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Surface passivity largely governs the bioaccessibility of nickel-based powder particles at human exposure conditions

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ABSTRACT

The European chemical framework REACH requires that hazards and risks posed by chemicals, including alloys and metals, are identified and proven safe for humans and the environment. Therefore, differences in bioaccessibility in terms of released metals in synthetic biological fluids (different pH (1.5–7.4) and composition) that are relevant for different human exposure routes (inhalation, ingestion, and dermal contact) have been assessed for powder particles of an alloy containing high levels of nickel (Inconel 718, 57 wt% nickel). This powder is compared with the bioaccessibility of two nickel-containing stainless steel powders (AISI 316L, 10–12% nickel) and with powders representing their main pure alloy constituents: two nickel metal powders (100% nickel), two iron metal powders and two chromium metal powders. X-ray photoelectron spectroscopy, microscopy, light scattering, and nitrogen absorption were employed for the particle and surface oxide characterization. Atomic absorption spectroscopy was used to quantify released amounts of metals in solution. Cytotoxicity (Alamar blue assay) and DNA damage (comet assay) of the Inconel powder were assessed following exposure of the human lung cell line A549, as well as its ability to generate reactive oxygen species (DCFH-DA assay). Despite its high nickel content, the Inconel alloy powder did not release any significant amounts of metals and did not induce any toxic response. It is concluded, that this is related to the high surface passivity of the Inconel powder governed by its chromium-rich surface oxide. Read-across from the pure metal constituents is hence not recommended either for this or any other passive alloy.
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1. Introduction

Inconel alloys, nickel-based heat-resistant super alloys, are used in a large variety of challenging engineering applications, including many elevated temperature environments and power plant systems (Choudhury and El-Baradie, 1998). The Inconel 718 alloy is the most commonly used alloy due to its high strength, mechanical properties and corrosion resistance (Choudhury and El-Baradie, 1998). Human exposure may occur at occupational settings via inhalation or dermal contact.

REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) is a European chemicals regulation first implemented in July 2007 (EC, 2007), in which metals are considered as substances for which registration dossiers have to be submitted. Such dossiers include the assessment of the toxicological and ecotoxicological hazard profile, for which the specific bioaccessibility (the pool of released and potentially available chemical species) in biological fluids plays an important role. Alloys are generally considered as special mixtures under REACH, for which no registration dossiers need to be prepared. Their assessments are hence predominantly based on their individual pure alloy constituents. So far, very few alloys on the market have been investigated individually. Potential health risks of alloys have in some cases been estimated via read-across from the properties of the respective pure metal components. Nickel metal is currently classified for several human health endpoints: *i)* as a skin sensitizer (Category 1,

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"may cause an allergic skin reaction"), *ii)* for causing specific target organ toxicity (STOT Repeated Exposure 1: H372: "causes damage to organs through prolonged or repeated exposure"; "route of exposure: inhalation"), and *iii)* as a suspected carcinogen (Category 2, "suspected of causing cancer") (EC, 2009). Alloys containing nickel are classified for skin sensitization if the release rate of nickel exceeds 0.5 μg/cm^2/week (EC, 2009), as measured by the European standard reference test method EN 1811 (CEN, 2011).

Recent studies highlight the importance of surface properties of alloys compared with their bulk composition, and show that read-across from pure metals is highly erroneous (Hedberg et al., 2013; Herting et al., 2005; Hillwalker and Anderson, 2014; Mörsdorf et al., 2015; Stockmann-Juvala et al., 2013). Suggestions have also been raised to group alloys and metals of similar bulk and surface characteristics to facilitate hazard classification of alloys (OECD, 2014; Santonen et al., 2010; Stockmann-Juvala et al., 2013).

The release of metals from metals and alloys in different fluids, without contribution of wear, is mainly governed by oxidation of the metal (corrosion) and of dissolution of the surface oxide. These processes depend on the passive properties of the surface oxide (hinders corrosion) and, among others, the pH and metal complexation capacity of the surrounding fluid (Hedberg and Odnevall Wallinder, 2016). Very low levels of released nickel and iron have previously been observed from the same Inconel powder as investigated in this study upon exposure in four different synthetic body fluids relevant for oral bioaccessibility (Henderson et al., 2012), and for another Inconel powder (74.2 wt% Ni, 15.8 wt% Cr) in three synthetic body fluids (Hillwalker and Anderson, 2014). Despite a relatively low nickel release of less than 0.5 μg/cm^2/week in artificial sweat, the Inconel alloy 600 (≥72 wt% Ni, 14-17 wt% Cr) caused skin reactions in 54% of 67 tested nickel-allergic individuals (Menné et al., 1987). This was most probably caused by an enhanced nickel release upon skin contact compared with immersion conditions (Hedberg and Odnevall Wallinder, 2016; Menné et al., 1987) and/or an elicitation threshold of less than 0.5 μg/cm^2 for nickel-allergic individuals (Andersen et al., 1993; Pedersen et al., 2004).

The aims of this study were to assess the bioaccessible fraction of metals released from an Inconel 718 powder in synthetic body fluids relevant for inhalation, ingestion and dermal contact, and to test the hypothesis that bioaccessibility cannot be predicted from the bulk alloy composition. The results are compared with corresponding, and similarly sized, powder particles of the main pure alloy constituents (nickel, iron, and chromium) and of stainless steel grade AISI 316L (17-19 wt% Cr, 10-12 wt% Ni). Furthermore, different *in-vitro* toxicity endpoints (cell viability, DNA damage, and reactive oxygen species generation) were investigated for the Inconel powder and compared with parallel measurements of nickel powder particles of similar size.

2. Materials and methods

2.1. Investigated particles

The Inconel alloy 718 powder, denoted Inconel, was supplied by NiPERA. The nominal alloy composition (from supplier information) is shown in Table 1. The powder is from the same batch as investigated in another published study (Henderson et al., 2012). The following powders are included in this paper for comparison: gas-atomized stainless steel grade AISI 316L sized <45 μm (denoted 316L-1) and sized <4 μm (denoted 316L-2). The 316L powders are composed of about 66–69 wt% Fe, 17-19 wt% Cr, and 10-12 wt% Ni. Detailed material and particle characterization, bioaccessibility and cytotoxicity information is given elsewhere (Hedberg et al., 2010, 2012). Metal powders of iron (Fe-1, Fe-2), chromium (Cr-1, Cr-2), and nickel (Ni-1, Ni-2) of two different sizes are included for comparison. The two size fractions of the iron and chromium powders have previously been investigated in a bioaccessibility and cytotoxicity study (Hedberg et al., 2010), and the two different nickel metal powders in an earlier bioaccessibility study (Mazinanian et al., 2013). Table 3 shows for all powders specific surface areas (measurement explained in section 2.3) and particle size distributions in phosphate buffered saline (PBS, pH 7.4, see sections 2.4 and 2.6). Particle morphologies, observed by means of scanning electron microscopy (section 2.5), are displayed in Fig. 1.

Table 1
Nominal bulk composition (wt%) of the Inconel powder.

Ni	Cr	Fe	Nb + Ta	Mo	Ti	Al	Cu	Si	Co	Others
57.3	18.3	14.6	5.0	2.9	0.92	0.4	0.2	0.16	0.1	O 0.06 C 0.04 N 0.01 B 0.006 S 0.005 P < 0.001

2.2. X-ray photoelectron spectroscopy

The outermost (approx. 5 nm) surface of the Inconel powder particles was investigated with X-ray photoelectron spectroscopy (XPS), using a Kratos AXIS UltraDLD x-ray photoelectron spectrometer (Kratos Analytical, Manchester, UK) driven by a monochromatic 150 W Al x-ray source. The analyzed areas were approximately sized 700 × 300 μm. Wide spectra measurements were performed to probe elements at the top surface of the powder particles. Measurements were performed on two different positions on unexposed powders and after exposure in artificial lysosomal fluid for 168 h. With a pass energy of 20 eV, detailed spectra for the main alloy elements of each powder, carbon (C 1s), oxygen (O 1s), iron (Fe 2p), chromium (Cr 2p), nickel (Ni 2p), molybdenum (Mo 3d), manganese (Mn 2p), and cobalt (Co 2p) were obtained. Molybdenum, manganese, and cobalt were not observed. The powders were mounted on copper tape to fix them against dispersion in the applied vacuum inside the ultra-high vacuum instrumental chamber. All binding energies were corrected to the carbon C 1s contamination peak at 285.0 eV. All peak areas were determined by assigning a linear base line. Iron (711.3 ± 0.55 eV) and chromium (576.6 ± 1.5 eV) were only observed in their oxidized states, both prior to and after exposure. Surface oxide compositional analyses presented in this paper consider the oxidized nickel peak at 855.1 ± 0.78 eV and the oxidized chromium and iron peaks. The metallic peaks of nickel, observed at 853.0 ± 0.66 eV and 858.8 ± 1.9 eV [satellite peak (Machet et al., 2004)] were used to assess the oxidized/metallic mass ratio.

2.3. Measurement of specific surface area by nitrogen adsorption

The surface area to weight ratios (specific surface area) of the Inconel and reference powders were estimated using the Brunauer-Emmet-Teller method (Brunauer et al., 1938) (Micromeritics GEMINI V) that measures the adsorbed amount of nitrogen at cryogenic conditions. The measurements were performed at five different partial pressures and at five different local positions for each powder (standard BET method). The mean value is presented in Table 3.

2.4. Light scattering

The particle size distribution of the Inconel powder was determined in phosphate buffered saline (PBS, pH 7.4, see section 2.6) by

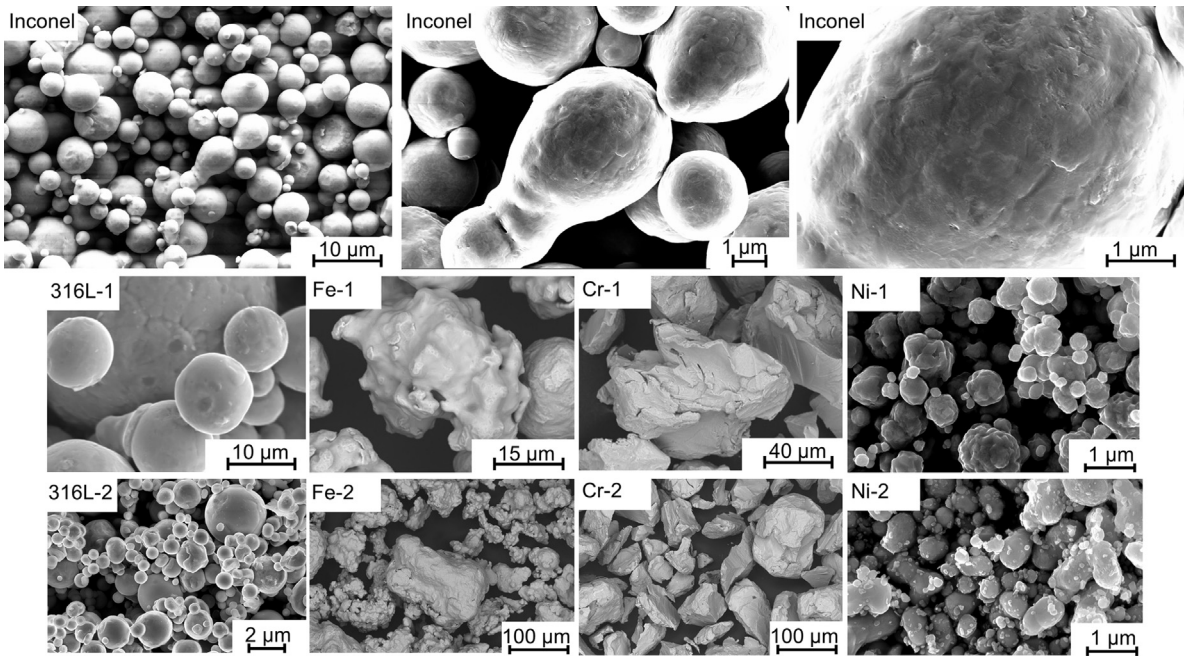


Fig. 1. SEM images of Inconel powder particles. Powders of AISI 316L, iron, chromium, and nickel metal of different size fractions are included for comparison.

means of low angle laser light scattering (Malvern Mastersizer 2000; with a Hydro SM dispersion unit). Refractive indexes for nickel (1.98) and water (1.33, the solvent of the test fluids) were used as input parameters for the calculation of the size distribution by volume. Data is presented as average numbers of at least three discrete measurements of each powder.

2.5. Scanning electron microscopy

Particle morphologies of the powders; Inconel, 316L-1, 316L-2, Ni-1, and Ni-2, were studied using a field emission gun scanning electron microscope (FEG-SEM Leo1530 upgraded to Zeiss Supra 55) using secondary electrons, and with a table-top scanning electron microscope (SEM) with backscattered electron analysis (Hitachi TM-1000) for the powders of Fe-1, Fe-2, Cr-1, and Cr-2. The powders were fixed on carbon tape to avoid their dispersion inside the instrument chamber and to assure appropriate conduction.

2.6. Exposure to synthetic body fluids

Triplicate samples of the Inconel powder were exposed for 2, 4, 8, 24, and 168 h to each of the seven synthetic body fluids (Table 2) at a ratio (loading) of 5 ± 0.5 mg particles in 50 mL solution (0.1 g/L) in closed PMP Nalgene® vessels positioned in a Stuart platform-rocker incubator (25 cycles/min of bi-linear shaking) set at 37 ± 0.5 °C. Blank samples (without powders) were run in parallel for each powder, solution, and exposure time. After exposure, the test fluid was separated from the powder particles by centrifugation at 3000 r.p.m. (704 g) for 10 min. The supernatant solution, free of particles, was acidified to a pH less than 2 (not needed in the case of artificial gastric fluid) with 65 vol% ultrapure HNO_3 prior to solution analysis (a standard procedure for metal analysis and conservation of samples). All vessels for exposure, centrifugation and storage were acid-cleaned in 10 vol% HNO_3 for at least 24 h, rinsed four times with ultra-pure water, and dried in ambient laboratory air to minimize the risk of contamination.

Seven synthetic physiological fluids were selected:

- Phosphate-buffered saline (PBS, pH 7.2–7.4), a standard physiological fluid that mimics the ionic strength of human blood serum.
- PBS containing 2.2 g/L lysozyme from chicken egg white (L6876, Sigma Aldrich, Sweden), denoted PBS + LSZ. Lysozyme is present in saliva and tear fluid, positively charged at pH 7.4, and a globular hard (high internal conformation stability) protein with a molecular weight of 14.1 kDa (Imoto et al., 1972).
- PBS containing 10 g/L bovine serum albumin (A7906, Sigma Aldrich, Sweden), denoted PBS + BSA. 10 g/L BSA is the same molar concentration as 2.2 g/L LSZ. BSA is a soft globular protein present in serum at high concentrations (30–50 g/L) (Roach et al., 2006) with a molecular weight of 66 kDa.
- Gamble's solution (GMB, pH 7.4), mimics the interstitial fluid within the deep lung under normal health conditions (Stopford et al., 2003).
- Artificial sweat (ASW, pH 6.5), simulates the hypoosmolar fluid, linked to hyponatraemia (loss of Na^+ from blood), which is excreted from the body upon sweating. The fluid is recommended in the available standard for testing of nickel release from nickel containing products (CEN, 2011).
- Artificial lysosomal fluid (ALF, pH 4.5), simulates intracellular conditions of lysosomes in lung cells occurring in conjunction with phagocytosis and represents relatively harsh conditions (de Meringo et al., 1994; Hillwalker and Anderson, 2014).
- Artificial gastric fluid (GST, pH 1.5), mimics the very harsh digestion setting of high acidity in the stomach (Hamel et al., 1998).

2.7. Atomic absorption spectroscopy and release data presentation

Total concentrations of released iron (Fe), nickel (Ni), chromium (Cr), molybdenum (Mo), cobalt (Co), and manganese (Mn) in solution were analyzed by graphite furnace atomic absorption spectroscopy (GF-AAS) for the Inconel powder exposed to the ALF

Table 2
Composition (g/L), initial, and final pH of the seven synthetic body fluids.

Chemical	GST	ALF	ASW	PBS	PBS + LSZ	PBS + BSA	GMB
$MgCl_2$	–	0.050	–	–	–	–	0.095
NaCl	–	3.21	5.0	8.77	8.77	8.77	6.02
KCl	–	–	–	–	–	–	0.298
Na_2HPO_4	–	0.071	–	1.28	1.28	1.28	0.126
KH_2PO_4	–	–	–	1.36	1.36	1.36	–
Na_2SO_4	–	0.039	–	–	–	–	0.063
$CaCl_2 \cdot 2H_2O$	–	0.128	–	–	–	–	0.368
$C_2H_3O_2Na \cdot H_2O$ (sodium acetate)	–	–	–	–	–	–	0.700
$NaHCO_3$	–	–	–	–	–	–	2.60
$C_6H_5Na_3O_7 \cdot 2H_2O$ (trisodium citrate)	–	0.077	–	–	–	–	0.097
NaOH	–	6.00	–	–	–	–	–
Citric acid	–	20.8	–	–	–	–	–
Glycine	–	0.059	–	–	–	–	–
$C_4H_4O_6Na_2 \cdot 2H_2O$ (disodium tartrate)	–	0.090	–	–	–	–	–
$C_3H_5NaO_3$ (sodium lactate)	–	0.085	–	–	–	–	–
$C_3H_5O_3Na$ (sodium pyruvate)	–	0.086	–	–	–	–	–
$CH_3CHOHCO_2H$ (lactic acid)	–	–	1.0	–	–	–	–
$(NH_2)_2CO$ (urea)	–	–	1.0	–	–	–	–
HCl (25%)	4.0	–	–	–	–	–	–
BSA	–	–	–	–	–	10	–
LSZ	–	–	–	–	2.2	–	–
Initial pH (prior to exposure)	1.7–1.8	4.5	6.5	7.2	7.2	7.2	7.4
Final pH (after exposure of 0.1 g/L Inconel powder up to 168 h)	1.5–1.6	4.3–4.4	5.3–6.7	7.2	7.2–7.4	7.2–7.3	7.9–8.5

GST – artificial gastric solution; ALF – artificial lysosomal fluid; ASW – artificial sweat; PBS – phosphate buffered saline; LSZ – lysozyme from chicken egg white; BSA – bovine serum albumin; GMB – Gamble's solution.

solution (pH 4.5). The limits of detection, calculated as average blank values plus three times the highest standard deviation of the blanks, were 3.5 μg Ni/L, 0.5 μg Cr/L, 4.0 μg Fe/L, 5.0 μg Mn/L, 1.0 μg Mo/L, and 6.0 μg Co/L in ALF. All sample concentrations for Mo, Mn, and Co were below the limits of detection in ALF. The total concentrations of Fe, Cr, Mn, and Ni in solution were analyzed in the other synthetic body fluids. All reported values of Fe, Cr, and Ni are based on concentrations that are significantly higher than their corresponding limits of detection, and higher than their corresponding blank concentrations.

Release data is presented as the measured total release of metals in solution normalized to the corresponding metal content in the powders, denoted as "released fraction of metal (%)", as follows:

$$\frac{\left(Sample\ concentration\left(\frac{g}{L}\right) - blank\ concentration\left(\frac{g}{L}\right)\right) * Volume(L)}{Particle\ mass(g) * x} * 100\%$$

Where x = 1 for the metal powders (Ni-1, Ni-2, Fe-1, Fe-2, Cr-1, and Cr-2); x = 0.573 (Ni), 0.183 (Cr), and 0.146 (Fe) for the Inconel powder; and x = 0.103 (Ni), 0.168 (Cr), and 0.689 (Fe) for the 316L-1 and 316L-2 powders.

Release data is also presented as normalized to the initial specific surface area (BET-area) of the particles, denoted as "released amount ($\mu g/cm^2$)", as follows:

$$\frac{\left(Sample\ concentration\left(\frac{g}{L}\right) - blank\ concentration\left(\frac{g}{L}\right)\right) * Volume(L)}{Particle\ mass(g) * BET\ surface\ area\left(\frac{m^2}{g}\right) * 10000}$$

Details on the atomic absorption spectroscopy measurements using graphite or flame mode for the Cr-1, Cr-2, Ni-1, Ni-2, Fe-1, Fe-2, 316L-1, and 316L-2 powders are given in the respective references cited in the figures and tables.

2.8. Cell culture and cell viability assay

A549 cells, from a human type II alveolar epithelial cell line (American Type Culture Collection, ATCC, Manassas, USA) were cultured in DMEM cell culture medium (Dulbeccos's Minimal Essential Medium, Cat. No. 41965-039, Gibco® Invitrogen). The medium was supplemented with 10% fetal bovine serum (European grade, Biological Industries), 1 mM sodium pyruvate (Gibco® Life Technologies), 100 units/mL penicillin and 100 μg/mL streptomycin (Pen Strep, Gibco® Life Technologies). The cells were cultured in cell culture flasks in a humidified (RH > 99%, 37 °C) 5% CO_2-atmosphere.

CuO nanoparticles (20–40 nm diameter, Sigma-Aldrich), dispersed in this cell culture medium at concentrations of 20 (DNA damage) or 40 (cell viability) $\mu g/cm^2$, were used as positive controls in the cellular assays, and resulted in all cases in significant differences compared with the negative control (data not shown). Since all cell tests were done in this cell medium, the released amounts of Ni, Cr, and Fe from the Inconel powder were also investigated in the cell medium at a particle concentration of 20 μg/mL (*i.e.* five times lower compared with the other release tests). No detectable amounts of released metals were observed (data not shown).

Cell viability was studied using the alamar blue assay, which indicates the level of cellular metabolic activity. This activity is affected both by cell viability and cell proliferation (the number of cells in the culture). A549 cells were exposed to particle suspensions (in the cell culture medium described above) of different concentrations, 0.1, 1, 5, 10, 20 and 40 $\mu g/cm^2$ (or μg/mL), for 24 and

48 h in duplicate wells of transparent 48 well plates. The particle suspensions were removed, after which the cells were incubated for 3 h in 200 μL of 10% alamarBlue® (Invitrogen, Life Technologies). Fluorescence was recorded under excitation and emission wavelengths of 560 and 590 nm (Molecular Devices SpectraMax® Gemini EM Microplate Reader). Possible interferences between the Inconel powder and the fluorescent dye (alamarBlue®) were examined by performing the assay at cell-free conditions. No interferences were observed (data not shown). Effects on cell viability were also studied using the released fraction of metals from the Inconel powder, by incubating the powder in the cell medium at similar conditions, separating the powder from the cell medium and exposing the cells to this medium. Three independent experiments were performed for each exposure.

2.9. DNA damage

The levels of DNA damage in A549 cells induced by the Inconel powder at 20 μg/cm^2 (μg/mL) were examined with the alkaline single cell comet assay. This version of the comet assay detects both DNA strand breaks and alkaline labile sites (Collins, 2004). The principle of the assay is that damaged DNA will migrate more in an electrical field creating an image of a comet with a head and a tail. A large tail size describes a large extent of DNA damage and vice versa. The cells were exposed to the Inconel powder dispersed in cell culture medium (described above) for 4 and 24 h in 24 well plates. Thereafter, the assay was completed as described in (Cronholm et al., 2013). The exposed cells were washed with PBS and trypsinized (0.25% Trypsin (Gibco®, Life Technologies) in PBS), after which they were suspended in cell medium. The cells were centrifuged (4 min, 210 g, 4 °C), washed in PBS and then mixed with 0.75% low melting point agarose (Sigma Aldrich, Sweden) before added on glass slides pre-coated with agarose (0.3%). The slides were treated with lysis buffer (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, pH 10) for 1 h, after which they were treated with alkaline electrophoresis solution (0.3 M NaOH, 1 mM EDTA) for 40 min. Electrophoresis was performed in the same solution (30 min, 17 V/1.15 V/cm, 600 mL). The slides were neutralized in 0.4 M Tris buffer (2 × 5 min) and in water (5 min), dried and fixed by soaking the slides in methanol (99.8%, Sigma-Aldrich) for 5 min. Ethidium bromide (0.5 μg/mL) was used to stain the DNA and make the comets visible with a fluorescence microscope (Olympus BH-2). The Komet 4.0 software (Kinetic Imaging Ltd, Liverpool, UK) was used for scoring the comets. The level of DNA damage was determined as "% DNA in tail". This represents the fraction of cellular DNA that is contained in the comet tail compared to the total cellular DNA. At least 100 comets were analyzed from each treatment. Three independent experiments were performed.

2.10. Reactive oxygen species

The inherent ability of the Inconel powder to generate reactive oxygen species (ROS) was assessed using an acellular version of the 2′7-dichlorodihydrofluorescin diacetate (DCFH-DA) assay (Rushton et al., 2010). Inconel powder dispersed in PBS was added on black clear bottom 96 well plates (25 μL/well), with DCFH (75 μL/well) either containing horseradish peroxidase (HRP) as a catalyst (+) or not (−). The possible background fluorescence of the powder was tested during each experiment by including wells that contained PBS (75 μL/well) instead of DCFH. The final Inconel powder concentrations were 5 and 20 μg/mL. Triplicate wells of each condition were included. The plates were kept in darkness during the preparation and the incubation (37 °C, 1 h). Fluorescence was measured using 485 nm excitation and 530 nm emission wavelengths (Molecular Devices SpectraMax® Gemini EM Microplate Reader). Three independent experiments were performed.

2.11. Statistical analysis

Data was analyzed with one-way analysis of variance (ANOVA), with significant differences for $p < 0.05$.

3. Results and discussion

3.1. Particle and surface characterization

Fig. 1 shows the morphology of the Inconel particles, compared with reference particles of stainless steel 316L, iron, nickel, and chromium metal powders of two different sizes, respectively. Both from Fig. 1 and from Table 3, showing the specific surface area at dry conditions and hydrodynamic sizes of each powder in solution, it is evident that the size distributions of the particles are different. Due to these differences, we decided to compare the Inconel powder to two different sizes of the reference powders. The size of the Inconel powder falls in between the sizes of the 316L, Fe, and Cr powders, but exceeds the size of both nickel powders, Fig. 1 and Table 3. However, the average hydrodynamic size ($d_{0.5}$ by volume in PBS) of the Inconel powder is similar to both nickel powders, Table 3.

XPS measurements revealed a metallic nickel peak at 853.0 ± 0.66 eV, both for unexposed Inconel particles and upon exposure to ALF for 168 h. No significant difference was observed in the oxide to metal peak ratio prior to and after exposure to ALF, Fig. 2a, which is indicative of a very thin (<5–10 nm) and passive surface oxide. In addition, a peak at 855.1 ± 0.78 eV was assigned to oxidized nickel. Trivalent oxidized chromium (Machet et al., 2004; McIntyre et al., 1978) was observed at 576.6 ± 1.5 eV both prior to and after exposure. The surface oxide was hence prior to exposure mainly composed of oxidized chromium (86 wt-%) and to a lesser extent of oxidized nickel (14 wt%), Fig. 2b. After exposure to ALF (168 h), the surface oxide was comprised of oxidized iron (35 wt%), most probably trivalent iron judged from a peak position at 711.3 ± 0.55 eV (McIntyre et al., 1979; Yamashita and Hayes, 2008), trivalent chromium (43 wt%), and oxidized nickel (22 wt%), Fig. 2b. Carbon peaks at 285.0, 286.4 ± 0.2, and 288.8 ± 0.07 eV were observed both prior and after exposure to ALF, predominantly indicative of surface contamination (Rubio et al., 2002). Accordingly, oxygen peaks positioned at 530.4 ± 0.25, 532.1 ± 0.48, and 533.2 ± 0.13 eV were observed both prior to and after exposure. In addition to the contribution from organic surface contamination, these peaks indicate the presence of metal oxides/hydroxides and/or oxyhydroxides and water (Biesinger et al., 2009; Machet et al., 2004). It is not possible in this study to rule out whether oxidized nickel, iron, and chromium of the surface oxide exist as single oxides or within mixed oxides (spinels). Spinels, such as $NiFe_2O_4$ and $NiCr_2O_4$, are likely components of the passive surface oxide of Inconel powders (McIntyre et al., 1978, 1979).

Table 3
BET specific surface area (m^2/g) of the different powders (relative standard deviation < 1%), and 10, 50, and 90 vol% cut-off values (μm) for each powder when immersed in PBS.

Powder	Inconel	316L-1	316L-2	Ni-1	Ni-2	Cr-1	Cr-2	Fe-1	Fe-2
BET (m^2/g)	0.16	0.069	0.70	1.05	2.15	0.035	0.91	0.046	0.84
$d_{0.1\ (\mu m)}$	4.2	11.7	2.4	2.5	1.5	57.5	59.7	37.1	36.6
$d_{0.5\ (\mu m)}$	6.9	22.5	5.0	4.8	3.0	104	104	101	83.1
$d_{0.9\ (\mu m)}$	11.3	41.4	34.1	11.3	59	170	173	181	167

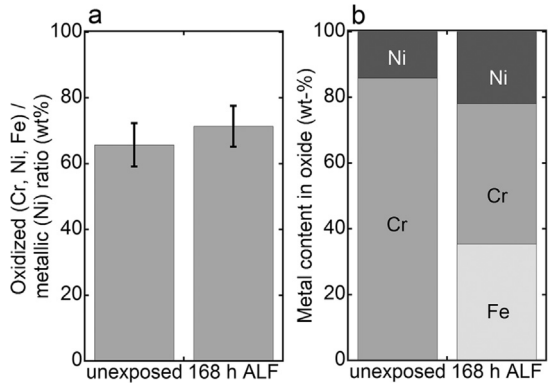


Fig. 2. Oxidized vs. metallic peaks (wt-%) of observed metals (a) within the outermost surface (5–10 nm) of unexposed Inconel powder and powders exposed for 1 week (168 h) in ALF (pH 4.5), and b) corresponding ratio of oxidized nickel, chromium, and iron (by wt-%). No iron was observed in the unexposed surface, and no manganese, molybdenum, or cobalt on any of the surfaces.

3.2. Metal release in synthetic biological fluids

Fig. 3 shows the released amounts of Ni, Cr, and Fe from the Inconel powder after 168 h into seven different synthetic body fluids of varying pH and solution composition (Table 2). The concentrations of the other main alloying constituents (Co, Mn, and Mo) were below their corresponding limits of detection. The release of metals after 2, 4, 8, and 24 h was lower or similar compared to observations made after 168 h, and in many cases close to their limits of detection or quantification (data not shown). Generally, the release of metals from the Inconel powder was very low. There was no clear trend in the extent of released amounts of metals among the different solutions, which indicates a very high surface passivity of the Inconel powder in the pH range between 1.5 and 7.4. The extent of released metals slightly increased with a reduced solution pH, Fig. 3. However it is not possible to judge whether this is an effect of precipitation of released metals at near neutral pH levels (Hedberg and Odnevall Wallinder, 2016), or because of an enhanced release of metals at more acidic conditions. The released amounts of Fe were close to the corresponding blank values and limits of quantification, which makes any comparison among the different solutions difficult for this element (Fig. 3c). For released Cr (Fig. 3b), solutions of low pH and presence of protein bovine serum albumin (BSA) resulted in a higher Cr release compared with solutions of higher pH or when containing lysozyme (LSZ). These observations are in accordance with literature findings for stainless steel (Hedberg and Odnevall Wallinder, 2016). When comparing released levels of Ni, Cr, and Fe in ALF (pH 4.5) from the Inconel powder with reference powders of stainless steel 316L, iron, chromium, and nickel metal, Fig. 4, it becomes evident that the Inconel powder behaves in a similar manner as similar sized powders of the biomedical stainless steel grade 316L. For all released elements (Ni, Cr and Fe), released levels of metals from the Inconel powder were in between observed findings for the smaller and larger (compared with Inconel powder) sized 316L powders, Fig. 4. When compared with slightly smaller sized nickel powders, the Inconel powder

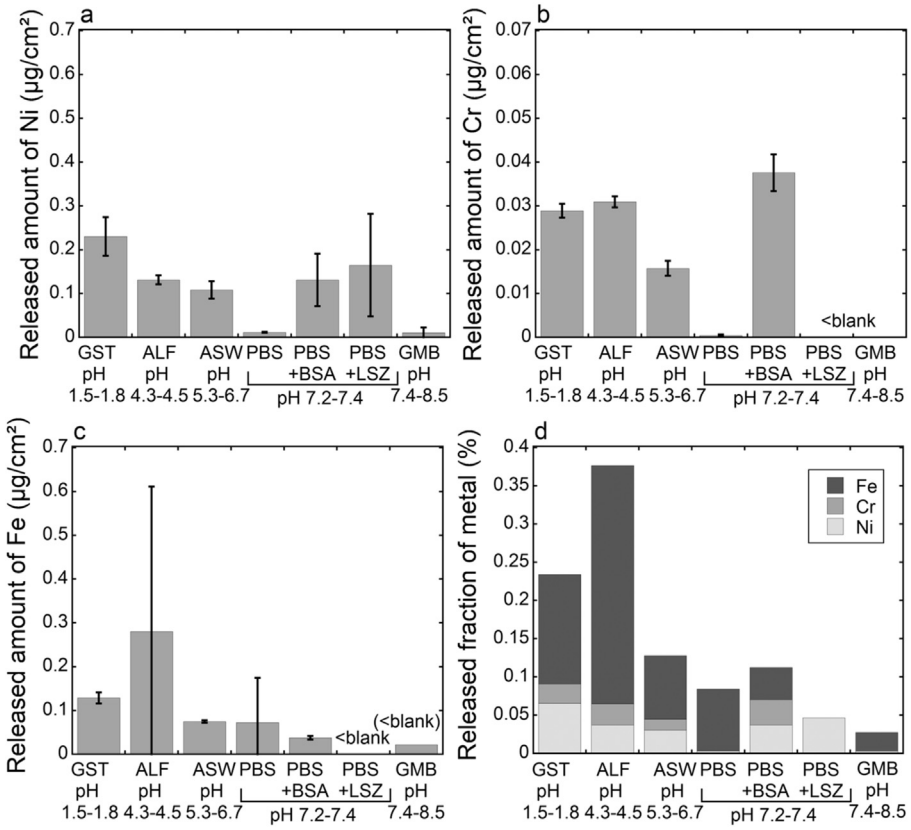


Fig. 3. Released amounts of Ni (a), Cr (b), and Fe (c), for the Inconel powder exposed for 168 h in different synthetic body fluids. Released fraction of Ni, Cr, and Fe, normalized to their bulk content in the Inconel powder (d). The pH range indicates the pH range during one week exposure in each solution. Note the different scales. <blank: measured sample concentrations were smaller than corresponding blank concentration. (GST – artificial gastric solution; ALF – artificial lysosomal fluid; ASW – artificial sweat; PBS – phosphate buffered saline; BSA – bovine serum albumin; LSZ – lysozyme from chicken egg white; GMB – Gamble's solution).

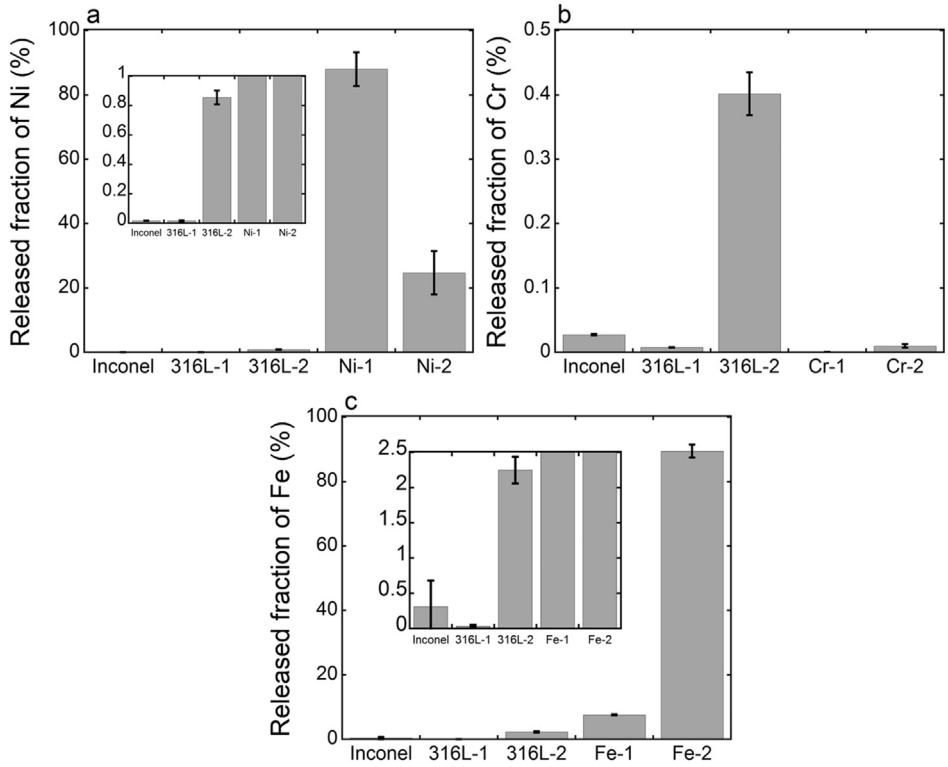


Fig. 4. Released fractions of Ni (a), Cr (b), and Fe (c), for the Inconel powder compared with reference powders of stainless steel 316L, nickel, chromium, and iron metal, and normalized to the nickel, chromium, and iron content in the powders. All release data is shown for ALF (pH 4.5) after 24 h (a) or 168 h (b and c) exposure. The reference powders were exposed at similar conditions. Data shown is from (Hedberg et al., 2011) for Ni release from the 316L powders (24 h in ALF), from (Mazinanian et al., 2013) for Ni release from the nickel powders, and from (Hedberg et al., 2010) for Cr and Fe release from the 316L, iron, and chromium powders. The error bars show the standard deviation of triplicate samples.

released significantly less Ni, Fig. 4a. Observed differences in released amount of Ni between the two nickel powders have previously been found to be caused by compositional differences in the surface oxide (Mazinanian et al., 2013). When compared with chromium metal powders of both smaller and larger size, Fig. 4b, the Inconel powder releases more Cr, since chromium metal is extremely passive at these conditions (Hedberg et al., 2010). Iron powders of both smaller and larger size (Hedberg et al., 2010) release significantly more Fe at similar conditions compared with the Inconel powder, Fig. 4c.

The amounts of released Ni and Fe from the Inconel powder correspond well with literature findings on the same powder (from the same batch) in GST (pH 1.5) and ALF (pH 4.5), *e.g.*, 0.00015 μg/μg Ni and 0.00040 μg/μg Fe after 2 h in GST (pH 1.5) in this study compared to 0.00014 μg/μg Ni and 0.00016 μg/μg Fe after 2 h in GST in (Henderson et al., 2012). The released amounts of metals from the Inconel powder in this study are lower (3–10-fold) compared with findings for another Inconel powder (74 wt% Ni, 15.8 wt% Cr) exposed in three synthetic body fluids that are similar to GST, ALF, and ASW (Hillwalker and Anderson, 2014).

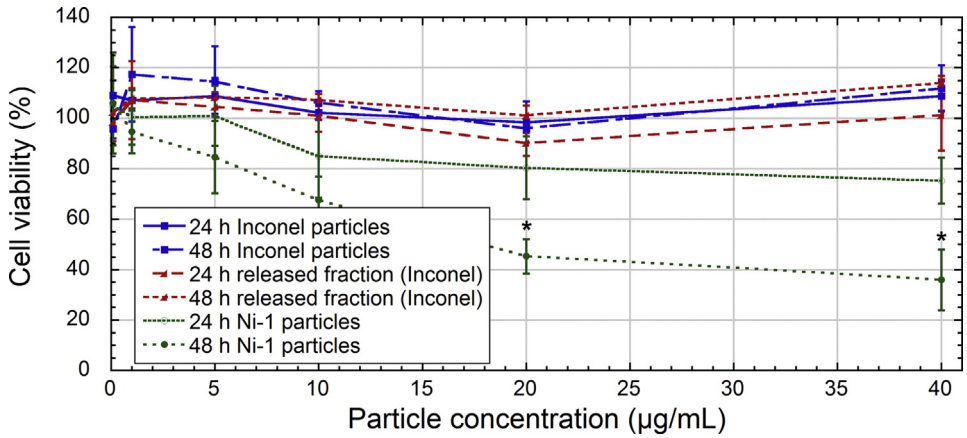


Fig. 5. Cell viability of A549 cells after 24 and 48 h of exposure to Inconel powder at concentrations of 0.1, 1, 5, 10, 20, and 40 μg/cm^2 (μg/mL) or to the corresponding released fractions (particles removed). Data for the Ni-1 powder after 24 and 48 h (Latvala et al., 2016) is included for comparison. The error bars show the standard deviation of three independent experiments. The asterisks mark a significant difference ($p < 0.05$) compared with the control.

Table 4
DNA damage (% DNA in tail) of Inconel powder (particle concentration 20 μg/cm^2) compared to the negative control at the different conditions tested. No significant difference between the control and the Inconel powder was observed. Data for Ni-1 powder (Latvala et al., 2016) is included for comparison.

Test conditions	Control[a]	Inconel powder	Ni-1 powder
4 h	5.36 ± 1.14	6.34 ± 1.13	6.03 ± 2.0
24 h	5.97 ± 0.20	5.46 ± 0.78	12.8 ± 7.1

[a] Control data is shown for the Inconel powder. Control values for the Ni-1 powder were very similar and given in (Latvala et al., 2016).

Table 5
Reactive oxygen species (ROS) generation by the Inconel powder at the different conditions tested (with and without HRP, and at two different particle concentrations), presented as the number of times increase compared to the negative control. No significant difference between control and Inconel powder was observed. Data for Ni-1 powder (Latvala et al., 2016) is included for comparison.

Test conditions	Inconel powder	Ni-1 powder
	Number of times increase compared to the control	
No HRP, 5 μg/cm^2	1.14 ± 0.11	0.89 ± 0.37
No HRP, 20 μg/cm^2	1.15 ± 0.11	0.91 ± 0.31
HRP, 5 μg/cm^2	0.94 ± 0.18	2.8 ± 1.6
HRP, 20 μg/cm^2	0.99 ± 0.23	3.8 ± 1.9

3.3. Cell viability, DNA damage, and generation of reactive oxygen species

No toxic effects were observed for the Inconel powder at the conditions tested in terms of cell viability and DNA damage, Fig. 5 and Table 4. There were also no signs of increased generation of reactive oxygen species, Table 5. The nickel powders Ni-1 and Ni-2 have recently been tested at similar conditions (Latvala et al., 2016). Some of the results for Ni-1 powder are included for comparison in Fig. 5 and Tables 4 and 5. The Ni-1 powder showed a significant reduction in cell viability at particle concentrations of 20 and 40 μg/cm^2 (μg/mL) after 48 h, whereas no such effects were observed for the Ni-2 powder. The Ni-1 powder also induced a significant DNA damage after 24 h, and generation of reactive oxygen species in the presence of HRP, but not the Ni-2 powder. This is in agreement with significantly more Ni released from Ni-1 compared with both the Ni-2 and the Inconel powders, Fig. 4. The 316L-2 powder has previously been investigated in terms of cell viability, DNA damage, and hemolysis using different assays (Hedberg et al., 2010), and in a 28-day in-vivo inhalation toxicity study on rats (Stockmann-Juvala et al., 2013). The 316L-2 powder did not induce any significant reduction in cell viability, increased hemolysis, or inhalation toxicity. It did, however, induce a significant DNA damage, which was later explained by the presence of a MnO_2-rich surface oxide (Hedberg et al., 2012, 2013). Judging from these results, the absence of toxic effects induced by the Inconel powder is mostly related to its low release of metals, its high surface passivity, and possibly its relatively large particle size, which may hinder cell uptake (Horie et al., 2009).

3.4. Read-across between metals and alloys

Table 6 compares the release of Ni, Cr, and Fe from the Inconel powder into ALF (pH 4.5) with the reference particles. Data is normalized to the initial particle surface area at dry conditions. From this table, the effective concentration, defined as the bioaccessible concentration of a constituent substance in a complex material (Henderson et al., 2012), can be calculated based on the average release of a given metal from reference metal particles (both sizes). The Inconel powder investigated in this study behaves hence like an alloy containing 0.12 wt% Ni (range 0.07–0.60 wt% when comparing with the two sizes of reference particles) and 0.21% Fe (0.17–0.26 wt%) when exposed to ALF, while it releases about 2–3 times as much Cr as compared with the chromium metal powder (though still very low levels). Therefore, it becomes evident that the effective concentration, and not the bulk composition, should be the basis of predictions, as previously suggested for 316L powders (Stockmann-Juvala et al., 2013) and for several other metals and alloys (Henderson et al., 2012).

4. Conclusions

Metal alloys are considered as special mixtures under the EU chemicals regulation REACH, and their risk and hazard assessments are hence based on the behavior of their individual pure alloy constituents. Since the intrinsic- and surface properties of alloys generally are very different from their pure constituents, their actual performance needs to be assessed. It also needs to be elucidated whether the nominal alloy composition can be used to predict either their bioaccessibility or any toxicological effects. The bioaccessibility of an Inconel alloy 718 powder (57.3 wt% nickel) was therefore investigated in seven synthetic body fluids covering a pH range of 1.5–7.4, relevant for the different exposure routes (inhalation, ingestion, and dermal contact at e.g. occupational settings). Several toxic endpoints were investigated (cell viability after 24 and 48 h, DNA damage after 4 and 24 h, and generation of reactive oxygen species). Data were compared with similarly sized powders of stainless steel AISI 316L (10.3 wt% nickel), and the pure metals of iron, chromium, and nickel. The following main conclusions are drawn:

- A very low released amount of Ni, Cr, and Fe from the Inconel powder was observed in the different synthetic body fluids. No release of Co, Mn, or Mo was observed. These findings are explained by the presence of a very thin and passive surface oxide of the Inconel powder particles. This surface oxide is predominantly comprised of trivalent chromium oxide (and of oxidized nickel) prior to exposure, and of a mixed oxide of oxidized chromium, nickel, and iron after exposure to ALF (pH 4.5).
- Nickel release from the Inconel powder was 0.1 μg/cm^2/week in artificial sweat (pH 6.5), *i.e.,* lower than the stipulated threshold values for piercing posts and direct and prolonged skin contact of 0.2 or 0.5 μg/cm^2/week, respectively.

Table 6
Comparison of the released amount of Ni, Cr and Fe (μg/cm^2) from the Inconel powder compared to the reference powders. The reference powders were exposed at similar conditions. Data shown is from (Hedberg et al., 2011) for Ni release from the 316L powders (24 h in ALF), from (Mazinanian et al., 2013) for Ni release from the nickel powders, and from (Hedberg et al., 2010) for Cr and Fe release (168 h in ALF) from the 316L, chromium, and iron powders.

Released element	Inconel	316L-1	316L-2	Ni-1	Ni-2	Cr-1	Cr-2	Fe-1	Fe-2
Ni (24 h ALF)	0.057 ± 0.0055	0.024 ± 0.0065	0.13 ± 0.0069	84 ± 5.0	9.6 ± 2.6	N/A	N/A	N/A	N/A
Cr (168 h ALF)	0.031 ± 0.0013	0.018 ± 0.0011	0.096 ± 0.0080	N/A	N/A	0.017 ± 0.0045	0.011 ± 0.0030	N/A	0.041 ± 0.0012
Fe (168 h ALF)	0.28 ± 0.33	0.22 ± 0.020	2.2 ± 0.19	N/A	N/A	N/A	0.060 ± 0.0052	165 ± 3.6	106 ± 2.4

N/A – not analyzed.

- Generally, solutions of lower pH and solutions containing bovine serum albumin induced a slightly higher amount of released metals compared with solutions of higher pH, lower metal complexation capacity, and/or lack of proteins. This effect was mostly pronounced for the release of Cr.
- The effective concentrations of iron and nickel in Inconel were 0.21 wt% and 0.12 wt%, respectively, in ALF (pH 4.5), when compared to corresponding bioaccessibility data observed for metal powders of the pure alloy components. The bulk composition (15 wt% iron and 57 wt% nickel) should hence not be used to assess hazards for this highly surface passive Inconel alloy.
- No significant toxic effects were observed for the Inconel powder at given *in vitro* conditions.

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References

Andersen, K.E., Lidén, C., Hansen, J., Vøeund, A., 1993. Dose-response testing with nickel sulphate using the TRUE test® in nickel-sensitive individuals. Multiple nickel sulphate patch-test reactions do not cause an “angry back”. Brit. J. Dermatol 129, 50–56.

Biesinger, M.C., Payne, B.P., Lau, L.W., Gerson, A., Smart, R.S.C., 2009. X-ray photoelectron spectroscopic chemical state quantification of mixed nickel metal, oxide and hydroxide systems. Surf. Interface Anal. 41, 324–332.

Brunauer, S., Emmet, P.H., Teller, E., 1938. Adsorption of gases in multimolecular layers. J. Am. Chem. Soc. 60, 309–319.

CEN, 2011. Reference Test Method for Release of Nickel from All Post Assemblies Which Are Inserted into Pierced Parts of the Human Body and Articles Intended to Come into Direct and Prolonged Contact with the Skin. EN-1811:2011.

Choudhury, I., El-Baradie, M., 1998. Machinability of nickel-base super alloys: a general review. J. Mater. Process. Tech. 77, 278–284.

Cronholm, P., Karlsson, H.L., Hedberg, J., Lowe, T.A., Winnberg, L., Elihn, K., Odnevall Wallinder, I., Möller, L., 2013. Intracellular uptake and toxicity of Ag and CuO nanoparticles: a comparison between nanoparticles and their corresponding metal ions. Small 9, 970–982.

Collins, A.R., 2004. The comet assay for DNA damage and repair. Mol. Biotechnol. 26, 249–261.

de Meringo, A., Morscheidt, C., Thélohan, S., Tiesler, H., 1994. In vitro assessment of biodurability: acellular systems. Environ. Health Persp 102, 1–6.

EC, 2007. REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals. European Commission Environment Directorate General. http://ec.europa.eu/growth/sectors/chemicals/reach_en.

EC, 2009. Commission Regulation (EC) No. 790/2009 Amending, or the Purposes of its Adaptation to Technical and Scientific Progress, Regulation (EC) No. 1272/2008 of the European Parliament and of the Council on Classification, Labelling and Packaging of Substances and Mixtures.

Hamel, S.C., Buckley, B., Lioy, P.J., 1998. Bioaccessibility if metals in soils for different liquid to solid ratios in synthetic gastric fluid. Env. Sci. Technol. 32, 358–362.

Hedberg, Y., Gustafsson, J., Karlsson, H.L., Möller, L., Odnevall Wallinder, I., 2010. Bioaccessibility, bioavailability and toxicity of commercially relevant iron- and chromium-based particles: in vitro studies with an inhalation perspective. Part. Fibre Toxicol. 7, 23.

Hedberg, Y., Hedberg, J., Liu, Y., Odnevall Wallinder, I., 2011. Complexation- and ligand-induced metal release from 316L particles: importance of particle size and crystallographic structure. BioMetals 24, 1099–1114.

Hedberg, Y., Mazinanian, N., Odnevall Wallinder, I., 2013. Metal release from stainless steel powders and massive sheet – comparison and implication for risk assessment of alloys. Env. Sci. Process. Impact 15, 381–392.

Hedberg, Y., Norell, M., Linhardt, P., Bergqvist, H., Odnevall Wallinder, I., 2012. Influence of surface oxide characteristics and speciation on corrosion, electrochemical properties and metal release of atomized 316L stainless steel powders. Int. J. Electrochem. Sci. 7, 11655–11677.

Hedberg, Y.S., Odnevall Wallinder, I., 2016. Metal release from stainless steel in biological environments: a review. Biointerphases 11, 018901–1-018901-17.

Henderson, R.G., Cappellini, D., Seilkop, S., Bates, H., Oller, A., 2012. Oral bioaccessibility testing and read-across hazard assessment of nickel compounds. Regul. Toxicol. Pharm. 63, 20–28.

Herting, G., Odnevall Wallinder, I., Leygraf, C., 2005. A comparison of release rates of Cr, Ni, and Fe from stainless steel alloys and the pure metals exposed to simulated rain events. J. Electrochem. Soc. 152, B23–B29.

Hillwalker, W.E., Anderson, K.A., 2014. Bioaccessibility of metals in alloys: Evaluation of three surrogate biofluids. Environ. Pollut. 185, 52–58.

Horie, M., Nishio, K., Fujita, K., Kato, H., Nakamura, A., Kinugasa, S., Endoh, S., Miyauchi, A., Yamamoto, K., Murayama, H., Niki, E., Iwahashi, H., Yoshida, Y., Nakanishi, J., 2009. Ultrafine NiO particles induce cytotoxicity in vitro by cellular uptake and subsequent Ni(II) release. Chem. Res. Toxicol. 22, 1415–1426.

Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C., Rupley, J.A., 1972. Vertebrate lysozymes. In: Boyer, P. (Ed.), The Enzymes, vol. 7. Academic Press, New York.

Latvala, S., Hedberg, J., Di Bucchianico, S., Möller, L., Odnevall Wallinder, I., Elihn, K., Karlsson, H.L., 2016. Nickel Release, ROS generation and toxicity of Ni and NiO micro- and nanoparticles. PloS One 11, e0159684.

Machet, A., Galtayries, A., Zanna, S., Klein, L., Maurice, V., Jolivet, P., Foucault, M., Combrade, P., Scott, P., Marcus, P., 2004. XPS and STM study of the growth and structure of passive films in high temperature water on a nickel-base alloy. Electrochim. Acta 49, 3957–3964.

Mazinanian, N., Hedberg, Y., Odnevall Wallinder, I., 2013. Nickel release and surface characteristics of fine powders of nickel metal and nickel oxide in media of relevance for inhalation and dermal contact. Regul. Toxicol. Pharm. 65, 135–146.

McIntyre, N., Zetaruk, D., Owen, D., 1978. XPS study of the initial growth of oxide films on Inconel 600 alloy. Appl. Surf. Sci. 2, 55–73.

McIntyre, N., Zetaruk, D., Owen, D., 1979. X-ray photoelectron studies of the aqueous oxidation of Inconel-600 alloy. J. Electrochem. Soc. 126, 750–760.

Menné, T., Brandrup, F., Thestrup-Pedersen, K., Veien, N., Andersen, J., Yding, F., Valeur, G., 1987. Patch test reactivity to nickel alloys. Contact Dermat. 16, 255–259.

Mörsdorf, A., Odnevall Wallinder, I., Hedberg, Y.S., 2015. Bioaccessibility of micron-sized powder particles of molybdenum metal, iron metal, molybdenum oxides and ferromolybdenum - importance of surface oxides. Regul. Toxicol. Pharm. 72, 447–457.

OECD, 2014. Series on Testing and Assessment, Number 194: Guidance on Grouping of Chemicals. In: Organisation for Economic Co-operation and Development, Environment Directorate, Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. ENV/JM/MONO(2014), second ed., p. 4.

Pedersen, L.K., Johansen, J.D., Held, E., Agner, T., 2004. Augmentation of skin response by exposure to a combination of allergens and irritants – a review. Contact Dermat. 50, 265–273.

Roach, P., Farrar, D., Perry, C.C., 2006. Surface tailoring for controlled protein Adsorption: effect of topography at the nanometer scale and chemistry. J. Am. Chem. Soc. 128, 3939–3945.

Rubio, C., Costa, D., Bellon-Fontaine, M.N., Relkin, P., Pradier, C.M., Marcus, P., 2002. Characterization of bovine serum albumin adsorption on chromium and AISI 304 stainless steel, consequences for the Pseudomonas fragi K1 adhesion. Colloid Surf. B 24, 193–205.

Rushton, E.K., Jiang, J., Leonard, S.S., Eberly, S., Castranova, V., Biswas, P., Elder, A., Han, X., Gelein, R., Finkelstein, J., 2010. Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. J. Toxicol. Env. Heal. A 73, 445–461.

Santonen, T., Stockmann-Juvala, H., Odnevall Wallinder, I., Darrie, G., Zitting, A., 2010. Use of read-across in the health risk assessment of ferro-chromium alloys under REACH. In: The Twelth International Ferro Alloy Congress (INFACON XII) Helsinki (FI).

Stockmann-Juvala, H., Hedberg, Y., Dhinsa, N.K., Griffiths, D.R., Brooks, P.N., Zitting, A., Odnevall Wallinder, I., Santonen, T., 2013. Inhalation toxicity of 316L stainless steel powder in relation to bioaccessibility. Hum. Exp. Toxicol. 32, 1137–1154.

Stopford, W., Turner, J., Cappellini, D., Brocka, T., 2003. Bioaccessibility testing of cobalt compounds. J. Environ. Monit. 5, 675–680.

Yamashita, T., Hayes, P., 2008. Analysis of XPS spectra of Fe2+ and Fe3+ ions in oxide materials. Appl. Surf. Sci. 254, 2441–2449.