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The influence of oxide-forming elements on corrosion and biocompatibility of experimental PFM alloys

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To my family.

Table of abbreviations

PFM	porcelain fused to metal
Ce	Cerium
Co	Cobalt
Cr	Chrome
Mo	Molybdenum
Saline/BSA	Saline/Bovine Serum Albumin
Ag	Silver
Pt	Platinum
mmol/L	micro molar masse per Litre
Ni	Nickel
Au	Gold
Pd	Palladium
Ir	Iridium
wt%	weight percent
In	Indium
Sn	Tin
Zn	Zinc
cm	centimeter
mm	millimetre
cm^2	square centimeter
W	Tungsten
Si	Silicon
Mn	Manganese
N	Nitrogen
ml	millilitre
min	minute
°C	degree Celsius
mol/l	molar masse per litre
NaCl	Sodium Chloride
ISO	International Organization for Standardization

ICP-OES	Inductively Coupled Plasma – Optical Emission Spectrometry
µg	microgram
SEM	Scanning Electron Microscope
EDX	X-Ray Diffraction Analyser
keV	kilo electron Volt
cps	counts per second
s	second
PVC	Polyvinyl Chloride
cells/cm²	cells per square centimeter
DMEM	Dulbecco's Modified Eagle Medium
Pen/Strep	penicillin and streptomycin
FCS	foetal calf serum
h	hours
CO_2	Carbon Sink
FDA	fluorescein diacetate
EB	ethidium bromide
M	molar masse
NaOH	Sodium Hydroxide
HEPES	hydroxyethyl-peperazine-ethane-sulphonic acid
SDH	succinic dehydrogenase
µl	micro litre
mg	milligram
nm	nanometer
ELISA	Scanning Multiwell Spectrophotometer plate Reader
ppb	part per billion
µm	micrometer
ppm	part per million

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1. Introduction

The porcelain fused to metal (PFM) restorations has been widely employed in Dentistry. The combination of metal and ceramic made it possible that some porcelain adverse factors such as low resistance impact and tensile failure could be improved. Such combinations made also possible appliances with acceptable resistance, aesthetics, and stability in the mouth with expansion in restoration longevity.

With the goal to improve the communication among the professionals, the American Dental Association established in 1984 a reviewed classification system for cast alloys (including the PFM alloys) [32]. This system didn't consider the alloy's performance, but facilitated the language in dental-procedure codes. Thus, the cast alloys had been divided in three groups based on noble-metal contents of alloys (Gold, Palladium and Platinum):

a) High-noble alloys ➔ noble-metal content is equal or more than 60% and Gold content is equal or more than 40%;
b) Noble alloys ➔ noble-metal content is equal or more than 25%; and
c) Predominantly base-metal alloys ➔ noble-metal content is less than 25%.

Years later, this classification system was substituted for a new one which is based on the alloy's mechanical and physical properties. In the current classification alloys are arranged according to their hardness and consequently to their base-metal content. The alloys are broken down into Type I (low-strength castings), Type II (medium-strength alloys), Type III (high-strength castings), and Type IV (extra-high strength alloys) [3].

In order to obtain a successful PFM restoration, the bond between metal and ceramic must be adequate, satisfactory and an optimal resistance has to be present [67, 73]. In this way, some oxide-forming elements were alloying to the

alloys to increase its mechanical properties as well as its adherence with the ceramic. Most of them are non-noble components like Indium, Tin, Copper and Iron. On the alloy surface at the moment of it's degassing and/or at the ceramic firing, an oxide layer is shaped [86, 87, 88, 116, 117]. Otherwise, the oxide layer resulting from the ceramic firing and from these common compounds may interfere with the corrosion properties of some alloys [130].

This oxide layer occurs also on metal without porcelain cover, mainly on its boards and metallic waist. This aspect must also be taken into consideration [13, 70, 131]. In this manner, the oxide-forming elements may lead to a significant increase in corrosion rate and release of ions from the oxide layer formed by the firing process. Such leakage processes may promote gingival discolorations or even biological risks to oral tissues [4, 35, 101, 102, 119, 131], even if the metal is well finished and polished [12].

Such occurrences are attributed to the incorporation of products from the metal corrosion [11, 12, 101]. Indeed, until no alteration happens visually; it's important to consider that some tissue responses may occur [11, 130].

Under certain oral conditions dental alloys may suffer increased corrosion [43, 59, 61, 99, 130]. Due to this fact and because this process is complex, it depends on many physical and chemical factors [17, 70, 78, 111, 119]; and also promotes changes in some alloy's properties, *e.g.* aesthetics, durability, efficacy and biocompatibility [33, 41, 96, 119, 128], many technical and biological aspects must be analyzed before an alloy becomes commercially available [57].

Technically an alloy has to be easy to handle and use in treatment; and must have a mechanical stability and a satisfactory adherence with the ceramic as well. Clinically, this alloy has to be compatible and present satisfactory applicability and corrosion behaviour [41, 59, 80]. Additionally it should not afford the appearance of side effects to the patient and should have relative

durability [47]. Such aspects have a straight relationship and are dependent on a stable and passive layer built on the metal's surface [74, 105, 123].

In order to satisfy these requirements, it is known that the high-noble alloys are the material of first choice. Since the beginning of the 1980s, the costs of such elements have increased, especially the price of Gold, and alternatives have been tried. With the introduction on the market of new alloys with low noble-elemental content, the main interest became to find an adequate composition of base-metal alloys that allows a material with more satisfactory properties [1, 4, 21, 119, 128]. In this manner, elements from the so called "Rare Earth" group of the periodic table, such as Lanthanum, Neodymium, Erbium, and Cerium (*Ce*) have been added to the base-metal alloys to assist the adhesion between the metal and the porcelain, and to enhance their corrosion resistance [47, 128]. Specifically, the element *Ce* is presumed to enhance the stabilization of the oxide layer in base-metal alloys. There is little knowledge about the correct amount for dental-casting techniques and its consequently corrosive behaviour and cytotoxic effects [46].

1.1 Purpose of this Study

Alloys, porcelains and opaque materials have been improved over the years. The constant introduction of new alloys and ceramic systems on the market has triggered the development of studies and works about their properties, their interaction with each other, and with the mouth as well.

The purpose of the present investigation was to analyse and to compare twelve non-commercial alloys (nine ceramic high-noble alloys and three ceramic base-metal alloys with and without Cerium) considering: 1) their corrosion resistance with and without oxide layers by analytical determination of type quantity of dissolved metallic ions; and 2) their cytotoxic effects, before and after simulated porcelain firing, with the same standardized cell-culture system, through out quantitative and qualitative biological assays.

2. Literature Survey

2.1 Interface Metal Porcelain - Oxidation's Kinetic

High-noble and noble alloys exhibit a chemistry adherence with porcelain through the formation of an oxide layer on the metal surface during the ceramic firing, which is developed by the oxide-forming elements [5, 28, 38, 86, 88, 91, 98, 114]. The most common oxide-forming elements described in the literature and/or commercially found are Indium, Tin, Gallium, Iron, Copper, Zinc, Silver, and Chrome [5, 16, 28, 34, 38, 98, 116].

The chemistry union that occurs between base-metal alloys and ceramic is created by one of the main elements of the alloy, which is concentrated in the oxide layer on the metal surface and thereby becomes oxidized. This oxide film is an interdigitates underlying that increases the superficial contact area and enhances the mechanical adherence with the porcelain. Such surface state could be improved through a pre-oxidation process [8, 16, 28, 29, 67]. Otherwise, VASCONCELLOS et al, 1999 [113] and DEKON et al, 1999 [36] said that the pre-heating process could damage this union, with loss in its strength, and should be employed only for the high-noble and noble alloys.

The compression forces that result from thermal-expansion difference between porcelain and metal play a significant role in this process [114]. The dynamic activity of atom transfer across the interface and diffusion within the metal and the oxide also contributes to this event [73].

On the other hand, pre-treatment procedure prior to metal-oxidation process, such as: sand blasting and mechanical retentions [39], insures optimal formation of oxide-layers phases [15]. Other aspects to be considered are that oxide formation has a fine line relationship with the oxidation-time exposure [5, 8, 28, 38, 116] as well as with the temperature through out the dental alloys are

submitted [8, 28, 116, 117]; and that the alloy solubility in oxygen influences the oxidation mechanism [8, 25, 116].

2.2 Alloys' Corrosion Behaviour

Corrosion can be defined as chemical or electrochemical reactions between a material, usually a metal, and its environment that produce a deterioration of the material and its properties [41]. Metals or dentistry alloys used to make restorations and prostheses will suffer some corrosion type when they are being used in the mouth. This happens because corrosion behaviour is not an internal-material property, but a feature, which depends on the milieu where it is found. This process may be greater or not in accordance with the changes in range of thermodynamic or kinetic factors occurring in the oral cavity. Indeed, if these events are constant and for a long period of time, it may result in dimensional changes and decrease in the material resistance [11, 41, 119, 128].

At most alloys, but also resins and ceramics, if exposed to aqueous milieu like body fluids, are able to suffer corrosion. When these fluids are the saliva and tissues liquids the metal corrosion is an electrochemistry process, where an oxidative milieu is necessary [2, 33]. In this process two reactions (an anodic and a cathodic) are noticed. In some cases the corrosion levels are not great enough to appear clinically. Nevertheless, the corrosion products (released metallic ions) may cause some biological effects and this aspect must be carefully considered [4, 11, 33, 48, 93, 119, 132].

Other aspects such as meal, temperature, water, blood, and saliva are factors that also play an important role in the corrosion susceptibility of a material [2, 41, 48, 59, 130, 132].

The most common corrosion types are corrosion caused by the surrounding, galvanic, stress, crevice, and pitting corrosion [48, 128, 130, 132]. An example

of the galvanic corrosion was reported by WIRZ et al, 1989 [131], who observed four clinical cases. In the moment of the substitution of the different materials for a common one, the signs and symptoms promoted by the corrosion process had disappeared in a few weeks.

2.2.1 Corrosion Behaviour of Porcelain Fused to Metal Alloys

Some studies demonstrated that PFM alloys are more corrosion resistant than conventional alloys [2]. It is a known fact that corrosion behaviour has a straight relationship to alloy noble degree. If an alloy contains more noble compounds, it provides a higher corrosion resistance [30, 37, 48, 55, 59, 95, 119, 123]. Such resistance is improved when an alloy also presents a higher amount of pure elements [92].

High-noble alloys are also able to release metallic ions, but their elements are dissociated in the first hours and their levels don't increase with time. On the other hand, noble and base-metal alloys suffer ion leakage in considerable levels, which have a tendency to increase with time. This fact is considered the main cause responsible for the cytotoxic increasing aspect of an alloy [63, 70, 124].

When an alloy is found in the mouth, the alloy/saliva phase tends to enhance an equilibrium stage. In an overview, high-noble and noble alloys are used extensively in dentistry works for this reason and due to their inertia properties, their noble atoms nature, their biocompatibility, and their longevity. Otherwise, predominantly base-metal alloys generally are not thermodynamically stable and have a great affinity for oxygen. The corrosion resistance of such alloys happens throughout a tin passivating oxide layer that covers their surface. This covering acts as a protective film. If this passivating film is broken, material degradation is able to occur by means of corrosion and metallic ions are released and may interact with the nearby biological tissues [4, 19, 70].

Based upon the current literature, it's possible to describe the nowadays dental alloys and their corrosion behaviour. Nickel-based alloys are corrosion sensible. Because of this they must have an addition of other compounds like Chrome, to achieve the formation of a protective oxide layer. Copper-based alloys are unable to be corrosion resistant. Nickel-Titanium alloys are susceptible to pitting and crevice corrosion. Cobalt-based alloys present a satisfactory corrosion resistance caused by the resistant oxide layer formed through the Chrome component. Iron-based alloys develop a protective oxide layer, but in some cases they are susceptible to pitting and crevice corrosion. Titanium and Titanium-based alloys are biocompatible and are the most corrosion resistant alloys [17, 35, 70].

In spite of the fact that, noble elements and their alloys are more corrosion resistant, the addition of precious elements such as Gold, Platinum, or Ruthenium to base-metal alloys has not been recommended. RECLARU et al, 2005 [95], testified to a decrease in the corrosion behaviour of *CoCr*-based alloys, which had been doped with such noble metals.

In order to obtain base-metal alloys with satisfactory properties and an improvement in the oxide layer with more stability, it was thought to add elements from the so-called Rare Earths group of the periodic table to work with the Chrome element in *CoCr*-based alloys. It's presumed that elements of this group can work by enhancing of the alloy's strength, resistance, and gain in temperature stability [47, 118, 128].

2.2.2 Other Aspects

Studies to compare corrosion behaviour of high-noble, noble and base-metal alloys have been made [42, 55, 123]. Some of them focused their work on the electrochemical procedure that happens between the alloy and the oral milieu [19, 37, 42, 44, 45, 59, 81]. Another study focused on the mass-loss property in different assay periods of time [43, 47, 58, 93, 112, 123]; or considered the

influence of some substrate such as mulcin [76], rhodanide – KSCN [44, 82], or proteins [50, 125].

In accordance with MÜLDERS et al, 1996 [81], the alloy-casting process doesn't cause significant effects upon the corrosion resistance. The aspects which play an important role might be listed as the metallic alloys properties, their structural composition [1, 2, 4, 42, 43, 45, 47, 59, 81, 93, 128], and the composition of the oxide layer [19, 48, 109].

It was discussed that frequent recasting increases the corrosion resistance. However, recasting also promotes loss of alloy elements with resultant changes in physical properties [59]. The corrosion resistance of a recasting alloy is favoured if noble metals are added [9]. Literature data affirmed that *CoCr*-based alloys could be reused four times successfully without representative change in corrosion behaviour [51, 60]. Nevertheless, other works affirmed that corrosion resistance had decreased after the recasting process of base-metal alloys [62].

The surface aspect may be an important factor in the corrosion behaviour of dental alloys [6, 56, 58, 101, 102]. KANEKO et al, 2000 [56], studied three ways of polishing and finishing procedures on base-metal alloys and found that such procedures promoted a significant difference in their corrosion resistance, while on noble alloys no representative effects are noticed. For these authors, the sandblasting surface condition showed the worse results, and a mirror-finishing procedure was comparable to a #600 grit abrasive-paper finishing.

In accordance with WATAHA et al, 2001 [125], the corrosion behaviour is affected by the biological surrounding and its composition [31, 48]. In this way, the pH value of the surrounding also plays a great role on the alloy's corrosion resistance [9, 48, 59, 71, 92, 103, 119, 127], as well as changes in the milieu temperature [129] and in the time test [1, 104].

It is indicated that PFM alloys should receive a simulated ceramic-firing procedure for corrosion-resistance tests [6, 58, 104]. Alloys which were heat treated were described in the literature to present a higher ion release, even after the finishing process [58].

2.3 Biocompatibility Aspects

The corrosion susceptibility of an alloy is an important property of its biocompatibility. An interaction between mouth tissues and released metallic ions is unavoidable. It happens due to their fine proximity and the straight relationship between these structures. In this manner, the professional must be familiar with the corrosion and biocompatibility levels of an alloy, in order to choose a correct material that causes minimal damage to the patient, with fewest side effects [4, 41, 49, 57, 79, 97, 119].

Materials that don't suffer corrosion processes, whether owing to their nobles' elements or due to a resistant oxide layer, provide a lesser risk to the surrounding tissues. However, it's known that all material exposed to the mouth milieu may suffer some type of corrosion in greater or lesser degrees. If this happens, elements of this process come in contact with the adjacent tissues or spread through out the organism. Other toxicity or allergic processes may not occur since the alloy's compound elements are biologically passive. Besides metal or ion biocompatibility, the biological risk also depends on the quantity of released elements and their toxicity levels [48, 57, 61, 119].

Alloys that release more metallic ions may be considered as more toxic [57, 102]. Nevertheless, this occurrence depends on others factors like elemental-toxic potential, exposing time, concentration, synergism, and antagonism of the released elements [119, 120]. Material, patient, dentist, and changes in the material manipulation by the dental technician are also additional factors [78].

As described in the literature, biological reactions may occur also by bacterial adhesion, toxic or sub-toxic effects as well as allergic processes; and not only in detriment of alloys' toxicity. The sub toxicity happens by the cell metabolism interference, which is not chained with the metallic ions release. The allergic reactions manifest clinically. On this aspect, metals like Gold, Palladium, Nickel and Cobalt play a great role. In this way, if some signal were noticed, the proposed diagnoses and treatment should be conduced in a multidisciplinary way [101].

Indeed, the reactions in the gingiva and subsequent tissues are more common than it appears to be. In accordance with BERSTEIN et al, 1992 [12], this process results from the alloys cytotoxicity that occurs even if the metal is well finished and polished. Some gingival tissues near dental alloys were analyzed to test this hypothesis. These works found localized chronic-inflammatory processes as well as incorporation of metal ions in these tissues [23, 94, 106]. This fact indicates that released metal ions are being stored by the neighbourhood tissues [101].

In accordance with NELSON et al, 1999 [84], the conditioning of an alloy also has to be considered before its employment in the mouth. It was possible to decrease the unnecessary exposure of oral tissues to the released elements. These authors concluded that alloys that were first exposed to saline/bovine serum albumin (Saline/BSA) solution had presented lower cytotoxic effects than those exposed to saline or cell culture medium by removing the superficial labile elements. The influence of Saline/BSA was also studied by WATAHA et al, 2001 [125].

The cytotoxicity of dental alloys has been tested "in vivo" [40, 57, 63] and "in vitro" [18, 20, 21, 34, 35, 99, 124]. Some studies were carried out to evaluate the citopatogenic effects of metallic ions which may be released from an alloy. BUMGARDNER et al, 1989 [21], said that significant levels of corrosion products were required to induce changes in the morphology and viability of

human gingival fibroblasts in direct contact assay, whereas H-thymidine incorporation was reduced at lower concentrations of released metal ions. Furthermore, SCHEDLE et al, 1995 [99], concluded that many ions, like Ag^{+}, Pt^{4+} and Co^{2+} at low concentrations (up to 0.01mmol/L) could inhibit the elements incorporated by L-929 fibroblasts and human gingival fibroblasts. They found that fibroblasts showed an increase of citoplasmatic as well as increases in the nucleus and organelles destruction. It induced a loss of fibroblast-cells function caused by necrosis (cell damage), but was not due to apoptosis (cell-death induction).

As the interest in the Rare-Earths elements for alloying base-metal alloys has increased, their toxic effects have also been studied. Such analyses took in consideration the local acute reaction of the oral mucosa [66], the “in vivo” toxic effect, through the sample implantation into the paraspinal muscles of mice [110], and Titanium-based alloys as well [133]. The influence of Cerium alloying to *CoCr*-based alloys had little reference in prior literature.

2.3.1 Biocompatibility of Porcelain Fused to Metal Alloys

It is general concern that high-noble alloys are more biocompatible than noble or base-metal alloys [34, 35, 57, 124]. Otherwise, KRATZENSTEIN et al, 1988 [63], after evaluation of prosthetic works from six months to eight years of use in the mouth, noticed that noble alloys might also promote hyperplasic reactions in oral tissues. However, the majority of sensitivity reactions, such as tongue or mucous-burning sensation, metallic taste, and mucous and prosthesis discoloration happened when noble and base-metal alloys were combined.

It was corroborated that Nickel-based alloys with low amounts of Chrome and Molybdenum and with Beryllium content must be avoided [12, 18, 20, 43, 57]. However, it must also be remembered that in spite of possible positive-skin reactions to Nickel, the oral mucosa reacted less and with low intensity to this and other elements [33, 40, 78, 128].

When metal-based PFM alloys are considered, their as-cast and polishing forms are both biocompatible, while other alloys with low levels of gold and base-metal alloys present no citotoxic effect only in their polished shape [34]. Moreover, single-phase alloys with a homogeneous surface characteristic are more corrosion resistant and consequently more biocompatible than multi-phase alloys [35, 41, 119, 120, 122, 128].

Can et al, 2004 [24], tested *NiCr*- and *CoCr*-based alloys and concluded that generally the alloys didn't promote changes in the exposed cells, but the sandblast form of these alloys have a mild to moderate response for the disintegration of the actin filaments of the cells' cytoskeleton.

3. Materials and Methods

3.1 Materials

Alloys

Twelve experimental different PFM alloys were used in this study (six samples of each alloy in each essay test). The testing alloys included high-noble alloys and metal-based alloys. The precious alloys were supplied in the as-cast form by Wieland Dental & Technik GmbH & Co. KG (Pforzheim, Germany), whereas the non-nobles alloys were supplied by Dentaurum J. P. Winkelstroeter KG (Pforzheim, Germany).

The nine high-noble alloys were provided in two groups “A” and “B”. The group “A” (*Au*84*Pt*9.9*Pd*5*Ir*0.1) with 1wt% *In* (#A1), *Sn* (#A2) and *Zn* (#A3) as oxide-forming elements; and the group “B” (*Au*81*Pt*9.9*Pd*5*Ir*0.1) with 4wt% *In* (#B1), 4wt% *Sn* (#B2), 2wt% *In* and 2wt% *Sn* (#B3), 3wt% *In* and 1wt% *Zn* (#B4) 3wt% *Sn* and 1wt% *Zn* (#B5) and 2wt% *In* and 2wt% *Zn* (#B6) as oxide-forming elements respectively. The three metal-based alloys were manufactured as bellow: without *Ce* content (#N1), with 0.19wt% *Ce* (#N2) and with 0.39wt% *Ce* (#N3). Their chemical compositions in weight percent (wt%), as reported by the manufacturers, are provided in Table 1.

They were supplied to the biological (direct contact) tests in the form of disks (1 cm in diameter and 1-1.5 mm in thickness) and in the form of ingots to the static corrosion measurements (mass loss test) with a surface area of approximately 10 cm² (34X13X1.5). Pure metals (*In*, *Sn* and *Zn*) in their as-cast state were also used in the form of disk samples to the direct contact test and in the form of ingots to the mass loss assay, as described above.

Alloy	*Au*	*Pt*	*Pd*	*Ir*	*In*	*Sn*	*Zn*
A1	84.0	9.9	5.0	0.1	1.0	x	x
A2	84.0	9.9	5.0	0.1	x	1.0	x
A3	84.0	9.9	5.0	0.1	x	x	1.0
B1	81.0	9.9	5.0	0.1	4.0	x	x
B2	81.0	9.9	5.0	0.1	x	4.0	x
B3	81.0	9.9	5.0	0.1	2.0	2.0	x
B4	81.0	9.9	5.0	0.1	3.0	x	1.0
B5	81.0	9.9	5.0	0.1	x	3.0	1.0
B6	81.0	9.9	5.0	0.1	2.0	x	2.0
In	x	x	x	x	100.0	x	x
Sn	x	x	x	x	x	100.0	x
Zn	x	x	x	x	x	x	100.0

Alloy	*Co*	*Cr*	*Mo*	*W*	*Si*	*Mn*	*N_2*	*Ce*
N1	63.94	22.54	7.36	4.24	1.55	0.21	0.16	x
N2	64.22	22.39	6.92	4.32	1.59	0.21	0.16	0.19
N3	63.89	22.39	6.99	4.34	1.64	0.20	0.16	0.39

Table 1: Chemical compositions of the test alloys in weight percent (wt%).

Sample preparation

The surfaces of each cast alloy (samples n=6) were wet polished with a series of SiC papers (#320, #600 and #1200 grit) in a polishing machine Buehler Metaserv® (Buehler UK Ltd, Dusseldorf, Germany). In order to avoid cross-contamination during polishing, fresh abrasive papers were used for each specimen. After being polished the samples were ultrasonically cleaned in 10 ml of 70% isopropyl alcohol for 5 min and then dried [42, 43].

Simulated Porcelain Fire

Three samples of each alloy were then heat-treated at 930°C (simulated porcelain-firing process) for a total time of 5 min (4 min in vacuum and 1 min in presence of room atmosphere) following the recommendations of the manufacturers. The oxidation treatment was carried out in a VITA VACUMAT® 500-porcelain furnace (H. Rauter GmbH & Co. KG, Bad Säckingen, Germany). This procedure was repeated before each test.

Three specimens were prepared for each experiment in each of the two surface states to allow three repetitions under the same conditions. The polished and polished and oxidized samples were then ready for the following tests.

3.2 Corrosion Behaviour Tests

A chemical corrosion test was selected to determine the corrosion behaviour of the experimental alloys. This assay was an immersion test through mass loss measurements. It concerned the analytical determination of the type and quantity of the dissolved metal ions.

Mass loss tests

For the static release measurements, the samples were stored for 1, 4, 7, 14, 21, 28 and 35-days at 37°C in sterile tubes with 10 ml of freshly made corrosion solution. In this manner, a little portion of the alloy surface was touching the tube (figure 1). An equimolar corrosion solution of 0.1 mol/l lactic acid and 0.1 mol/l NaCl with a pH value of 2.3, was used as recommended by ISO 10271 [42, 43, 52, 58, 93, 107, 108, 109, 111, 112].

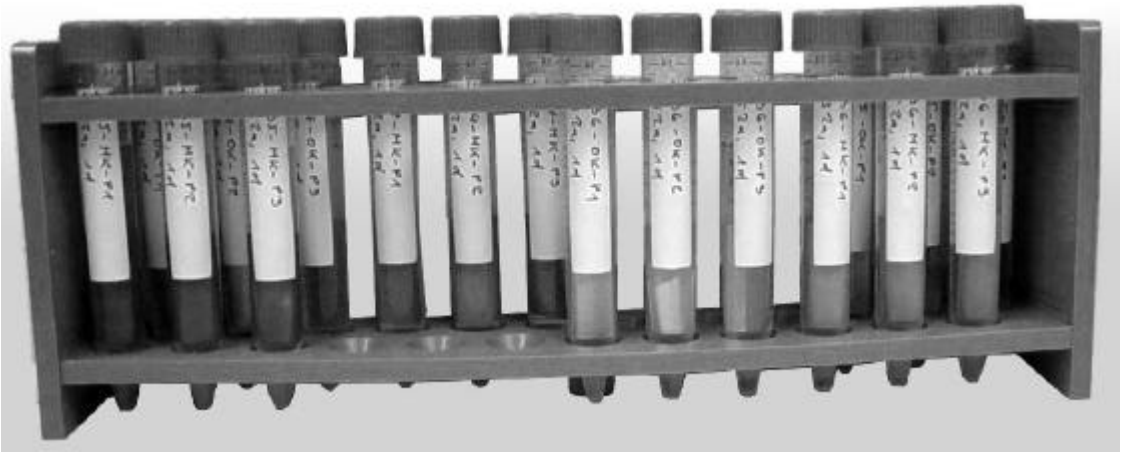


Figure 1: Example from the support and the tubes before incubation (samples from #B4 to #B6 alloys).

After each period of time the solutions were replaced with freshly made electrolytes (figure 2). With the help of a Teflon pincer, the specimens were transferred rapidly from the old recipient to the new one. The samples and the new solution were stored again at 37°C for the subsequent period. The changing of the solutions also simulated the constant replacement that happens in oral fluids [121, 122].

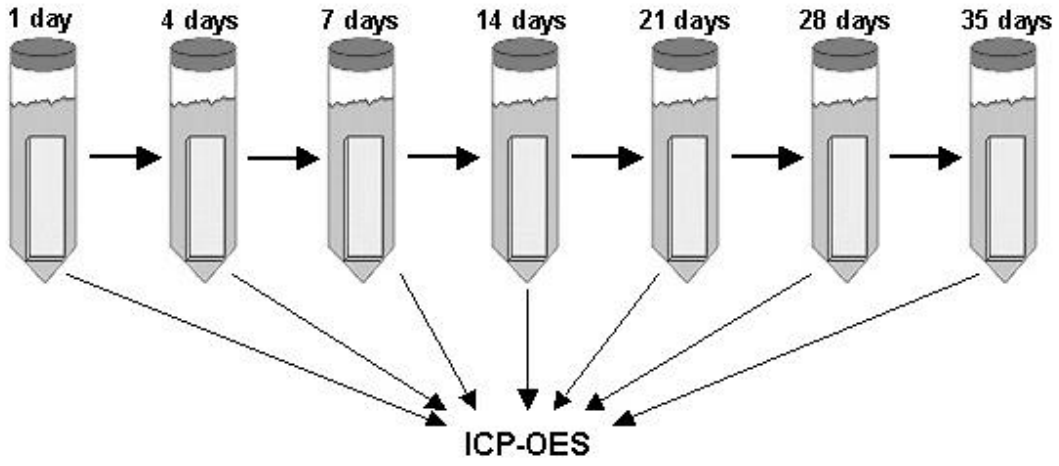


Figure 2: Experimental procedure diagram. With help of Teflon pincer, the specimens were transferred rapidly from the old recipient to the new one. After each incubation time the solutions were analyzed using ICP-OES.

The conditioning media from those exchange periods were analyzed for the presence of *Au, Pt, Pd, Ir, In, Sn, Zn*, to the high-noble alloys; and *of Co, Cr, Mo, W, Si, Mn* and *Ce*, to the base-metal alloys, using an Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) [Optima 4300 DV - The PerkinElmer™ Instruments, Norwalk, USA]. The results from aqueous-standard solutions, prepared with known amounts of the studied ions (1.0, 5.0 and 10.0 µg/ml) in aqueous solutions by addition of salts, gave results which were used as calibration curves (figure 3).

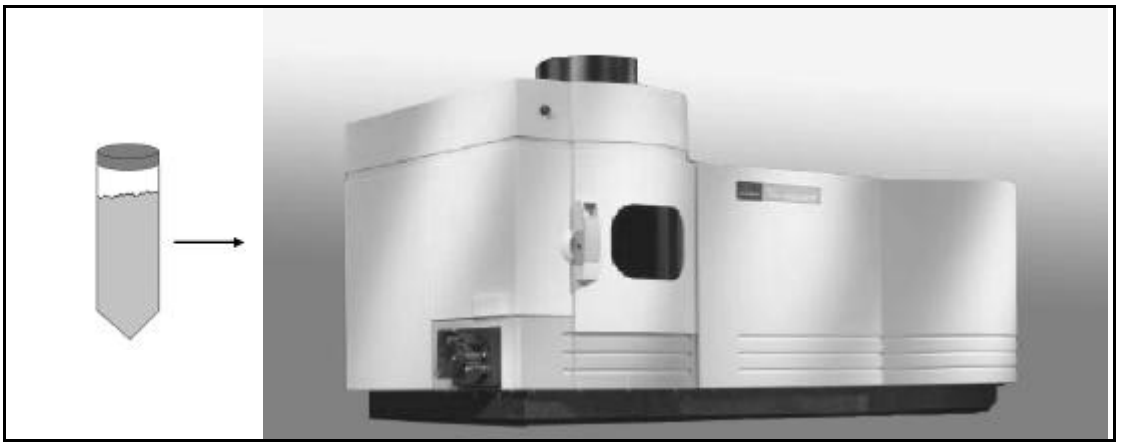


Figure 3: The conditioning media was analyzed by ICP-OES for the presence of the elements of interest.

Triplicate-absorbance readings per element were made for each sample and standards. The mean value for these three readings was used. Each reading was used to determine the mean of concentration (**x**) of the different elements in µg of mass loss released from the exposed surface alloy per cm^2 to the corrosion solution ($\mu g/cm^2$) at each period of measurement time, through the equation:

$$x = c * (v / A)$$

where **c** is the released element measured in the solution (µg/ml), **v** the volume of the corroded solution (10 ml) and **A** the surface area of the specimen (~10 cm^2). Standard deviations and coefficients of variation were calculated for the respective average of the means [82].

Briefly, ICP-OES is used for multielemental analysis due to its low-detection limits, large linear-dynamic range, and high precision. In comparison to other forms of atomic excitation, ICP-OES have the advantage of a multielemental determination with high-analytical frequency and the versatility to be interfaced with other analytical techniques. The selectivity of ICP-OES in analytical spectrometry makes it suitable for elemental analysis in a variety of different matrixes for simultaneous or sequential metal determination in a wide-concentration range. Like other techniques in atomic spectrometry, this method also requires that the samples must be dissolved. This solution is transformed into an aerosol during the sample analysis, *i.e.* the sample is decomposed by

intense heat into a cloud of hot gases containing free atoms and ions of the elements of interest. Due to the high temperatures in the equipment, the elements are ionised and the intensity of the light emitted at specific wavelengths is measured, and then the concentration of an element is determined. Thus, it allows a specific detection of the trace elements as well as quantitative (what element is present) and qualitative (concentration) multielemental determination [14].

Results of three independent-experimental series were tested for statistical significance for the surface state and among alloys using Student's t-Test ($p<0.05$).

SEM and EDX analyses

In addition a study was done of the alloys' surface by scanning electron microscope (SEM) combined with an X-ray diffraction (EDX) analysis. Specimens in the form of disks (1 cm in diameter and 1-1.5 mm thick) were handled as described and immediately analyzed. For the microscopic surface analysis from the polished and polished and oxidized samples was used a high-sensible scanning electron microscope [1430, Firma Leo] which is combined with a X-ray diffraction [Edwin NT, Firma Rontec] for the surface chemical composition determination as elemental mapping. It was analyzed specimens from group #A of the high-noble alloys and the base-metal alloys.

SEM specimens were viewed at 4000X at 20 keV. EDX specimens were investigated using an X-ray source at 20 keV and 3000 cps for 60 s. One sample of each alloy was observed. For EDX analysis all elements from the alloys were investigated.

3.3 Cytotoxicity Assays

Due to the fact that only one biological assay is not enough to give a secure result whether a material is biocompatible or not [21, 71]; a combination of two "in vitro" biological assays, a direct contact assay and a non-specific test by extracts, based on XTT-test, was selected.

Direct Contact Test

The specimens were processed as described before. The non-oxidized samples were ultrasonically cleaned in 1 ml of 70% isopropyl alcohol for 10 min, while the specimens with oxide layer were only submerged in 70% isopropyl alcohol for 10 min, to avoid any disruption of the oxide film. After this time the alcohol was suctioned off. All samples were then washed with Ampuwa Wash® and after that carefully dried with sterile touch paper. Specimens were positioned in a six–well sterile culture plates and fixed with sterile wax at two points. Titanium samples served as negative control, while PVC and Copper specimens were used as positive controls. L-929 mouse fibroblasts were used to evaluate the cellular response to the alloys [7, 27, 64, 77, 96, 100].

Cells were seeded on top of specimens with a density of approximately 30.000 cells/cm², and incubated in DMEM (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) medium [1% Pen/Strep (Gibco), 1% L-Glutamine (Gibco), 10% FCS (PAA)] at 37°C for 24h in a humid air atmosphere containing 5% CO_2, according to ISO 10993-5 [53]. One test-alloy specimen per well was placed in direct contact with the fibroblasts in 9 ml of complete media. Alterations in morphology, viability, and proliferation were used to evaluate the toxicity effects of the test alloys on the cultured cells in comparison with the negative controls for morphological changes [18, 20].

After 24h, alterations of cell morphology and vitality were microscopically determined. First, morphology of the cells close to the samples in the wells in

the unstained state were examined and documented by light microscopy [7, 77]. Then culture media was removed and viability of the cells on top of the specimens was determined quantitatively by photo documentation and cell counting using a vital-fluorescence staining technique, *i.e.*, 0.2% fluorescein diacetate (FDA) and 0.2% ethidium bromide (EB) [Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany] to determine the viability of the cells [64, 85]. Viable cells, with intact plasma membranes, were stained by the FDA pigment and showed green colour. Dead or damaged cells were penetrated by the EB and therefore appeared red.

Three polished specimens and three polished and oxidized samples were tested for each alloy in every experiment. The amount of viable cells was determined in two areas per sample, resulting in n=6 viability counts per surface. Viability on each sample was related to the mean viability on the negative-control material, which was set as 100%. Experiments were repeated a minimum of three times.

XTT-Test

The cytotoxicity effect of the corrosion solutions with released metal ions from the oxidized samples was assessed on L-929 mouse fibroblasts. After the mass loss measurements, the solutions were frozen and at the opportune time utilised. As the highest levels of ion release occurred in the first hours, it was selected to investigate the citotoxic effects of the extracts after 1-day of immersion test. In order to compare results, two groups were established. The first one was of extracts from the alloys incubated in corrosion solution; while the second one was of extracts of the alloys in conventional cell culture medium (DMEM). The solutions of the oxidized samples were used for the alloys and solutions of the as-cast for pure metals. By the second group, extracts of the heat-treated alloys and *Zn* element were conduced in conventional medium culture at 37°C for 24h.

Due to the acid pH value of the corrosion solution (2.3) a “neutralization’s mix” was first employed. This “mix” was prepared using 2.25 ml of 10M NaOH, 2.5 ml HEPES buffer (hydroxyethyl-peperazine-ethane-sulphonic acid) [SERVA Electrophoresis GmbH, Heidelberg, Germany] and 0.25 ml distilled water to make 5 ml of solution with a final pH value of approximately 13.8. Finally 2 ml of the corrosion solution was adjusted to pH 7.0 with the help of this “neutralization mix”. After this adjustment, ICP-OES measurements of the solutions were conduced to confirm the lack of precipitation of metallic ions. In order to make the solutions physiologically compatible with the cell cultures, 9 parts of this neutral-corrosion solution were mixed with 1 part of ten concentrated culture medium. This procedure was conduced right before each individual experiment to allow freshly-made extracts. The specimens in the culture medium needed no further preparation before testing. ICP-OES analyses of the ions in the cell culture milieu were also conduced.

Briefly, XTT-test is a non-specific cytotoxic assay of extractable and diffusing compounds of a material. Its principle is based on measuring the metabolic activity of “in vitro” cultured cells. The metabolic active cells cleave the yellow tetrazolium-salt XTT (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide) to form orange formazan-crystals dye, which are measured spectrophotometrically. The activity of the mitochondrial succinic dehydrogenase (SDH) of viable cells is correlated by incubation with the prepared corrosion-solution extracts and the extracts of the conventional culture medium. A variance in cell metabolism results in a change in the volume of formed formazan, and this fact indicates the cytotoxicity degree of the tested material [22].

First, the cells (approximately 5.000 cells/well) were grown in microtiter plates with a final volume of 100µl culture medium per well (96-wells tissue culture grade) for 24h in normal culture medium (DMEM [Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany] medium with 1% Pen/Strep [Gibco], 1% L-Glutamine [Gibco], 10% FCS [PAA]) in a humidified 5% CO_2 atmosphere at 37°C.

After this time, the medium was suctioned off and the prepared extracts were then added to the cultured cells. For a dose response relationship in this cytotoxicity assay, dilution were established of 1:0, 1:3 and 1:15 (respectively 150µl, 50µl and 10µl) of the neutralizing solution with ten concentrated culture medium in normal medium; and from the conventional medium, with a final volume of 150µl per well. DMEM without cells was used as a blank control, culture medium only with cells was used as 100% negative control, PVC extract was used as positive control, and extracts of the corrosion solution were used as parameter to prove whether this solution itself could induce some cell damage.

The cells and the eluates were then incubated for 24h at 37°C with 5% CO_2. After the incubation period, the cytotoxicity of the extracts was assessed using the XTT assay (Roche Diagnostics GmbH, Mannheim, Germany). In this way, 50µl of the XTT-labelling mixture (5 ml XTT-labelling reagent and 0.1 ml electron-coupling reagent) was added to each well with a final XTT sodium-salt concentration of 0.3 mg/ml.

The cells were incubated at 37°C for 2h, and the resulting formazan stored by the mitochondria in the living cells was analysed. The absorbance was measured with a wavelength reference of 492/620 nm, using a Scanning Multiwell Spectrophotometer plate Reader (ELISA) [SLT-Tecan]. The highest optical absorbance (1.2) had been not exceeded.

The results obtained by the spectrophotometer were compared to each other and with the negative and positive controls. Graphic presentations were then employed in % of viability, where the corrosion solution was set as 100% of cell vitality, for the extracts from the first group; as well as the negative control for the second one. Error bars were four standards of the means. Student's t-Test ($p<0.05$) was used to analyse the statistical significance of the results.

SEM analysis

SEM was also used to evaluate the presence or absence of some cell on the alloys' surface. Cells were grown as described previously. To avoid cell explosion or degradation during the SEM analysis some criteria had to be set. After 24h incubation's time, the medium was carefully removed and the specimens within the cell culture were 24h fixed with 2% glutaraldehyde, followed by twice rising in 0.1M cacodylate buffer. The cells were dehydrated with a series of ethanol washes from 50% to 100%, with a final dehydration in 100% isoamylacetate. The specimens and the cells were air dried with the help of a Critical Point Dryer® (Quorum Technologies Ltd., Watford, UK). The critical point-drying protocol was respected. Finally, the samples were fixed in the appropriate base and *Au-Pd* coated under argon gas atmosphere [18, 75]. Samples were analyzed by SEM at 20 keV. One sample in each surface state was evaluated and Titanium sample was used as negative control.

4. Results

4.1 Corrosion Behaviour

The results of ICP-OES analysis per element in the testing periods (1, 4, 7, 14, 21, 28 and 35-days) were calculated. The total mass loss results in solution per studied alloy in µg/cm² after 35-days of immersion are given in Table 2 (high-noble alloys group #A and group #B) and 3 (base-metal alloys group #N). Overall, it should be noted that non-oxidized alloys demonstrated lower corrosion rates compared to polished and oxidized surfaces. In Table 4 the ion-leakage levels of pure-metals samples (*In*, *Sn* and *Zn*) can be viewed.

Total substance loss over 35-days of immersion for the high-noble alloys ranged from 0.5 µg/cm² (#B6, polished) to 238.4 µg/cm² (#B6, polished and oxidized). Generally the highest ion release was from oxide-forming elements for the oxidized samples. Exception were #A2 and #B2, where *Au* was the element most released, with amounts up to 2.4 µg/cm² for both #A2 and #B2; while *Sn* release reached 0.3 µg/cm² and 0.1 µg/cm² respectively.

For the base-metal alloys the cumulative mass loss after 5-week of assay period varied between 3.1 µg/cm² (#N2, polished) to 54.7 µg/cm² (#N1, polished and oxidized). The results of the non-noble alloys showed comparable behaviour to the high-noble alloys.

Time	Alloys/Element					
	#A1 P	#A1 OL	#A2 P	#A2 OL	#A3 P	#A3 OL
1 day	0.7 (0.5)	1.0 (0.4)	0.4 (0.3)	0.4 (0.3)	2.2 (1.2)	89.2 (16.4)
4 days	1.6 (0.6)	2.6 (0.4)	1.4 (0.9)	1.6 (0.8)	6.7 (5.2)	91.4 (1.6)
7 days	1.9 (0.2)	3.4 (0.6)	1.7 (0.5)	2.0 (0.3)	10.3 (4.5)	92.8 (1.7)
14 days	2.9 (0.8)	6.0 (1.5)	2.7 (0.4)	2.7 (0.6)	11.4 (1.7)	93.8 (0.4)
21 days	3.9 (0.8)	8.7 (1.0)	3.4 (0.6)	3.4 (0.6)	12.9 (1.7)	94.7 (0.7)
28 days	4.4 (0.5)	10.8 (0.3)	3.6 (0.4)	3.7 (0.4)	13.8 (1.1)	95.0 (0.3)
35 days	4.6 (0.2)	12.1 (1.0)	4.1 (0.2)	4.1 (0.2)	14.6 (0.8)	95.5 (0.5)

Time	Alloys/Element					
	#B1 P	#B1 OL	#B2 P	#B2 OL	#B3 P	#B3 OL
1 day	1.6 (1.0)	2.1 (1.2)	0.9 (0.7)	0.8 (0.7)	0.6 (0.6)	1.2 (0.9)
4 days	3.4 (0.4)	5.0 (0.9)	1.7 (0.6)	1.8 (0.6)	1.5 (0.3)	4.5 (0.2)
7 days	5.1 (0.6)	8.0 (0.6)	2.6 (0.6)	2.9 (0.5)	1.9 (0.3)	7.8 (0.7)
14 days	9.0 (1.1)	20.5 (6.0)	2.6 (0.1)	2.9 (0.1)	1.9 (0.1)	26.2 (8.6)
21 days	13.0 (1.4)	34.2 (6.1)	2.7 (0.01)	2.9 (0.01)	2.2 (0.2)	46.9 (10.1)
28 days	16.4 (1.1)	45.7 (5.4)	2.7 (0.01)	2.9 (0.01)	2.3 (0.2)	64.4 (6.4)
35 days	20.7 (1.3)	59.4 (6.9)	2.7 (0.01)	2.9 (0.01)	2.4 (0.1)	83.0 (7.5)

Time	Alloys/Element					
	#B4 P	#B4 OL	#B5 P	#B5 OL	#B6 P	#B6 OL
1 day	0.3 (0.2)	102.5 (34.9)	0.5 (0.4)	131.1 (17.3)	0.3 (0.2)	221.6 (24.1)
4 days	0.6 (0.3)	111.4 (7.5)	0.6 (0.2)	133.1 (2.0)	0.4 (0.1)	225.9 (3.5)
7 days	0.7 (0.1)	118.6 (6.6)	0.8 (0.1)	134.4 (1.0)	0.4 (0.02)	226.8 (0.7)
14 days	0.7 (0.03)	145.2 (4.6)	0.8 (0.05)	135.2 (0.4)	0.4 (0.1)	231.4 (1.4)
21 days	0.7 (0.02)	169.1 (4.5)	0.9 (0.04)	135.3 (0.1)	0.5 (0.03)	234.2 (1.4)
28 days	0.7 (0.02)	189.6 (6.5)	0.9 (0.04)	135.3 (0.02)	0.5 (0.03)	236.3 (1.0)
35 days	0.8 (0.05)	209.6 (4.6)	0.9 (0.01)	135.4 (0.04)	0.5 (0.03)	238.4 (0.7)

Table 2: Ions' release results from #A- and #B-group experimental alloys in µg/cm² after immersion assay (test solution: 0.1 mol/l NaCl + 0.1 mol/l lactic acid; pH=2.3), pre-oxidized according to manufacturer's indications. P: polished, OL: oxide layer, ( ): ±standard deviation.

Time	Alloys/Element					
	#N1 P	#N1 OL	#N2 P	#N2 OL	#N3 P	#N3 OL
1 day	1.0 (0.8)	5.9 (4.8)	0.4 (0.2)	0.4 (0.4)	0.8 (0.8)	0.4 (0.3)
4 days	2.4 (1.5)	13.2 (4.9)	0.4 (0.1)	0.9 (0.8)	1.2 (0.7)	0.9 (0.8)
7 days	2.5 (0.2)	17.1 (5.0)	1.2 (0.8)	1.8 (0.7)	1.6 (0.4)	1.6 (0.5)
14 days	2.7 (0.2)	27.1 (8.3)	1.3 (0.1)	2.7 (1.1)	1.7 (0.1)	2.1 (0.6)
21 days	2.8 (0.2)	37.2 (8.3)	2.4 (1.6)	4.6 (1.2)	2.8 (1.6)	4.0 (1.2)
28 days	2.9 (0.1)	46.7 (7.6)	3.1 (0.9)	6.1 (0.9)	3.5 (0.9)	5.5 (0.5)
35 days	3.3 (0.3)	54.7 (6.4)	3.1 (0.1)	7.0 (1.3)	3.5 (0.03)	6.2 (0.7)

Table 3: Ions' release results from experimental #N alloys in µg/cm² after immersion assay (test solution: 0.1 mol/l NaCl + 0.1 mol/l lactic acid; pH=2.3), pre-oxidized according to manufacturer's indications. P: polished, OL: oxide layer, ( ): ±standard deviation.

In general, the highest ion release occurred within the first day. After 7-days of study the amount of ion leakage from oxidized samples appears to reach a recognizable passivation. Exceptions to this statement were those heat-treated alloys within *In* component. For these alloys (#A1, #B1, #B3, #B4 and #B6) the release of Indium showed a pattern of increase after 7-days, and did not decrease over time. The oxidized #N1 showed the same performance due to the increase of *Co*, *Cr* and *Mo* ion leakage. Pure metals demonstrated the same corrosion behaviour that they showed while components of the high-noble alloys.

Time	Alloys/Element		
	In	*Sn*	*Zn*
1 day	80.8 (10.8)	52.4 (16.1)	3211.3 (296.0)
4 days	151.4 (13.2)	151.4 (11.4)	7371.0 (165.2)
7 days	285.3 (18.3)	282.7 (26.5)	11517.9 (193.6)
14 days	876.4 (24.1)	534.3 (86.1)	15.652.1 (558.6)
21 days	1486.5 (37.9)	723.7 (75.2)	19618.5 (599.4)
28 days	2139.9 (129.3)	891.2 (36.5)	22986.0 (313.7)
35 days	2784.0 (40.8)	1072.5 (62.2)	26812.9 (285.2)

Table 4: Ions' release results from the pure metals *In*, *Sn* and *Zn* in µg/cm² after immersion assay (test solution: 0.1 mol/l NaCl + 0.1 mol/l lactic acid; pH=2.3). ( ): ±standard deviation.

High-Noble Alloys

Much larger levels of ions were leached from the alloys with *Zn*-content elements (#A3, #B4, #B5 and #B6), specifically after the pre-heating treatment in comparison to the others. Such release happened in the first 24h. After this time the mass loss amount had a tendency to decrease. Analysis of *Zn* levels noted that, of the experimental dental alloys used throughout the test period, alloy #A3 released the statistically lowest amount of Zinc.

Alloys content *In* element built up representative ascending polynomial curves after 7-days immersion's test, with no identifiable passivation over time. Alloys with 1wt% and 4wt% *Sn* (#A2 and B2 respectively) shaped a flat-release curve. The final substance loss for the alloys from group #A comparing the surface state (polished and head treated respectively) can be described as bellow: 4.6 µg/cm² and 12.1 µg/cm² for #A1 alloy; 4.1 µg/cm² for both #A2 alloy surface condition; and 14.6 µg/cm² and 95.5 µg/cm² for alloy #A3.

As the most released ions were from oxide-forming elements, the following ranking shows their influence on the alloy's substance loss in descending order: *Zn>In>Sn* for the #A-group alloys. When the pure metals of these elements were studied, they showed the same descending magnificence order of release. For the #B-group alloys, where the oxide-forming elements are combined, they were ranked as: *In/Zn>Sn/Zn>Sn/In>In>Sn*.

Significant differences ($p<0.05$) were noticed between polished samples and polished and oxidized specimens from the #A1 alloy in each interval of the experimental time test overall the 35-days. There were no significant differences ($p>0.05$) on *In* released from #A1 alloy in the studied surface state in the first day of immersion. However, significant difference ($p<0.05$) was noticed after 4-days of immersion for alloy #A1. The quantity of Indium released from the oxidized samples increased up to 8.4 µg/cm² after 35-days, while for the non-oxidized specimens values were 1.0 µg/cm². There were no significant

differences (p>0.05) in the amount of *Sn* released from #A2 alloy after 35-days with a total mass loss of 4.1 µg/cm² for both surface conditions. Indeed, no statistical differences were noticed among specimens of the #A2 polished and oxidized and polished surfaces throughout the assay period.

Further significant difference (p<0.05) was noticed in the amount of Zinc released from alloy #A3 oxidized and polished samples after immersion for 1-day in the corrosion solution with mean values of 88.1 µg/cm² and 2.2 µg/cm², respectively. Significant difference (p<0.05) was continual for #A3 alloy in the 4, 7 and 14-days of immersion. After this period both surface states presented no significant difference (p>0.05) in total ion release. Moreover, samples of alloys #A1 and #A3 polished, and #A2 (both surface states), presented a low overall ion release. The results of ICP-OES analysis of the corrosion solution exposed to the #A-group alloys by the total mass loss are presented in figure. 4. Figure 5 shows the ion leakage distributed among the elements of the alloys' composition.

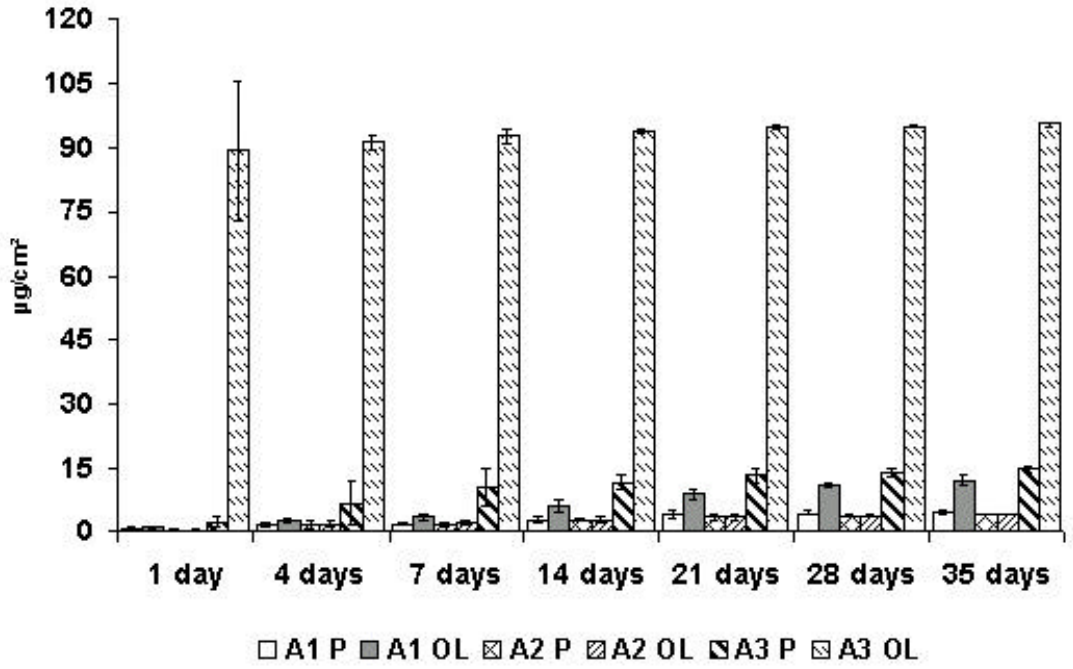


Figure 4: Total mass loss over 35-days of immersions test in corrosion solution (lactic acid/NaCl pH value 2.3) at 37°C from experimental alloys (group #A). Comparison between polished samples and polished and oxidized specimens. Values in µg/cm². P: polished OL: with oxide layer

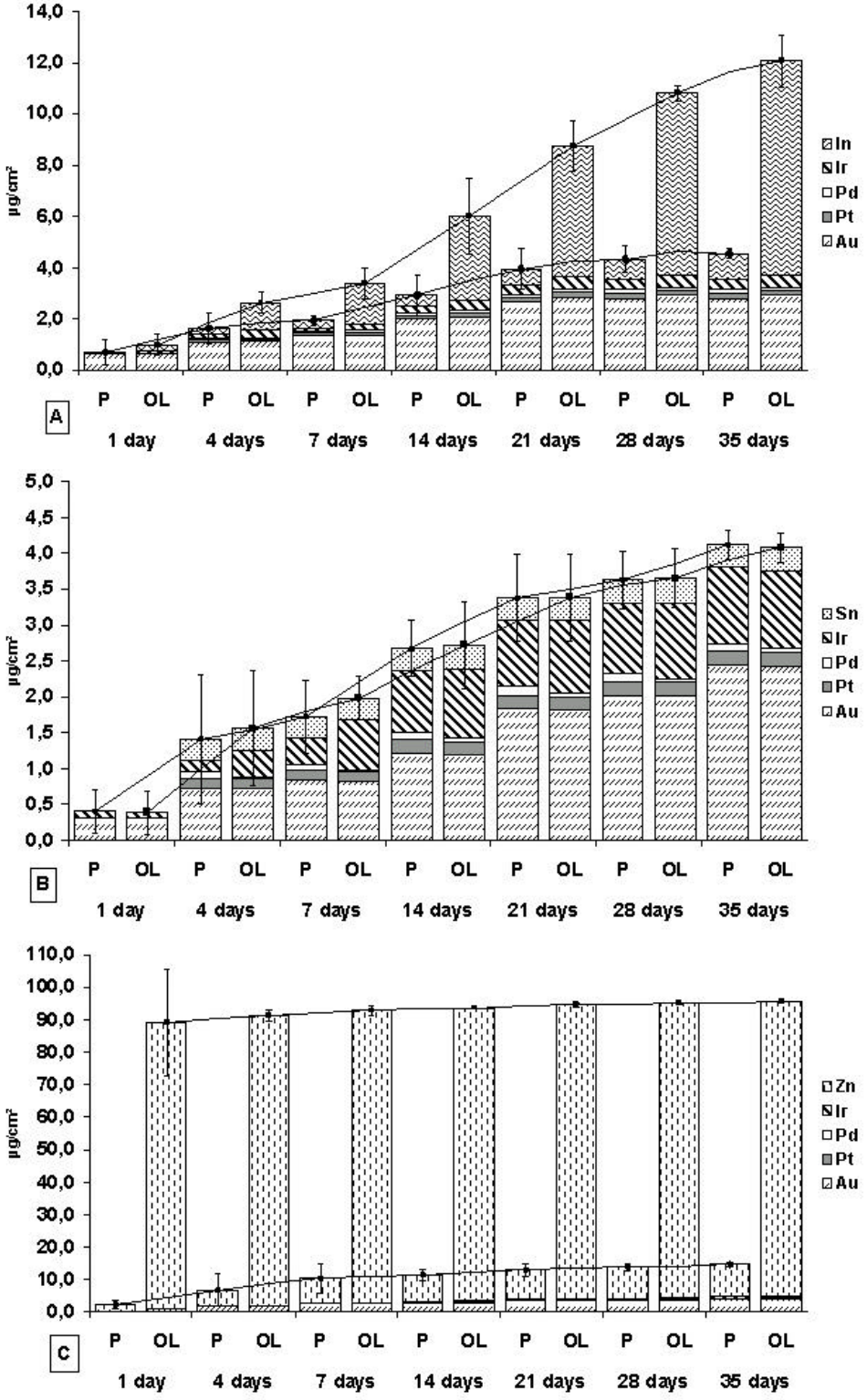


Figure 5: Ion leakage from experimental #A-group alloys after 35-days of immersion test in corrosion solution (lactic acid/NaCl pH value 2.3) at 37°C. The total mass loss (curve) is expressed in the columns by the elements of each alloy. A) alloy #A1; B) alloy #A2; and C) alloy #A3. Comparison between polished samples and polished and oxidized specimens. Values in µg/cm². P: polished OL: with oxide layer

The total substance loss after 35-days of immersion test in corrosion solution from experimental #B-group alloys is presented in figure 6 for each alloy type. In the alloys from group #B, Student's t-Test analysis showed that there were no significant differences ($p>0.05$) for #B1 alloy only in the first day and after the fourth day of immersion assay. A significant difference ($p<0.05$) was noticed, however, after seven days of test, caused by the increase in Indium ion release by the #B1 polished and oxidized. The mean values of total mass loss in the first day were 2.1 µg/cm² and 1.6 µg/cm² for #B1 oxidized and non-oxidized respectively; while the total mass loss after 35-days was up to 59.4 µg/cm² and 20.7 µg/cm² respectively. The great amount of the released ions was from *In* element (16.3 µg/cm² and 54.9 µg/cm² for polished and heat-treated samples respectively) for #B1 alloy.

Alloy #B2 showed no statistical difference ($p>0.05$) over the course of the immersion test, with a total substance loss after 35-days up to 2.9 µg/cm² and 2.7 µg/cm² for the oxidized and non-oxidized samples respectively. Tin-ion release was from 0.1 µg/cm² for both #B2 alloy surface states.

Further significant differences ($p<0.05$) occurred at all immersion interval periods by the #B3 alloy. The total mass loss after the assay time was from 2.4 µg/cm² and 83.0 µg/cm² for #B3 polished and polished and oxidized respectively. Heat-treated #B3 alloy released Indium element up to 73.3 µg/cm² and Tin ions up to 7.5 µg/cm² after 35-days of immersion.

Significant differences ($p<0.05$) could also be seen in the values of *Zn* ion release in the first day of immersion for the Zinc-content alloys (values ranged between 89.4 µg/cm² and 0.1 µg/cm²; 121.8 µg/cm² and 0.2 µg/cm²; and 205.6 µg/cm² and 0.2 µg/cm² for #B4, #B5 and #B6 oxidized and non-oxidized respectively). After 7-days of immersion, alloys #B4 and #B6 presented significant differences ($p<0.05$) between oxidized and non-oxidized samples due to the increase of Indium leakage. For these alloys the total ion release from the oxide-forming elements in the polished and oxidized samples was up

to 114.2 µg/cm² from Indium and 94.9 µg/cm² from Zinc for the #B4 alloy; while for the #B6 alloy the values were respectively at amounts of 29.0 µg/cm² and 209.2 µg/cm². Alloy #B5 presented a total of *Sn* ion leakage up to 9.5 µg/cm² and *Zn* ion release of 125.7 µg/cm² over 35-days of immersion. The total ion release results, separated by the alloy elemental composition, over 35-days of corrosion immersion test are given in figure 7 and figure 8.

Comparisons between the high-noble alloys with similar oxide-forming element's constitution (#A1 and #B1; #A2 and #B2; #B4 and #B6) demonstrated that they were significantly different ($p<0.05$) in the corrosion behaviour after the immersion test. The ion release was significantly different ($p<0.05$) in all time analysis for alloys #A1 and #B1. Student's t-Test showed that alloys #A2 and #B2 presented significant ion leakage difference ($p<0.05$) after 7, 14, 21 and 35-days of immersion, for both surface conditions.

There was significant difference ($p<0.05$) only after 7-days of testing for alloys #B4 and #B6 in the polished surface state. When the heat-treated samples of these alloys were compared there was a significant difference ($p<0.05$) in the first day of immersion due to the higher value of *Zn* ion release from alloy #B6. After 4-days of testing there was no significant difference ($p>0.05$) in the mass loss for these alloys. Moreover, after 7-days and over 35-days of immersion the total substance release was significantly different ($p<0.05$); mainly caused due to the increase of Indium release.

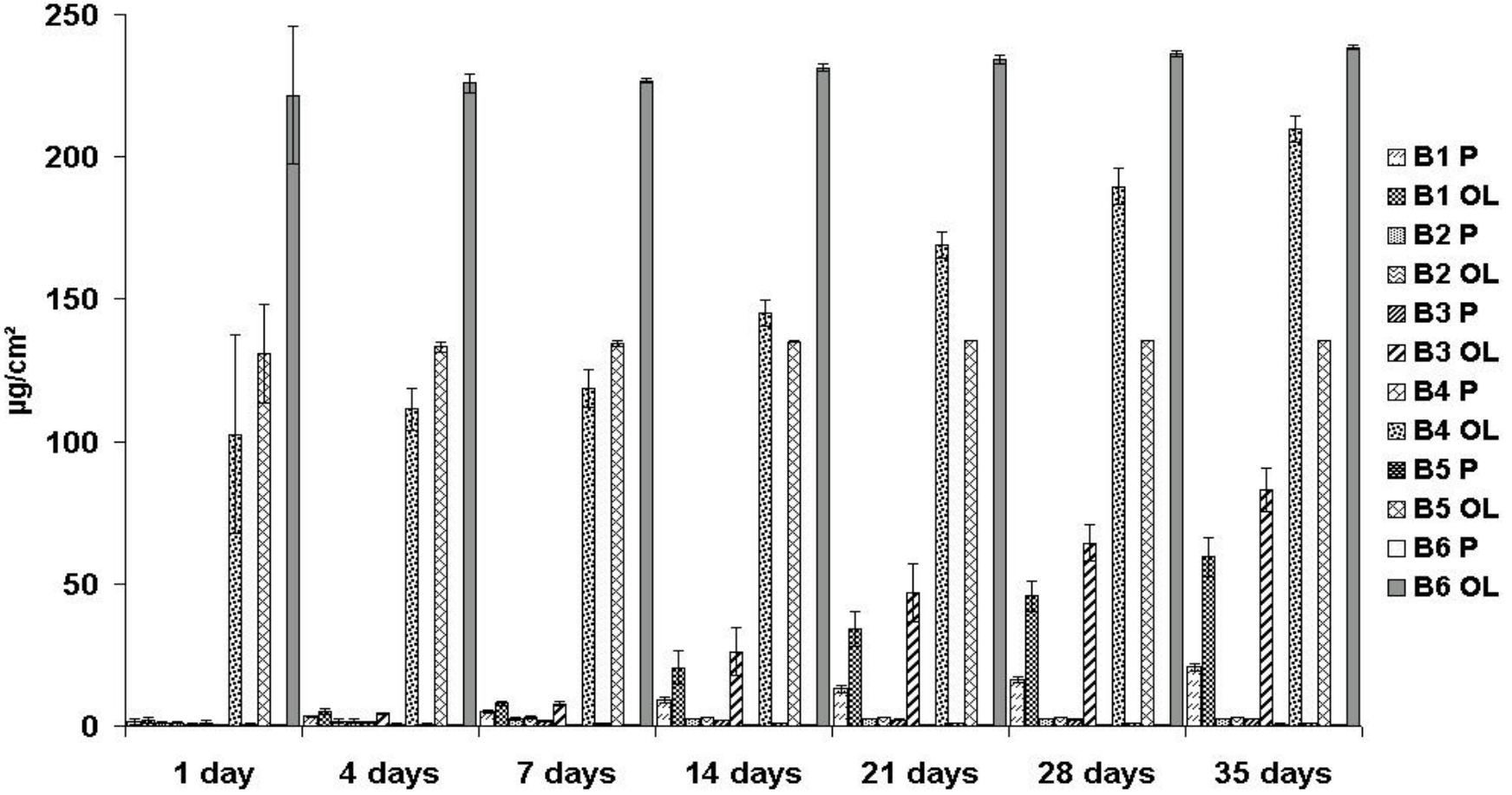


Figure 6: Total released mass over 35-days of immersions test in corrosion solution (lactic acid/NaCl pH value 2.3) at 37°C from experimental #B-group alloys (#B1-#B6). Comparison between polished samples and polished and oxidized specimens. Values in µg/cm². P: polished OL: with oxide layer

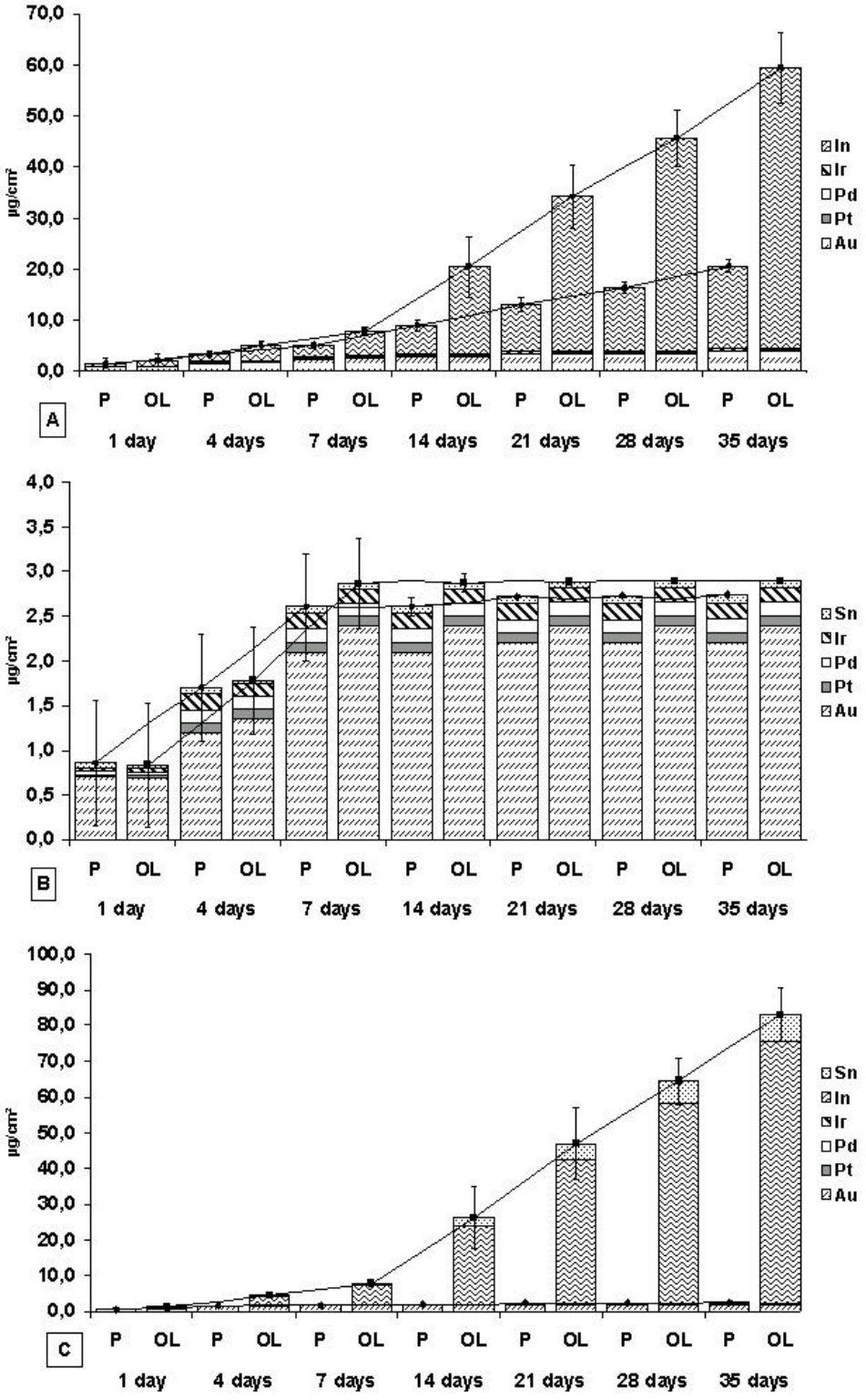


Figure 7: Ion leakage from experimental #B-group alloys after 35-days of immersion test in corrosion solution (lactic acid/NaCl pH value 2.3) at 37°C. The total mass loss (curve) is expressed in the columns by the elements of each alloy. A) alloy #B1; B) alloy #B2; C) alloy #B3. Comparison between polished samples and polished and oxidized specimens. Values in µg/cm². P: polished OL: with oxide layer

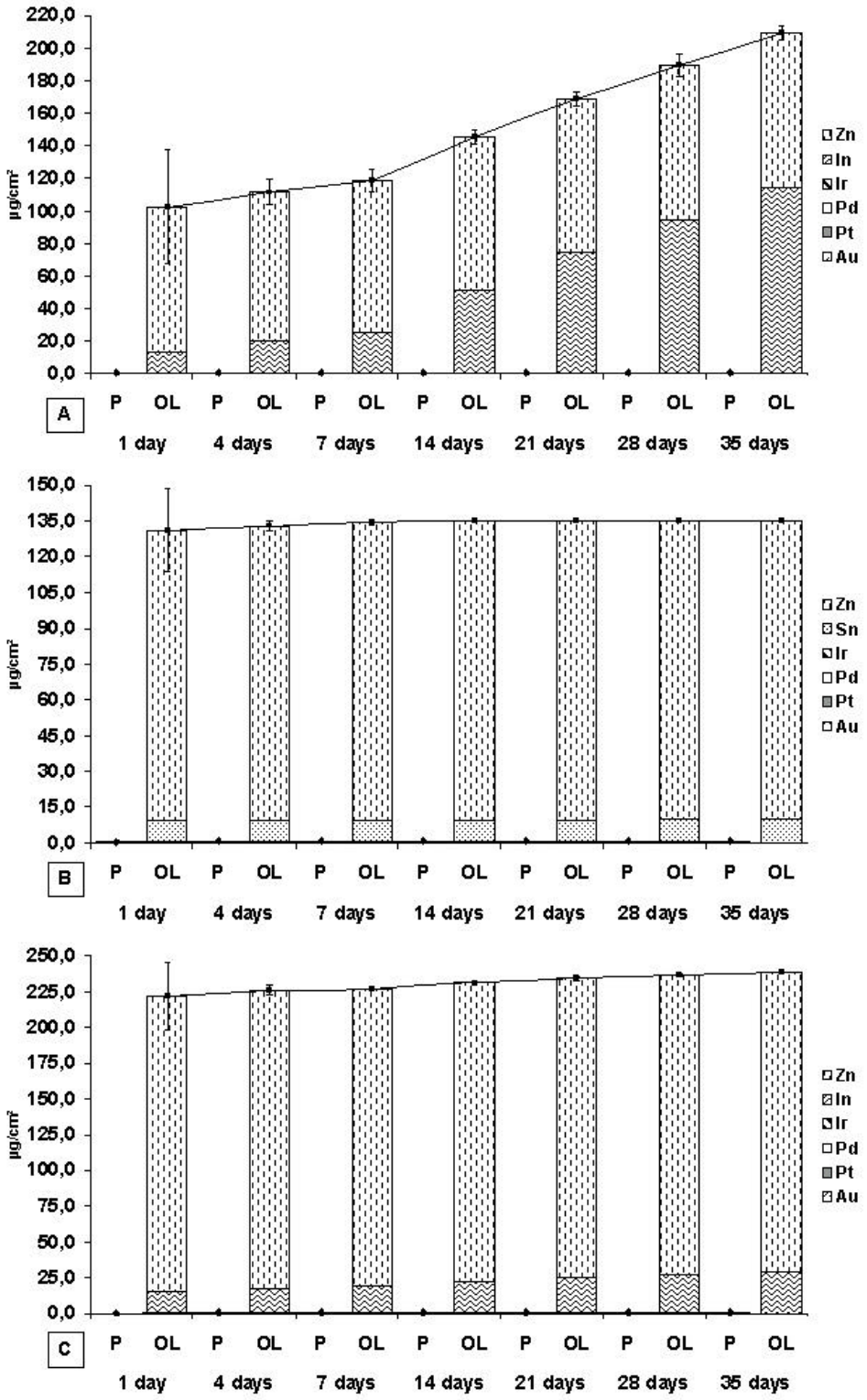


Figure 8: Ion leakage from experimental #B-group alloys after 35-days of immersion test in corrosion solution (lactic acid/NaCl pH value 2.3) at 37°C. The total mass loss (curve) is expressed in the columns by the elements of each alloy. A) alloy #B4; B) alloy #B5; C) alloy #B6. Comparison between polished samples and polished and oxidized specimens. Values in µg/cm². P: polished OL: with oxide layer

The release rates of the pure metals after 35-days of immersion test were much higher than by the greatest cumulative peak of these elements from the high-noble alloys (figure 9). The element *Zn* as pure metal and in the as-cast stage was released 128 times more than by the #B6 oxidized alloy (26812.9 µg/cm² and 209.2 µg/cm² respectively). The release of pure *Sn* was 1072.5 µg/cm² while #B5 oxidized alloy showed a leakage of *Sn* up to 9.5 µg/cm² that means 113 times more. Element *In* demonstrated 25 times more release as pure metal than by the #B4 oxidized alloy (2784.0 µg/cm² and 114.2 µg/cm² respectively).

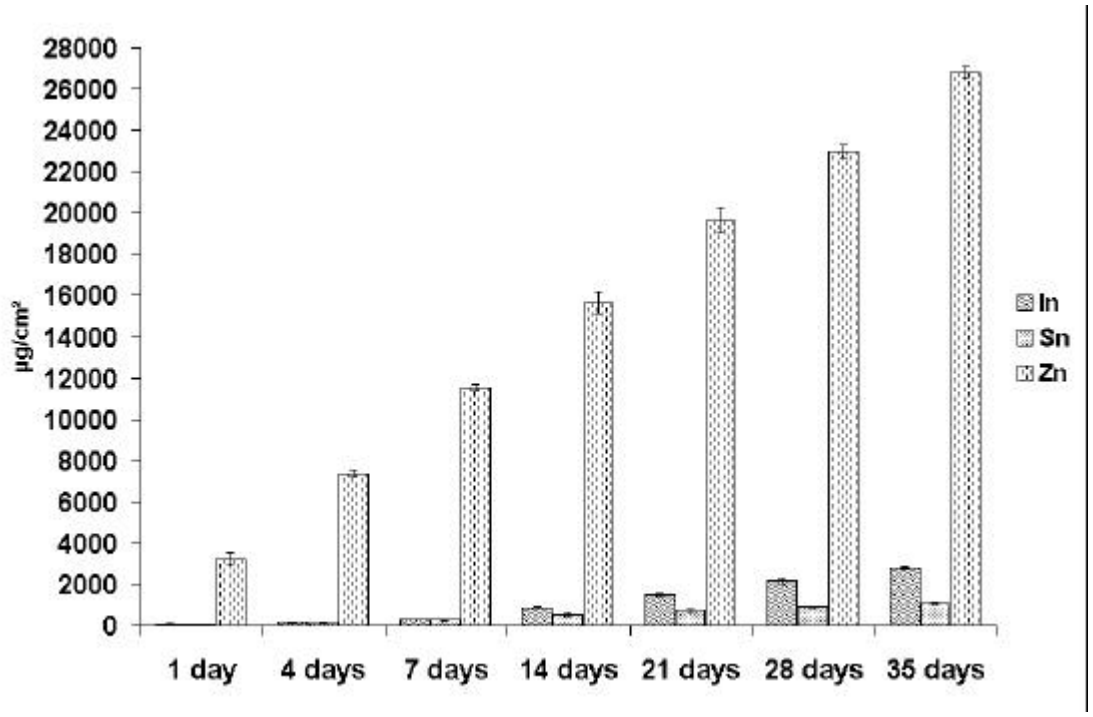


Figure 9: Total mass loss from as-cast pure metals *In*, *Sn* and *Zn* after 35-days immersion test. Values in µg/cm².

Base-Metal Alloys

Oxidized #N1 alloy exhibited the greatest substance loss for the *CoCr*-based group, which increased with no recognizable passivation over time. After 35-days, a mean total ion release of 54.7 µg/cm² was recorded. The most representative released elements from heat-treated #N1 were its main compounds: *Co* with a total leakage up to 39.2 µg/cm², followed by *Mo* with a

mean value of 7.9 µg/cm² and *Cr* with levels of 3.6 µg/cm². Total mass loss results of the *CoCr*-based alloys are reported in figure 10 regarding the immersion test period.

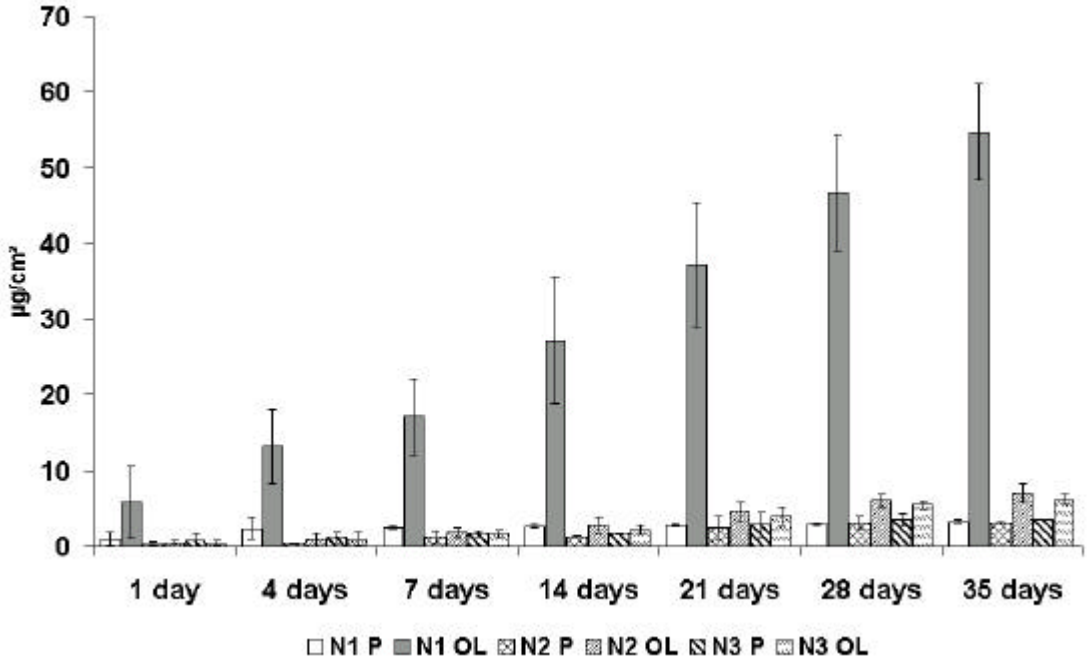


Figure 10: Total ion release from experimental alloys (group #N). Comparison between polished samples and polished and oxidized specimens. Values in µg/cm². P: polished OL: with oxide layer

The alloys #N1 polished and #N2 and #N3 in both surface states demonstrated a rapidly increasing substance loss during the first day. In the subsequent periods the mass loss decreased, and after 35-days it reached a mean total mass loss of 3.3 µg/cm², 3.1 µg/cm² and 3.5 µg/cm² for the polished samples #N1, #N2 and #N3 respectively.

For the heat-treated specimens' #N2 and #N3 a total substance loss respectively of 7.0 µg/cm² and 6.2 µg/cm² after 35-days of immersion test was noticed. For these alloys Cobalt was the most released element at 3.9 µg/cm² and 3.1 µg/cm² respectively, Molybdenum was secondly at 1.1 µg/cm² and 1.3 µg/cm² respectively, followed by Chrome with an ion release of 0.8 µg/cm² and 0.7 µg/cm² respectively. Element Cerium had released amounts under the

detection limit and therefore could not be identified by the ICP-OES analysis for the #N2 and #N3 alloys corrosion solutions.

A relationship among the heat-treated #N2 and #N3 alloys and the oxidized #N1 specimens could be determined in the proportion that alloys with *Ce* element had released respectively 8 and 9 times less metallic ions than alloy #N1. The results of the elemental release in relation with the alloys' base-metal compounds correlated with the immersion test period are presented in figure 11.

It could be corroborated that #N1 alloy presented a significant difference ($p<0.05$) between polished and polished and oxidized samples over the course of the test. Alloys #N2 and #N3 showed no significant difference ($p>0.05$) in both surface states. Meanwhile, comparison among these alloys showed that alloys #N2 and #N3 demonstrated no significant difference ($p>0.05$) for all test periods in the two surface conditions.

In addition, Student's t-Test showed that polished alloys #N1 and #N2 presented significant difference ($p<0.05$) in ion release after 4, 7 and 35-days of immersion test. Otherwise alloys #N1 and #N3 demonstrated no significant difference ($p>0.05$) over the period assay. Further significant differences ($p<0.05$) in ion leakage, mainly of Cobalt, was evidenced between heat-treated #N1 and #N2 samples as well as among polished and oxidized #N1 and #N3 specimens after 4, 14, 21, 28 and 35-days of immersion test.

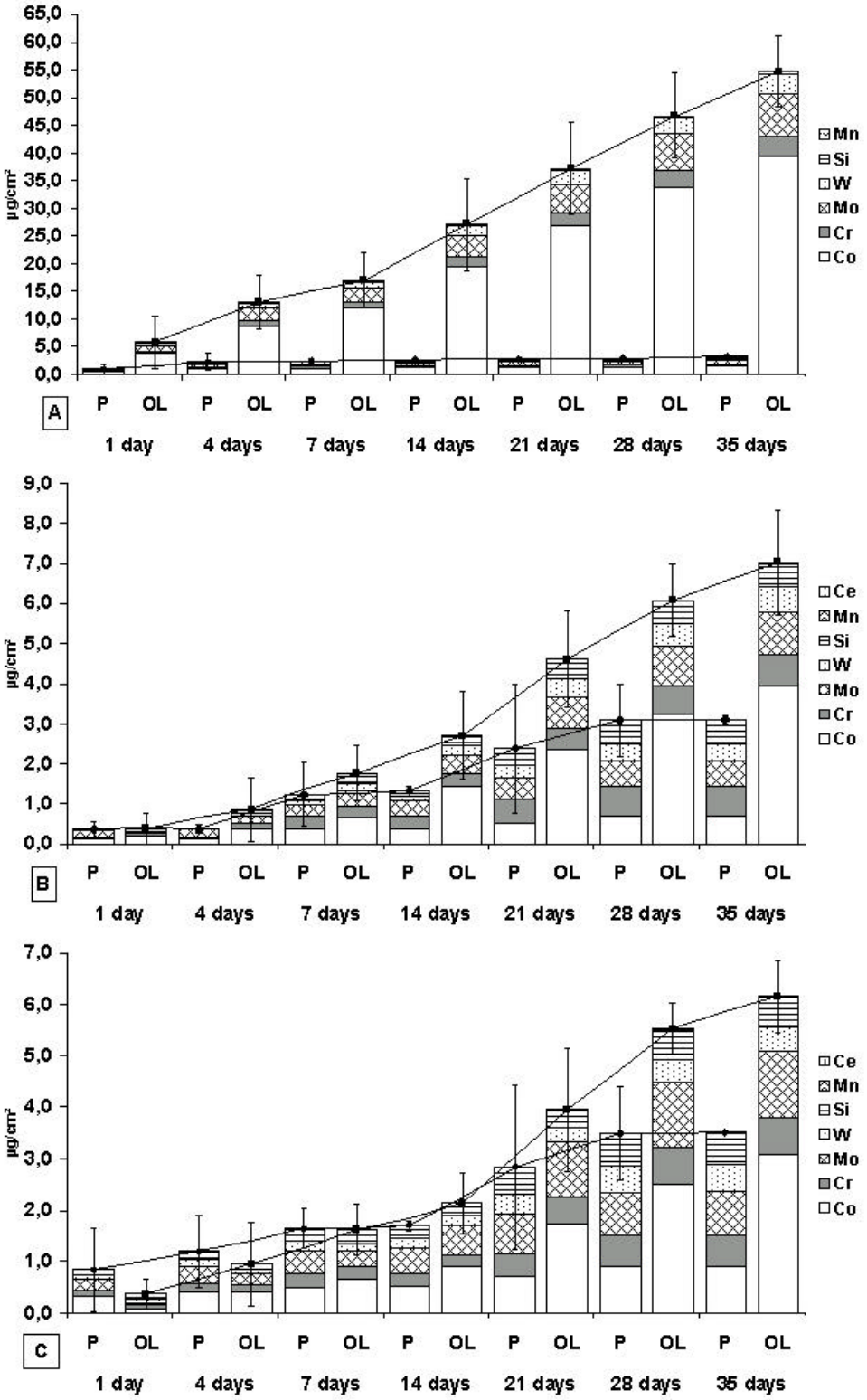


Figure 11: Ion leakage from experimental #N-group alloys after 35-days of immersion test in corrosion solution (lactic acid/NaCl pH value 2.3) at 37°C. The total mass loss (curve) is expressed in the columns by the elements of each alloy. A) alloy #N1; B) alloy #N2; and C) alloy #N3. Comparison between polished samples and polished and oxidized specimens. Values in µg/cm². P: polished OL: with oxide layer

SEM and EDX Analysis

Polished and polished and oxidized samples from the experimental high-noble alloys group #A and from the base-metal alloys were analysed. SEM analysis demonstrated differences in the aspect on the alloys' surface up to 4000X magnifications before and after pre-heated treatment.

The results obtained from SEM examination clearly indicated the simulated oxidation of the tested alloys showed the formation of an oxide layer on the surface of all alloys. A SEM micrograph of the polished #N3-specimen surface is shown in figure 12. A typical example from all alloys which have their surfaces finished with a wetting #1200 SiC paper. In figures 13 and 14 are presented the SEM micrographs of the studied alloys #A3, #N1, #N2 and #N3 after pre-heated treatment.

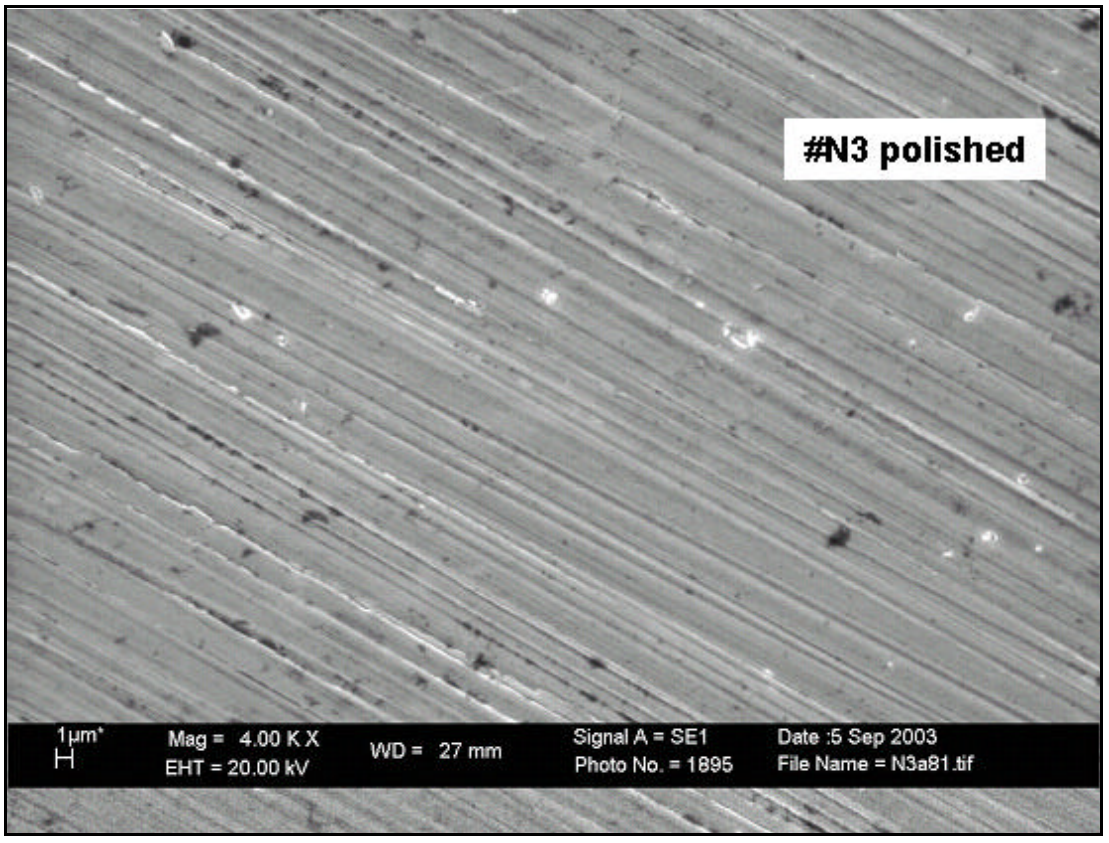


Figure 12: SEM micrograph of experimental #N3 non-oxidized alloy as a typical representation of the polished specimens with the roughness of the finishing #1200 SiC paper. 4000X.

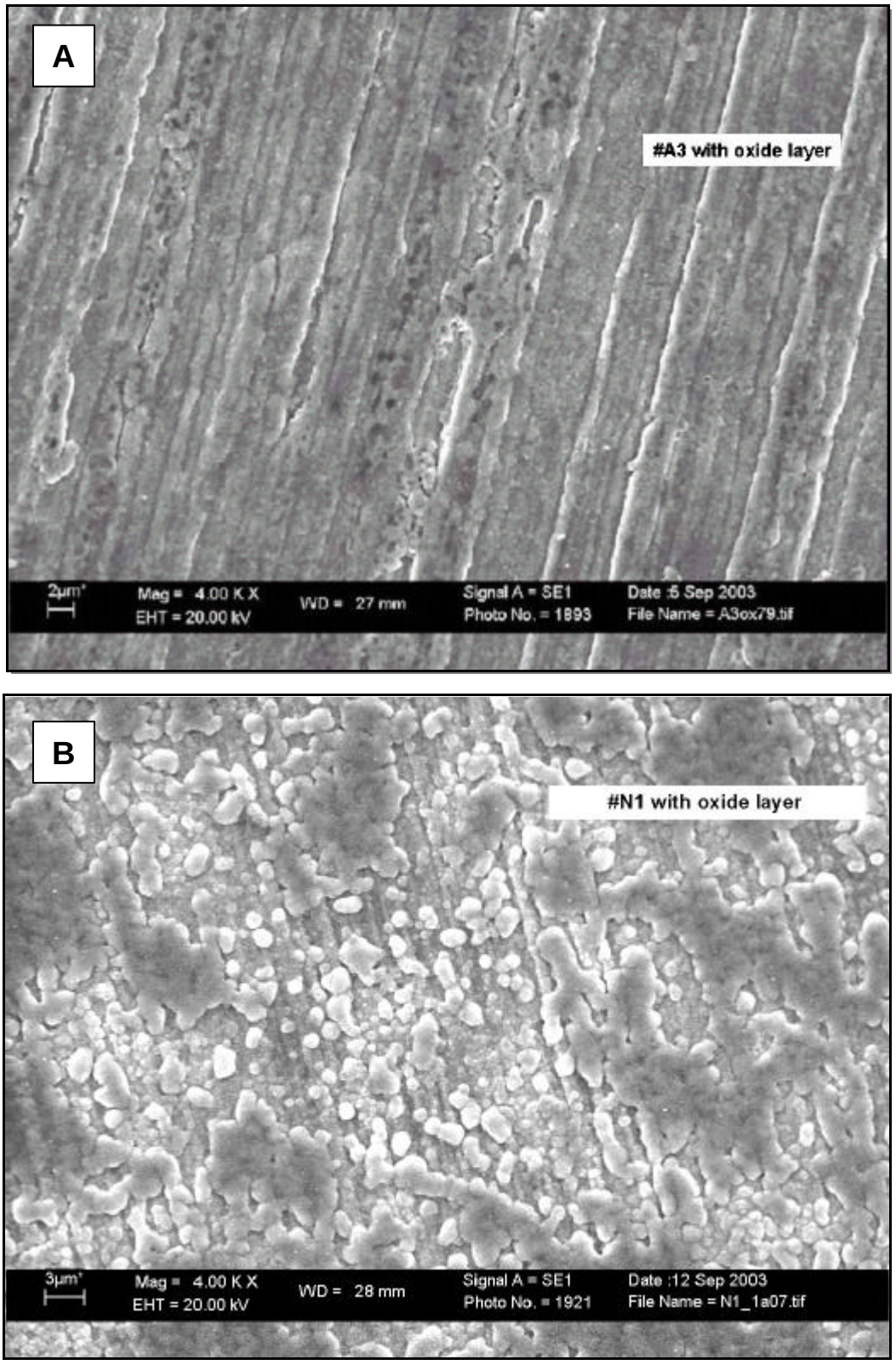


Figure 13: SEM micrograph of oxidized specimens. A) #A3 alloy, with the representative aspect for all the pre-heated high-noble alloys with overall homogeneous distribution of elements. B) #N1. 4000X.

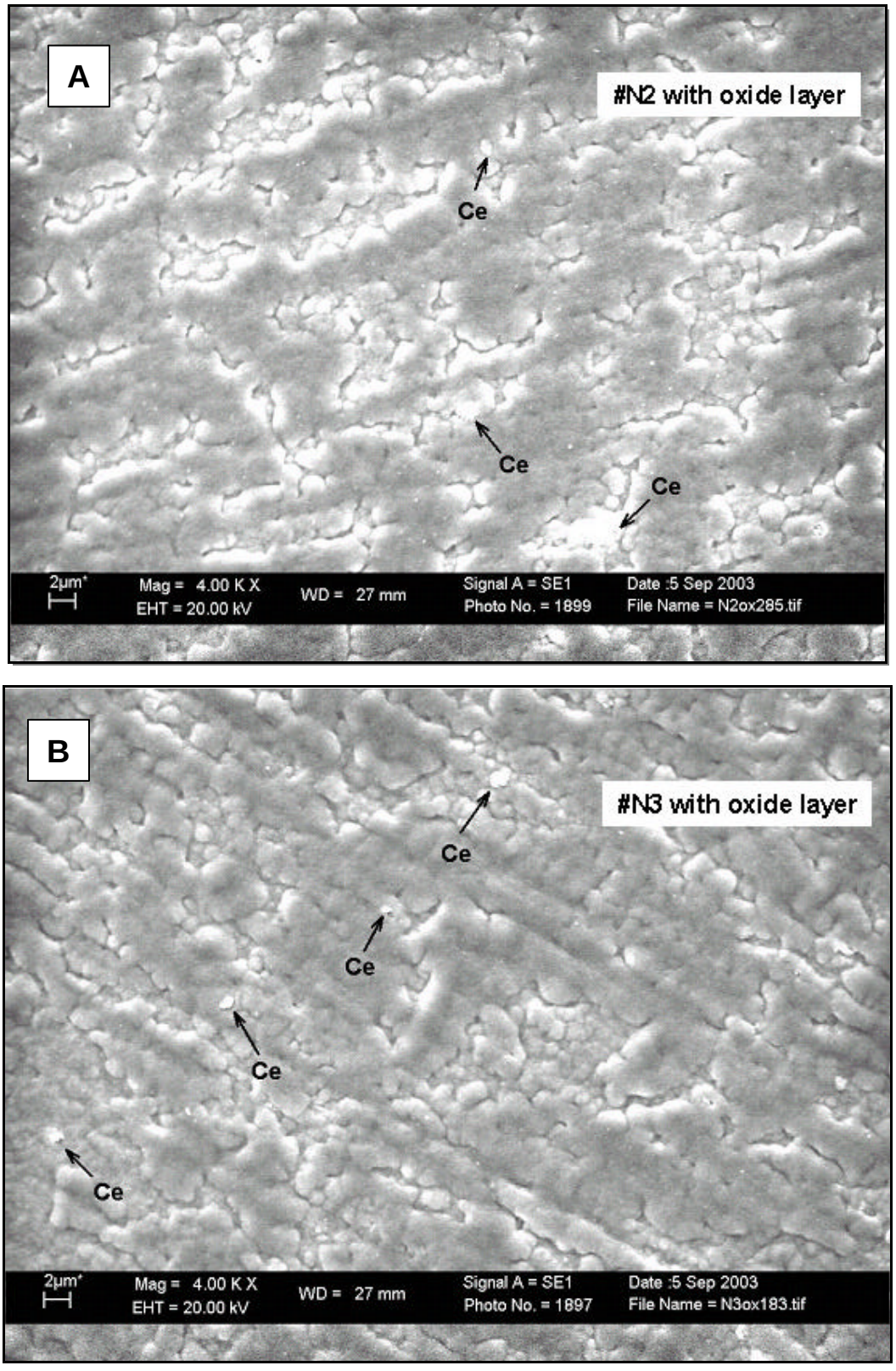


Figure 14: SEM micrograph of some oxidized specimens. A) #N2 alloy and B) #N3 alloy, where the bright particles represent the concentration of Cerium element on the alloy surface. 4000X.

Ridges, crevices, and pits characterized the surface of the oxidized alloys. Particles were found fused to the surface or incorporated into the alloy and protruding less above the surface at various points and largely concentrated in the crevices as fused particle masses.

The EDX analysis showed that the polished alloy surface of the experimental high-noble alloys consisted mainly of *Au*, *Pt* and *Ir* with small traces of *Pd* and oxide-forming elements. The peaks of the three first elements appear to be over positioning due to the intensive energy-emission difference between *Au*, *Pt* and *Ir*, which are close to each other. For the experimental base-metal alloys it should be noted that the *Co* element was most identified in all polished specimens, since this is the main element of such alloys.

Representative diafractograms of diverse alloys' composition surfaces, analyzed through the EDX are shown in figure 15.

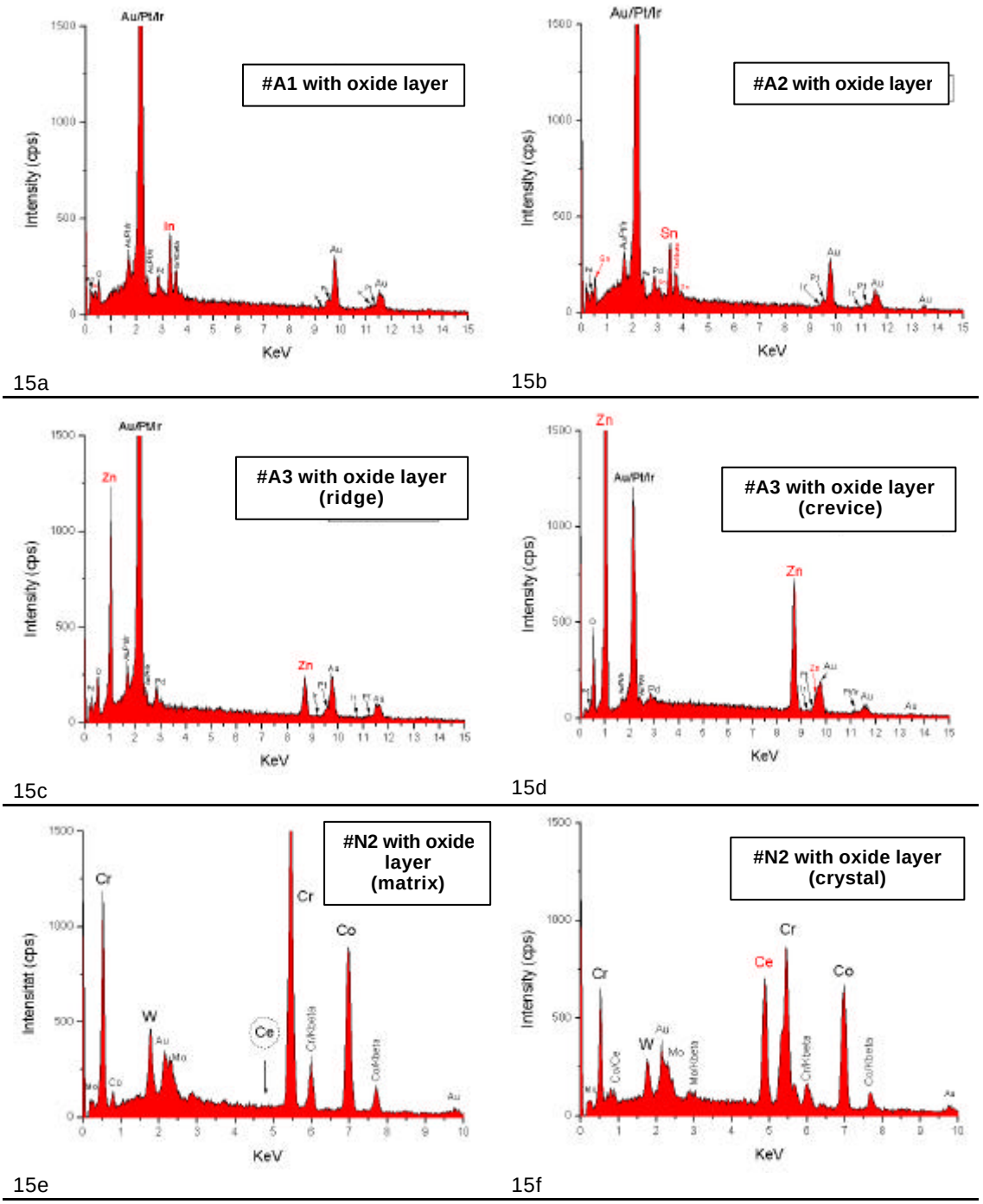


Figure 15: EDX spectrum from the studied alloys after simulated porcelain fire. a) #A1, b) #A2, c) #A3 on the ridge, d) #A3 in the crevice with most concentration of Zinc element, e) #N2 in the matrix; f) #N2 in the bright particles where there was the high concentration of Cerium. Samples were investigated for 60 s, using an X-ray source at 20 keV and 3000 cps.

The elemental concentration of the alloys' surface composition varied considerably between polished samples and heat-treated specimens. After the oxidation process, an oxide layer was noticed on the alloys' surface. In the trace taken from the high-noble alloys after the fire cycle, it was found that the oxide-forming elements lines became more prominent; indicating that *In*, *Sn* and *Zn* content on the alloy surface was increased. This suggests the elements had diffused toward the alloy onto the surface during the firing and became oxidized.

EDX surface mapping of the oxidized samples revealed a homogeneous distribution of all elements for the high-noble alloys on its surface. The oxide-forming elements were found for the #A1 and #A2 alloys spread through the alloy surface, but also concentrated as particles partially fused to the surface. By the #A3 a preferential diffusion of *Zn* could be seen. On this alloy two areas could be distinguished: one on the ridges with higher content of noble elements, and other in the crevices where *Zn* was present at high ranges.

The oxidized #N1 showed *Cr* as the main element on the alloy surface, followed by *Co*. The pre-heated #N2 and #N3 specimens also presented two areas: a matrix where *Cr* was also the higher concentrated element followed by *Co* and some bright particles like crystals where *Ce* appeared in considerable concentrations.

4.2 Biocompatibility

Direct Contact

In the 24h direct contact test, light-microscopic evaluation showed that there were no differences in morphology between cells exposed to the respective alloys compared to the negative-control samples. The cells exposed to the negative control, to the samples from alloy groups #A, #B, #N, and to pure *In* and *Sn* exhibited a normal morphology and demonstrated normal growth towards the samples (normal cell densities around the specimens). Cell viability

on the alloys ranged from 98.1% (oxidized #N1 alloy) and 99.9% (heat-treated #B2 and #B3 alloys), compared to the Titanium control (figure 16). Cells exposed to the positive controls and pure Zinc were rounded and detached with an area of reduced cell density in the near vicinity of the samples, in some cases also at a distance from the specimens (figure 17).

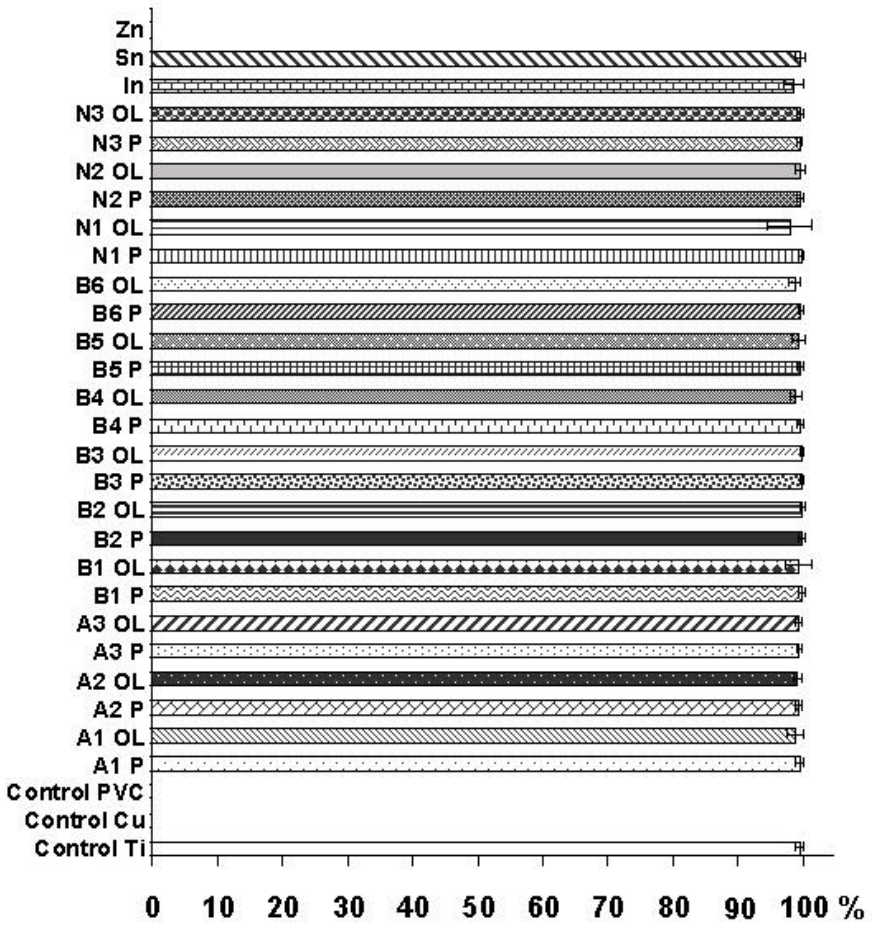
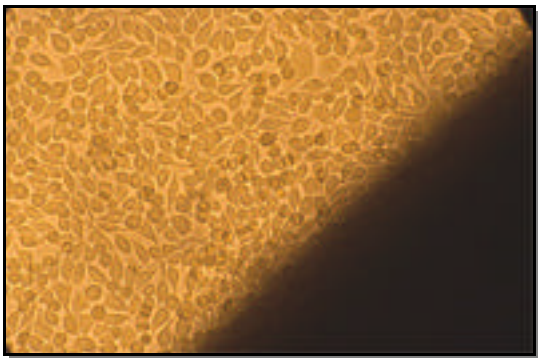
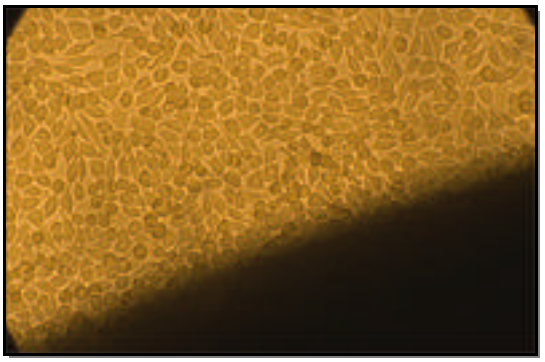


Figure 16: Viability of L929 mouse fibroblasts after direct contact with 12 experimental PFM-dental alloys according to ISO 10993-5, tested before (P) and after simulated porcelain firing (OL). Data represent means and standard deviations of three experiments, expressed as percentage of viability in comparison to the negative control (100% viability).



17a



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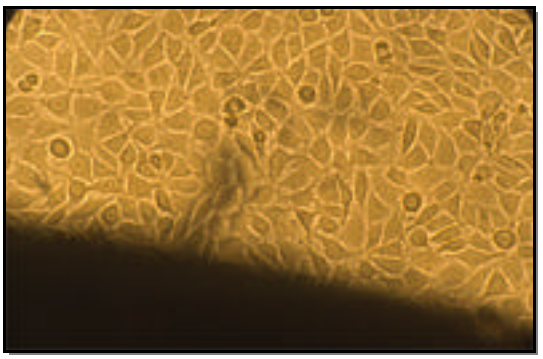

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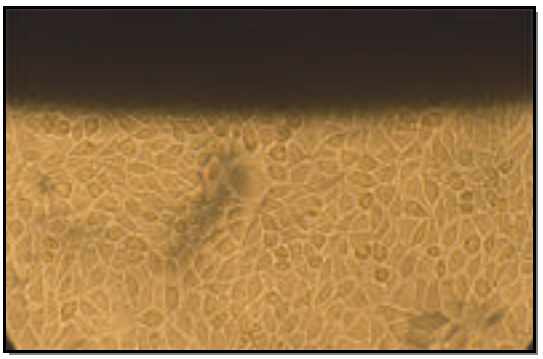

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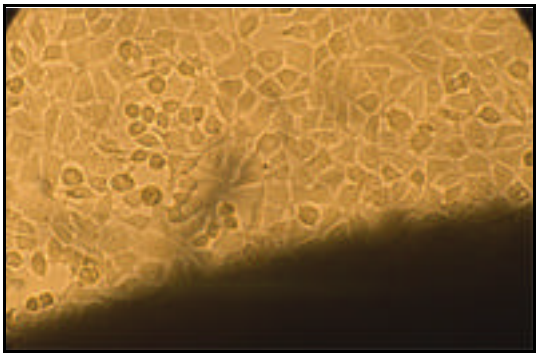

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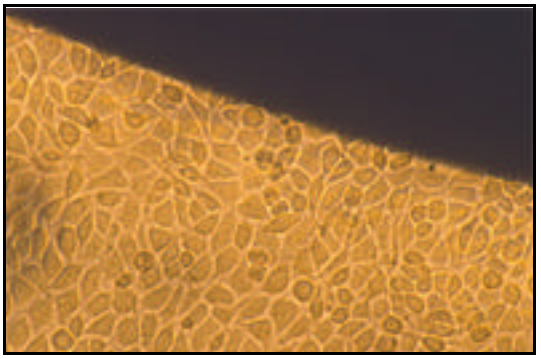

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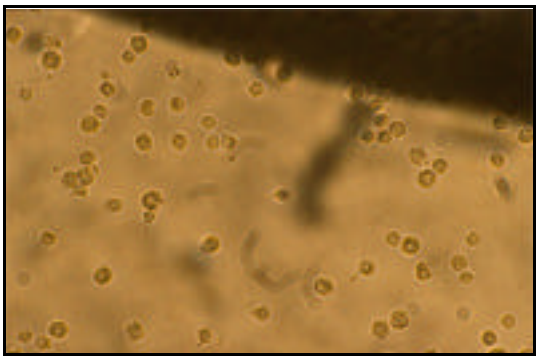

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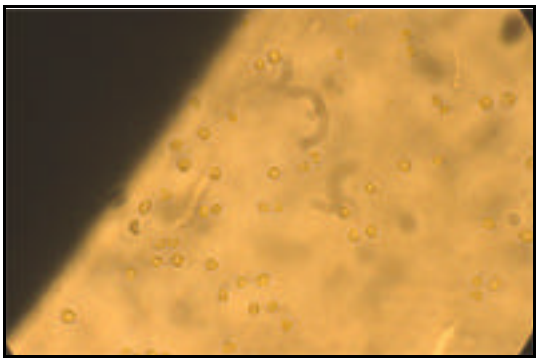
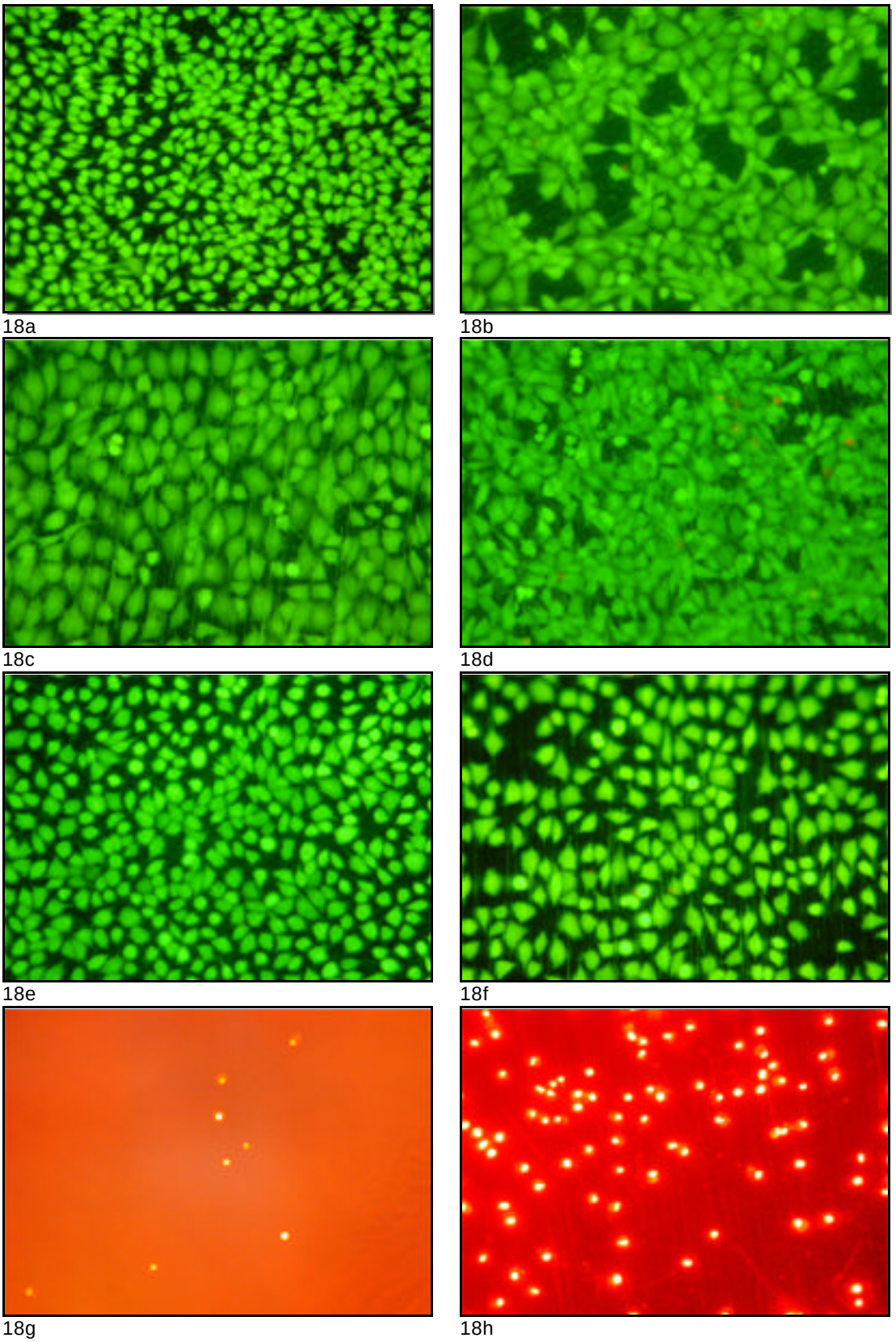


17h

Figure 17: Microscopic evaluation of cell morphology after 24h of direct contact to: a) #A2 oxidized; b) #A3 polished; c) #B1 polished; d) #B3 oxidized e) #B5 after simulated porcelain firing; f) #N3 after simulated porcelain firing; g) PVC; h) pure Zinc. 200X



18a 18b

18c 18d

18e 18f

18g 18h

Figure 18: Viability of L-929 mouse fibroblasts after 24h of direct contact to: a) #A1 after simulated porcelain firing; b) #B4 oxidized; c) #B6 polished; d) #B6 after simulated porcelain firing; e) #N1 oxidized; f) #N2 oxidized; g) Copper; h) pure Zinc. Vital staining with FDA (green/viable) and EB (red/dead), 100X.

After staining, it was noted that the polished samples presented on average a slightly higher number of cells in comparison to the polished and oxidized specimens, especially on those from experimental alloys #A1, #B4, #B6 and #N1. However, viability of the cells did not appear to be affected (Fig. 18).

XTT-Test

Cytotoxicity of alloy extracts in concentrations of 1:0, 1:3 and 1:15 was tested by the exposition of L-929 fibroblasts to these eluates for 24h. All cell cultures were conduced in 4 replicates per time. It was chosen to use extracts made from corrosion solutions of the oxidized samples after the first day of immersion tests on the basis of their higher release ions concentration content (data corroborated by the mass loss assay).

Optical absorbance read from treated cell cultures were correlated to the cells exposed to the neutralized corrosion solution (=100%) for the eluates from corrosion solution; and to the untreated cell cultures (=100%) for the extracts made from the conventional culture cellular medium. Finally all results were expressed in viability percent.

In the XTT-test a curve graphic is expected which increases the cellular viability with the reduction of the concentration of the extract sample. As shown in figure 19, however, it was possible to notice that the corrosion solution itself had demonstrated an influence in the SDH activity of the L-929 fibroblasts, with an uncommon cell response. At the concentration of 150µl, the solution of lactic acid/NaCl promoted a cell depression by an order of 43%. The middle and low concentration of the corrosion solution presented no significant toxic effect.

This aspect of low cell viability in the higher eluate concentration was constant for all extracts and attributed to the corrosion solution and not to the function of the metal ion's presence. h this way, for a clear analysis of the results, it was determined to avoid the concentration of 150µl by the corrosion solution

extracts. Otherwise, as by the DMEM cell culture eluates such aspect was not relevant, all concentrations were considered.

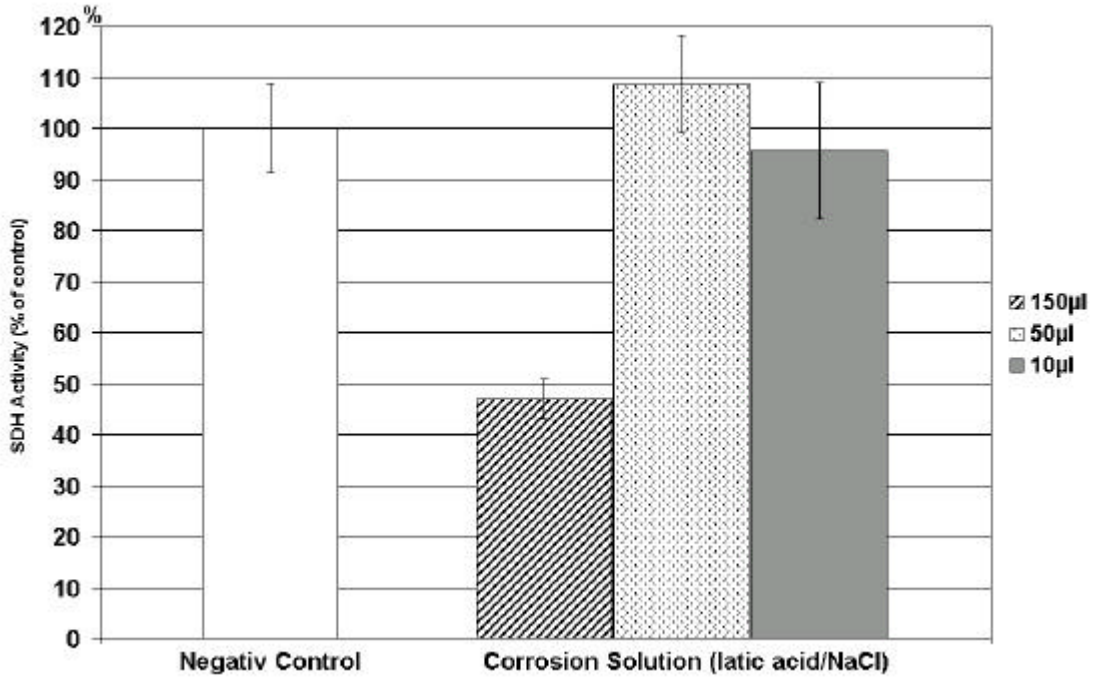


Figure 19: Cytotoxicity results of the corrosion solution extract (Lactic acid/NaCl) in three concentrations (1:0, 1:3 and 1:15), after 24h incubations time with L-929 fibroblasts. The mitochondrial activity (SDH) was measured and expressed as a percentage of the negative control (=100% of viability). Error bars represent standard deviations.

The results of the XTT-test from the corrosion-solution extracts are shown in figure 20. Signs of slight to no cytotoxicity were noticed with the extracts of #A1, #A2, #B1, #B2, #B3, #N1, #N2, #N3 alloys and pure *In* and *Sn*, with a cell viability variance between 86,6% (#A1 at a concentration of 1:15) and 104.7% (pure *Sn* in 1:15 of dilution) in comparison with the corrosion-solution group control. Alloys #A3, #B4, #B5 and #B6 showed toxic effects in the concentration of 1:3 (50µl) with clear increases in cell viability at the low eluate concentration. Cells exposed to the pure *Zn*, in all extract concentrations, presented low viability, with poor uptake of the XTT reagent when compared to the control.

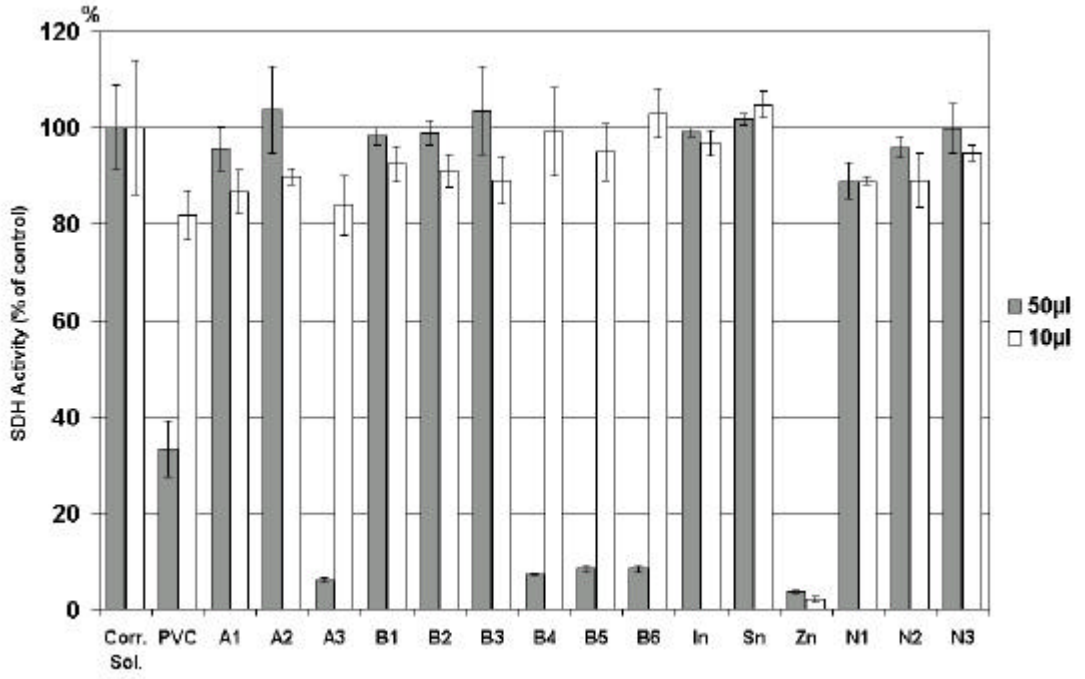


Figure 20: Cytotoxicity of alloys and metals extracts in corrosion solution milieu (lactic acid/NaCl), in two concentrations (1:3 and 1:15) after 24h incubations time with L-929 fibroblasts. The mitochondrial SDH activity was measured and expressed as a percentage of cell viability relative to the control (corrosion solution – lactic acid/NaCl) for the extracts of the samples in terms of time of the prior immersion test. The higher extract concentration (150µl) was not considered. Standard deviations were expressed by the errors bars.

The cytotoxicity of #A3, #B4, #B5 and #B6 alloy extracts were significantly greater than the control at the concentrations of 1:3, where the cell viability was decreased at means of 93.9%, 92.7%, 91.5% and 91.6% respectively of the corrosion-solution control. While at a dilution of 1:15 their eluates presented mild to no damage influence on cell proliferation. Moreover, extracts from pure Zinc showed continuous and significant toxicity over the experimental alloys and pure metals in all concentrations. The cytotoxicity of pure *Zn* increased from 96.3% of depression at 1:3 dilutions to 97.9% cell damage at 1:15 of concentration, compared with the control.

Student's t-Test ($p<0.05$) demonstrated that the difference in cytotoxicity of the #A1, #A2, #B1, #B2, #B3 and the base-metal alloys extracts, as well as eluates of pure Indium and Tin, were not significantly different ($p>0.05$) in cytotoxicity

from the corrosion-solution control at either extract concentration. Otherwise extracts from #A3, #B4, #B5 and #B6 alloys showed significant difference from the control group ($p<0.05$) at the concentration of 1:3. Meanwhile pure Zinc eluates were statistically different ($p<0.05$) from control in both 1:3 and 1:15 of solution dilution.

In the present work it was clear that the higher ion leakage occurred in the first day of immersion assay. To compare the substance loss after 1-day of immersion test of the heat-treated samples in corrosion solution and its influence on cell proliferation, the same standard test was performed in cell culture medium. The total mass loss results after 24h of immersion test in corrosion solution and in DMEM medium of the alloys of interest are noted in figure 21.

The selected alloys were the base-metal alloys, those who demonstrated cytotoxic effects in the XTT-test with corrosion extracts (alloys with Zinc compound) and pure *Zn*. The specimens exposed to the acid pH value (2.3) of the lactic acid/NaCl solution presented a higher ion release, which ranged between 226.6 µg/cm² and 89.2 µg/cm² for the high-noble alloys #B6 and #A3 respectively. Pure Zinc showed an ion release in order of the 3211.3 µg/cm²; while the *CoCr*-based alloys a mean value of 5.9 µg/cm² for #N1 alloy and 0.4 µg/cm² for both #N2 and #N3 alloys.

The total mass loss of the alloys and pure *Zn* was much lower in DMEM milieu. ICP-OES analysis showed that alloys with *Zn* content released only this element, with a variance between 4.3 µg/cm² for the #A3 alloy and 1.5 µg/cm² for the #B4 alloy. Moreover pure Zinc presented a mass loss up to 17.0 µg/cm² in cell culture medium. The elemental-ion leakage detected by ICP-OES of the alloys #N1, #N2 and #N3 was below the detection limit, and hence their release amounts were not reported.

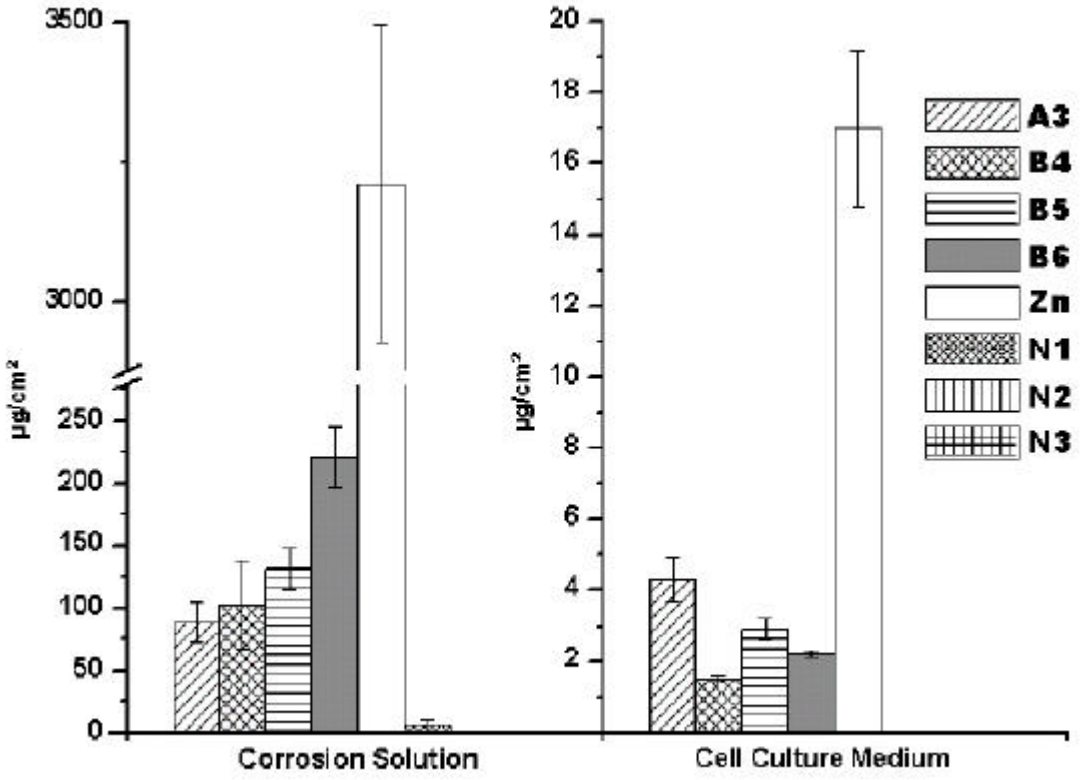


Figure 21: Comparison between the total substance loss after 1-day of immersion test of the heat-treated alloys #A3, #B4, #B5, #B6, #N1, #N2, #N3 and pure Zn; in two different corrosive media: lactic acid/NaCl (corrosion solution) with pH value 2.3 and DMEM (cell culture medium) with pH value 7.0.

The citotoxic effects of the extracts from DMEM cell culture medium are shown in figure 22. With these results it was possible to determine that there was considerable decrease in cytotoxicity of the extracts of pure *Zn* compared to the control and all other extracts for the 1:0 concentrations. Pure Zinc presented a cell-proliferation depression up to 43.8% at the higher concentration.

The alloys #A3, #B4, #B5 and #B6 showed slight toxic response at the concentration of 1:0. Cell depression ranged between 24.4% for the #A3 alloy to 0.4% for both #N2 and #N3 alloys. At the concentration of 1:3 and 1:15 alloys #A3, #B4, #B5 and #B6; and pure *Zn* noticed middle to no cytotoxicity effects between 18.7% for #A3 alloy and 5.8% for #A3 alloy of cell depression in comparison with the control whereas the *CoCr*-based alloy #N1 presented a

slight effect of 5.4% when compared to the control at all concentrations despite its low ion release. Alloys #N2 and #N3 showed no toxic effect.

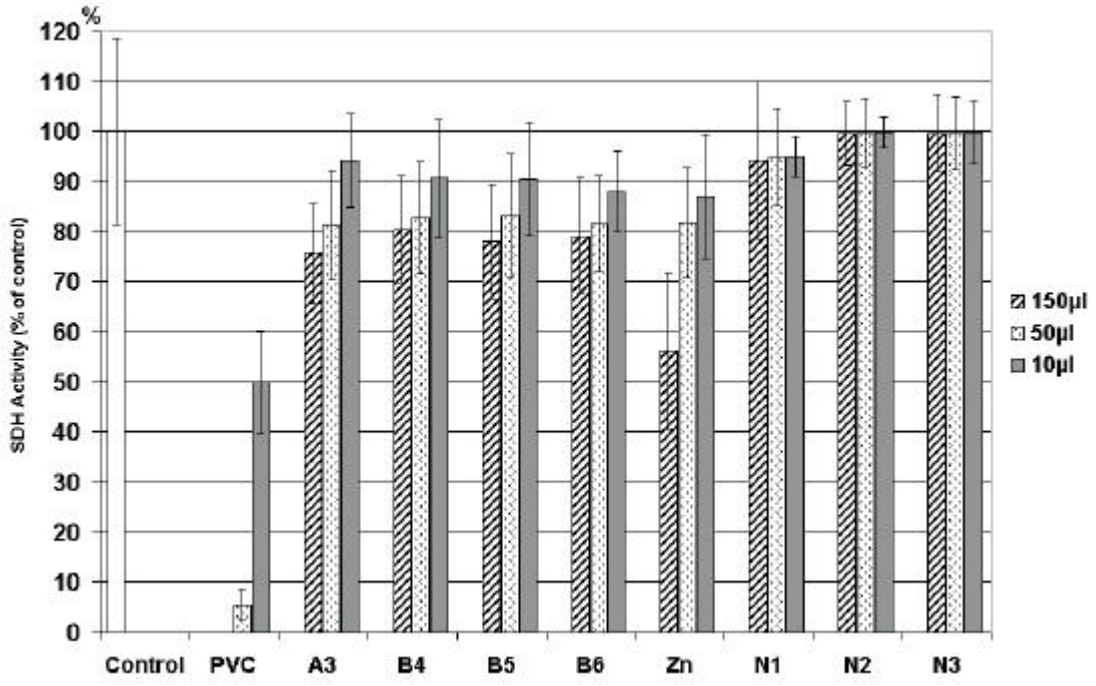
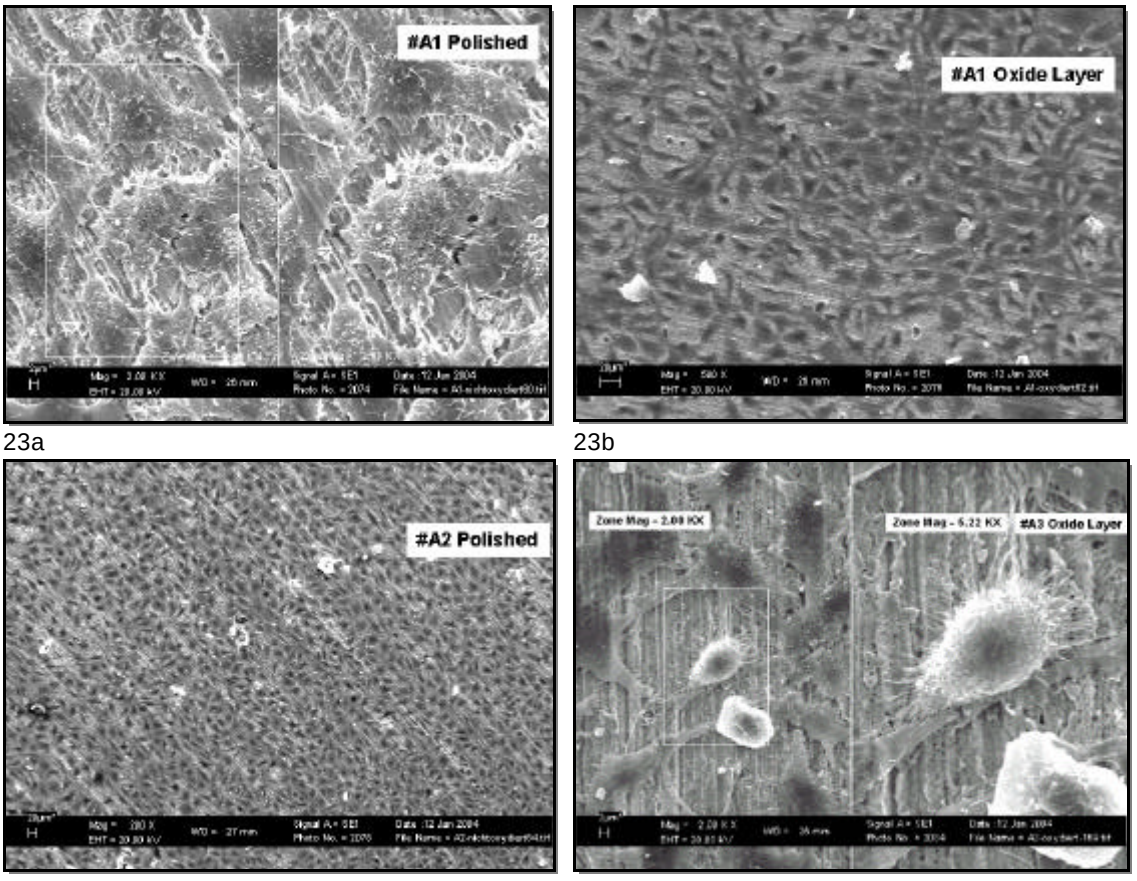


Figure 22: Cytotoxicity of alloys and metals extracts in DMEM conditioning media after 24h incubations time with L-929 fibroblasts. The mitochondrial SDH activity was measured and expressed as a percentage of the negative control (=100% of viability).

Student's t-Test ($p<0.05$) demonstrated that the base-metal alloys #N1, #N2 and #N3 showed no significant difference ($p>0.05$) at any extract concentration in comparison with the negative group. Alloy #A3 was statistically different ($p<0.05$) from control when its eluates were added to the cell culture in the concentration of 150µl and 50µl. All other alloys (#B4, #B5 and #B6) and pure *Zn* presented significant difference from control ($p<0.05$) in all extract concentrations.

SEM Analysis

There were no differences in the appearance of the control cells and the cells exposed to the testing alloys as observed by SEM. The cells on top of the negative control as well as on the surface of all specimens (figures 23 and 24) exhibited normal well-defined morphology with a textured surface. It should be noted that cells presented a satisfactory adhesion with alloys' surface. No change in size or disruption in their configuration was noticed.



23a 23b

23c 23d

Figure 23: SEM micrograph of some experimental alloys specimens after cell culture incubation. Cells presented no changes in size or membrane disruptions. a) alloy #A1 polished; b) alloy #A1 polished and oxidized; c) alloy #A2 polished; d) alloy #A3 heat-treated.

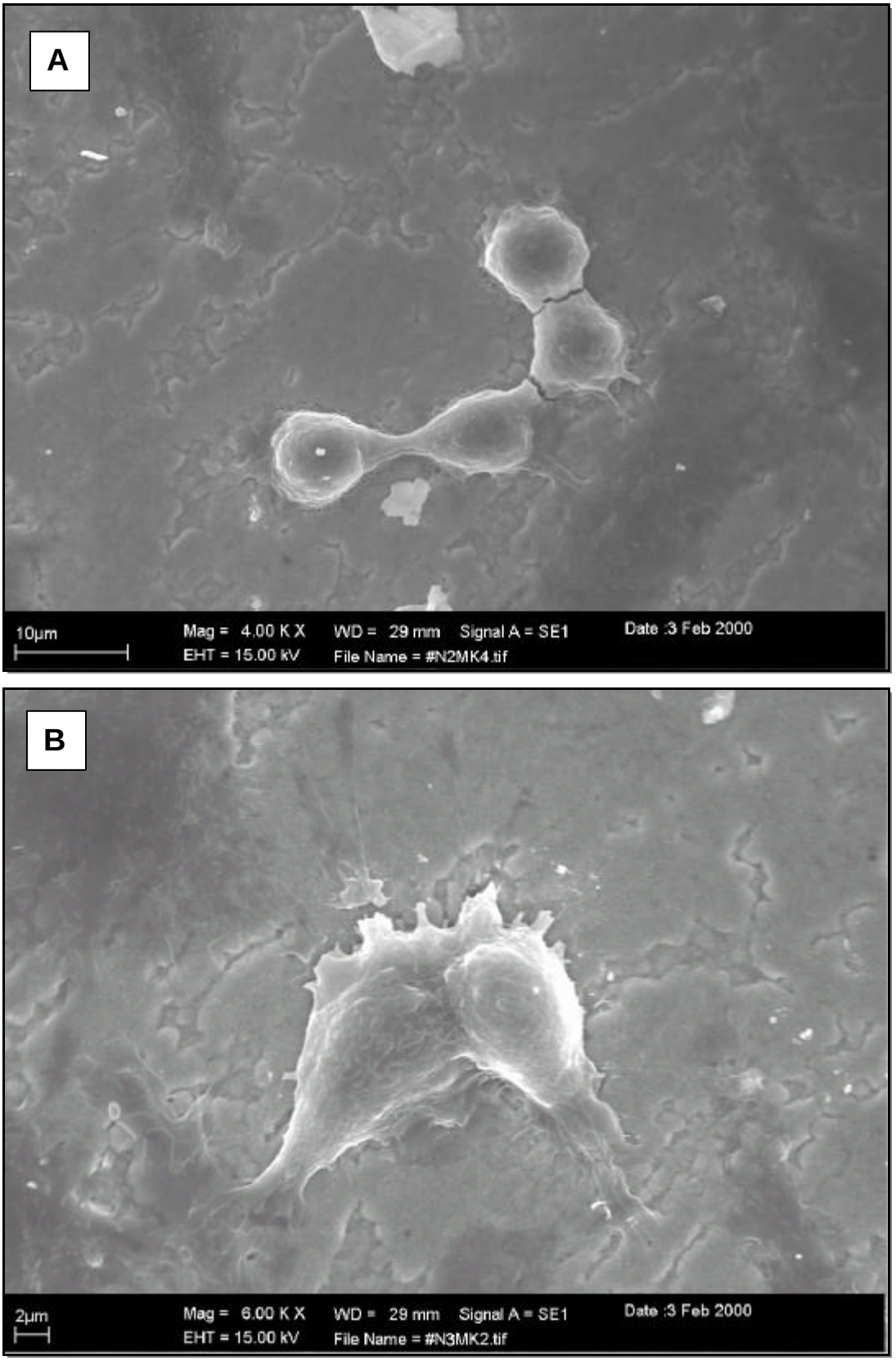


Figure 23: SEM micrograph of some experimental alloys specimens after cell culture incubation. A) alloy #N2 heat-treated; B) alloy #N3 polished and oxidized.

5. Discussion

The present work drew attention to the corrosion behaviour and the consequently biocompatibility of 12 experimental PFM alloys. The tested alloys consisted of two groups: high-noble alloys (9 variants) and *CoCr*-based alloys (3 types). Although they were experimental materials, these alloys were selected in order to represent the PFM alloys most often used in dentistry appliances, and to enable a comparison among the high-noble and base-metal alloys.

As specified in the ISO 10271 [52] and frequently described in the literature, an alloy has to be carefully polished before a corrosion test is carried out [37, 58, 93, 112]. For this study, however, polished samples as well as polished and oxidized specimens were used. Moreover, it was presumed that this condition would simulate a current clinical situation of no success by the finishing process, where some porcelain-uncovered parts of the prosthesis may contain some remains of the oxide layer [102, 104, 109]. Except for the pre-heating process, all the other aspects (cleaning, sterilization, dimensions, and the ratio between surface area of the specimens to the volume of the corrosion media) were in accordance with the ISO dental standard [52].

As related from previous investigations, portions of the appliances with an oxide-layer remaining may lead to a greater ion release under aggressive acid conditions [58, 102]. In this way, for a correct analysis and comparison of the corrosion assay findings, it's necessary to employ a pre-heating process before the test begins without removing the formed oxide layer on the sample surface. This procedure was described in earlier works [6, 102, 104], although some authors claim that such process is irrelevant in the final results [65].

The ion leakage from PFM alloys through the mouth, into the gingival and nearby tissues, is of high clinical relevance, and might be taken as an important oral heath-risk factor to the patient [4, 11, 71, 93, 119, 123, 124, 132]. In this

manner, it is known that the corrosion resistance of an alloy is the aspect of most influence on its biocompatibility property. The corrosion behaviour has a great importance in the alloy's biological response and its change as well [33, 48, 111, 119, 122].

The local toxicity of an alloy, but also its systemic toxic effects and carcinogenicity, depends on the elemental leakage into the mouth during the corrosion process [20, 119, 128]. The reactions occurring in the alloys and surrounding tissues are believed to be related to these ion releases. Therefore knowledge about these circumstances and the alloys' corrosion rates are essential to understand their biocompatibility [1, 11, 12, 97, 101, 102, 111].

5.1 Corrosion Behaviour

To access the corrosion behaviour of the experimental alloys, the mass loss test was utilized, through the long term assay up to 35-days of immersion. A long period of testing was selected over a short term one (seven days) because longer-term studies are required to better simulate "in vivo" conditions in which release is expected to be continued for extended periods [43, 104, 108, 112, 121, 122]. The advantage of this method, associated with its posterior evaluation, enables the amount determination of different types of metallic ions, which presents distinct-biological responses. It also makes possible an overview of the passivation tendency of an alloy, by the multiple condition media-elemental analysis [41, 58].

In order to simulate the aggressive situation presents in the mouth which occurs from eating and drinking, and due to the fact that corrosion levels are decided by the surroundings [31, 48, 59, 121, 125], a corrosion solution composed of 0.1 mol/l lactic acid and 0.1 mol/l sodium chloride with a low pH value (2.3) was selected [41, 58, 93, 103, 111, 119, 121]. Some previous studies have used different conditioning media like cell culture medium [102, 120, 121, 124, 125], 0.9% NaCl [56], artificial saliva [55], and distilled water [1]. The selection of

other corrosion milieu was based on the different goal of each study. In the present investigation it was intended to expose alloy samples at extreme cases of oral acidification, which happen under plaque films and in the crevices of restorations.

Metal-element levels in the analytic tests of the experimental PFM alloys were analyzed by ICP-OES. This method had been used in previous studies [46, 109]. The results obtained through ICP-OES analyses in µg/cm² were related with time in days. It enabled the achievement of an accurate view of the time dependant corrosion process, as well as the alloy ion release average throughout the test corrosion period [47, 111].

The results of ICP-OES showed that the alloys released more ions in the first day of immersion, with the greater range for alloys with *Zn* content. After four days of assay high-noble alloys with *In* and base-metal alloys without *Ce* content demonstrated a continuous increase in ion release without sign of passivation. Literature data mentions concerns of initial higher ion release from alloys [103, 111, 124]. Furthermore, as reported in previous investigations, the metallic ion leakage was not in proportion to its presence in the alloy composition [20, 48, 101, 119, 120, 121].

As described in the literature the satisfactory corrosion behaviour of a material is indicated by an element-release curve that drops in time, with representative decrease in elemental leakage over the curve. It means a fast and stable passivation. On the other hand, alloys more susceptible to corrosion build up a representative curve that demonstrates a non-decrease in the substance loss with time [24, 41, 43, 47, 58].

The substance loss occurring in the initial stage with posterior passivation happened most probably due to the selective dissolution of non-precious metal components [43, 58]. Such relation is also to be noted in the mouth, where the

elemental ion release is great at first, drops with passivation, and remains constant over time [24].

The surface state (with or without oxide layer) is also an important aspect of influence in the corrosion susceptibility of an alloy, as well as the polishing and finishing processes [6, 56, 58, 101, 102]. SCHMALZ et al, 1998 [102], studied the ion release of polished and polished and oxidized noble alloys with *Zn* content. Their findings showed that Zinc leakage was increased by a factor of 2 to 3.5 after oxidation process, due to the fact that their samples were immersed in cell culture medium, with less aggressive characteristics. In the present study this proportion was greater for the *Zn* content alloys once the pH value of the corrosion solution was 2.3.

The variation of total mass loss over the period assay among the polished and polished and oxidized surfaces for the *Zn* content alloys was 6.5 times greater for the #A3, while for the #B6 this difference was increased up to 476.8 times. These results are in agreement with previous studies that stated the finishing of the alloy surface influences better corrosion behaviour. This means that alloys with oxide layers from pre-heating treatments release considerably more ions than the polished alloys [6, 58, 104, 108, 109]. However, by the #A2 and #B2 alloys the difference between the two surface conditions was not significant.

High-Noble Alloys

As discussed before, the correct surface polishing is set by the ISO 10271 for the corrosion behaviour test through mass loss assay [52]. The present investigation demonstrated that samples wet finished with a series of SiC papers until #1200 grit showed low total substance loss after 35-days of immersion test. Polished samples were enabled to build up an ion-release curve, which reached a characteristic passivation in few days. Such corrosion behaviour of high-noble and noble alloys with Indium, Tin and Zinc in the polishing surface state correspond well with previous studies [47, 58, 127].

In the present work, the heat treated high-noble alloys with Zinc showed the great release of non-noble elements, mainly of *Zn* itself. The noble components of the alloys were detected in small traces in the analyzed corrosion solution or were under the detection limit. These results are in accordance with the literature [6, 48, 58, 63, 101, 102, 120]. However some other studies made note of low *Zn* ion release [1, 123, 124]. The difference suggests that the ISO-corrosion test solution used in the present study (an aqueous lactic acid/NaCl corrosion solution with an acid pH value) enabled the greater release of Zinc.

Moreover what also plays a significant role is the lability of each element. Such property is intrinsic to each metal, but when this element is alloying with another one, the specific lability depends on the total alloy composition, due to the interaction that happens among their compounds [1, 68, 84, 119, 120, 121].

These tests were aimed at assessing the corrosion behaviour of experimental alloys in situations that simulate real oral conditions where the mouth milieu is subjected to numerous influences [41, 58, 93, 103, 119, 127]. Such conditions may lead both to a short-term pH change, through intake of acid beverages (pH 2-3) or secretion of gastric acid (pH 1), and to a long-term pH variance which occurs under plaque films (pH 4-4.5) or in the crevices of restorations (pH 1.5-4.5) [41].

Nevertheless the findings of previous reports agreed well with the findings of the present study, where the highest ion release means happened in the first day of the immersion regardless of which conditioning media had been used [41, 43, 58, 93, 104, 122, 123, 124, 126, 127]. The posterior ion release decrease is also well documented and in agreement with earlier investigations which carried out the subsequent immersion corrosion test and replacement in freshly made corrosion solution [58, 104, 111, 121, 122, 123, 126].

High amounts of Zinc were released from oxidized alloys #A3, #B4, #B5 and #B6, at least on the first day. The *Zn* leakage from these alloys was most

probably caused by the type of selected corrosion media and due to the high lability of this element, resulting in greater release as shown by the pure Zinc corrosion behaviour [84]. This means Zinc tends to be easily released. In the currently investigation, this fact is easily noted by the comparison between the results of the tested alloys and the pure metals. Whereas the experimental alloys presented a high metal release in the first day with a relative decrease in the following days, the pure metals showed a constant increase in the ion leakage over the assay period greater than 100 times for pure *Zn* and *Sn*, with no regards of passivation.

On the other hand, the experimental alloys with *Sn* as oxide-forming element presented an overall metallic ion release at levels too low to be relevant. Similar results of slight Tin release from high-noble and noble alloys were also found in previous investigations [58, 109, 112, 125].

After the first day it was noticed that the mean range of released *Zn* ion of the respective alloys decreased at low levels, while alloys with *In* showed an increase in mass loss with time. The increase in the release of the element Indium from the corresponding high-noble alloys was also noted by the pure *In* mass loss property. After 7-days of immersion test the rates of substance loss of the samples within *In* were caused by this element, with no signs of decrease over the assay period. Such aspect was not in agreement with the literature, which affirms that generally Indium does not dissolve into the medium [120, 125]. It's believed that this behaviour was most probably due to the corrosion solution employed by these previous works, a cell culture medium with a pH value of 7.

Base-Metal Alloys

Earlier corrosion studies reported that ion leakage has a straight relationship to alloy noble degree, which means that noble alloys and in specially base-metal alloys may release more elements than traditional high-noble alloys [30, 37, 48,

55, 59, 76, 95, 119, 123, 124]. However, the present work showed that base-metal alloys with *Ce* content were less susceptible to corrosion than high-noble alloys alloying with *In* and *Zn*.

The experimental base-metal alloys also demonstrated difference in total substance loss depending on their surface state (with or without oxide layer). Moreover the experimental alloy #N1 (without Cerium) exhibited considerably higher mass loss after the simulated porcelain-firing process in comparison with the polished samples and with the experimental alloys #N2 (0.19wt% *Ce*) and #N3 (0.29wt% *Ce*). However, results from previous investigations found that non-oxidized *CoCr*-based alloys released more ions than after exposure to a ceramic-firing process [6].

Cobalt was reported to provoke corrosion and to produce products that dissolve in fluoride solutions [59]. A better corrosion resistance of polished *CoCr*-based alloys, with low Cobalt release, was corroborated after analysis of the ion release behaviour in many solutions (alpha-medium, PBS[-], calf serum, 0.9% NaCl, artificial saliva, 1-2 mass% L-cysteine, 1 mass % lactic acid and 0.01% mass HCl) for 7-days by OKAZAKI & GOTOH, 2005 [89]. A satisfactory corrosion resistance of *CoCr*-based alloys with comparable results to noble alloys was also reported in recent investigations [115].

The results of total mass loss of the *CoCr*-based alloys obtained in the present investigation are in agreement with previous studies. Literature data also demonstrates that Cobalt-metallic ions are preferentially released from such alloys in the polished surface state during immersion tests, where *Co* is the main element of the tested alloys. As the results evaluation of the experimental base-metal alloy #N1, Cobalt was the most preferred labile element; followed by Chrome and Molybdenum [43, 93, 108]. Non-oxidized *CoCr*-based alloys were reported in previous studies to be corrosion resistant after immersion test in modified Fusayama artificial saliva, in which was added rhodanide (KSCN) [44].

In the 35-days immersion test a tendency of continuous Cobalt release from the experimental heat-treated #N1 alloy with no regard of passivating was noted. These findings were in agreement with earlier investigations, which corroborated the constant *Co* ion leakage [63]. Meanwhile, literature data made note of a decrease in the ion leakage of Cobalt alloys with a reach of passivating after 20-weeks of immersion assay [108]. Nevertheless, no significant difference was noticed between specimens of *CoCr*-based alloys with Cerium content (#N2 and #N3) in both surface conditions.

Results data of mass loss from experimental alloys #N2 and #N3 showed low ion leakage over the test period with satisfactory passivation. A slight increase in elemental release was noticed in the polished and oxidized samples than in the polished specimens, without statistical significance. In this way, the Rare Earth element Cerium alloyed to these alloys may play an interesting role on their corrosion resistance. Literature data made also concern on the decrease in the ion release from polished *CoCr*-based alloys with *Ce* content [43, 47, 93].

Previous investigations demonstrated that polished *CoCr*-based alloys with *Ce* content between 0.15wt% to 0.4wt% presented a low ion release (0.43µg/cm² and 0.9µg/cm² respectively), while those alloys without Cerium showed a total mass loss up to 13µg/cm² and 34.9µg/cm², mainly of Cobalt element [43]. Further studies showed that non-oxidized *CoCr*-based alloys with 0.5wt% of *Ce* presented an increase in the ion release after 7-days of immersion test in distilled water with a total mass loss of 358 ppb [1]. While in a corrosion solution of 0.1 mol/l lactic acid and 0.1 mol/l sodium chloride (pH value 2.3), a total ion release up to 3.9 µg/cm² was noted [107]. Moreover it was demonstrated in an early long-term mass loss assay a first Cobalt ion leakage of 2.1 µg/cm² from *CoCr*-based alloys with Cerium compound, with further decrease and reach of passivating characteristic curve after 5-days of immersion [93].

Earlier comparison between the substance loss of polished high-noble alloys and *CoCr*-based alloys with 0.5wt% *Ce* content corroborated no difference, and

showed that both were corrosion resistant through their lower ion release [107]. Previous investigations of heat-treated *CoCr*-based alloys with 0.15wt% *Ce* content also demonstrated satisfactory and better corrosion-behaviour results compared to polished Cobalt alloys without Cerium [108]. The findings of these previous studies are in accordance with the current one.

In the present study, no significant difference among the alloys #N2 and #N3 was corroborated. Furthermore, their ion leakage amounts were comparable to the high-noble alloys with Tin content. It is to suppose that a Cerium concentration of 0.19wt% (experimental alloy #N2) is enough to increase the corrosion resistance of *CoCr*-based alloys to satisfactory levels. Although these differences, the oxidized experimental base-metal alloys with Cerium reached a satisfactory passivation in the ion release curve after 7 or 14-days.

These experimental *CoCr*-based alloys and other alloys with similar compositions have to be cast under vacuum processes. Such procedures also contribute to achieve a material more homogeneous with less final porosity. All these aspects are important and play a significant role on the corrosion behaviour and biocompatibility of base-metal alloys [43].

SEM micrographs and EDX analyses of the experimental alloys' surfaces confirmed the presence of an oxide layer after the simulated porcelain-firing procedure. In the high-noble alloys, as expected, a higher concentration of oxide-forming elements was present. In the base-metal alloys the concentration of *Cr* was noted, since this element is the main one responsible for the chemistry adherence between the metal and the porcelain. Concentrations of Indium, Tin, Zinc and Chrome oxide on heat-treated alloys' surfaces were previously described in the literature [28, 67, 72, 87, 88].

5.2 Biocompatibility

In the present work the effects of a variety of experimental PFM alloys on L-929 fibroblasts were investigated with the standardized test system recommended by the ISO 10993-5 [53]. Other authors have used other cell cultures, such as: 3T6 mouse embryo fibroblasts [10], Balb/c 3T3 fibroblasts [2, 34, 35, 84, 124], human gingival fibroblasts [12, 18, 21, 99], human tissue mast cells [99] or human fibroblasts [24]. The selection for working with L-929 fibroblasts was due to their relative ease in handling and because of the higher experience of reproducibility with these cells [7, 27, 64, 77, 96, 97, 99, 100, 102, 109].

Direct Contact Tests

The cellular-membrane integrity, as well as its support, is an aspect of high relevance on cell viability. Bioluminescence assays provide a rapid method for the determination of the mitochondrial activity levels in cells [18]. Previous studies using the same staining method showed a satisfactory correlation between mitochondrial activity and cell viability [64, 85]. Moreover it was also correlated that mitochondrial depression preceded decreases in viability, and in changes in morphology and release of intracellular organelle [24, 64].

Addition of non-noble components like *In*, *Sn* or *Zn* to PFM alloys enhances the adhesion strength of the porcelain facing. Nevertheless, it also leads to a significantly increased corrosion rate and liberation of alloy components from the oxide layer formed by the firing process. This raises concern because it may promote toxic effects in the surrounding tissues [4, 11, 12, 35, 101, 102, 119, 131]. In the corrosion tests shown before, at acid pH values (2.3), ion dissolution after simulated porcelain firing was found to be greater. However, no significant toxic effects caused by this increased ion leakage could be detected. Such condition may be the result of the differences among the condition media. The direct contact was carried out in a neutral pH value, which may lead to a low ion release [9, 59, 103].

Overall the direct contact between the samples and the monolayers corroborated no significant decrease in the cell number as well as the cell growth area in comparison with the Titanium control group. In this study, the slightly better biocompatibility occurred in the polished high-noble alloys was attributed to their better stability. However, the small decrease in this biocompatibility when the specimens are pre heated can be explained by the elemental release from the alloy surface. Although such difference was too small to be considered significant, this might happen through the elemental leakage from the oxide-forming elements (*In*, *Sn* and *Zn*) by the medium [124].

The potential toxicity of the *Zn*-compound, which was added in concentrations up to 1wt% to the experimental alloy #A3; and up to 2wt% to the alloys #B4, #B5 and #B6, was demonstrated by the virtually total lethality of the pure metal for the fibroblasts. Cytotoxicity of *Zn*-containing dental alloys has been also described in the literature [35, 61, 68, 126]. However, the absence of cytotoxicity shown by the alloys indicates that the amount of Zinc released into the medium in the direct contact test was too low to impede viability of the cells. Similar results were also reported by other authors [1].

The results of the light-microscopy evaluations indicated that the polished and oxidized samples showed a slight depression in cell number on the alloy surface in comparison with the polished specimens. Literature data concerning the biocompatibility of PFM alloys containing *In* and *Sn* are also consistent with the results of the current study [12, 34, 35], whereas Indium has been described as cytotoxic [99]. Moreover the absence of cytotoxicity of Tin is also in agreement with previous works [99]. In accordance with previous investigations, these base-metal elements (Indium and Tin) promote an adherent oxide layer, whose passivation contributes to the biocompatibility of the alloy [34, 35].

The absence of alterations in cellular morphology observed by SEM analysis is in accordance with the direct contact results, in which depressions on cell viability by metal ion release after 24h incubation were not large enough to

promote alterations in cell morphology. However, previous investigations demonstrated the incorporation of metal ions by cells with resulting alterations on cell ultra structure may appear more well-defined after long-term assay periods [49, 124].

On the other hand, polished base-metal alloys were also compatible; while the ranking to *CoCr*-based alloys with oxide layer in spite of their small decrease remained in the normal range. The small or non-existent difference among the experimental base-metal alloys could be attributed to a possible non-stable oxide layer [35, 124]. When this occurs some Co^{2+} should be leached and may lead to a depression in cell growth due to its direct inhibitory effect on basic cellular metabolism [10, 24, 119]. Moreover the ion leakage by the cell culture medium from polished and oxidized #N1 (without *Ce*) alloy was probably too low to promote any significant decrease in cell viability.

Indeed *CoCr*-based alloys proved to have excellent biocompatibility in the present work after direct contact assay in both surface states (with and without oxide layer). Although different results were described by BERSTEIN et al, 1992 [12], who noticed that specimens of *CoCr*-based alloys had inhibited completely the growth of primary human fibroblasts, maybe due to the presence of Nickel and Beryllium elements. In “in vivo” assays through subcutaneous implantation of polishing samples in mice, KANSU & AYDIN, 1996 [57], found that *CoCr*-based alloys caused severe response with the most rigorous tissue reaction due to the non-favourable biological effects of Cobalt and Chrome compounds. In addition, it was affirmed that such metallic ions may promote hypersensitivity reactions with a broad range of clinical manifestations [49].

Metal-based alloys containing *Ce*, as well as Cerium as pure metal are described in the literature as cytotoxic materials [1, 90]. These findings are not in agreement with the present investigation. Can et al, 2004 [24], also studied commercial *CoCr*-based alloys within Cerium in a proportion ranging between 0.3wt% and 1wt%. They noticed that these alloys did not promote great

alterations in the cell morphology, with best results in the samples alloyed with 0.3wt% of *Ce*. They tested polished and sandblasted samples (with 50µm Al_2O_3). They found mild to moderate degradation of actin-based filaments of human gingival fibroblasts. These results were indicative of a progressive cell depression over time and were mostly motivated due to the Co^{2+} ion release by the samples. They concluded that a polished surface state was the most favourable condition.

Sandblast surface state of metallic-based alloys was reported by other authors as more toxic, by the promotion of cell morphology alterations compared to polished samples [34, 35]. In the present study, *CoCr*-based alloys with up to 0.19wt% and 0.29wt% of *Ce* were also reported to present no inhibition effect with no significant difference between #N2 (0.19wt% of *Ce*) and #N3 (0.39wt% of *Ce*) alloys. These results are in agreement with previous works that corroborated the non-cytotoxicity of Cerium element by the direct contact to the oral mucosa of mice in the proportion of 0.2% and 1% [66] and after muscle implantation as well [110].

It is known that some metal cations are the main cause of diverse effects or inhibitions on cell metabolism [99]. Nevertheless the small decrease between the alloy groups observed in the present work was not statistically significant in the direct contact test. This can be explained most probably due to the neutral pH value of the cell culture medium, which promoted a low ion leakage. Thus, released ion levels would to be far below the necessary amounts to induce some cell response. In addition the stable protective layer and consequently its passivation on the alloys surface also contributed to the biological rank shown by the experimental high-noble and base-metal alloys after the direct contact assay.

XTT-Tests

In this study the use of the corrosion solution from the analytical assay was selected due to its real metallic ions content. In some critical situations where a material, in this case a PFM alloy, may be exposed to an acid pH value, alterations in its surface composition are able to occur and its damaged passivating layer may lead to an enhancement in the corrosion rates. Such circumstances may be noticed in marginal crevices, in pits or under plaque, and where the pH can reach acid values due to higher hydrogen ion concentration [11, 33, 119, 132], as well as on account of mechanical variations and forces, *e.g*. chewing or abrasion [2, 17, 48], where the released metallic ions may cause some adverse biological effects in the surrounding [33, 48].

In this way, the findings of the present investigation enabled the relationship between the possible cellular damage that these alloys could have promoted and the cellular damage to the released elements from the experimental oxidized alloys in the corrosion solution from the immersion test. As the higher ion release from the alloys happened in the first hours, in this investigation the extracts of corrosion solution after 1-day of immersion test was selected [104, 124, 126].

As a general rule a conventional cell culture medium is used to make extracts for such a biological assay based upon its neutral pH value. The addition of alkali metal ions in order to neutralize the acid pH of corrosion solution may lead to a dilution of the ions in the corrosion solution, and promote precipitations of the metallic ions of interest as well as adverse cell response [48, 99, 102, 124]. In the present study, however, the unfavourable acid pH value from the corrosion solution was neutralized by mixing with HEPES puffer and sodium hydroxide (the “neutralization mix”). Furthermore this procedure presented no problems in the present investigation, due the small substance amounts necessary to neutralize the corrosion solution. No ion precipitation was noticed, and the metallic ion concentration was verified by ICP-OES analysis of the

neutralized corrosion solution. The results were compared with the previous findings by the mass loss assay of the tested eluate and no statistical significance was noted. In addition the cell response results were in accordance with the XTT-test standards. The influence of the extract in two concentrations (1:3; 1:15) could be distinguished.

Other authors have used another art of assay to determine the cytotoxicity in terms of depression on cell metabolism, such as the MTT-test [1, 83, 97, 102, 109, 124, 126]. In the present study the XTT assay method to access the cell SDH activity was selected, due to the simplicity and time saving factors of this technique in comparison to other cell analysis procedures [22, 26, 54, 69, 71, 107].

In the elution of the corrosion solution, products released from Zinc-content alloys (#A3, #B4, #B5 and #B6) were in amounts capable of inhibiting the SDH activity, and cause decreases in both cellular capability and proliferation, with a cell depression about of 93% of the control at the concentration of 1:3. However, it was at much lower levels than those of individual metal salt solutions of pure Zinc required to inhibit similar cellular response, whose amounts exhibited cell damage in all extracts concentration.

Statistical analysis of the achieved data revealed that experimental alloys with *In* and *Sn* (#A1, #A2, #B1, #B2 and #B3), *CoCr*-based alloys and pure Indium and Tin showed no significant difference with the corrosion solution media control at any concentration. These data are not in agreement with previous investigations, which showed the decrease viabilities and alterations in morphology and ultra structure of cells exposed to *CoCr*-based alloys, due to the releasing of Cobalt ions [24, 57, 61]. The non-toxic effect of alloys with Indium and Tin elements in non-specific tests through extracts was corroborated in earlier investigations [22, 83].

The greater cytotoxicity of the eluates from oxidized Zinc content alloys was most probably due to the presence of high amounts of *Zn* in the extracts. Zinc as pure element promoted an inhibition of over 96% of the corrosion solution control in L-929 fibroblasts cell culture. This element was reported in previous works on present toxic effects. This effect is believed to be caused by its ionic form, and could be an explanation of the appearance of gingivitis or discoloration observed in some cases near to PFM works made with high-noble and noble alloys [61, 63, 101, 124].

It was also reported that pure *Zn* and alloys with this element inhibited the metabolic activities of Balb/c 3T3 cells and cortical cell cultures, with a settlement of dependent cytotoxicity and inhibition of cellular proliferation [68, 126]. In the current study, the extracts of the alloys within Zinc were found to cause a cytotoxic effect and reduce the succinic dehydrogenase activity to about 93% of the control. It was corroborated that concentrations of *Zn* ranged between 88.1 µg/cm² and 205.6 µg/cm² in the eluates from oxidized #A3 and #B6 respectively, after 1-day of immersion, and were enough to inhibit the cell viability.

High-noble and noble alloys are more biocompatible than base-metal alloys [34, 35, 57, 124]. However, this depends on the composition of the alloys, the release behaviour of each individual element, its amounts, and the individual toxic effect capability [10, 61, 97, 119, 120]. As reported in previous works, noble alloys are able to promote cell damage [63]. In the present investigation it was clear that heat-treated high-noble alloys with Zinc element induced a cell depression in the concentration of 1:3, and were as toxic as pure *Zn* in the same concentration.

In spite of the base-metal alloys satisfactory results, other investigations have reported that a concentration of 7.5 µg/ml of *Co* is able to promote mild alterations on the cell structures, but doesn't lead to sever cytotoxicity effects. These works also noticed that the effects of *Co* on cell growth appear fast and

are depending on the metal concentration [10]. SCHEDLE et al, 1995 [99], also made note that the concentration of Co^{2+} ions had a straight relationship with the cell depression and inhibited the spontaneous incorporation of H-thymidine as well (due to toxic cell damage – necrosis).

Reduction in the succinic dehydrogenase activity up to 50% is described in the literature for Co^{2+} release to be about 6.2 ppm [97]. Meanwhile the degree of growth inhibition for *CoCr*-based alloys is dependent upon the concentration of corrosive Cobalt ions in the eluate. In the present study, experimental *CoCr*-based alloys, which extracts presented concentrations of Cobalt means of 3.9 µg/cm², 0.2 µg/cm² and 0.1 µg/cm² for the heat-treated alloys #N1, #N2 and #N3 respectively, were found to be not enough to cause a cytotoxic effect and reduce the SDH activity, which was about 11%, 4% and 0.2% respectively in the concentration of 1:3. These alloys were able to build up a resistant surface layer, which was not disrupted after the immersion test despite the acid pH exposure.

No inhibitory processes of metals solutions of Co^{2+} and Ce^{3+} was also described in the literature [6]. The element Cerium was supposed to enhance the corrosion resistance of base-metal alloys due to low ion release, and this fact is extremely favourable to the biological aspect. The present results are in agreement with previous studies that compared the toxic effects of Titanium-based alloys with 0.3wt% Cerium and without *Ce*. After analysis it was concluded that *Ce* was innocuous in the concentration of 0.3wt% in the alloy [133]. The concentration of *Ce* up to 0.39wt% in the experimental alloy #N3 showed also no influence on the cellular vicinity, with no significant difference with the experimental alloy #N2 (0.19wt% of Cerium).

After the assessment of the results of the XTT-test of extracts from the corrosion solution and its comparison with the findings from the direct contact test, some differences were noticed. In contrast to the direct contact cell assay, Zinc-content alloys (#A3, #B4, #B5 and #B6) caused a decrease in the SDH

activity in XTT-test at much reduced metal ion concentrations, at least in the concentration of 1:3 as compared to the controls.

Additionally, with the higher concentration of released metallic ions in the acid corrosion media, a synergistic effect of the element Zinc with the other elements of the alloy as well as with the compounds of the cell culture media may be the reason for the non-cytotoxic effect noticed by the direct contact assay [119]. Differences in biological results between direct contact test and elution assay is described in the literature, with the proposition that adverse changes in DNA synthesis may be able to occur at much lower ion concentration than changes in morphology and viability [21].

In this way, it was determined to test the experimental alloys, which showed satisfactory biological effect in the direct contact assay, but presented toxicity in the extracts from corrosion solution in other elution in conventional cell culture medium. This group was compounded of those Zinc content high-noble alloys (#A3, #B4, #B5 and #B6) and pure Zinc. In addition, the biological response of the base-metal alloys was also verified.

Otherwise, alloys that demonstrated a cell depression in the eluates from corrosion solution, exhibited slight to no toxic effect after extraction in a neutral pH value. For these alloys, most with *Zn* content, the amounts of released Zinc were higher in the acid milieu than in the cell culture medium. These metallic ion levels were supposedly the main reason for the citotoxic effects evidenced by these alloys. These results correspond to previous investigations in which metal ion release in acid pH medium were greater than in cell culture milieu or another conditioning media with a pH value of 7 [1, 102, 109].

It was corroborated that a decrease on the amounts of ion release after immersion test in cell culture medium presented a factor variation between 21 and 189 considering the tested alloys, the elements of interest and pure Zinc. Literature data also noted differences in the amounts of elemental leakage in

two different corrosion media with a pH value 2.3 and a pH value of 7, with a variance factor up to 10 and 300 by the studied alloys [109].

After ICP-OES analyses of the extracts from the oxidized high-noble alloys in cell culture medium, it was noticed that only Zinc element was present. The noble compounds and the oxide-forming elements Indium and Tin were not present in the solution, or their amounts were under the detection limit. The preference of Zinc release and the lack of detectable levels of Indium and noble elements into the cell culture medium are also reported in the literature [120, 122, 123].

In the DMEM extracts of alloys with *Zn* element reductions in the intracellular SDH activity as compared to the negative control were also noticed. However, these depressions, which were up to 24.4% of controls for #A3 (1wt% *Zn*) at the higher concentration, were not in the same proportion as the eluates made with the corrosion solution (93.9%). The amounts of Zinc ion release from oxidized #A3 samples in cell culture medium was 4.3 µg/cm², far bellow its level in the heat-treated sample in corrosion solution media, up to 88.1 µg/cm². Experimental alloys #B4, #B5 and #B6 showed less Zinc ion leakage (1.5 µg/cm², 2.9 µg/cm² and 2.2 µg/cm² respectively) and therefore low cell depression. Otherwise cytotoxic effects of extracts from oxidized dental alloys with *Zn* content in cell culture medium were demonstrated in previous investigations, where Zinc releasing ranged between 3.3 µg/cm² and 3.7 µg/cm² after three days of immersion test and promoted over 50% of cell depression [102].

The mitochondrial activity of Balb/c 3T3 in extracts of high-noble, noble and based-metal alloys after immersion test in cell culture medium for 6, 24, 48, 72 e 96h was carried out by WATAHA et al, 1995 [124]. Their findings demonstrated that the depression of the SDH activity may be correlated with the release of Copper and Zinc from the alloys. In the current investigation the toxic effect of pure Zinc at higher concentrations in the corrosion solution extracts

became slight after elution in cell culture medium. The concentration of 17 µg/cm² found by the ICP-OES analysis from the pure *Zn* extract in DMEM was sufficient to inhibit the cell proliferation with a depression of 43.8% at the dilution of 1:0. At other dilutions the cell depression was not significant. BUMGARDNER et al, 2002 [22], corroborated that DMEM extracts of alloys with Zn (0.6 to 2.0wt%), In (0.3 to 8.5wt%) and Sn (5.0wt%) after seven days of immersion showed no cytotoxic response either in primary human gingival fibroblasts or in 3T3 mouse fibroblast cell line cultures after XTT-test.

The findings of the present work about the cell metabolic activity from extracts in cell culture medium of *CoCr*-based alloys with *Ce* are in agreement with previous investigations [107]. The tested base-metal alloys showed no significant cell depression in comparison to the control. In addition the ICP-OES analysis of the conditioning media showed that the ion concentration of the released elements in DMEM were under the detection limit and therefore were not identified.

6. Conclusions

This “in vitro” study investigated the elemental release from experimental porcelain fused to metal alloys into a corrosion solution of 0.1 mol/l lactic acid and 0.1 mol/l NaCl, with a pH value of 2.3; and their cytotoxic effects throughout a direct contact assay and through extracts made of these corrosion solutions as well as eluates of these alloys in normal cell culture medium. Under the limits and conditions of this work, the following conclusions have been drawn:

Generally polished surfaces samples showed a lower ion release than the polished and oxidized surfaces specimens. The higher ion leakage was noticed in the first day of immersion.

The experimental heat-treated high-noble alloys with Zinc compound showed the highest mass loss levels, mainly of Zinc itself, at least after one day of immersion test. The experimental alloys within Tin (#A2 and #B2) demonstrated satisfactory corrosion behaviour and along with the Zinc content alloys reached a relatively passivating characteristic after 7 to 14-days of experiment. Alloys with Indium evidenced a constant and progressive ion release with no regard of passivating over time.

By the direct contact biological assay, and within the limitations of this test system, all tested alloys showed no toxic effect. Moreover high-noble alloys with Indium and Tin were also biocompatible even after elution in an acid media. In contrast, Zinc content alloys extracts with higher amounts of release ions promoted several cell depressions in lower dilutions. Otherwise, alloys in corrosion solution extracts with toxic effects presented none to slight effects after elution in cell culture medium.

After the mass loss test, the *CoCr*-based alloys demonstrated satisfactory corrosion behaviour with low ion leakage. Both #N2 and #N3 alloys (Cerium content) showed results comparable to the best of the experimental high-noble

alloys. Together with the high-noble alloys with Tin (#A2 and #B2), these alloys were capable of reaching a passivating with a resistant surface layer even at acid pH values. The addition of up to 0.19wt% *Ce* to the *CoCr*-based PFM alloy (#N2) decreased the corrosion rate, whereas the 0.39wt% *Ce* content alloy (#N3) showed no statistically significant further effect.

CoCr-based alloys and their corrosion products demonstrated no significant toxic effect, as measured by the biological assays, without affecting viability or morphology of the cell cultures. There was a slight enhancement in the cell response to base-metal samples alloying with Cerium. This element appears to play an important role in the corrosion and in the biocompatibility property of such alloys. These alloys presented no difference of biocompatibility after their elution in corrosion solution or cell culture medium.

The correlation between the immersion test and biological assays was effective. The use of a neutralized corrosion solution with the real amounts of released ions after a mass loss test was possible. The comparison between these results with those of the assays employed with a cell culture medium is important, once the pH value difference among these milieus enable several substance loss findings with different biocompatibility response.

Taken together, the corrosion rates were one order of magnitude higher after the oxidation process than in the polished surface state for the high-noble alloys and were caused by the oxide-forming elements, with exception for Tin element. The toxic effect demonstrated by the high-noble alloys was associated with the high amounts of released Zinc by the corrosion media. The addition of Cerium to *CoCr*-based PFM alloys enhanced the corrosion resistance of the heat-treated surfaces, whereas the biocompatibility was not affected. The element Cerium showed no influence on the biocompatibility of the experimental base-metal alloys. Results of the present investigation indicate that a total removal of the oxide layer is recommended in the clinical practice to avoid unnecessary ion release and risks to the patient's oral health.

7. Summary

Introduction

Under certain conditions dental alloys as prosthetic works, in the mouth, can suffer corrosion process and some of their elements can be released. The release of elements from remaining oxide layers caused by corrosion processes may promote gingival discolorations or even biological risk to oral tissues.

Aim of the study

The purpose of this study was to investigate the corrosion resistance of twelve experimental PFM alloys with and without oxide layer, as well as to compare their substance loss to their cytotoxic effects with the same standardized cell-culture system, through out quantitative and qualitative biological assays.

Materials and methods

Nine experimental high-noble alloys with Indium, Tin and Zinc as oxide-forming elements, and three experimental *CoCr*-based alloys with *Ce* contents of 0, 0.19 and 0.39wt% were tested after wet-polishing to SiC #1200 and simulated porcelain firing at 930°C for a total time of 5min (4min in vacuum and 1min in air). Three pure metals (*In*, *Sn* and *Zn*) were also tested. Samples of each alloy and surface state were stored in 0.1 mol/L lactic acid/sodium chloride corrosion solution (pH=2.3, 37°C) as recommended by the ISO 10271. After 1, 4, 7, 14, 28 and 35-days solutions were analyzed using ICP-OES. Results of three independent experimental series were tested for statistical significance using Student's t-Test ($p<0.05$). The biological assays of the experimental alloys with and without oxide layer and pure metals were first carried out with cells grown on the material in direct contact. Viability and morphology analysis together with stains by means of fluorescein diacetate and ethidium bromide were performed by optical microscopy. The alloys toxic effects were determined by XTT-test following incubation of L-929 fibroblast cell cultures with extracts from the

corrosion test and in cell culture medium for 24h at 37°C. Cytotoxicity assays were both in accordance with ISO 10993-5.

Results

High-noble polished alloys demonstrated lower mass loss rates compared to polished and oxidized surfaces. Total substance loss over 35-days ranged from 0.5 µg/cm² (#B6, polished) to 238.4 µg/cm² (#B6, oxidized). The highest ion release with #A1, #A3, #B1, #B3, #B4, #B5 and #B6 oxidized samples was from oxide-forming elements (*In* and *Zn*). Zinc was released in concentrations up to 88.1 µg/cm² and 205.6 µg/cm² in the first day for heat-treated #A3 and #B6 respectively. Oxidized high-noble alloys with Indium content revealed constant ion leakage over the test period. Alloys with Tin (#A2 and #B2) demonstrated low overall ion release.

The addition of 0.19wt% *Ce* to the *CoCr*-based alloy decreased the corrosion rate, whereas 0.39wt% *Ce* showed no statistically significant further effect. The accumulated mean total substance loss values after 35-days of immersion revealed that #N1 polished released 3.3 µg/cm², #N2 up to 3.0 µg/cm², and #N3 amounts of 3.5 µg/cm². Meanwhile the polished and oxidized alloys released 54.7 µg/cm², 7.1 µg/cm² and 6.5 µg/cm², respectively.

The results showed that the PFM high-noble alloys as well as the base-metal alloys are biocompatible by the direct contact assay. The pure metal presented a ranking (from the most to the less toxic) of: *Zn>In>Sn*. Moreover, it was found that there isn't any great difference between polished and pre-heating specimens. However a substantial difference was noted after the XTT-test. Alloys with Zinc content promoted cell depression after elution in corrosion media, whereas all other alloys showed no significant effect. Differences were to be noticed among the extracts of corrosion solution and in cell culture milieu. Zinc-content alloys demonstrated slight to no toxic effect after elution in cell

culture media. No influence of the *Ce* content could be found for the polished as well as for the polished and oxidized samples.

Conclusions

Highest ion release was within the first day in most cases. The following ranking shows the influence of oxide-forming elements on the alloy's substance loss in descending order: *Zn>In>Sn*. The corrosion rates were in general one order of magnitude higher after oxidation than in the non-oxidized surface state and were caused by the oxide-forming elements. Toxicity was associated with the released Zinc element. The addition of Cerium to *CoCr*-based PFM alloys enhanced the corrosion resistance of oxidized surfaces, whereas the biocompatibility was not affected. Taken together, a total removal of the oxide layer is recommended to avoid unnecessary ion release.

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