based on the chemical dissolution mechanism. Particle and fibre dissolution and breakdown in the body are important determinants of their biopersistence. The physical and chemical mechanisms whereby particles and fibres may release ions through dissolution and/or degrade in biological tissues and organs have been studied extensively *in vitro* using "simulated biological fluids" (see Table A.1 to Table A.5). Such *in vitro* tests provide information on the biodurability which impacts the biological effects of particles and fibres[50]. *In vitro* dissolution tests have also been suggested for screening for the biodurability of nanomaterials in environmental media (natural freshwaters, simulated seawater, and simulated estuarine waters) (see Table A.8)[51][52].

10.2 *In vitro* acellular methods

In vitro acellular methods are comprised of two components, the simulated biological fluid and the system to hold and retain the study material. In this first section, various simulated biological fluids are described and in the next section available test systems are reviewed.

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Over the last several decades, numerous publications have used various simulated biological fluids to assess dissolution as a measure of biodurability of particles and fibres. Several studies have tried to establish a relation between physicochemical characteristics such as chemical composition, density, and diameter of a synthetic vitreous fibre and their dissolution rate in physiological fluids[53][15]. Several publications have also assessed the impact of different properties of biological fluids on the dissolution of particles and fibres. These properties include pH 7,3 of the extracellular lung fluid and pH 4,5 of macrophage phagolysosomal fluid, presence of surfactants (e.g lung lining fluid), and presence of certain biological compounds, including organic chelators such as sodium citrate that selectively bind to surface ions (see Tables A.1 to A.5).

10.3 Description of different simulated physiological media

10.3.1 General

In this section, simulated biological fluids are described that are intended to mimic the lung and oral as well as dermal exposure pathways. For the lung, generally there are two main compartments to consider – the extracellular airway lining fluid having near neutral pH and the phagolysosomal fluid of macrophage cells having acidic pH. For the oral route, particles will briefly come into contact with saliva followed by gastric and interstitial fluids. To describe adequately the dissolution behaviour of materials in the body, it is imperative that the simulant reflects the biochemical composition of the fluids in the organ or tissue being modelled. The most common simulants used were therefore based on the ionic composition of lung extracellular fluid described by Gamble[54] which is near neutral pH (7,2 to 7,4). Gamble's simulant fluid was also called Ringer's solutions, simulant ultrafiltrate fluid (SUF), or simulant lung fluid (SLF)[55][56]. The majority of experiments have, therefore, been conducted at physiological pH (7,3), either at 25 °C or 37 °C. With evidence that dissolution of insoluble particles occurs predominantly in phagolysosomes, investigators began to adjust the pH of lung extracellular fluid-simulants to match that of the phagolysosome, i.e. pH 4,5 to 5.

Therefore, the simulated physiological fluids (SPF) that are discussed below have included: (1) the lung airway lining simulant, (2) the phagolysosomal stimulant fluid, (3) the simulated saliva, (4) the gastrointestinal fluids, as well as (5) sweat the composition of all of which are presented in Annex A. For more information on the composition of various biological fluids and their simulants, the reader is referred to Marques et al.[57].

While these SPF are considered useful analogues of human biofluids, they all suffer from the same basic limitations. Firstly, SPFs have defined compositions and lack the dynamic conditions present *in vivo*. For example, none of these fluids contain enzymes or oxidative cascades which can be important in dictating the properties (composition, pH) of a SPF or biodurability of nanomaterials such as carbon nanotubes[58]. Additionally, unless specifically noted, proteins are omitted from most SPF for pragmatic reasons (see 10.5.3); however, *in vivo*, proteins might serve as important binding molecules for dissolved ions and influence their concentration at nanomaterial surfaces.

10.3.2 Simulated lung airway lining fluids

Aerosol materials that deposit in the lung are quickly immersed in the extracellular fluid that is excreted by the lung tissue. The original composition of physiological fluids described by Gamble in 1967[54] has served as a basis for a number of experimental derivations for human extracellular fluid, including the experimental solvents used by Scholze and Conradt[59] and Kanapilly et al.[60].

Gamble's Solution is a type of simulated lung airway lining fluid that is intended to mimic the surfactant fluids released by Type II alveolar cells. The fluid fills the space between alveolar cells and acts to reduce the surface tension of the water in the lungs, facilitating gas exchange. The composition of this solution was compared to the fluid in human lungs and found to be identical in terms of major components. The original Gamble's Solution was a mixture of water and inorganic salts including chlorides, carbonates and phosphates. Most researchers have however modified the solution to include proteins and other organic components, chelators and additives[61] (see Table A.1).

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10.3.3 Simulated lung macrophage phagolysosomal fluid

Particles that deposit in the non-ciliated alveolar region of the lung are initially immersed in extracellular airway lining fluid but are rapidly phagocytized by scavenger cells such as macrophages. Upon engulfment by a macrophage cell, the particle-laden vesicle (lysosome) fuses with a phagososome to form a phagolysosome which is rich in oxidizers and has acidic pH to promote degradation of foreign materials.

The original composition of SPF described by Gamble in 1967[54] served as a basis for human macrophage phagolysosomal fluid (PSF). The fusion occurs when neutrophil or macrophage encounters a foreign substance, including nanomaterials, with ensuing destruction or degradation of the foreign substance in the phagolysosome. The intracellular fluid within alveolar macrophages is considerably more acidic (pH approximately 5 to 6) than the extracellular fluid (pH approximately 7 to 7,5). Some particles that do not dissolve efficiently in extracellular lung fluid are degraded within the cellular phagolysosomes of alveolar macrophages[62][63][64][65][66][67][46][68][69] (see Table A.2).

10.3.4 Digestive system (saliva, gastric and intestinal fluids)

10.3.4.1 General

The main fluids of the digestive system may include saliva, gastric and intestinal fluids[57][70]. The stomach produces gastric juice while the intestine is a mixture of bile from liver (digestive enzymes) and pancreatric juice. The assessment of biodurability in these fluids may be of relevance when considering the development of nanomaterials for drug delivery or for mouth cleansing formulations[71][72].

10.3.4.2 Simulated saliva (SS)

It is almost impossible to duplicate the properties of human saliva because of its particular characteristics. Saliva is a mixture of fluids secreted by several salivary glands, with numerous constituents, which varies depending on time of day and diet. The pH of the normal healthy saliva is between 6,7 to 7,4 which may drop to pH 5 when sweets and carbohydrates are consumed. Simulated saliva (SS) was developed for different applications[73][74][75][76] (see Table A.3) and was also used to test the dissolution of nickel nanoparticles[77].

10.3.4.3 Simulated gastric fluid

Conditions in the gastric environment, such as pH, enzyme concentration, water content, temperature, pressure, microbial load, and emptying rate are continually changing during the fasting and fed states[78][79]. Acellular *in vitro* digestion assays using simulated gastric fluids (see Table A.4) have been used to determine how human gastric conditions impact the behaviour of nanomaterials. For example, AgNPs in simulated gastric fluid aggregated significantly and released ionic silver from their surface that re-precipitated and coated the aggregates as silver chloride[80][81]. The aggregation rate was higher for smaller (10 nm) AgNPs compared with larger (75 nm) nanoparticles (NPs). Additionally, the ionic silver released from the NPs could generate new AgNPs that might differ in size and morphology from the NPs that the system was initially exposed to[82][83]. The low pH of the gastric fluid could also cause degradation of the surface coating of nanomaterials, which could alter their downstream toxicity[70][84]. These studies demonstrate the importance of using isolated acellular systems such as gastric fluids to understand behaviour of nanomaterials in these fluids. In addition to exposing nanomaterials to simulated gastric fluids separately, there are systems that simulate sections of the gastrointestinal system, from the mouth to intestine, to allow sequential exposure of test materials to different compartments[78][85]. These systems can be useful in studying how nanomaterials are affected by different conditions that change substantially from one compartment to another.

10.3.4.4 Simulated intestinal fluid

The fasted-state simulated intestinal fluid and fed-state simulated intestinal fluid (see Table A.4) were introduced to simulate additional important aspects of the GI fluids, including bile salts and lecithin[71].

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