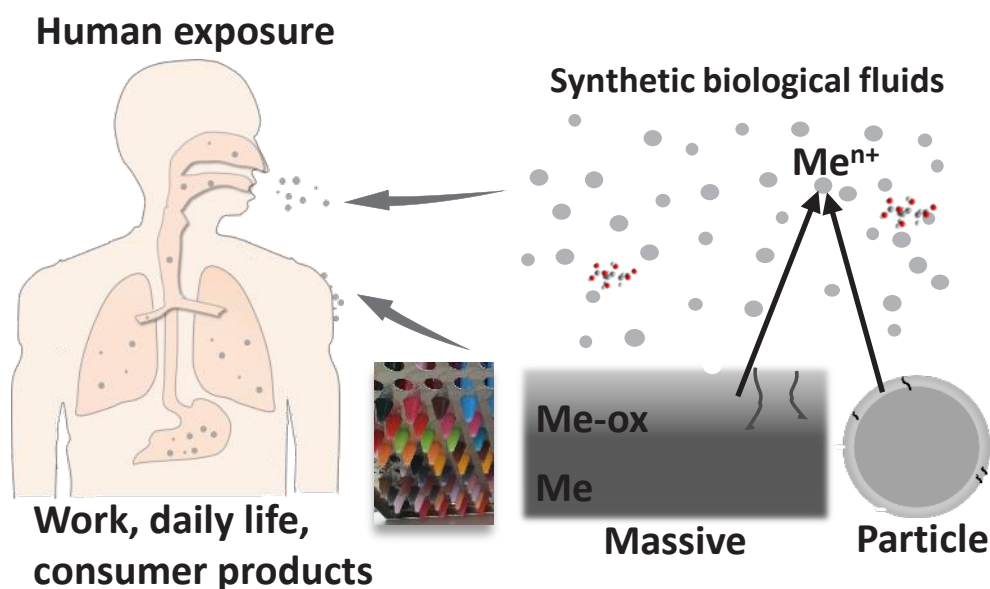


Doctoral Thesis in Chemistry

Bioaccessibility, corrosion and surface properties of metals, alloys and metallic powder in biological fluids of relevance for occupational and consumer health hazard assessment

XUYING WANG



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XUYING WANG

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*Perfection is not attainable,
but if we chase perfection,
we can catch excellence.*

- Vince Lombardi

Abstract

When assessing hazards of metals, alloys, and metal-containing items, the metal release (bioaccessibility) is of high importance. Triggered by increasingly stricter hazard classifications for metals, especially cobalt (Co), and limited knowledge, the aim of this doctoral thesis is to assess correlations between the metal/alloy surface characteristics, corrosion and metal release pattern in different simulated biological fluids relevant to human exposure scenarios (dermal, ingestion and inhalation, Papers I-V). In addition, occupational alloy powders (additive manufacturing, Paper III) and tattoo inks (Paper VI) were assessed in relation to potential health hazards. A reliable and reproducible in-vitro bioaccessibility test methodology was elaborated (Paper I). Generated nickel (Ni) and Co bioaccessibility data correlates with the surface and corrosion properties of the metals/alloys in the synthetic fluids, suggesting that the bioaccessible concentration, rather than bulk content, should be used for hazard assessment of Ni and Co in alloys and metals (Papers I-IV). Metallic Co was homogeneously distributed along with metallic Ni beneath the surface oxide of stainless steel 316L under different exposure conditions (Paper V). Exposure in a phosphate-containing neutral fluid resulted in the precipitation and incorporation of both Co and phosphate in the oxide (Paper V). Reused alloy powder during laser additive manufacturing processes revealed, depending on alloy type, different physicochemical properties including corrosion properties (Paper III). A large majority of the investigated tattoo inks violated existing regulations on labelling and substances of health concern (Paper VI), and Co, Ni, and other impurities were associated with iron-rich pigments. In all, this thesis presents bioaccessibility findings connected to surface characteristics relevant for hazard assessments of alloys and powders, and points towards physicochemical reasons behind the release of certain metallic impurities.

Keywords: Metal and alloy, metal release, synthetic biological fluids, surface oxide, corrosion resistance, hazard assessment

Populärvetenskaplig sammanfattning

Olika hälsorisker som till exempel hudallergi kan uppstå vid exponering för nickel och kobolt. Det är därför viktigt att förstå om olika metalliska föremål, metaller och legeringar kan frisätta dessa element i kontakt med olika kroppsvätskor. I den här doktorsavhandlingen har en metod utvecklats för att bestämma mängder av frisatt nickel och kobolt från massiva ytor och pulver av olika metalliska legeringar och rena metaller på ett jämförbart sätt och för olika exponeringsvägar (hudkontakt, inandning, förtäring). Ett annat mål har varit att undersöka om metaller som kobolt, som ofta endast befinner sig som föroreningar i legeringar, förekommer i ytoxiden eller endast i bulkmaterialet och hur det påverkar frisättningen (kobolt har nyligen omklassificerats då metallen kan orsaka cancer). Studierna har också omfattat samband mellan ytegenskaper och frisättning av metaller från metalliska pulver som används i tillverkningsindustrin och metalliska föroreningar samt undersökt hälsorisker med tatueringsfärger. Resultaten har visat att frisättningen av nickel och kobolt till olika syntetiska kroppsvätskor från metaller och legeringar ofta korrelerar väl med deras yt- och korrosionsegenskaper. Det medför att frisättnings- eller korrosionstester skulle kunna användas för att gruppera olika metaller och legeringar och bedöma deras hälsorisker, i stället för att eventuella risker baseras på legeringarnas bulksammansättningar. Legeringarnas ytoxider har ofta en skild sammansättning och en förmåga att minska frisättning av metaller på ett mycket effektivt sätt. Återanvänt pulver vid additiv tillverkning (3D-printning) kan erhalla ändrade fysikaliska och kemiska egenskaper på grund av exponeringen för laser. Innehållet av nickel, kobolt och kromföroreningar i tatueringsfärger var kopplat till andra metaller, främst järnrik röd pigment. Den här avhandlingen bidrar till att förfina riskbedömningar av metaller, legeringar, och metallinnehållande produkter.

Nyckelord: Biotillgänglighet, metaller, legeringar, pulver, additiv tillverkning, tatueringsfärger, korrosion, syntetiska kroppsvätskor, ytoxid, riskbedömning

List of summarized papers

This thesis is a summary of the following papers:

- I. **Bioaccessibility of nickel and cobalt in powders and massive forms of stainless steel, nickel- or cobalt-based alloys, and nickel and cobalt metals in artificial sweat.**
Xuying Wang, Gunilla Herting, Zheng Wei, Inger Odnevall Wallinder, Yolanda Hedberg
Regulatory Toxicology and Pharmacology, 106: 15-26 (2019)
- II. **Bioaccessibility of nickel and cobalt released from occupationally relevant alloy and metal powders at simulated human exposure scenarios.**
Xuying Wang, Inger Odnevall Wallinder, and Yolanda Hedberg
Annals of work exposures and health, 64(6): 659-675 (2020)
- III. **Bioaccessibility and reactivity of alloy powders used in powder bed fusion additive manufacturing.**
Xuying Wang, N. V. Srikanth Vallabani, Alix Giboin, Johan Lundqvist, Kim Färnlund, Hanna L. Karlsson, Yolanda S. Hedberg, Inger Odnevall
Materialia, 19: 101196 (2021)
- IV. **Metal bioaccessibility in synthetic body fluids – a way to consider positive and negative alloying effects in hazard assessments.**
Xuying Wang, James J. Noël, Inger Odnevall Wallinder, and Yolanda S. Hedberg
Materials & Design, 198: 109393 (2021)
- V. **Cobalt release process from stainless steel 316L under exposure to different synthetic biological fluids - Localization of cobalt impurities in the surface.**
Xuying Wang, Jonas Hedberg, Heng-Yong Nie, Mark C. Biesinger, Inger Odnevall, Yolanda S. Hedberg
Submitted manuscript
- VI. **Analytical survey of tattoo inks—A chemical and legal perspective with focus on sensitizing substances.**
Xuying Wang, Leila Josefsson, Silvia Meschnark, Marie-Louise Lind, Åsa Emmer, Walter Goessler, Yolanda S. Hedberg
Contact Dermatitis, 85(3): 340-353 (2021)

Author's contribution to the papers

Paper I

Part in experimental work (bioaccessibility studies, particle characterization and OCP). Major part in data visualization and paper writing.

Paper II

Major part in experimental work, except for XPS, BET and SEM measurements. Major part in data visualization and paper writing.

Paper III

Part in experimental work (bioaccessibility studies and electrochemical measurements). Major part in data visualization and paper writing.

Paper IV

Major part in experimental work, except for XPS measurements. Major part in data visualization and paper writing.

Paper V

Part in experimental design, and major part in data visualization and paper writing.

Paper VI

Major part in data visualization and paper writing.

Work not included in this thesis

Papers

- VII. Corrosion and metal release investigations of selective laser melted 316L stainless steel in a synthetic physiological fluid containing proteins and in diluted hydrochloric acid.**
Masoud Atapour, Xuying Wang, Kim Färnlund, Inger Odnevall Wallinder, Yolanda Hedberg
Electrochimica Acta, 354: 136748 (2020).
- VIII. Corrosion of binder jetting additively manufactured 316L stainless steel of different surface finish.**
Masoud Atapour, Xuying Wang, Mats Persson, Inger Odnevall Wallinder, Yolanda S. Hedberg
Journal of The Electrochemical Society, 167(13): 131503 (2020).

Reports

- IX. Nickel and cobalt release testing from several stainless steel grades in several synthetic body fluids (Bio-elution testing).**
Xuying Wang, Inger Odnevall Wallinder, Yolanda Hedberg
commissioned by Team Stainless, Sweden, March 2018.
- X. Nickel and cobalt release testing from three alloy grades in four synthetic body fluids (bioelution testing).**
Xuying Wang, Zheng Wei, Inger Odnevall Wallinder, Yolanda Hedberg
commissioned by Aperam, France, August 2018.
- XI. Nickel release from mascara into artificial sweat.**
Xuying Wang, Yolanda Hedberg
commissioned by Miild A/S, Denmark, March 2019.
- XII. Nickel release from nine cosmetic samples into artificial sweat.**
Xuying Wang, Yolanda Hedberg
commissioned by Miild A/S, Denmark, April 2019.
- XIII. Nickel release from five cosmetic samples into artificial sweat.**
Xuying Wang, Yolanda Hedberg
commissioned by Miild A/S, Denmark, June 2019.

- XIV. Nickel release from Chameleon Foundation 002 Light into artificial sweat.**
Xuying Wang, Yolanda Hedberg
commissioned by Gosh, Denmark, December 2019.
- XV. Nickel release from Foundation #03&06 into artificial sweat.**
Xuying Wang, Yolanda Hedberg
commissioned by Miild A/S, Denmark, January 2020.
- XVI. Bioaccessibility testing and characterization of five molybdenum compounds.**
Zheng Wei, Nanxuan Mei, Xuying Wang, Jonas Hedberg, Inger Odnevall Wallinder, Yolanda Hedberg
commissioned by the international molybdenum development association, August 2019.

Presentations

- XVII. Bioaccessibility and reactivity of alloy powders used in powder bed fusion additive manufacturing.**
Poster presentation, A Symposium on Surface Chemistry and Materials Science, October 2019, Malmö, Sweden.
- XVIII. Electrochemical testing – a potential tool complementing bioaccessibility assessments of alloy- and metal sheets and powders.**
Oral presentation, 2020 Western University ECS Student Chapter Symposium, December 2020, Virtual.
- XIX. Hazard assessment of alloys in biological environments - The surface matters: study on Co impurities in the oxide film of 316L stainless steel.**
Oral presentation, NACE International Corrosion 2021, April 2021, Virtual.

List of abbreviations

ALF	Artificial lysosomal fluid
ASL	Artificial saliva
ASW	Artificial sweat
As	Arsenic
BET	Brunauer Emmet Teller
Ba	Barium
CA	Citric acid
CLP	Classification, Labelling and Packaging Regulation
Co	Cobalt
Cr	Chromium
Cu	Copper
CV	Cyclic voltammetry
DNA	Deoxyribonucleic acid
DNEL	Derived No Effect Level
EBSD	Electron backscatter diffraction
ECVAM	European Centre for the Validation of Alternative Methods
EIS	Electrochemical impedance spectroscopy
EU	European Union
Fe	Iron
(GF/Flame -) AAS	(Graphite Furnace / Flame -) Atomic absorption spectroscopy
GHS	Globally Harmonized System for Classification and the Labelling of Chemicals
GST	Artificial gastric fluid
Hg	Mercury
ICPMS	Inductively coupled plasma mass spectrometry
JESS	Joint expert speciation system
LD	Laser diffraction
MALDI-TOF-MS	Matrix assisted laser desorption/ionization time of flight tandem mass spectrometry
Mn	Manganese
Mo	Molybdenum
N/A	No data available
Ni	Nickel
OCP	Open-circuit potential
OM	Optical microscopy
OEL	Occupational exposure limit
PBS	Phosphate buffered saline
Pb	Lead
PIGE	Paraffin impregnated graphite electrode
RAC	Committee of Risk Assessment
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
ToF-SIMS	Time of flight secondary ion mass spectrometry
XPS	X-ray photoelectron spectroscopy

Table of Contents

1. Motivation and objectives	1
2. Background.....	5
2.1 Daily human exposure to metal-containing products and particles	5
2.2 Existing regulations/limits of metals for risk assessment and management.....	6
2.3 Bioelution testing.....	9
2.4 Metal release mechanisms.....	11
2.5 Impact on sustainability	12
3. Experimental	13
3.1 Materials	13
3.2 In-vitro bioaccessibility (bioelution) testing	14
3.2.1 Simulated biological solutions and exposure parameters	14
3.2.2 Bioelution method modified from the EN 1811 standard	15
3.3 Analytical techniques.....	16
4. Summary and discussion of results	20
4.1 Surface characterization of alloys and metals - effects of alloy grade, material forms (powders versus massive surfaces) and changes upon exposure (Papers I - V).....	20
4.2 Investigation on Ni and Co release from alloys and metals in conditions relevant for human exposure scenarios (Paper I, II, IV).....	24
4.2.1 Most investigated alloy grades passed the compliance test related to Ni release as stipulated in the EN 1811 standard....	24
4.2.2 Effects of material forms (powder versus massive surfaces), alloy grade, solution, and exposure duration on the	

release of Ni and Co following the modified bioelution methodology.....	24
4.2.3 Bioaccessible concentration rather than bulk content should be used for hazard assessments of alloys	29
4.3 Corrosion resistance determines the extent of metal release in synthetic body fluids (Paper I - IV)	31
4.4 Studies on metal-containing substances used and collected at occupational settings and present in consumer products (Papers III and VI).....	33
4.4.1 Virgin and reused alloy particles used in additive manufacturing vary in particle and surface characteristics ...	33
4.4.2 Analytical survey on sensitizing metal-containing substances in tattoo inks	35
4.5 In-depth study on the location of Co impurities and the Co release process of stainless steel 316L in different synthetic fluids (Paper V).....	36
5. Main conclusions	39
6. Outlook.....	41
7. Acknowledgements.....	42
8. References	44

1. Motivation and objectives

When and how are humans exposed to metals? It could for instance be via contact with metallic daily high-touch surfaces, consumer products, jewelry, cosmetics, or metal-based powders used or formed at industrial workplaces. Human exposures via skin contact, ingestion or inhalation exposure paths can under certain conditions induce adverse health effects including allergy and DNA damage, and/or pose carcinogenic and respiratory risks [1-3]. To protect human health and the environment from risks posed by chemicals (including metals and alloys), the chemical regulation of the European Union (EU) - “Registration, Evaluation, Authorisation and Restriction of Chemicals” (REACH), entered into force in 2007. REACH requires companies including manufacturers and importers to provide solid data that show that their used chemicals and products to be placed on the market are safe to both humans and the environment [4] – ‘no data, no market’. Such information includes, e.g., physico-chemical characteristics and toxicological hazard information, properties which are related to the bioaccessibility (total amount of released metal) for specific exposure scenarios. In the lack of alloy-specific information, the legislative approach of REACH is to treat alloys as mixtures of their individual metal components, i.e., their nominal bulk alloy content. This is highly problematic as alloys cannot be treated as a chemical compound and the intrinsic properties are very different compared to their individual components. Another important aspect is that the surface oxide composition generally is very different from the nominal bulk alloy composition, e.g., chromium (Cr) in stainless steel reacts with oxygen in the environment to form a Cr-rich oxide layer on the surface of the steel [5]. Alloys are treated as mixtures also in the Globally Harmonized System for Classification and the Labelling of Chemicals (GHS) [6] and in the Classification, Labelling and Packaging Regulation (CLP) [7]. Hazard classification of metallic alloys is hence today often based on their bulk content and extrapolated from the behavior of the pure metal. Since Nickel (Ni) metal according to CLP is classified as skin allergen (Skin Sens.1), this means for example that alloys containing more than 1 wt.% Ni are classified for skin sensitization under CLP, regardless of their Ni content at the surface or their actual released amounts of Ni in

relevant conditions [8]. Cobalt (Co)-containing products with a bulk content exceeding 0.1 wt.% are from October 1st, 2021, classified as carcinogenic (Carc. 1B) [9]. Discussions by EU are under way to reduce this concentration limit even further to 0.01 wt.% Co, which would basically apply to most stainless steel and steel grades. However, it is well known to chemists and materials scientists that alloys often behave differently from their corresponding pure metals, and that even if present, certain alloy constituents will remain within the bulk of the material and neither be present at the surface nor be released to the environment. Since bioaccessibility findings and toxicological data of alloys are scarce, the regulatory approach based on simple mixtures remain. It is hence crucial to improve the scientific understanding of these aspects and to generate data that can be used to modify given regulatory perspectives on alloys.

Knowledge on the extent of metal release is of highest concern in sensitive environments, such as in the human body, and it is hence essential that bioaccessibility aspects are considered in hazard assessment and management. Triggered by the limited knowledge, reliable and reproducible in-vitro test methodologies (bioelution) were developed [10-12] to assess metal bioaccessibility of different materials (as massive surfaces and as powder particles) in synthetic biological fluids of relevance for different exposure scenarios, without the need for animal testing. However, to date few established and standardized test methods exist concerning metal release relevant to the dermal, ingestion and inhalation routes of exposure. Based on the existing methods, recent studies show that the generated bioaccessibility data for metals such as iron (Fe), Ni, Co, Cr, and molybdenum (Mo) from alloys were considerably lower than those based their bulk content proportions and that the bioaccessibility is mainly governed by the surface properties and the chemical environment in given exposure conditions. This is especially true for alloys of high corrosion resistance such as stainless steel [13-16]. Since such materials are commonly used in different societal applications and are a prerequisite for our industrialized society, it emphasizes the need to investigate correlations between alloy surface properties, corrosion resistance and bioaccessibility in conditions relevant for human exposure scenarios. Generated data can be used to

improve and refine hazard identification and classification of alloys without animal testing.

The main objectives of my PhD studies were therefore to:

- 1) elaborate a modified bioelution test procedure and protocol to enable reproducible testing of massive sheets, independent of transport and storage history, and to be applicable to powders;
- 2) quantify the extent of Ni and Co release from different alloys (both in massive and powder forms), including stainless steels and high/low-alloyed steel, compared with the behaviour of Ni and Co metals, upon exposure in different synthetic biological fluids simulating dermal, ingestion and inhalation exposure scenarios;
- 3) investigate and compare the surface characteristics and corrosion resistance of stainless steels and high/low-alloyed steel under the different exposure conditions in relation to the bioaccessibility, i.e., the extent of Ni and Co release;
- 4) assess differences between virgin and reused metal alloy particles used in additive manufacturing (selected laser melting) from a physicochemical, microstructural, surface composition, bioaccessibility, and in-vitro toxicity perspective;
- 5) provide in-depth understanding of the Co release process from stainless steel (grade 316L) by locating Co impurities in and underneath the surface oxide upon exposure to three synthetic biological fluids of varying composition and pH;
- 6) evaluate potential hazards with consumer products - tattoo inks, by measuring total contents of metals in pigments, and whether the labelling of the products fulfilled current legal requirements.

These objectives were accomplished using a combined fundamental and applied multi-analytical and interdisciplinary approach in close collaboration with international industry associations and academic partners, schematically shown in Figure 1.

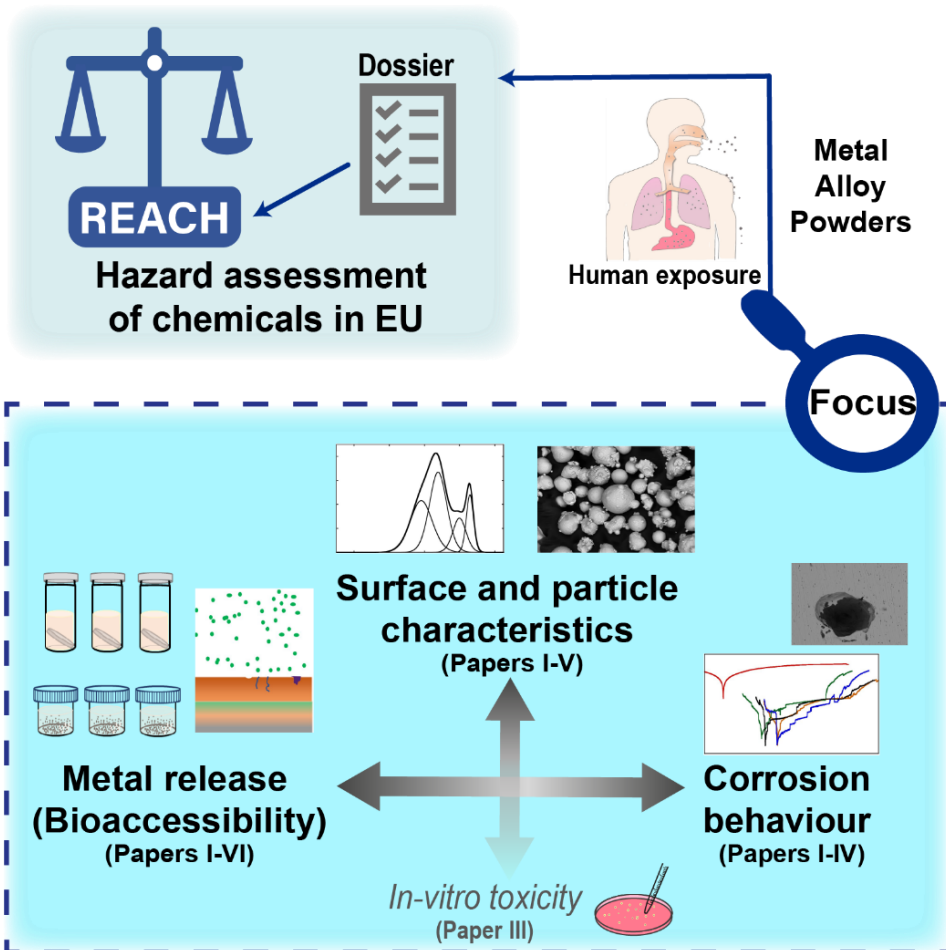


Figure 1 Schematic illustration of research approach conducted to provide scientific data to be used for hazard assessment within REACH. The *in-vitro* toxicity (italic in figure) measurements were performed by colleagues at Karolinska Institutet.

2. Background

2.1 Daily human exposure to metal-containing products and particles

The use of metals in the society exploded when the Industrial Revolution took off in the middle of the 18th century [1]. Metals and alloys are indispensable constituents in infrastructure, construction, electronics, as well as in consumer products such as cosmetics and jewelry. Human exposure to metal-containing products and/or particles is thus inevitable, e.g., via occupational exposures, during handling and disposal of metals during production and transport, and via contact with consumer products like mobile phones and earrings [17-23]. Human exposure scenarios mainly include skin contact (dermal), ingestion (oral and digestive) and/or inhalation (respiratory) [14], schematically shown in Figure 2.

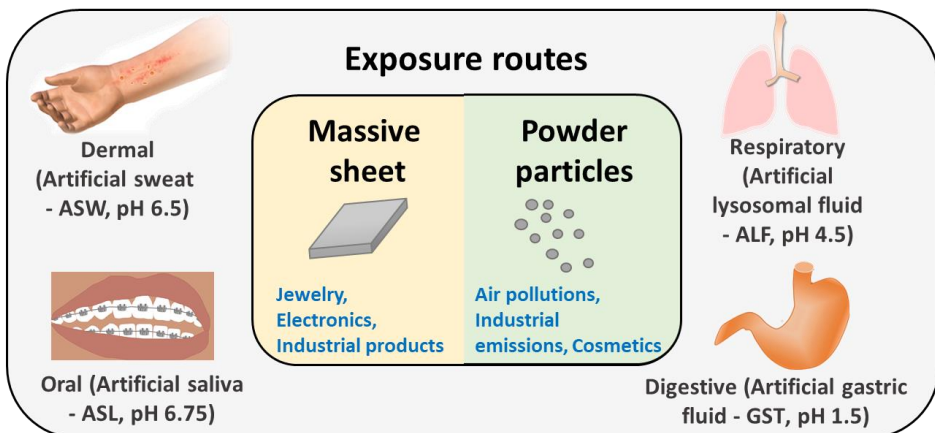


Figure 2 Schematic illustration of main human exposure routes to metal-containing products and/or particles.

As no material is inert and all materials to different extent interact with the environment, metals may be diffusely released into the surrounding environments. Knowledge of such dispersions is important [14, 15]. If the released amount of metal species exceeds certain values, they may result in adverse health effects [1, 3, 17, 18, 23] such as allergy, inflammation, DNA damage, carcinogenic and respirable risk. However, the potency is highly metal and environment specific.

2.2 Existing regulations/limits of metals for risk assessment and management

To protect human health and the environment from risks posed by chemicals, several laws or regulations, which require safety information on used chemicals, have been elaborated and established over the years. Among these, REACH is a chemical regulation within the EU adopted to ensure that chemicals and products on the EU market are safe for humans and the environment. It promotes alternative methods to animal testing for hazard assessments of substances [4]. Figure 3 presents an overview of the overall process of collecting and assessing existing information of a substance, followed by a final risk assessment under REACH. Some parts of my doctoral studies were an evident consequence of the need to generate novel data on physiochemical and toxicological information of substances by in-vitro studies and performed in close collaboration with companies, industry associations, or relevant organizations.

As previously mentioned, metals are described as substances under REACH for which registration is required, but registration dossiers are not required for all alloys, which, if data is lacking, are considered as simple mixtures of metals from a legislative perspective. Therefore, the hazard identification and classification of an alloy is often based on the intrinsic properties of individual metal constituents. The same interpretation is applied under the GHS [6] and the CLP [7]. Ni and Co are two important metals to consider in health risk assessment, and were therefore studied (Papers I, II and IV). These two metals are the most common metal skin sensitizers, affecting about 10-20% and 1-2% of the general population for Ni and Co, respectively [1]. Ni is regulated by the Nickel Directive, adopted in 1994 in Europe and subsumed into REACH Regulation since 2009, consisting of several parts for different types of products and their intention of use [24]. The Nickel Directive covers metal items but is insufficiently precise for metal powders. The Ni release restriction limit is $0.2 \mu\text{g}/\text{cm}^2/\text{week}$ for items inserted into pierced body parts and $0.5 \mu\text{g}/\text{cm}^2/\text{week}$ for items intended to come into direct or prolonged skin contact [25]. Except for causing contact allergy, Co is classified as being carcinogenic, mutagenic, and a reproduction hazard [1]. The Committee of Risk Assessment (RAC) has therefore suggested

an even stronger hazard classification for Co. Since October 1st, 2021, all metals and alloys containing 0.1 wt.% Co, i.e., basically most Fe- and Ni-based alloys (a threshold that will probably be reduced to 0.01 wt.% in the future) are classified as carcinogenic [9]. The current hazard identification and classification of Ni and Co under CLP, and concentration limits set for workers and/or general population under REACH, or recommended by RAC, are summarized in Table 1.

Metals are also risk-assessed within cosmetic legislative frameworks in the EU. Under the EU Commission, the Cosmetics Regulation No. 1223/2009 [26] (replacing the old Cosmetics Directive 76/768/EEC [27]) lists more than 1,000 chemical substances which shall not be used in cosmetic products due to their toxicological properties, including several metals such as arsenic (As), Cr, Co, mercury (Hg), Ni and lead (Pb). Tattoo inks or permanent make-up, which are neither directly legislated as cosmetics nor drugs, have been confirmed to contain harmful impurities and are regulated in the EU [28] to limit the risks posed by unsuitable ingredients.

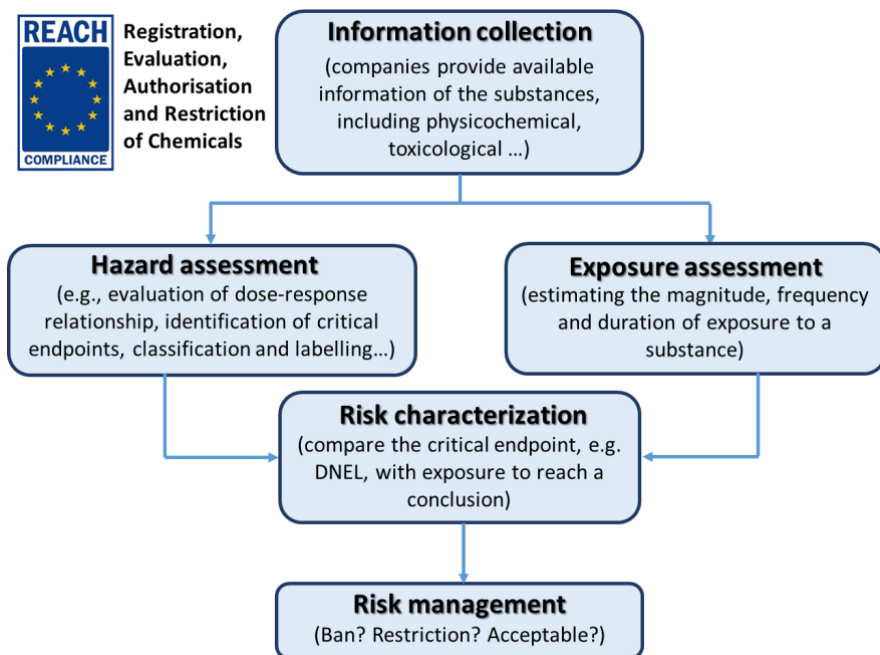


Figure 3 Overall process related to chemicals safety assessment under REACH.

Table 1 Summary of hazard classification and concentration limits of Ni and Co metals according to CLP, REACH and suggestions from RAC.

Metal		Ni		Co	
Form		Massive sheet	Powder particles	Massive sheet	Powder particles
CLP Classification		Skin Sens.1 (may cause an allergic skin reaction); STOT RE 1 (causes damage to organs); Carc. 2 (suspected of causing cancer)	Skin Sens.1; STOT RE 1; Carc. 2; Aquatic Chronic 3 (harmful to aquatic life with long lasting effects)	Skin Sens.1; Resp. Sens. 1B (may cause asthma or breathing difficulties if inhaled); Carc. 1B (may cause cancer); Muta. 2 (suspected of causing genetic defects); Aquatic Chronic 4 (may cause long lasting harmful effects to aquatic life); Repr. 1B (may damage fertility)	N/A
Toxicological value under REACH	Skin contact	a DNEL value of 0.035 mg/cm ²		N/A	
	Inhalation	a DNEL value of 0.05 mg/cm ³		a DNEL value of 40 µg/cm ³	
	Ingestion	a DNEL value of 0.011 mg/kg bw/day		a DNEL value of 29.8 µg/kg bw/day	
Recommended occupational exposure limit by RAC		0.005 mg/m ³ for respirable dust 0.03 mg/m ³ for inhalable dust		N/A	

* REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals; RAC- Committee of Risk Assessment; CLP - Classification, Labelling and Packaging Regulation; DNEL-Derived No Effect Level; N/A – no data available; bw – body weight.

2.3 Bioelution testing

REACH requires companies that want to place substances or products on the European market to provide comprehensive information on their toxicological hazards. The toxic potency of most metal-containing substances is related to metal bioavailability which is connected to the chemical form in a given environment [12]. The metal bioavailability is generally defined as the extent to which the released metal can be absorbed in the body and made available in the target organ. Information on bioavailability can be obtained by in-vivo studies in body fluids and/or target organs. To minimize the use of animal tests and support the use of alternative in-vitro methods for testing the safety of materials required by the REACH, bioaccessibility may be used as a conservative measure of bioavailability. Bioaccessibility is defined as the total amount of released metal species under surrogate physiological conditions. How bioaccessibility and bioavailability are connected is illustrated in Figure 4. The bioaccessibility of different metals can be assessed by in-vitro bioelution testing, a methodology to determine the extent of metal release from metals and alloys in relevant synthetic biological fluids in a simple, rapid and reproducible way. In-vitro bioelution testing of metal-containing materials (either in massive or powder forms) have in recent years been conducted and test methods elaborated with the aim to generate short or long-term quantitative metal release data to predict relative bioavailability for hazard identification [14, 16, 29-31]. Some bioelution methods have been formalized in the EU and elsewhere as standards for product testing, such as EN 1811 [25] (testing Ni release from consumer articles intended for prolonged and direct skin contact), EN 71-3 [32] and ASTM F-963 [33] (determining the migration of metals from toy materials), and ASTM D-5517 [34] (determining extractability of metals from art materials). However, there is in the EU not yet any standardized bioelution test protocol applicable for metal substances following dermal, ingestion and/or inhalation exposure. A test protocol focusing on ingestion is though currently under regulatory acceptance by European Centre for the Validation of Alternative Methods (ECVAM) [35], a protocol partly based on accomplishments of the research team at KTH in close collaboration with International metal

associations (Eurometaux, Nipera, International Stainless steel forum, International Chromium Development Association, Eurofer and others).

Several synthetic biological fluids have been used to mimic relevant human exposure routes and to estimate the extent of metals that can be released under simulated human body conditions. As an example, artificial sweat (ASW) simulates dermal contact following a standardized test method (EN1811) for products [25]. This method was approved in 1998 and later slightly revised for compliance with the Ni restriction in REACH. Artificial saliva (ASL) represents the environment of the oral cavity [36], with a modified formula in which calcium chloride (CaCl_2) was replaced by potassium chloride (KCl) (similar corrosivity) [37] since Ca ions interfere with trace metal analysis of Cr. Diluted hydrochloric acid is used as a surrogate of gastric fluid (GST) and mimics the very harsh digestion environment in the stomach with high acidity. This fluid has been used to evaluate metal solubility in the standard bioelution method (EN 71-3) for the safety of toys since 1982 [32], and was then adopted in the United States Standard ASTM D-5517 for testing art materials [34]. When compared to synthetic gastric fluid containing organic acids and enzymes, measurements in diluted hydrochloric acid show equivalent results [38]. Artificial lysosomal fluid (ALF) is a solution that in a simple way simulates the intracellular environment (lysosome, pH 4.5) after particle cell uptake or in inflammatory conditions within lung cells at a low pH [39], and provides for many metals (e.g., Fe, Ni, Co) a “worst-case” scenario from a metal release perspective. Citric acid in the fluid mimics the organic components of intracellular fluid. Phosphate buffered saline (PBS) [29] is a standard physiological solution that closely mimics the pH and ionic strength of human blood serum, and is widely used in biological research as a reference test solution for simulated physiological conditions [29, 40, 41]. The above mentioned synthetic biological fluids have been studied in this thesis. More details are given in Section 3.2.1. Although real body fluids are very complex and the artificial solutions can only simulate physiological conditions to a limited extent, in-vitro findings in the synthetic biological fluids can nevertheless provide useful and comparable information relevant for the real conditions.

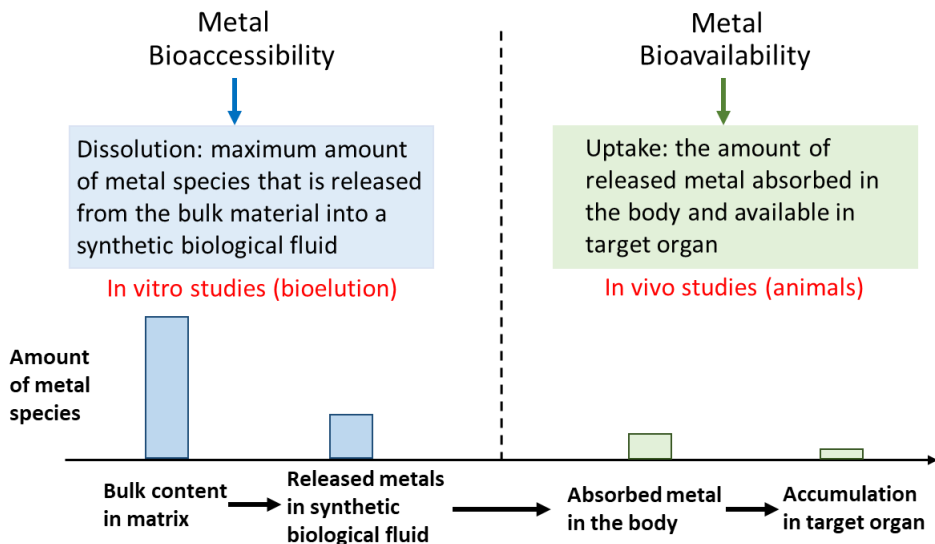


Figure 4 Illustration of the relationship between bioaccessibility and bioavailability.

2.4 Metal release mechanisms

Metal release from most alloys exposed to a biological environment is usually governed by different processes taking place at/within the surface oxide. As a largely used alloy group, stainless steel has been widely investigated and prevailing metal release mechanisms have been assessed [14] including i) electrochemical processes (e.g., localized or uniform corrosion) by metal oxidation, ii) chemical/electrochemical dissolution of the surface oxide (e.g., surface complexation – ligand induced dissolution), and/or iii) physical process (e.g., wear), schematically illustrated in Figure 5. The predominating metal release mechanism may change with time, and generally, more than one mechanism takes place at the same time. The extent of metal release from different materials is influenced by many factors [14-16, 42-44], such as alloy grade, surface finish, exposure conditions (pH, temperature and exposure time), and presence of organic species.

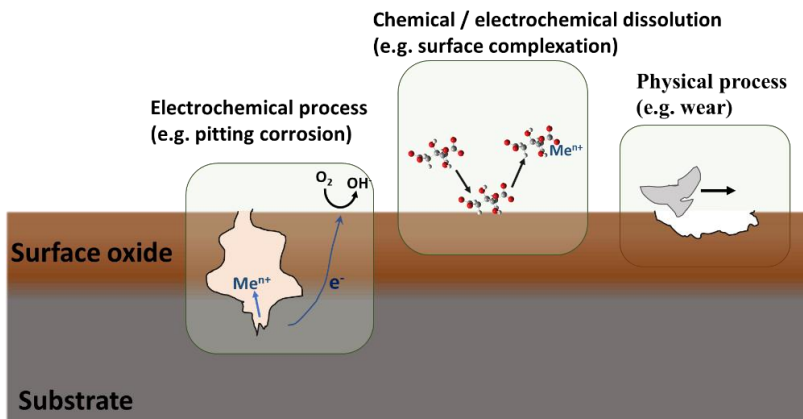


Figure 5 Schematic illustration of metal release mechanisms for stainless steels exposed to a biological environment, summarized and inspired by [14].

2.5 Impact on sustainability

The European Green Deal, EU’s new growth strategy, has set goals to better protect human health and the environment as part of its ambitious approach on zero pollution and moves towards a toxic-free environment by 2050 [45]. Over the last few years, several regulations, such as REACH and CLP, have been successful in protecting worker and consumer health and the environment by providing a legislative framework for companies to operate to reduce the risks posed by certain hazardous chemicals. Under these regulations, animal testing is usually limited and can only be used as a last resort. Alternative in-vitro methods are encouraged for the chemical industry to demonstrate the safety of a specific substance. The investigations of this PhD study aimed at correlating materials and surface properties with metal bioaccessibility and toxicity in simulated biological environments using an in-vitro methodology (bioelution testing). A modified bioelution method was elaborated and used. It was proven to be an excellent example of a reliable alternative method for animal testing to support grouping and read-across of metal-containing materials relevant to various exposure routes in risk assessment and management (Papers I-IV, [46]). With the aim to improve and refine hazard identification and classification of alloys, our research is directly linked to at least two of the UN global sustainability development goals – SDG 3: Good Health and Well-being; and SDG 12: Responsible Consumption and Production.

3. Experimental

3.1 Materials

A list comprising the investigated alloys in Papers I-V, both as massive sheets and powder particles is given in Table 1.

Table 1 Summary of alloys and pure metals as massive sheet and powder particles investigated in the different papers (I-V). The elemental contents >2 wt.% and Paper number are given for each material.

Material (massive sheet)			
316L (FeCr18Ni15, FeCr17Ni10, FeCr17Ni13) <i>Papers I, IV, VI</i>	630 (FeCr15Ni5) <i>Paper I</i>	FeCr20Ni9 <i>Paper I</i>	CoCr28Mo6 <i>Paper I</i>
FeCr11 <i>Paper I</i>	304 (FeCr18Ni9) <i>Papers I, IV</i>	FeCr21Ni11 <i>Paper I</i>	Low-alloyed Steel <i>Paper IV</i>
430 (FeCr16) <i>Papers I, IV</i>	303 (FeCr17Ni8) <i>Paper I</i>	IN718 (Ni53Cr18Mo3Fe18) <i>Paper I</i>	Ni metal <i>Papers I, IV</i>
FeCr18 <i>Paper I</i>	LDX2101 (FeCr21Mn5) <i>Papers I, IV</i>	R30008 (Co39Ni16Cr20Mo7Fe16) <i>Paper I</i>	Co metal <i>Papers I, IV</i>
FeCr21 <i>Paper I</i>	FeCr23Ni3 <i>Paper I</i>	Alloy 605 (FeCo50Cr20Ni11) <i>Paper I</i>	-
Material (powder particle)			
316L (FeCr17Ni11Mo2, FeCr17-19Ni13- 15Mo2-3) <i>Papers I, II, III</i>	304 (FeCr19Ni9) <i>Papers I, II</i>	430 (FeCr17) <i>Papers I, II</i>	Ni metal <i>Papers I, II</i>
IN625 (NiCr21Mo9Fe2) <i>Papers I, II</i>	IN718 (NiCr19Mo3Nb5Fe) <i>Paper III</i>	18Ni300 (FeCo9Ni18Mo5) <i>Paper III</i>	Co metal <i>Papers I, II</i>

The investigated materials are available on the market and relevant for human exposure scenarios. Differences between massive sheets and powder particles were highlighted in Paper I. Papers II and III mainly focused on the powders, while Papers IV and V focused on massive sheets. The bioaccessibility of Ni and Co were studied in Papers I, II and

IV for different alloys and compared with pure Ni and Co metals. In Paper VI, 73 different tattoo inks currently available on the market were investigated to assess potential hazards.

3.2 In-vitro bioaccessibility (bioelution) testing

3.2.1 Simulated biological solutions and exposure parameters

Table 2 Summary of investigated simulated biological solutions and exposure parameters in Papers I-V.

Solution	Simulated environment	Composition	pH	Temperature	Exposure period	Paper
ASW	Skin (sweat)	5.0g/L NaCl, 1.0g/L (NH ₂) ₂ CO (urea), 1.0g/L CH ₃ CHOHCO ₂ H (lactic acid)	6.5	30 °C	168 h (1 week)	I
					2 h, 4 h, 8 h, 24 h, 168 h	II; IV
ASL	Mouth (saliva)	0.4g/L NaCl, 1.21g/L KCl, 0.78g/L NaH ₂ PO ₄ ·2H ₂ O, 0.005g/L Na ₂ S·9H ₂ O, 1.0g/L (NH ₂) ₂ CO (urea)	6.75	37 °C	2 h, 4 h, 8 h, 24 h, 168 h	II; IV
ALF	Lung (lysosome in cells)	20.8g/L C ₆ H ₈ O ₇ (citric acid), 6.00g/L NaOH, 3.21g/L NaCl, 0.128 g/L CaCl ₂ ·2H ₂ O, 0.090g/L C ₄ H ₄ O ₆ Na ₂ ·2H ₂ O (disodium tartrate dihydrate), 0.086g/L C ₃ H ₃ O ₃ Na (sodium pyruvate), 0.085g/L C ₃ H ₅ NaO ₃ (sodium lactate), 0.077g/L C ₆ H ₅ Na ₃ O ₇ ·2H ₂ O (trisodium citrate dihydrate), 0.071g/L Na ₂ HPO ₄ , 0.059g/L H ₂ NCH ₂ COOH (glycine), 0.0497g/L MgCl ₂ , 0.039g/L Na ₂ SO ₄	4.5	37 °C	2 h, 4 h, 8 h, 24 h, 168 h	II
					24 h	III; V

GST	Stomach (gastric fluid)	4g/L 25% HCl	1.5	37 °C	2 h, 4 h, 8 h, 24 h, 168 h	II; IV
					24 h	V
PBS	Blood (serum)	8.77g/L NaCl, 1.28g/L Na ₂ HPO ₄ , 1.36g/L KH ₂ PO ₄	7.4	37 °C	24 h	V

3.2.2 Bioelution method modified from the EN 1811 standard

The European EN 1811 standard [25] exists to assess Ni release from articles of metals and alloys in massive forms intended for direct and prolonged skin contact. There is no corresponding test for Co, for powders, or for hazard assessment of material/alloy grades following other exposure routes. Testing according to the EN 1811 standard is to be done for the as-received surface condition of the test items. However, as previously shown [14, 43, 47], the extent of metal release can largely differ for as-received surfaces based on storage, surface finish etc., which affect the characteristics and composition of the surface oxide and hence the metal release process. Although testing of as-received products on the market is highly relevant from a legislative perspective, it is less relevant when comparing different alloys and metals. Further, the EN 1811 protocol does not stipulate any agitation or movement of the fluid during the exposure period, which is required for testing of powders to avoid extensive agglomeration. Therefore, a modified bioelution method was elaborated based on the EN 1811 standard adding a defined sample preparation procedure and agitation conditions to realize a reproducible and reliable in-vitro bioaccessibility test, and to enable a comparison between massive surfaces and powders of a given alloy or metal (Paper I). Detailed procedures of the EN 1811 and the modified method are illustrated in Figure 6. Differences in results between the four stainless steel grades tested using the two methods were relatively small (from 1.2 to 5.3-fold) (Paper I), suggesting that the surface preparation method in the modified protocol resulted in comparable surfaces as for as-received surface conditions, and that the use of gentle agitation (movement of fluids) used for massive surfaces did not largely influence the results compared with the non-agitated conditions. As a result, the modified bioelution method was applied for both massive sheets (Papers I, IV and V) and powder particles (Papers I, II and III), in different fluids

simulating human exposure scenarios. In Paper V, the modified method was employed to assess the extent of metal release from stainless steel 316L in different fluids, but without the 2 h pre-passivation (in a similar solution as the later test solution), since the study did not require as-received surfaces.

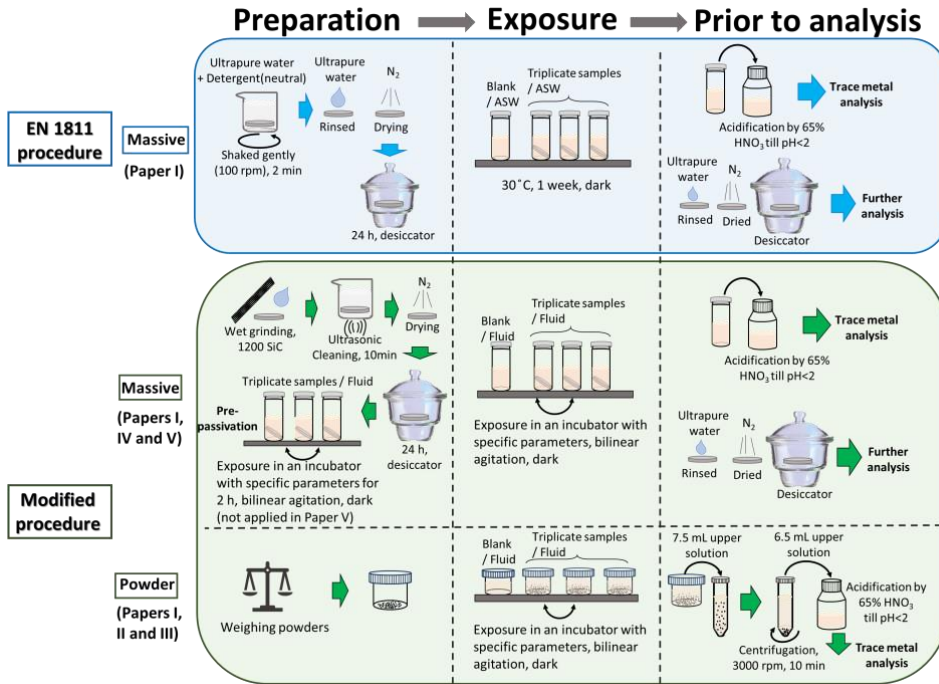


Figure 6 Overview of experimental schemes stipulated in the EN 1811 standard and the elaborated bioelution protocol for the modified test procedure (Paper I).

3.3 Analytical techniques

Several complementary analytical techniques were employed to provide a deep understanding of metal release, surface changes, particle characteristics, and corrosion resistance of different materials under simulated human exposure conditions. An overview of the main applied techniques is given in Table 3. Specific methodologies and information for each technique are given in the respective papers. It should be underlined that several techniques were employed in close collaboration with different colleagues, as indicated in Table 3.

Table 3 Summary of main techniques (and initials of operators) used within the appended Papers of this thesis.

Analysis	Technique	Purpose of use	Used in Papers	Operators
Trace metal / chemical speciation analysis in solutions	(GF/Flame -) AAS	Total concentrations of Ni and Co in solution	Papers I, II, IV	XW, YH, GH, ZW
	ICP-MS	Total concentrations of various metal in solution	Papers III, V, VI	Biotron staff, ALS Scandinavia staff, SM
	MALDI-TOF-MS	Identification of organic pigments	Paper VI	LJ
Surface analysis	XPS	Chemical speciation and elemental composition of outermost surface	Papers I, II, III, IV, V	IO, MB
	SEM	Surface morphology and particle size-particles and massives	Papers I, II, III, IV	XW, YH, GH
	OM	Surface morphology – massives	Paper IV	XW
	ToF-SIMS	Elemental depth distribution	Paper V	JH, HYN
	EBSD	Bulk microstructure	Paper III	OK
Particle specific characterization	LD*	Size characterization of particles in solutions	Papers I, II, III	YH, XW
	BET	Specific surface area in dry conditions	Papers I, II	Sandvik Heating Technology AB
	JESS	Chemical speciation modelling in solution	Paper IV	YH
Electrochemical measurements	OCP	Corrosion potential	Papers I, II,	XW
	Potentiodynamic polarization	Corrosion resistance	Papers II, III, IV	XW
	EIS	Corrosion resistance and barrier properties of surface oxide	Paper IV	JJN, XW
	CV	Chemical speciation of the surface oxide on particles	Paper II	YH, XW
Toxicological tests	Cytotoxicity	In-vitro toxicity of powders	Paper III	NS
	ROS generation		Paper III	NS

**In Papers I and II, this technique was denoted static light scattering, but a more accurate term is laser diffraction for the measurements conducted herein.*

XW-X. Wang; YH-Y. Hedberg; GH-G. Hering; ZW-Z. Wei; NS-N.V. Srikanth; JH-J. Hedberg; IO-I. Odnevall; OK-O. Karlsson; HYN-H.Y. Nie; SM-S. Meschark; MB-M.C. Biesinger; JJN-J.J. Noël; LJ-L. Josefsson

Electrochemical measurements

In the electrochemical measurements, both massive sheets and micron-sized powder particles were investigated to obtain information on passive and corrosion properties as well as surface composition. For the massive sheets, a normal experimental set-up with a three-electrode system was used, see Figure 7a (Papers I and IV). In the case of powder particle electrochemistry, conventional experimental set-up is difficult to apply and hence a special electrode set-up was used as illustrated in Figure 7b (Papers II and III). Electrochemical studies on the powder particles were performed by using a paraffin-impregnated graphite electrode (PIGE) as working electrode onto which the particles were immobilized. PIGE was shown sufficient to provide electrical conductance to the powders, with a very low background current compared with alternative working electrodes suitable for powders [48, 49]. PIGE was pre-grounded using 1200 grit SiC paper, cleaned with ethanol and ultrapure water, and shortly heated before pressing the particles onto its surface. Although the exact surface area or mass of the attached powders cannot be accurately determined and exact current densities are hence impossible to calculate, each powder covered the same geometric surface area of the PIGE. This set-up was used for OCP measurements (generally independent of surface area) in different solutions (Paper II), for potentiodynamic polarization studies to compare corrosion properties of different powder particles (Papers II and III), and for CV measurements in a strongly alkaline buffered solution to assess information on surface oxide composition (Paper II).

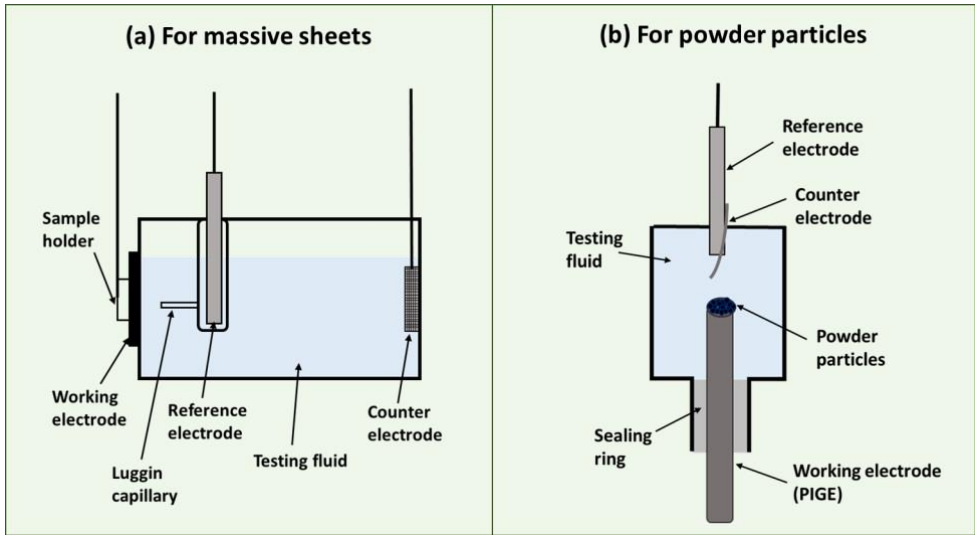


Figure 7 Experimental set-up of electrochemical measurements for (a) massive sheets and (b) powder particles.

4. Summary and discussion of results

4.1 Surface characterization of alloys and metals - effects of alloy grade, material forms (powders versus massive surfaces) and changes upon exposure (Papers I - V)

Knowledge of bulk and surface properties of powder particles and massive sheets is important, as many factors, such as particle size, shape/morphology, and surface composition, largely govern the extent of metal release in different synthetic biofluids [13-16, 49, 50]. Furthermore, physiochemical characteristics of substances are required in human health risk assessment under REACH [4]. In this study we employed a multi-analytical approach for detailed bulk and surface oxide characterization.

For the powder particles, our main focus was placed on stainless steels (grades 430, 304 and 316) and a Ni-based alloy (IN626), compared with pure metals (Ni and Co) (Papers I and II), schematically summarized in Figure 8. The alloy powders were all relatively spherical and showed a similar particle size both under dry and wet conditions, as judged from SEM imaging and specific surface areas in dry conditions (BET) and in the three synthetic body fluids (ASW, ASL and ALF). However, the Ni and Co metal powders were smaller-sized in dry conditions, and agglomeration was evident in the solutions with an up to 5-fold reduced specific surface area in solution compared to dry conditions.

In agreement with previous literature findings [49, 51-53], the strong enrichment of oxidized Mn(III/IV) (48-55 wt.% of the oxidized metals compared to 0.5-1.4 wt.% of the bulk content) was observed within the outermost surface of the stainless steel powders (grades 430, 304 and 316L) (Paper II), followed by oxidized Fe(II/III) and Cr(III). The enrichment of Mn was observed for the inert-gas-atomized stainless steel particles and was explained by the large affinity of Mn to oxygen and its tendency to diffuse to the surface during the gas-atomization process [49, 54]. The same observation was made for the 316L particles during additive manufacturing using selective laser melting (Paper III). The Ni-base alloy, IN625 was predominantly composed of oxidized Cr(III) and Ni (presumably NiO/Ni(OH)₂) (Paper II). All investigated alloy powders

showed a surface oxide rich in Cr(III), providing good corrosion resistance [5]. In contrast, the surface oxides of the Ni and Co metal powders provided less protective properties with a substantially higher corrosion susceptibility compared to the alloy powders in the synthetic test fluids (Paper II).

The presence of Mn(III/IV)-phases on the surface of the stainless steel powders (grades 430, 304 and 316) induced a strong surface ennoblement, i.e., a relatively higher OCP, compared with the Ni-based alloy and the pure metals (Paper II), and the water-atomized powder and massive sheet of the same grade [49]. This ennoblement could be because Mn oxides catalyze the reduction of oxygen by chemical oxidation of Mn(III)-ions [55]. The presence of Mn-oxides could also cause the higher corrosion potential (E_{corr}) displayed for stainless steels compared with the Ni-based alloy and the pure metals (Paper II) [49, 56].

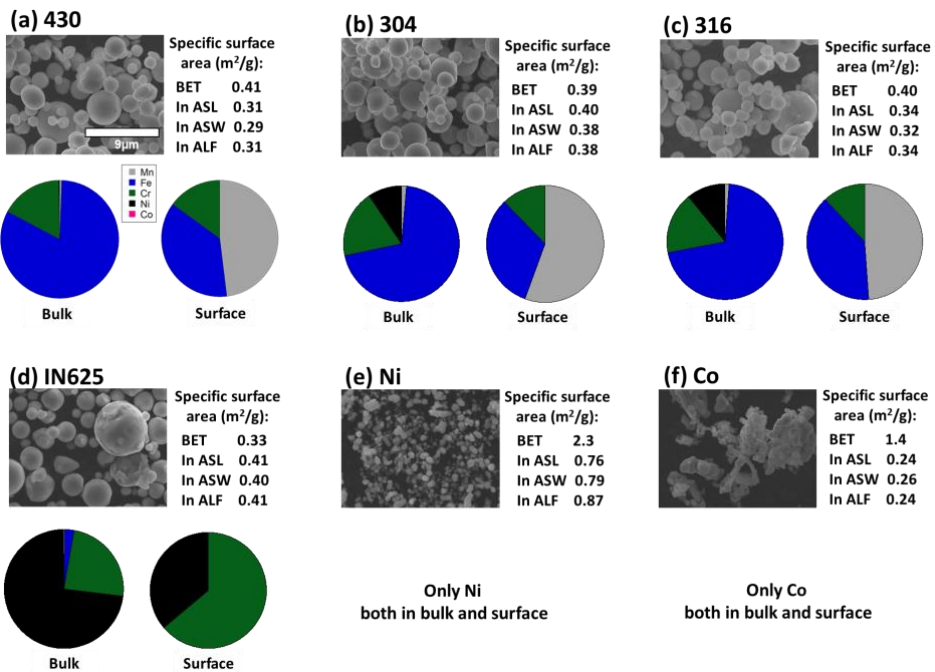


Figure 8 Overview on the particle size, morphology and bulk and surface composition (nominal composition and surface composition based on XPS) of unexposed alloy (stainless steels – 430, 304, 316L, Ni-based alloy - IN625), and pure metal (Ni and Co) powders investigated in Papers I, II. All SEM images in same scale as in (a).

Changes in surface oxide composition with time and solution were investigated primarily for the massive sheets. Similar studies on powders are much more difficult since the exposed powder needs to be successfully separated from the solution in a sufficient amount and by different and complicated washing methods. Figure 9 shows differences in surface oxide composition of representative massive alloys investigated in Papers I and IV, prior to and after exposure to ASL (pH 6.75), ASW (pH 6.5) and GST (pH 1.5) for one week. Both the solution chemistry and the alloy grade play important roles for observed changes in the surface oxide composition. The outermost surface oxide was for all investigated stainless steel grades composed of Fe(II/III)- and Cr(III)-oxides. Exposures in the pH-neutral solution ASW (pH 6.5) induced slightly or negligible changes in composition. A reduced Cr content in the surface oxide was observed after exposure in ASL (pH 6.75), probably due to the presence of sulfide ions in ASL. These are adsorbed onto the surface or even enter into the film, accelerating metal or metal oxide dissolution, thus inhibiting the formation of Cr-oxides in the passive film [57]. A strong enrichment of Cr within the outermost surface of stainless steels was observed in the acidic GST fluid (3-4-fold enrichment compared with the unexposed surface). This was explained by preferential release of Fe in acidic conditions, which is expected for Cr-rich alloys [14]. Similarly, an increased oxidized Cr content in the surface oxides of the stainless steel grades was observed after exposure in ALF (pH 4.5) for 24 h (Paper V). No oxidized Ni in the outermost surface oxide was observed for any grade, but metallic Ni was identified for 316L and 304 (Paper IV), in line with its expected enrichment beneath the surface oxide [14, 58]. Different from the powder particles, all investigated massive stainless steels showed no or negligible amounts of oxidized Mn in the surface oxide (Papers I and IV). In the case of the low-alloyed steel (0.017 wt.% Cr), only oxidized Fe was observed on the surface, and that steel corroded actively after the potentiodynamic scan (Paper IV). Besides oxidized Fe and Cr, Ni-rich oxides were observed within the surface oxide of IN718, which could explain the relatively higher Ni release from IN718 into ASW (Paper I).

In general, it is evident that the surface oxide characteristics of massive sheets are different from powder particles of the same alloy. While we

attempted to achieve as comparable surface conditions as possible by grinding, polishing, cleaning, and pre-passivating (pre-exposing) massive materials, the surface compositions of massive sheets and powders were not comparable. The surface composition of powder particles is determined by the rapid cooling process and the environment during manufacturing [47, 49].

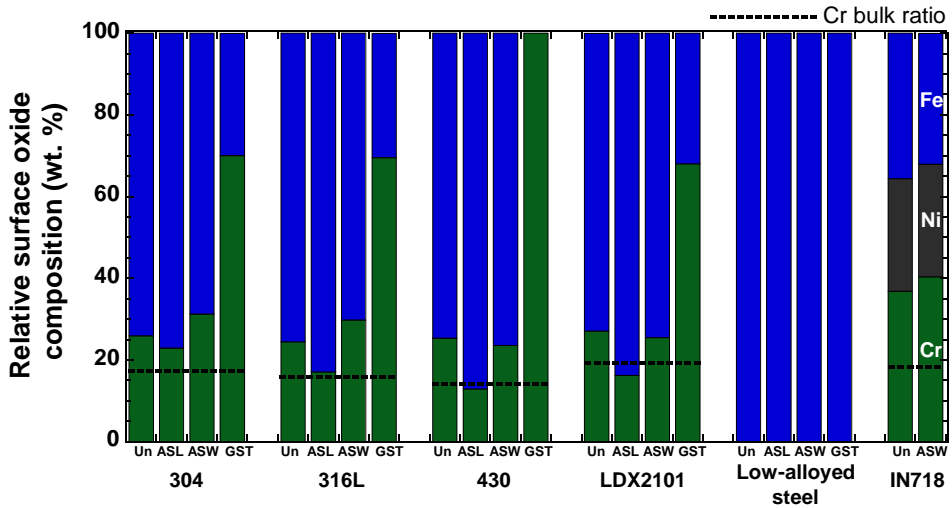


Figure 9 Relative mass content (wt.%) of oxidized metals (Fe, Ni and Cr) in the outermost surface oxide of the massive stainless steels (grades 304, 316L, 430, and LDX2101), the low-alloyed steel, and a Ni-based alloy (IN718) determined by means of XPS, prior to (unexposed – Un) and after exposure to ASL (pH 6.75), ASW (pH 6.5) and GST (pH 1.5) for one week. (Papers I and IV)

In all, alloy powders have a very different surface composition as compared to ground, polished, cleaned, unexposed or exposed massive sheets of the same alloy. This is primarily related to differences during manufacturing, e.g., rapid cooling during the atomization process for the powders. The composition of the surface oxide changes upon exposure to synthetic body fluids.

4.2 Investigation on Ni and Co release from alloys and metals in conditions relevant for human exposure scenarios (Paper I, II, IV)

4.2.1 Most investigated alloy grades passed the compliance test related to Ni release as stipulated in the EN 1811 standard

Ni is the most common metal allergen upon skin contact, and the release of Ni from consumer products and articles has been regulated under REACH since 2007 [4]. 15 common stainless steels and 4 high-alloyed steels were assessed for their Ni release following the EN 1811 standard (Paper I). The released amount of Ni from all investigated alloys was below $0.5 \mu\text{g}/\text{cm}^2/\text{week}$, the restriction limit for items intended to come into direct or prolonged contact with the skin [4], but two grades, 303 ($0.29 \mu\text{g}/\text{cm}^2/\text{week}$) and IN718 ($0.30 \mu\text{g}/\text{cm}^2/\text{week}$), exceeded a stricter restriction limit of $0.2 \mu\text{g}/\text{cm}^2/\text{week}$ set for items inserted into pierced body parts [4]. These two exceptions were related to surface oxide characteristics (Mn-sulfide inclusions in the surface of 303 causing metastable pitting) and differences in bulk composition (high Ni bulk content in IN718). The other tested alloy grades released very low amounts of Ni into the ASW, about 5-100-fold lower than the lower restriction limit ($0.2 \mu\text{g}/\text{cm}^2/\text{week}$), related to their high corrosion resistance.

4.2.2 Effects of material forms (powder versus massive surfaces), alloy grade, solution, and exposure duration on the release of Ni and Co following the modified bioelution methodology

Both Ni and Co are metals of concern within hazard assessment and management. The modified bioelution test procedure was employed to assess and compare Ni and Co release from some selected powders and massive alloys.

As discussed above, the surface characteristics and composition play important roles in the metal release and corrosion behavior of a metal and an alloy. An important aspect that needs to be considered is the surface area, as metal release is an interfacial reaction that takes place at the surface. It is evident that there is a large difference between the

surface area of massive forms and powder, e.g., about 8,000-fold larger for 5 mg 316L powder compared with 5 mg massive sheet of 316L. From a chemical and material perspective, it is more relevant to normalize the amount of released metals to the surface area. However, the specific surface area of powders often varies for different exposure conditions and is not easy to determine in an accurate way (e.g., the low pH in GST prohibits particle size measurements, Paper II). Further, normalization to the mass is more relevant from a toxicological perspective. Figure 10 illustrates the comparison between the release of Ni from massive and powder samples exposed to ASW for 168 h following the modified bioelution method (Paper I).

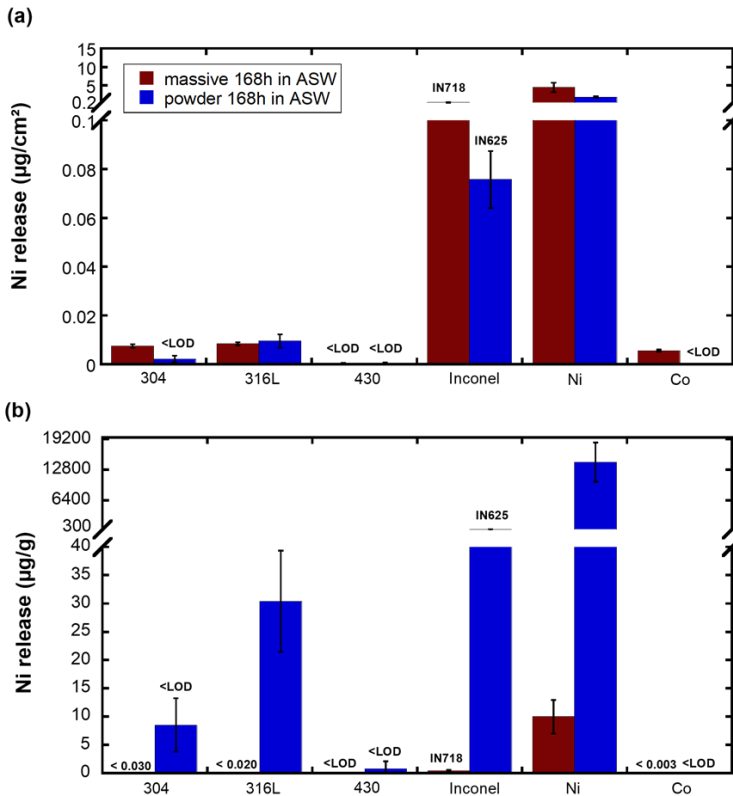


Figure 10 Comparison of Ni release from massive sheet and powders of different alloys and pure metals (after exposure to ASW for 168 h) - (a) normalized to surface area, $\mu\text{g}/\text{cm}^2$, and (b) normalized to mass, $\mu\text{g}/\text{g}$. The error bars represent the standard deviation of three independent samples. Please note the massive and powder Inconel grades are not identical but included for comparison. <LOD – below limit of detection. (Paper I)

As clearly elucidated, the massive forms showed a higher or similar Ni release when normalized to the surface area compared with the powders, but lower when normalized to the mass. The former can be explained by the possible agglomeration of particles in solution, which prohibit the total surface area to be exposed to the solution, and by the presence of relatively passive surface oxides on the powder particles. When normalizing to mass, it is common with considerably higher metal release from powders compared with corresponding massive forms due to a larger surface area (per mass) exposed to solution, providing a larger possibility of surface reactions and metal release. In the following studies on released amounts of metals from different powders, normalization to mass was preferentially used to avoid the limitations caused by particle size measurements and to enable comparisons from a toxicological perspective.

Total amounts of released Ni and Co into synthetic body fluids from different powder and massive forms of alloy grades and pure metals are presented in Figures 11 and 12, respectively (Papers II and IV). Ni was released at a higher extent than Co for all alloys and fluids, which is primarily due to substantially higher Ni bulk alloy content (0.034 - 63.7 wt. %) compared with Co (0.01 – 0.24 wt. %).

Compared to pure Ni and Co metals, all alloys released a lower amount of Ni (up to 20,000-fold for the powders, up to 300,000-fold for the massive surfaces) and Co (up to 300,000-fold for the powders, up to 1,600,000-fold for the massive surfaces) in all test fluids. This is mainly caused by the surface characteristics and composition. The differences between the stainless steel grades were relatively small in most fluids, but the release of both Ni and Co from the 316L powder was higher compared to the other stainless steel grades in the more acidic fluids (ALF and GST), and higher than observed for the IN625 powder (Figure 11). This was suspected to be a consequence of a higher extent of Ni enriched beneath the surface oxide of 316L [49], and the presence of unstable Mn-phases within the surface oxide of the powder particles facilitating Ni release under acidic conditions [59]. In general, an increased corrosion resistance reduces the amount of Ni and Co release. For example, as illustrated in Figure 12, the low-alloyed steel released considerably higher amounts of Ni and Co, despite lower Ni and Co bulk

content, into all fluids after 168 h exposure compared with all stainless steel grades. This is related to the less protective surface oxide (Figure 9) and lower corrosion resistance (Paper IV) of the low-alloyed steel. In contrast, the duplex stainless steel LDX2101, with the highest corrosion resistance among all investigated alloys (Paper IV), released the lowest amounts of both Ni and Co for most exposure conditions and fluids.

In addition to alloy surface properties, the extent of metal release was highly pH/solution and time dependent. For all grades, released amounts of Ni and Co were as expected considerably higher in the acidic fluids (ALF and GST) compared with the near-neutral fluids (ASL and ASW) (Papers II, IV and V, [14, 44, 50]). These observations are in line with previous findings of increased metal release with increasing acidity of the test fluids. When comparing the released amounts of Ni and Co with exposure time for the stainless steel grade 316L, it was in most cases increasing but not proportionally as expected from exposure time. Hence, strongly reduced metal release rates with time were observed in all fluids (Papers II and IV), which means that some extent of surface passivation, mass transport limitation or solution saturation effects took place with time. In the acidic fluids ALF and GST, there was no evident increased amounts of Ni and Co release from the 316L powder after 4 h of exposure, indicative of improved passive properties. The measured amounts of released Ni and Co in ASL and released Co in ASW were after a certain initial period slightly reduced with exposure time, which suggests precipitation (sedimentation) of Ni and Co from solution at that pH. These findings were consistent with chemical speciation modelling (JESS) results (Paper IV), which predict Ni and Co ions to form solid γ -NiS and β -CoS as predominant phases in ASL (at pH 6.75) and Co as solid Co_3O_4 in ASW (at pH 7). It was noted in Paper II that the release rate of Ni and Co in ALF increased between 2 h and 8 h before declining to slower rates. This is associated with a delayed complexation-induced metal release process related to the high content of citric acid in ALF [29, 59]. Such a release behavior was also observed in ASW, supported by the formation of Ni/Co-lactate and Ni/Co-Cl complexes in ASW predicted by JESS.

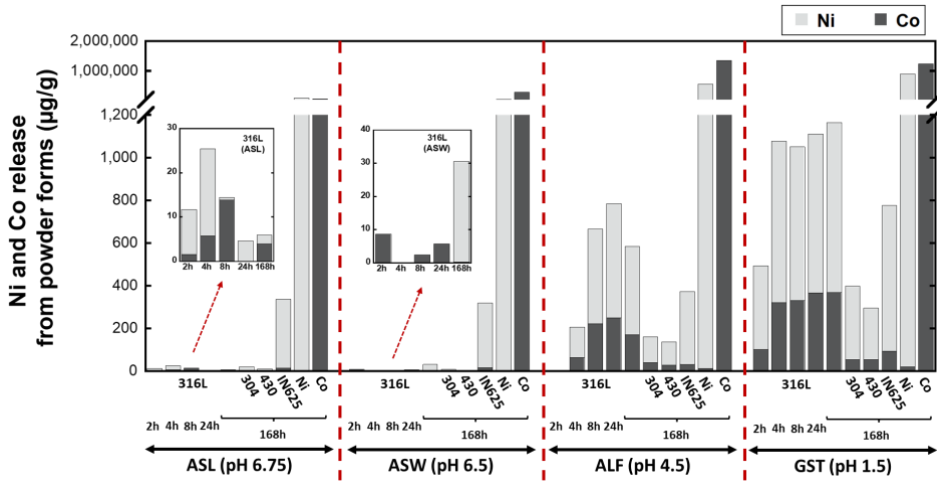


Figure 11 Overview of the total amount of released Ni and Co per particle mass (µg/g) from different alloy and metal powders immersed in ASL (pH 6.75), ASW (pH 6.5), ALF (pH 4.5) and GST (pH 1.5) for 2 h-168 h, following the modified bioelution method. The inset graphs show the magnification of released amounts of Ni and Co from 316L powder immersed in ASL and ASW for 2, 4, 8, 24 and 168 h. (Paper II)

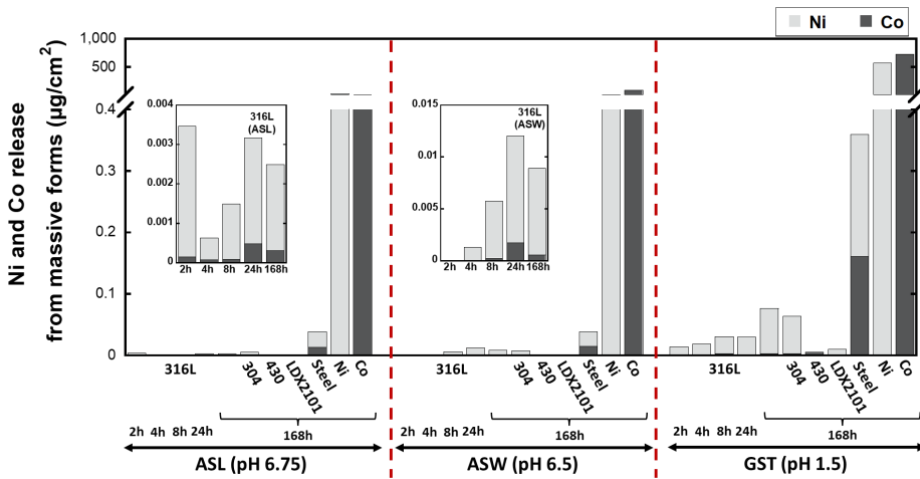


Figure 12 Overview of total Ni and Co release per surface area (µg/cm²) from different massive alloys and metals immersed in ASL (pH 6.75), ASW (pH 6.5), and GST (pH 1.5) for 2 h-168 h, following the modified bioelution method. The inset graphs show the magnification of released amounts of Ni and Co from 316L immersed in ASL and ASW for 2, 4, 8, 24 and 168 h. (Paper IV)

In all, a bioelution test was elaborated to enable a fair comparison between the extent of metal release into synthetic body fluids from

different alloys, pure metals, and massive and powder forms. Since metal release is a surface-governed process, the surface area is important. This was illustrated for the powders which showed a higher extent of released Ni and Co when normalized on their mass and when compared to their massive counterparts. The surface characteristics and composition, the solution composition and pH strongly influenced the bioaccessibility of Co and Ni released from alloys and metals in synthetic body fluids.

4.2.3 Bioaccessible concentration rather than bulk content should be used for hazard assessments of alloys

It has been shown in several papers of this thesis (Papers I, II and IV), as well as in previous studies [60-62], that stainless steel, ferrochromium, and Ni-based alloys (Inconel) behave considerably different compared with their corresponding pure metals from a metal release perspective. Measurements of total amounts of released metals are often approximated as the bioaccessible metal fraction of which some fraction may be bioavailable and absorbed by the human body. Information of the bioaccessible fraction is hence important for hazard assessment. To clearly describe the specific alloy effect, the bioaccessible concentration was calculated, eq. (1), based on a relative comparison of the amount of the released metal (normalized on surface area or mass) from the alloy with the released amount from the corresponding pure metal exposed under identical conditions.

$$\text{Bioaccessible concentration (wt. \%)} = \frac{\left(\text{Released amount}_{\text{alloy}} \left(\frac{\mu\text{g}}{\text{cm}^2} \text{ or } \frac{\mu\text{g}}{\text{g}} \right) \right)}{\left(\text{Released amount}_{\text{metal}} \left(\frac{\mu\text{g}}{\text{cm}^2} \text{ or } \frac{\mu\text{g}}{\text{g}} \right) \right)} * 100 \text{ wt. \%} \quad \text{eq. (1)}$$

where the released amount_{alloy/metal} is the amount of Ni or Co released per surface area (for massive) or per mass (for powder) from the alloy or the metal.

Figure 13 summarizes the bulk and bioaccessible concentration of Ni and Co released from the different alloys after 168 h exposure in the synthetic body fluids. All stainless steel grades and IN625 exhibited lower bioaccessible concentrations of Ni and Co in all test fluids as compared to their bulk alloy contents. This means that less Ni or Co was released from the alloys than could be expected if considering their bulk content,

an effect related to their beneficial surface passivity. For example, the 316L alloy which contains 10 wt.% Ni and 0.24 wt.% Co, behaves like an alloy containing 0.01-0.19 wt.% Ni and 0.0006-0.02 wt.% Co in the different fluids. One exception was observed for IN625 in ASL, in which the bioaccessible concentration of Co was higher than the bulk content. This is possibly explained by the precipitation of released Co from the Co metal powder in ASL resulting in overestimated bioaccessible concentration of Co. In the case of the low-alloyed steel, all bioaccessible concentrations for different solutions were significantly higher than, or similar to, the corresponding bulk content. This is expected for materials of lower corrosion resistance than the pure metals. Different from a simple metal mixture, a linear relationship between the fraction of released metals and the total metal content in the material [50] does not exist. The matrix effect of an alloy induces a different relative metal release from the alloy compared to the reference material. The observed findings suggest that the bioaccessible concentration, rather than bulk content, should be considered for hazard assessments of alloys, such as stainless steels, Ni-based alloys, and some low-alloyed steels, taking consideration into the positive or negative alloying effects. It should be noted that the prediction from the bulk alloy composition in some cases could be valid. For example, released amounts of Co, copper (Cu), Ni and Pb from different copper alloys into GST and ASL have been reported to be similar to released amounts from corresponding pure metals [63].

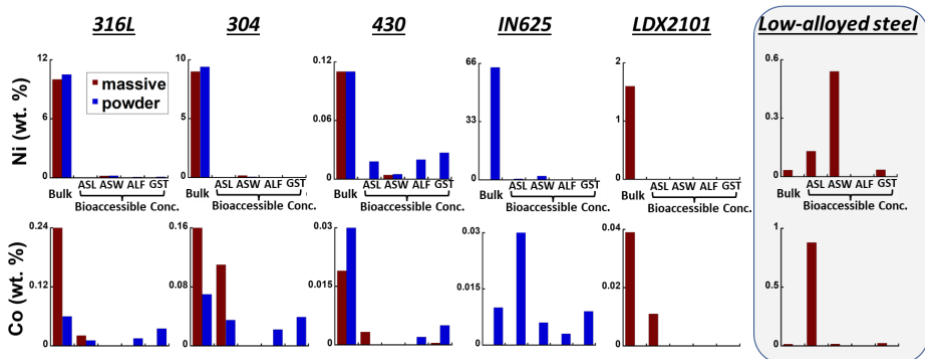


Figure 13 Comparison between the bulk alloy content and the bioaccessible concentration of Ni and Co released from the different alloys (including massive and powder forms) exposed to ASL, ASW, ALF and GST for 168h. (Papers II and IV, Report IX)

In all, alloys are not just simple mixtures of metals and will, depending on their corrosion resistance and surface properties, either reduce or increase the bioaccessibility of Ni and Co, when compared to pure metals.

4.3 Corrosion resistance determines the extent of metal release in synthetic body fluids (Paper I - IV)

Several studies, previously [15, 42, 49, 56] and within this thesis (Papers I-IV), show that the bioaccessibility (metal release) data correlates very well with the surface and corrosion properties of the metals/alloys in synthetic body fluids. Alloys of high corrosion resistance release less metals than expected from their bulk content in given conditions, and vice versa.

In Paper I, OCP measurements on stainless steel 303 exposed in ASW for 168 h showed several OCP fluctuations indicative of metastable pitting events, which explained its relatively higher Ni release into ASW among the investigated stainless steels. Potentiodynamic polarization measurements were also carried out for all investigated powders (Papers II-III) and massive sheets (Paper IV) in different fluids. As illustrated in Figures 14 (a) and (c) and in agreement with the corrosion resistance estimations, the release of Ni increased with increasing acidity of the test fluids. Although 316L showed higher corrosion potential (E_{corr}) in ASW than in ASL, more Ni release was evident in ASW. This could be a result of precipitation of Ni in ASL and complexation-induced metal release in ASW. In the most corrosive fluid, GST (pH 1.5), the low-alloyed steel corroded actively through the polarization scan and showed, as expected, the lowest corrosion resistance compared with the stainless steels (Figure 14b), which is in accordance with the highest amounts of released Ni (Figure 14d). Similarly, in Paper III, the high strength steel powder 18Ni300 showed an evident active corrosion behavior in ALF, contributing to its complete dissolution in ALF after 24 h exposure.

These comparisons elucidate that the corrosion properties of some alloys govern the extent of metal release into the synthetic body fluids, effects not possible to predict from the bulk alloy content. It is thus suggested that electrochemical testing could be used as a complementary screening

tool in bioaccessibility assessments, at least for the alloy classes and experimental conditions investigated herein.

It is noteworthy that the extent of Ni and Co release from the investigated alloys and metals mostly was in accordance with the electrochemical findings. However, this is not always true for all conditions. In a previous study in ALF, the release of Ni, Cr and Mo from different stainless steel grades was not correlated to the corrosion resistance, e.g., the highest Ni release was observed for 316L even though it was not the alloy of lowest corrosion resistance in that study [14]. In agreement with this study, a previous study on simulated rain events showed the release rate of Cr from stainless steels to be similar to that from pure Cr, which has a very high corrosion resistance, while the release rates of Ni and Fe were evidently higher from pure metals than from stainless steels due to the presence of protective surface oxides [64].

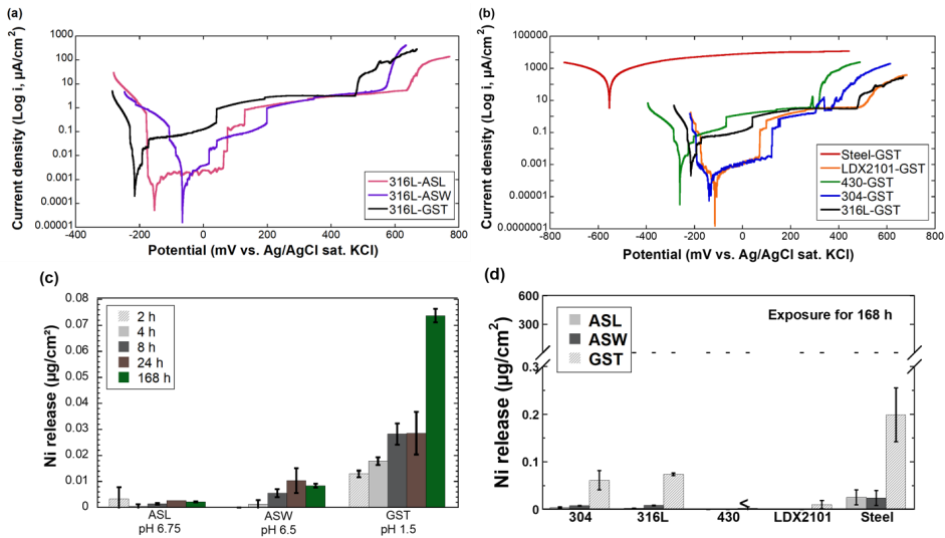


Figure 14 Potentiodynamic polarization curves of massive sheet of (a) 316L exposed to ASL, ASW and GST; (b) 316L, 304, 430, LDX2101 and low-alloyed steel in GST; (c) Ni release from 316L exposed to ASL, ASW and GST from 2 h to 168 h; (d) Ni release from 316L, 304, 430, LDX2101 and low-alloyed steel into ASL, ASW and GST after 168 h exposure. (Paper IV)

In all, released amounts of Ni and Co into different synthetic body fluids correlated well with electrochemically determined corrosion resistance values for the alloys investigated here.

4.4 Studies on metal-containing substances used and collected at occupational settings and present in consumer products (Papers III and VI)

4.4.1 Virgin and reused alloy particles used in additive manufacturing vary in particle and surface characteristics

A large number of alloy powders of relevance for several occupational settings and different exposure scenarios have been investigated in Papers I and II. Commonly used Fe and Ni-based alloy powders (316L, IN718, 18Ni300) used and collected at additive manufacturing settings (with selective laser melting, also named laser powder bed fusion) were investigated from a physico-chemical and toxicological perspective relevant to the inhalation exposure route (Paper III).

The focus was to assess differences between virgin and reused (non-fused) powders, as well as on dust powder of 18Ni300, by assessing their bioaccessibility in ALF, corrosion behavior, surface morphology and composition, microstructure, hydrodynamic size distribution in ALF, and in-vitro toxicity towards cultured human lung cells. Less than 1% of the powder mass was released into ALF for the passive alloys (316L and IN718), while 18Ni300 showed an active behavior and was almost completely dissolved after 24 h. No or minor differences in metal release pattern and surface characteristics were observed between virgin and reused (and dust) powders of IN718 and 18Ni300. In contrast, the reused 316L powder showed, after the laser melting process, different characteristics with an increased fraction of ferrite (magnetic) in the microstructure and thus increased particle agglomeration and increased hydrodynamic size in ALF, an increased corrosion current, increased amounts of released Fe, and Mn enriched within the outermost surface oxide compared with the virgin powder (summarized in Figure 15). However, these differences are not generally observed for all 316L particles used in additive manufacturing, and depend on aspects such as particle size, history and/or manufacturing process. For smaller and differently pre-treated 316L particles, no such differences were observed between virgin and reused powders (unpublished data). Furthermore, all powders showed low, or negligible, cytotoxic effects and the ability to generate ROS in in-vitro conditions. This is possibly attributed to their

size distribution as particle sizes smaller than 4 μm would be required for efficient cell uptake [65, 66], their slow transformation/dissolution and changed chemical speciation of released metals in contact with cell media which may hinder uptake [67, 68]. Further studies are required to determine long-term cytotoxic effects and other potential health hazards, especially for 18Ni300 powders which show high corrosion rates and metal bioaccessibility.

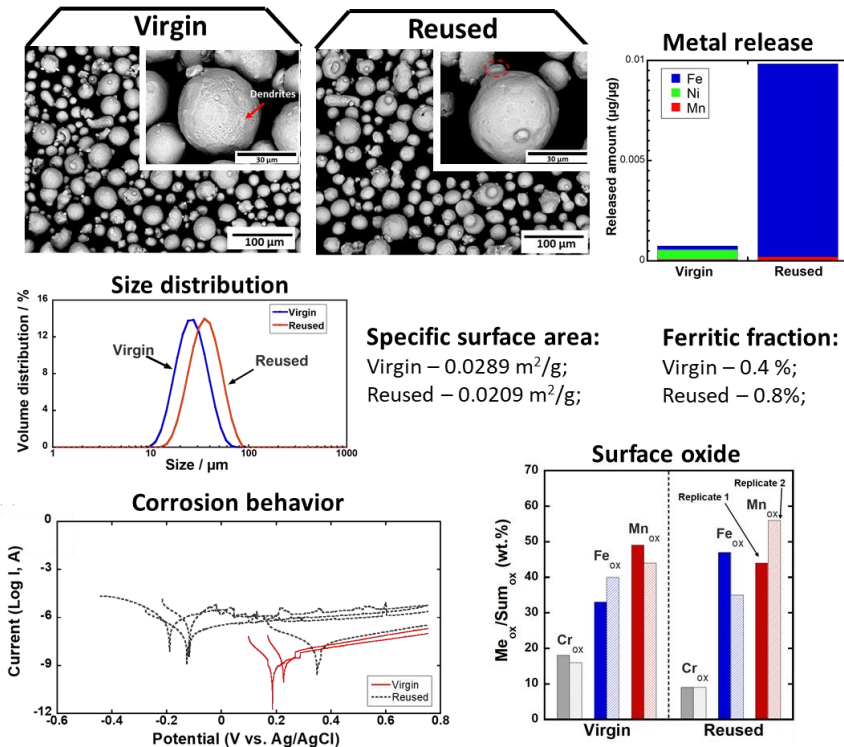


Figure 15 Summary of different physicochemical characteristics, corrosion, and metal release in ALF of stainless steel 316L powder before and after use in additive manufacturing with selective laser melting. (Paper III)

In all, the reuse of alloy powders in additive manufacturing might induce a change in physicochemical properties, including corrosion resistance and bioaccessibility. These changes are highly dependent on aspects such as alloy, history of the powder, and manufacturing processes.

4.4.2 Analytical survey on sensitizing metal-containing substances in tattoo inks

Triggered by the rising incidence of allergic reactions to tattoos, 73 tattoo inks, collected or purchased from the web and tattoo stores in Sweden, were evaluated in relation to their labelling and contents of metals and pigments from a legal requirement perspective (Paper VI). The investigation resulted in the following main findings:

- 93% of the investigated tattoo inks violated legal requirements on labelling in the European resolution ResAP 2008(1), varying among different brands;
- 50% of the tattoo inks declared at least one ingredient wrongly on the label;
- 61% inks contained pigments of concern from a sensitizing and cancer perspective, especially for red inks;
- The concentrations of most metals in tattoo inks were below or slightly exceeding current restriction limits, but relatively high levels of Cr and Ni were detected in almost all samples.

Metal contents of Fe, Cr, Mn, Co, Ni, zinc (Zn), Pb, and arsenic (As) in the tattoo inks were shown to significantly correlate with each other. This means that, e.g., if a tattoo ink contains Fe, it most likely also contains Cr, Mn, Co, Ni, Zn, Pb, and As. We therefore concluded that Fe-containing pigments (a common red pigment) might be an important source of common sensitizers, such as Ni and Cr. The observed correlation between the different metals is probably related to the difficulty to separate metals and purify metal oxides, ores, and metals and alloys. This observation is also applicable to impurities in alloys (more information in next section on Paper V).

Paper VI also showed a positive correlation between the total (through microwave digestion with concentrated HNO₃) and water-soluble metal concentrations (extracted in 0.9% NaCl, more details in Paper VI) in tattoo inks, at least for Cu and barium (Ba). Soluble amounts were lower than the total amounts in the inks. As shown in several studies and discussed above, the concentrations of bioaccessible metals for metals

and alloys, are not necessarily correlated to the bulk content (Papers I-V, [13, 14, 44, 59, 64]).

In all, consumers and regulators should be more careful with tattoo inks, as the majority of investigated inks contained mislabeled ingredients, substances of health concern, and did not comply with existing regulations. Fe-oxide pigments might be a source of other metal impurities.

4.5 In-depth study on the location of Co impurities and the Co release process of stainless steel 316L in different synthetic fluids (Paper V)

In light of the novel legislation in Europe [9], which stipulates that alloys and metals containing more than 0.1 wt.% Co (later possibly 0.01wt.%) in the bulk from October 1st 2021 are classified as carcinogenic (Carc. 1B), an in-depth investigation was conducted to gain an interfacial understanding of the Co release process from stainless steel. The Co release behaviour (bioaccessibility) of different stainless steel grades had previously been investigated in different simulated body fluids ranging from pH 1.5 to pH 7.4 (Papers I, IV, [50]), and the results clearly showed that the released amounts of Co from stainless steels were considerably lower (up to 1,000-fold) than what would have been expected from the bulk Co content (< 0.3 wt.-%). The metal release is usually governed by different processes at/within the surface oxide such as metal oxidation processes, surface complexation-induced dissolution and/or physical processes (see Figure 5). However, no prior studies on the location of Co impurities within the stainless steel have been conducted since Co impurities generally have no impact on either the function or corrosion resistance of the stainless steel, except if used in the nuclear industry [69].

Triggered by the new regulation and the limited knowledge, three complementary techniques – ICPMS, XPS and ToF-SIMS – were used to determine the location of Co impurities in the surface of 316L to explore their involvement in the metal release process during 24 h of exposure to three synthetic biological fluids of varying pH (GST, ALF

and PBS) (Paper V). The approach and results are schematically illustrated in Figure 16.

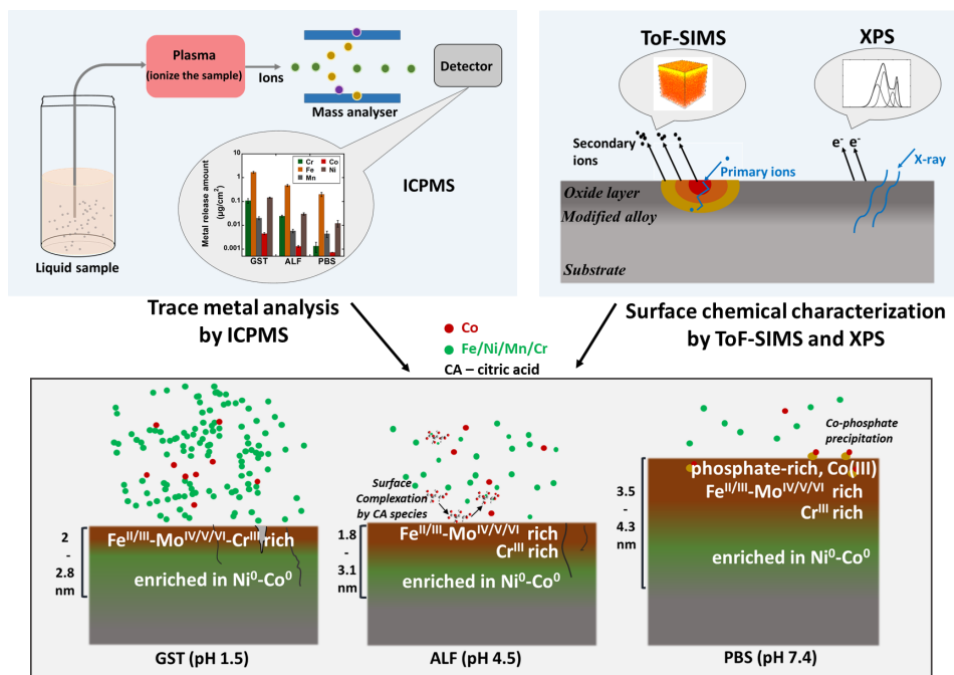


Figure 16 Overview of the extent in metal release and changes in surface oxide characteristics (composition and thickness) of stainless steel 316L after 24 h of exposure to GST (pH 1.5), ALF (pH 4.5) and PBS (pH 7.4) at 37 °C. The study used a combination of solution trace metal analysis and semi-quantitative and quantitative surface analytical methods. (Paper V)

Co was homogeneously distributed along with metallic Ni beneath the surface oxide and co-released with other metals upon processes such as surface reformation and passivation. In GST, the surface defects, such as formed pits, made it easier for Co and other elements to be released into solution (Paper IV). In ALF, surface complexation by citric acid species is probably the main metal release mechanism (Papers II, [54, 70]). The observation of Co(III) by XPS and strong phosphate signals by ToF-SIMS within the surface oxide when exposed to PBS, suggests the possible incorporation of Co-phosphate precipitates in the outermost surface [71, 72]. However, whether Co from such precipitates could be released in any subsequent exposure in a more corrosive medium requires further investigations. When comparing the changes in surface

oxide thickness by means of XPS and ToF-SIMS with the corresponding dissolved surface oxide thickness (assuming uniform thickness reduction and calculated based on ICPMS data after 24 h), it was evident that oxide formation was favoured over dissolution in PBS. The reduction in oxide thickness was equal to the oxide dissolution in ALF. The reduced oxide thickness was four-fold lower than the oxide dissolution in GST, suggesting the importance of surface defects.

In all, Co impurities in 316L were homogeneously co-located with metallic Ni beneath the surface oxide. If prone to precipitation (in neutral, phosphate-containing solutions), Co could along with phosphate be incorporated in the surface oxide.

5. Main conclusions

The objectives of my PhD-studies were to improve the knowledge on the metal release behavior in relation to surface and corrosion characteristics of relevance for hazard assessments of variety of metals and alloys, occupational alloy powders (additive manufacturing), and tattoo inks. Quantitative bioaccessibility data were generated and correlated with findings on surface properties and corrosion resistance under different exposure conditions by using a multidisciplinary approach. The tattoo inks were assessed on their label compliance with legal requirements, the contents of metals, and the prevalence of pigments of health concern. The following main conclusions were drawn:

- A modified bioelution test protocol, including gentle solution agitation and well-defined sample surface conditions prior to testing, has been elaborated able to assess bioaccessibility data for metals and alloys exposed in synthetic body fluids. The methodology enables fair comparisons between alloys and metals in massive and powder forms for different simulated human exposure scenarios. (Papers I-V)
- The metal release process from metals and alloys is highly dependent on bulk and surface characteristics, solution composition and pH, as well as on experimental factors such as exposure time. (Papers I-V)
- All investigated alloys, with the exception of low-alloyed steels, in massive or powder form released substantially less amounts of Ni and Co, attributed to the passive surface oxide characteristics, compared to pure Ni or Co metals in the investigated synthetic body fluids (artificial saliva (ASL), sweat (ASW), lysosomal (ALF) and gastric (GST) fluids). (Papers I, II, IV)
- For highly corrosion resistant alloys (such as stainless steels and Ni-based alloys), the bioaccessible concentrations of Ni and Co in the synthetic biological fluids were considerably lower than corresponding bulk alloy contents. In contrast, low corrosion-resistant alloys (such as low-alloyed steels) showed higher bioaccessible concentrations than their bulk content. The results elucidate that the bioaccessible concentration, rather

than the bulk content, should be used for hazard assessment of alloys. (Papers I, II, IV)

- The extent of released metals (Ni, Co, Fe, Cr, Mn) from the different alloys and metals was higher in the acidic (GST) and complexing (ALF) fluids compared with the neutral fluids (ASL, ASW and PBS). The relative content of oxidized Cr in the surface oxide of stainless steels increased after exposure to GST and ALF, due to the preferential dissolution of Fe compared to Cr during exposure in acidic conditions. (Papers II, IV and V)
- The release rates of Ni and Co from 316L strongly decreased with increasing exposure time in the acidic fluids, due to improved passive properties. (Papers II, IV)
- Metallic Co was homogeneously co-located with Ni beneath the surface oxide of 316L under different exposure conditions, and Co was released with other metals upon surface reformation and passivation. Exposure in PBS resulted in the precipitation of released Co along with phosphate on/within the surface oxide. (Paper V)
- The laser melting process in additive manufacturing induced changes in the surface oxide composition, microstructure, corrosion behaviour and metal release pattern of 316L in ALF, but did not have any impact on the IN718 and 18Ni300 powders. (Paper III)
- A large majority of investigated tattoo inks violated legal requirements on labelling and contained metals and pigments of health concern, varying among different brands. A positive correlation was observed between the metals of Fe, Cr, Mn, Co, Ni, Zn, Pb, and As, as well as between soluble and total concentrations of Cu or Ba in tattoo inks. (Paper VI)

6. Outlook

In this work, bioaccessibility data, surface properties and corrosion behavior have been investigated for different metals, alloys and metallic powders under different exposure conditions. Several metal-containing substances used in occupational settings and in consumer products were also analyzed regarding their potential health hazards. Future work should in my view be conducted on the following aspects:

1. Investigations of any surface oxide changes with time and solution during exposure are highly recommended for powder particles. Such knowledge can contribute to an improved understanding of possible metal release processes/mechanisms. This could be achieved by developing a methodology to separate exposed powders from the solution and to wash the powders after exposure.
2. Future studies should further investigate chemical speciation of released metals in different synthetic fluids. Chemical speciation of metals, such as the oxidation state and complexation with ligands (including the number and type), plays a crucial role in determining toxicity in human health [67, 68, 73]. Chemical speciation modelling (such as JESS in this study) could be used for predictions of chemical speciation of metals of interest and to assess risks of metal precipitation in given solutions.
3. This study has generated bioaccessibility data ranging from 2 h to 168 h (1 week). Since exposure to particles and certain chemicals may result in chronic effects, further long-term immersion studies are suggested to facilitate potential health hazards.
4. The possible correlation between released metals and interactions with biomolecules (e.g., proteins) and cells (toxicity) should be further studied for improved risk assessment and management.

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Stockholm, October, 2021

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